Author’s response to reviews

Title: Immunomodulatory effect of water soluble extract separated from mycelium of Phellinus linteus on experimental atopic dermatitis

Authors:

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Author’s response to reviews: see over
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The BioMed Central Editorial Team

Re: MS: 9985955336339504 Decision Letter

Dear Editor

Thank you for your help in reviewing the above manuscript, “Water soluble extract of Phellinus linteus modulates experimental atopic dermatitis” by Ji Sun Hwang et al., for the *BMC Complementary and Alternative Medicine*. We are very grateful for the inputs from the reviewers. By following the comments or suggestions raised by reviewers we have carefully revised our manuscript and accordingly incorporated changes (marked in yellow) in the revised manuscript.

Point-by-point responses to reviewers’ comments are also attached. We hope that this revised manuscript is now suitable for publication in the *BMC Complementary and Alternative Medicine*.

Sincerely yours,

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A point-by-point response to reviewers’ comments

Reviewer: Shuichi Segawa

Comment #1
You induced atopic dermatitis in mice by painting DNCB and mite extract for 4 weeks. After 2 weeks of AD induction, you started P. linteus WA or ceramide treatment. At the 2 weeks of AD induction, nevertheless at this point of time you did not start the intervention, the ear thickness of control group was significantly increased compared to P. linteus WA or ceramide treatment group. I am concerned about the possibility that the grouping of mice in your experiment might be inappropriate.

Response #1:

As pointed out by reviewer, ear thickness of control group seemed to little bit thicker than WA or ceramide treated group. However, the difference was not statistically significant (Fig. 3C). In addition, total serum IgE as well as mite specific IgE levels were measured before dividing the treatment and confirmed no significant differences between the groups as shown in Fig. 4. To clarify this issue, new sentences were introduced to describe the dividing criteria for each treatment group in Materials and Methods part of the revised manuscript.

Comment #2
You investigated the effect of P. linteus on cell viability by MTT assay. The administration of P. linteus significantly increased the cell viability at 0.5 mg/ml, however at the concentration of 0.75 mg/ml, cell viability was significantly decreased. You had better explain this drastic change in cell viability by the administration of P. linteus.

Response #2:
To confirm the cytotoxicity of P. linteus, we performed trypan blue staining as well as MTT assay. In both experiments, treatment of P. linteus up to 0.5 mg/ml did not alter the cell viability and even enhanced cell proliferation. However 0.75 mg/ml of P. linteus significantly decreased cell viability indicating that the drastic changes of cell viability was not due to our systemic mistake. Although we did not measure the cell viability in a range of 0.5 mg/ml to 0.75 mg/ml, but it seems that threshold for cytotoxicity of P. linteus is located between 0.5 to 0.75 mg/ml. So based on the cytotoxicity result, we performed all the in vitro and ex vivo
experiments with 0.5 mg/ml of extracts of *P. linteus*.

**Reviewer: Naoki Inagaki**

[major]

**Comment #1:**

The title suggests that the main subject of this manuscript is *in vivo* examination on mouse dermatitis model. The impression is not the case.

**Response #1:**

By following the reviewer’s comments, we modified the title of this manuscript to “Immunomodulatory effect of water soluble extract separated from mycelium of Phellinus linteus on experimental atopic dermatitis” and we changed in modified manuscript.

**Comment #2:**

Human B cell line and mouse B cells were used for examining IgE production. Why were human B cells employed? It seems to be inconsistent. Why was IgE production induced by LPS and IL-4? Antigen-induced IgE production using mouse B cells should be examined.

**Response #2:**

U266B1 cell line produce human IgE constantly (Kim et al. 1999) and widely used to screen the candidate molecules that could inhibit IgE expression. Before examining the effect of each fraction of *P. linteus in vivo*, we tested inhibitory effect on IgE production *in vitro* cell line system and selected WA as the best candidate. Although U266B1 cell line constantly produces large amount of IgE compare to other B cell lines, LPS/IL-4 stimulation is optimal condition for IgE class switching. So we examined the anti-IgE effect of each fraction of *P. linteus* under LPS/IL-4 stimulation. In addition, since cell lines have limitations to mimic the *in vivo* situation, we applied WA to mouse primary B cells to mimic the *in vivo* relevance.

Regarding the issue of Antigen-induced IgE production in mouse B cells, we examined the effect of WA on the expression of pathogenic cytokines and chemokines from ear cells under the antigen induced stimulation condition from AD induced mice. We added the explanation about this result in Supplement figure. Under the mite extract stimulation condition, WA treatment significantly decreased expression of pathogenic cytokine (IL-4, IL-5,
IL-13, IFN-γ and TNF-α) and chemokine (CCL22, CCL17 and CCR4).

Comment #3:

Dermatitis was induced by repeated exposure to DNCB and mite antigen. Why was so complex model employed? What was the merit of the model? Characteristics of the dermatitis model as an atopic dermatitis model have to be mentioned. Has antibody production against dinitrophenyl residue evaluated?

Response #3:

Combination of DNCB and mite extract induced atopic dermatitis model was firstly developed by our laboratory [1] and other group adapted our AD model to examine the therapeutic effect of their extract [2]. Based on our own result, only DNCB challenge increased both IgE and IgG levels, which does not mimic the human atopnic dermatitis. In our model, treatment of DNCB breaks skin barrier to initiate the immune response then mite extract breaks gap junctions to amplify the immune response. Our combined method induced higher serum IgE not IgG level, increase of pathogenic cytokine expression and severe scratching behavior compared to only DNCB or mite extract challenge method. As indicated by reviewer, we also measured DNCB specific IgE levels. However it was not detectable. Usually previous reports also mainly measured total IgE levels in the serum not DNCB specific IgE levels when they applied DNCB induced AD model [3-6].

Comment #4:

Chemokine and cytokine production was investigated using cells from ear tissues. Why were the cells stimulated with PMA and ionomycin? Antigen-induced chemokine and cytokine production should be examined.

Response #4:

According to reviewer’s comments, we also examined antigen-induced chemokine and cytokine production by real time-PCR. Similar with PMA/ionomycin stimulation, WA treatment significantly decreased pathogenic cytokine and chemokine levels including CCL17, CCL22, CCR4, IL-4, IL-5, IL-13, IFN-γ and TNF-α. We included this result as a Supplement figure in revised manuscript.

Comment #5:

P. linteus is well known to exhibit anti-cancer effects through immuno-potentiating effects. Present results, however, seem to be immuno-suppressing nature. The relationship
should be discussed.

Response #5:

As indicated by reviewer, anti-cancer effect is most well known function of *P. linteus*. Previous studies have shown the anti-cancer effect of *P. linteus* is mediated by diverse mechanism [7] [8] [9]. Anti-tumor effect of *P. linteus* is mainly mediated by polysaccharide. The biological activities of polysaccharides are influenced by their different solubility in water, molecular weights, degrees of branching, and by their different triple helical confirmations [10]. Different regulation mechanisms seem to be mediated by different kinds of polysaccharides, purity of polysaccharide and preparation process.

Anti-inflammatory effect of *P. linteus* was also suggested [11][12]. However, active compound involving anti-inflammatory effect was not clearly identified. As we described, exact action mechanisms of anti-tumor and anti-inflammatory effect might be mediated by different active component. In addition, routes of treatment such as oral administration or topical application and target cells of *P. linteus* under the certain disease environment can mediate the differential effects of *P. linteus* treatment. We included the above sentences in the Discussion part.

[minor]

Comment #6:

*P. linteus* extract was used in experiment shown in figure 1B. However, there is no description on the preparing method and yield.

Response #6:

By following reviewer’s comments, we included the preparation method for mycelium of *P. linteus* in Materials and Methods section of revised manuscript.

Comment #7:

Origin and characteristics of human B cell line U266B1 should be shown. Do the cells secrete IgE constantly?

Response #7:

Human U266B1 multiple myeloma cells (ATCC TIB-196™, American Type Culture Collection, USA) were established from the peripheral blood of a patient with an IgE myeloma (epsilon2, lambda2). These cells constantly secret IgE and IgE secretion is more
induced upon LPS plus IL-4 stimulation. According to reviewer’s comments, we included the description about origin and characteristics of U266B1 cells in Materials and Methods.

**Comment #8:**
Were P. linteus fractions other than water soluble fraction ineffective for the dermatitis?

**Response #8:**
Our *in vitro* cell line result showed that WA was the most effective and other extracts such as ME and BW failed to decrease IgE production. When we compared serum total IgE levels after 2 weeks of treatment of each fraction, consistent with *in vitro* result, ME and BW did not decrease IgE levels, while WA significantly reduced total serum IgE concentration as well as ear thickness. So based on this finding, we could conclude that among the fractions of *P. linteus* only WA has the therapeutic effect against atopic dermatitis. We included this explanation in Discussion part.

**Comment #9:**
Water soluble fraction of P. linteus was examined by topical application. Why was such route selected?

**Response #9:**
Until now, topical application of moisturizing emulsion or ointment is the most widely used therapies for atopic dermatitis. Topical application is easier than oral intake for babies. In addition, topical application is considered as safer than oral administration. So we selected this route instead of oral feeding to mice. Even though we did not include data, topical treatment of ointment containing extract of *P. linteus* significantly decreased total serum IgE levels. Furthermore, not only topical treatment of WA, we also tested the effect of oral administration of *P. linteus* on AD symptoms. We found that oral administration could also reduce IgE levels, pathogenic cytokine expression and immune cell infiltrations. We addressed this issue in Discussion part.

**Comment #10:**
Ceramide was used as a reference drug. Why was ceramide selected? The mechanism for its effectiveness should be mentioned.

**Response #10:**
By following reviewer’s suggestion, we included the sentences for therapeutic effect and mechanism of ceramide in AD situation in Result part. There are many reports showing that reduced ceramide contents in the AD patient skin compared to normal healthy skin [13-15]. To improve this phenotype many ointments or emulsions for AD therapy include diverse types of ceramide. Several reports showed that ceramide treatment can down-regulate IL-4, TNF-α expression level, ear thickness and cell infiltration [16]. We add the sentences for the mechanism of ceramide in discussion section.

Comment #11:
Introductory descriptions in result section can be deleted.

Response #11:
According to reviewer’s comments, we deleted introductory descriptions in Results as well as Materials and Methods sections.

Comment #12:
There is no figure 3E.

Response #12:
We apologize for causing the confusion in our figure. In the original version, Figure 3E was located below the Figure 3B so it looked like figure 3B and 3E are combined figure. To exclude this misunderstanding, we changed the location of figure 3E and 3D in revised figures.

Comment #13:
Student’s t-test does not seem to be appropriate for evaluating present results. It should be considered.

Response #13:
Even though reviewer mentioned Student’s t-test does not seem to be appropriate for evaluating present results, this analysis also has been well accepted for many in vivo studies. Many publications regarding in vivo study from our lab also applied this program for the statistical analysis [17-21].

Comment #14:
In figure 1, spontaneous IgE production should be shown. In the legend for figure 1, explanation for asterisks is missing.

Response #14:
We apologize for missing the explanation for asterisks for Figure 1 and we added explanations in Figure 1 legend. We also changed Figure 1B result including inhibitory effect of total extract of *P. linteus* in W/O stimulation condition. Total extract of *P. linteus* inhibited IgE levels compared to control in the presence or absence of stimulation however, inhibitory effect was more efficient upon LPS/IL-4 stimulation (W/O: 20 %, LPS/IL-4: 50 %). We have described these results in the Result and Figure legend section accordingly.

Comment #15:
In relation to the in vivo experiments, mouse B cells should be employed for experiments on IgE production. Why were mouse B cells used only for dose-response study shown in figure 2c?

Response #15:
Firstly, we tried to find most effective extract among three fractions using widely used *in vitro* screening system with U266B1 cell lines then to confirm the anti-IgE effect of the best candidate extract using mouse primary B cells. Consistent with cell line result, when we performed same experiment with mouse primary B cells, only WA significantly reduced secretion of IgE.

Comment #16:
Supplemental figure 1 can be added to figure 4. Figure 5 should be replaced with supplemental figure 2.

Response #16:
By following reviewer’s suggestion, we included supplement figure 1 as Figure 4C and Figure 5 was replaced with supplement Figure 2.

Comment #17:
There are some errors and inappropriate descriptions in the text.

Response #17:
We apologize for the mistakes in our manuscript and also thank to reviewer for
pointing out these errors thus we have corrected them as suggested by the reviewer as highlighted in bold letters here.

1. Line 11 of page 2, total *P. linteus* extract -?
   ➔ total extracts of *P. linteus*

2. Line 12 of page 2, in the presence of various concentrations of *P. linteus* extract. -?
   ➔ Deleted

3. Line 15 of page 2, isolated from atopic mice -?
   ➔ Deleted

4. Line 18 of page 2 and Line 2 of page 3, Balb/c -?
   ➔ BALB/c

5. Line 25 of page 2, dose dependently -?
   ➔ Deleted

6. Line 9 of page 3, water extract of *P. linteus* -?
   ➔ water soluble extract of *P. linteus*

7. Line 23 of page 7, *P. linteus* extract (0.5 mg/ml) -?
   ➔ various concentration of WA

8. Line 12 to 16 of page 8, After 2 weeks ---- 4 weeks induction. -?
   ➔ Revised to “After 2 weeks of AD induction, based on the serum IgE levels, mice were divided into 3 groups. Then AD mice were treated daily with PBS (Cont), 1 mg/each ear of water soluble extract of *P. linteus* (WA) or 15 μg/each ear of ceramide (Cera) (C2-ceramice, Cayman chemical, Ann Arbor, Michigan, USA) until end of 4 weeks induction.”

9. Line 16 of page 8, Only tape stripping and PBS-painting without AD induction were performed as a control group (without AD induction). -?
   ➔ Changed to “Only tape stripping and PBS-painted group was used as a control (W/O).”

10. Line 22 of page 10 to line 24 of page 10, For the detection of total IgG level, ---- 200 fold for ELISA. -? This sentence was reduced with shorten description.
For the detection of total IgG level, serum was analyzed with mouse IgG ELISA kit (BETHYL, Montgomery, TX, USA) by following the manufacturer’s protocol.

11. Line 22 of page 11, PMA (20 nM) and ionomycin (2 μM) -?
   ➔ PMA (0.5 μg/ml) and ionomycin (1 μM)

12. Line 6 of page 12, To find out the optimal ---- extracts was tested. -?
   ➔ “First of all, cytotoxicity test was examined to find out the optimal concentration of *P. linteus* extract for *in vitro* efficacy test.”

13. Line 15 of page 12, *P. linteus* extract (PL)(0.5 mg/ml) or PBS (Cont) -?
   ➔ *P. linteus* extract or PBS as a control

14. Line 2 of page 13, fraction -?
   ➔ extract

15. Line 3 of page 13, WA extract -?
   ➔ WA

16. Line 6 of page 13, WA could -?
   ➔ WA can

17. Line 6 of page 13, WA was applied ---- with PBS control (Fig. 2C). -?
   ➔ “WA was applied to mouse primary B cells. Indeed, WA treatment significantly decreased IgE production in a dose dependent manner (Fig. 2C).”

18. Line 13 of page 13, To test the potential immune modulatory function of *P. linteus* extract on IgE production *in vivo*, -?
   ➔ Then to evaluate the immunomodulatory function of *P. linteus in vivo*,

19. Line 20 of page 13, Therapeutic effect of WA ---- with the PBS-treated control group.-?
   this sentence was reduced with shorten description.
   ➔ “Painting of WA significantly reduced the AD symptoms including erythema, horny substance, dryness, and scaling (Fig. 3B) by reducing ear thickness (Fig. 3C) and clinical score (Fig. 3D) compared with the PBS-treated control group.”
20. Line 17 page 14, even compared with -?
   ➔ even better than

21. Line 3 of page 15, Total ear cells ---- and ionomycin (2 μM) for 4 hours -?
   ➔ “Total ear cells isolated from each group were stimulated with PMA (0.5 μg/ml) and ionomycin (1 μM) for 4 hrs”

22. Line 5 of page 15, In line with the result of histological analysis -?
   ➔ In line with the histological analysis

23. Line 16 of page 16, Clinical trials ---- for a long time. -?
   ➔ “These plant derived products have been used as drugs and food additives for a long time.”

24. Line 5 of page 17, its -?
   ➔ inhibitory

25. Line 9 of page 17, that the extract significantly ---- extracting diverse solvents (Fig. 2A). -?
   ➔ Modified to this sentence “that the extract significantly inhibited IgE production under the LPS and IL-4 stimulation which is the well known IgE class switching condition [22, 23] as well as w/o stimulation (Fig. 1B). To identify potent inhibitory fraction we further fractionated P. linteus by diverse solvents including chloroform, methanol, water and boiling water (Fig. 2A).”

26. Line 15 of page 17, both in -?
   ➔ in both

27. Line 19 of page 18, Several chemokines ---- site of inflammation. -? Revised with shorten description.
   ➔ “Several chemokines have association with AD phenotype and among them, CCL17, CCL22, CCR4 have pivotal roles in migration of pathogenic immune cells (mainly CD4+ T cells) to the site of inflammation [24].”

28. Line 4 of page 19, Th2 like cells -?
Th2 cells

29. Line 10 of page 20, water soluble fraction of *P. linteus* -?
   ➔ water soluble extract of *P. linteus* (WA)

30. Line 18 of page 21, In this study we ---- chemokines (CCL17 and CCL22). -?
   ➔ “In this study we have shown that topical application of water soluble extract of mycelium of *P. linteus* (WA) inhibits the development of experimental AD by reducing the leukocytes and granulocytes infiltration mediated by inhibition of chemokine expression (CCL17, CCL22 and CCR4) and by decreasing serum IgE levels mainly through the down regulation of Th2 type cytokines including IL-4, IL-5 and IL-13”

31. Line 4 of page 23, Lipopolysachharide -?
   ➔ Lipopolysaccharide

32. Line 2 of page 35, 0.2mg/each ear -?
   ➔ 0.2 mg/each ear

Reviewer: Shang-tzen Chang

Comment #1:

The authors should provide the detail information for *P. linteus* in materials and methods section.

Response #1:

By following reviewer’s comments, we included the sentences for preparation method for mycelium of *P. linteus* in Materials and Methods section of revised manuscript.

Comment #2:

In this study, the PBS was used as vehicle for water extract, did the solution absorb easily (completely) by mouse skin?

Response #2:

Since atopic dermatitis induced mouse skin showed severe dryness due to loss of ceramide contents and increased water loss in the regional skin, PBS was very easily
absorbed.

Comment #3:

Is it possible to provide the composition for your water extract? I think it is very important for the natural medicine research.

Response #3:

We totally agree with reviewer’s suggestion. Among the subfractions, the water soluble fraction appeared to be most effective in anti-inflammation, implying that *P. linteus* would contain active anti-inflammatory component(s) with relatively hydrophilic characters. To isolate the active component from this fraction, we performed HPLC analysis. Identification of active components which we got based on HPLC analysis is under investigation. However we had difficulty to characterize the properties of each fraction. We described this content in Discussion section.

Comment #4:

AS the author mentioned in discussion section" Atopic dermatitis is a kind of inflammatory immune disorder which causes severe economical and social problem due to significantly increased incidence in developed countries. Atopic dermatitis (AD) is thought to be a typical Th2 type immune disorder which shows elevated serum IgE level and increment of Th2 type cytokines such as IL-4, IL-5 and IL-13. Th1 type response also plays key role in pathogenesis and maintenance of AD [25]. Clinical trials have been performed to modulate Th2 and Th1 type responses as well as chemokines levels “. Although the effects of *P.linteus* on IgE expression had not been reported, other cytokines modulated by the same material have been proved. Thus, I think the relationship between your IgE results with other cytokines (the data obtained by your study and compared with the same study by others) should be addressed.

Response #4:

We appreciate for reviewer’s comment. As indicated by reviewer, the effects of *P. lindeus* on IgE production had not been reported but other cytokines modulated by *P. lindeus* have been proved. In this study we found that WA mainly down-regulated the expression of IL-4, IL-5 and IL-13 from inflamed tissue residual CD4+ T cells as well as ear cells. Since IL-4, IL-5 and IL-13 are typical Th2 type cytokines which are crucial for IgE class switching from B cells [22, 23], down regulation of these cytokine by WA treatment may lead to reduction of serum IgE levels. We added this description in Discussion section.
**Comment #5:**
Is there any side effect for your extract?

**Response #5:**
During 2 weeks of topical treatment period of water soluble extract of *P. linteus* (WA), any side effect was not detected. In addition, oral administration of total extract of *P. linteus* up to 3 weeks did not cause any side effects based on the measurement of body weight changes and internal organ size examination.

**References**


