Author’s response to reviews

**Title:** Immunomodulatory activity of polysaccharides isolated from Clerodendrum splendens: Beneficial effects in experimental autoimmune encephalomyelitis

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**Author’s response to reviews:** see over
June 2, 2013

Tom Rowles, Ph.D.
Executive Editor
BMC Complementary and Alternative Medicine

Dear Dr. Rowles:

With this letter, we are submitting our revised manuscript entitled “Immunomodulatory activity of polysaccharides isolated from Clerodendrum splendens: Beneficial effects in experimental autoimmune encephalomyelitis.” We have revised the manuscript according to the Reviewer’s comments and provide a point-by-point response for each of the comments. We thank the Reviewers for helpful comments and feel these revisions have improved this manuscript.

Thank you for your consideration of this revised manuscript.

Sincerely,

Mark T. Quinn, Ph.D.
Professor
**Responses to Reviewer #1 Comments**

1. Authors should correct the mismatches between the symbols of Figure legends and Figures.
   
   **Response:** We went back through the figures and legends to confirm the correct symbols. Mistakes may have occurred when the manuscript was converted by the journal. We are going to submit a PDF so hopefully these conversion problems do not occur.

2. Authors used a lot of monocyte/macrophage cell lines including PBMC. However, there are no explanations why authors used them. Authors should explain them. I think some of results using monocyte/macrophage cell lines should be deleted.
   
   **Response:** The basic mechanism of the immunostimulatory effects of botanical polysaccharides is thought to occur via macrophage stimulation. We tested samples in murine and human cell lines, as well as primary human cells because we often see differences in macrophage responses between species. Thus, it is important to confirm similar responses in murine phagocytes when considering future in vivo studies using a murine model. In addition, confirmation of cell line responses in primary cells is important to confirm cell line responses are also relevant to primary cells. We now include this information on page 18, lines 18-19, and page 19, lines 2-7, of the revised manuscript.

3. I cannot understand CSP-AU1 is the most potent activator of TNF, IL-6, and GM-CSF production. CSP-NU1 is also potent activator of them.
   
   **Response:** We agree that CSP-NU1 is also a potent activator of cytokine production. We have added additional data on CSP-NU1 immunomodulatory activity *in vivo*. A description of results and discussion related to CSP-NU1 is now provided on pages 22-23, 25, and Figure 10 of the revised manuscript.

4. In Fig.11, authors should examine GM-CSF production.
   
   **Response:** We examined GM-CSF secretion in serum and did not find significant increases in the level of this cytokine in serum after injection of CSP-AU1 or CSP-NU1 sub-fractions. Thus, we conclude that the mechanisms of cytokine production could be different in *in vitro* and *in vivo* systems. Data for GM-CSF levels in serum are now shown in Figure 10 for the revised manuscript.

5. In effect of CSP-AU1 on EAE, I think CSP-AU1 have only delayed the onset of EAE. Authors should examine the effect of CSP-AU1 after 17 days. In addition, author should examine the effects of CSP-NU1 as an immunomodulator and CSP-AU2 as a negative control on EAE. Moreover, authors should examine the effects of polysaccharides by oral administration for estimating clinical usefulness.
   
   **Response:** We examined the effects of other fractions, including CSP-NU1 and CSP-AU2 as an immunomodulators *in vivo* by evaluation in mice and received a similar cytokine profile for both high activity fractions CSP-NU1 and CSP-AU2. However, the low activity sub-fraction CSP-AU2 was also minimally active in ability to induce serum cytokines *in vivo*. At the present time, we do not have enough material to start new experiments with animal models of EAE, including different applications (oral) and doses. We are planning to do it in next rounds of our collaboration.
Minor Essential Revisions:
1. In Fig.9, please mark panel A and panel B.
   **Response:** As suggested, we revised panels in the Figure (see new Figure 8).

2. In Fig.12, the symbols of statistical significance should be marked on drug-treated group.
   **Response:** As suggested, we changed position of the symbols of statistical significance in the Figure (see new Figure 11).

Responses to Reviewer #2 Comments
1. Introduction: The last part of the introduction is results from the paper. I would recommend moving this part to the results/discussion part.
   **Response:** As suggested, we moved these details to the Results section of the revised manuscript.

2. Field studies from 2010-2012 are missing a reference.
   **Response:** Field studies are now included in the Results section on page 15, first paragraph, of the revised manuscript.

3. Page 6: Specify where a specimen has been deposited and under which number.
   **Response:** In the revised version of the manuscript, we now included details that this plant was identified and authenticated by Dr. Aké-Assi, Emeritus Professor of Botany, using voucher specimens deposited at various periods, including voucher specimen #16877, deposited at the National Herbarium of the National Centre of Floristique (CNF) of the University of Cocody-Abidjan. These details are now included on page 6, lines 3-7, of the revised manuscript.

4. Specify how the plant material was dried.
   **Response:** Parts of plants tested were air-dried over 7–10 days at room temperature and powdered under laboratory conditions. This description is now included on page 6, lines 7-8, of the revised manuscript.

5. Centrifuged at 80,000xg. To my knowledge this seems very high. Specify the device capable of doing this.
   **Response:** Thank you for pointing out this typographical error. The precipitates were pelleted by centrifugation at 8,000×g. The sentence was corrected on page 6, line 13, of the revised manuscript.

6. The column was washed (…) (Eluted?)
   **Response:** Thank you for pointing out this typographical error. Yes, the column was eluted. The sentence was corrected on page 6 of the revised manuscript.

7. Page 7: Homogeneous means that the polymers present are geometrically similar. This information is best found with SEC-MALS-VISC (conformation plot). HP-SEC is based on the polymer size, but it is difficult to tell whether it is homogeneous or not with SEC only. It may contain different polymers with similar size. It is not possible to give
the relative molecular mass (Mr) unless the sample is monodisperse (PDI=1). With the
method mentioned (gelfiltration) it is not possible to say if the sample is monodisperse
or not, I would therefore recommend MW (molecular weight).

Response: We agree with the Reviewer and have deleted descriptions of the
fractions related to “homogeneous” on pages 7, and page 15. We also indicate
molecular weight instead of Mr.

9. Reference missing for Yariv test.
Response: We now include reference a reference for the Yariv test (see
Reference 16 of the revised manuscript.

10. “LAL test” is suggested to be moved to a more relevant place.
Response: The description of the LAL test was moved to separate section of
the Materials and Methods (see page 9, lines 15-18, of the revised manuscript).

11. Page 12: I could not find information about the control animals in the methods
section. It also unfortunately that only one dose was measured (dose-response not
possible to measure). If the person evaluating the animals was not blinded, this will give
rise to a bias in the results which should be mentioned in the discussion. A reference is
missing. Positive control?
Response: We added information about the control animals in the methods
section and also included the appropriate reference (see page 13, lines 3-20, of
the revised manuscript). We now expanded the description of this model,
including control group and its evaluation. We also added details that “mice were
monitored and scored daily by a technician blinded to treatments for clinical signs
of EAE…” and that control group was treated with PBS only (page 13).

12. Page 14: Type of statistical analysis is not mentioned under the “statistics”
subsection. (Only the program used is mentioned)
Response: We now describe the types of statistical analyses used in this
manuscript on page 14, lines 8-13, of the revised manuscript.

Results:
13. Page 15: See information on “homogenous sample” above.
Response: We agree with the Reviewer and have deleted descriptions of the
fractions related to “homogeneous” on page 15. We also indicate molecular
weight instead of Mr.

14. Page 16: The amount of LPS should be related to the material, not to a solution.
Response: The amount of endotoxin now is recalculated as ng of endotoxin/mg
of polysaccharide (see page 16, lines 13-15, of the revised manuscript.
15. Page 17: “Common back bone”. Do you mean a RG-I type of backbone?
Response: As suggested, we revised this sentence for clarification.

16. All bar plots should have error-bars in both directions.
Response: All bars now have error-bars in both directions.

17. Figure 1 should be removed.
Response: Figure 1 was removed as recommended by the Reviewer.
18. Figure 2: Error bars missing for some of the graphs. LPS signal is difficult to comprehend and to me it seems as it is not correctly put into the graph (200ng/ml should be more to the left?) (this also counts for figure 3, 4 and 5).
   **Response:** All graphs have error bars; however, some bars are too small to be seen beyond the size of the symbols. We also revised the graphs to clarify the LPS-induced signal so that it is clearly indicated as 200 ng/ml LPS.

19. Table 1: Clearify “Methoxyl content” and “DM” in the footnotes of the table.
   **Response:** We now included more information about “Methoxyl content” and “DM” in the footnote of Table 1.

20. Table 2: Include information on CSP-AU2 and NU2 for comparison Discussion.
   **Response:** Unfortunately, yields of these fractions were too small, and we were unable to submit them for this analysis. We now include a footnote that sugar analysis was not performed on these fractions.

   **Response:** We now include Ethnopharmacological information as a separate section in the Results and present the data obtained (see page 15).

22. Page 25: The CSP-AU1, I believe, was the polysaccharide with the highest biological activity, and this had a fairly high tanning-content.
   **Response:** This sentence was corrected.

23. Conclusion: To my opinion, the authors are claiming too much of their results. (i.e. “provide a molecular basis of the therapeutic effect in treatment of autoimmune disease”). In my opinion this is wrong as the injections only delay the onset of the inflammation. (After day 17 the control and the polysaccharide injection showed similar symptoms.)
   **Response:** We agree with the reviewer and have removed overstatement regarding the results. We revised the Conclusion accordingly to remove claims regarding treatment of autoimmune disease.

24. References: Some misprints and missing information. (Journal abbreviation)
   **Response:** We have corrected all of the references and journal abbreviations.