Author's response to reviews

Title: Effects of Payena dasyphylla (Miq.) on hyaluronidase enzyme activity and Metalloproteinases protein expressions in Interleukin-1beta stimulated human chondrocytes cells

Authors:

Kamini Citalingam (kaminicitalingam@gmail.com)
Seema Zareen (seema_zareen@yahoo.com)
Shamsul Khamis (shamsul@ibs.upm.edu.my)
Khozirah Shaari (khozirah@yahoo.com.my)
Syahida Ahmad (syahida@biotech.upm.edu.my)

Version: 3 Date: 6 March 2013

Author's response to reviews: see over
Dear Sir/Madam,

Our research groups have discussed the comments that you have sent to us and agreed to consider several corrections on the article. The corrections are highlighted in red.

Below are our responses toward the reviewer’s comments:

**Reviewer 1:**

**Major compulsory revisions**

1. This is a very interesting work with very promising data. The only missing element, for me, is data on cell toxicity of the extract. Without that, we could think that all effects report in the paper could be due to a toxicity or cell suffering, resulting in a decrease of protein production by the cells.

   **Answer:**

   Data on cell toxicity for both methanolic and ethyl acetate extracts have been added (Figure 2).

2. An other point is the choice of IL-1β concentration. The authors used 100 ng/ml for some experiments, 10 ng/ml for other experiments. Why? 100 ng/ml is a huge supra-pathological stimuli.

   **Answer:**

   IL-1β is a proinflammatory cytokine that is known to stimulate the production of hyaluronidases and MMPs. In this study, the high concentration of 100 ng/µL was used to stimulate hyaluronidase activity and the gene expressions study. Based on our optimization results (data not shown), both hyaluronidase enzyme activity and gene expressions were found to be undetectable in the chondrocyte cells at lower concentration of IL-1β as reported. Besides, we had also shown that the 100 ng/ml of IL-1β could stimulate both enzyme activity and gene expression without affecting the cells viability (Figure 2).

   While, 10 ng/µL was used to stimulate MMP-3 and MMP-13 protein expressions because that lower concentration of IL-1β was able to show detectable amount of both protein expressions.
Minor essential corrections

1. Material and method, Cell culture: could you precise how many passages did you used for NHAC.

   Answer:

   Cell passage numbers have been added under the material and method, cell culture section.

2. Discretionary Revisions
   Background:
   change "is the pathological characteristic of this disease" by "is the mean pathological feature in this disease"
   paragraph 2: other factor too are known: other factors are also known
   metalloproteases: metalloproteinase
   paragraph 4: MMP is also describe

   Answer:

   a) Background – the phrase “is the pathological characteristic of this disease” have been rephrased to “is the main pathological feature in this disease” as suggested by the reviewer.
   b) Paragraph 2: “other factor too are known” already corrected to “other factors are also known” and “metalloproteases” had been changed to “metalloproteinase”.
   c) Paragraph 4: the sentence “MMP is also described” has been corrected.

Reviewer 2:

This paper describes that the extract of Payena dasyphylla suppresses an activity of testicular hyaluronidase, gene expressions of Hyal1 and Hyal2, as well as protein expression of MMP-3 and MMP-13 in cultured chondrocyte. In addition the extract has an antioxidant activity. The author claims that the extract of this plant may be useful for development of the new medicine for OA.

Major compulsory revisions

1. Basically this paper contains some interesting results, however, the explanation of this research does not look logical. This paper starts with the description of the inhibitory activity of bovine testicular hyaluronidase by the extract. Then the authors moved to investigate whether the extract inhibits hyaluronidase produced by cultured chondrocyte. However, authors actually demonstrated the effect of the extract on gene expression of Hyal1 and Hyal2 in cultured chondrocyte. They did not examine the effect on the activity of Hyals. The results concerning these hyaluronidases are difficult to be linked. Therefore, the reviewer would like to recommend that authors should remove the
The first part of the results in this manuscript was to show that 20 plants extracts (bark and leaf) from 10 different plant species had been screened on their inhibitory effect toward bovine testicular hyaluronidase enzyme. From the results, we showed that *P. dasyphylla* methanolic bark extract demonstrated the highest or potential anti-hyaluronidase activity. From here, we would like to validate whether the plant could show the same results toward human hyaluronidase enzyme by testing the sample on normal human articular chondrocyte derived from the knee (NHAC-kn), which was known to express hyaluronidase enzyme upon stimulation with IL-1β. Therefore, the zymography method was used to determine the hyaluronidase activity in the chondrocyte cells.

The hyaluronidase is synthesized through the expression of two known genes in the cultured chondrocytes cells. Thus, we would like to clarify whether the inhibition of the hyaluronidase enzyme was due to the inhibition of both *HYAL1* and *HYAL2* gene expressions. Hence, the effect of the *Payena dasyphylla* was studied on the hyaluronidase activity in general then followed by the two principles *HYAL1* and *HYAL2* gene expressions using RT-PCR method.

2. As to the effect of the extract on MMPs, authors showed the protein expression by western blots. On the other hand, the gene expression and the enzyme activity were shown about Hyals. Therefore, reviewer feels that why they did not show the effect on the gene expression and the activities of MMPs. Especially it is not difficult to show the activity of MMPs using zymography as they did with Hyals.

**Answer:**

Yes, it is possible to look at the effect of *Payena dasyphylla* on MMPs enzyme activity using zymography test. But, the zymography results could only show the total enzyme activity. We can’t determine which MMP enzyme had been inhibited because there are more than one isoform of MMPs had been reported in the stimulated chondrocytes cells. Thus by doing the western blot technique, we are able to look at the plant effect on the specific MMP protein expression (MMP-3 and MMP-13), that are also involved in the degradation of the cartilage in OA disease progression.

To strengthen the mechanism of action of *P. dasyphylla* towards hyaluronidase enzyme in stimulated chondrocytes cells, we tested its effect on both genes that involved in the synthesis of hyaluronidase enzyme, which are *HYAL1* and *HYAL2* genes.
Minor Essential Revisions

1. In Background: Hyaluronidase is not a proteolytic enzyme. Author should check the sentence “proteolytic enzyme activity such as hyaluronidases and several matrix metalloproteases”.

   Answer:

   Background – “proteolytic enzyme...” have been replaced with “hydrolytic enzyme...”

2. In Background: MMP-13 can degrade type I, II, and III collagen, too. Author should check the sentence “MMP-13 has specific affinity for type II collagen but is ---”.

   Answer:

   Background – “MMP-13 has specific affinity for type II collagen...” have been replaced with “MMP-13 cleaves type II collagen and aggrecan at particular sites”.

3. In Methods, Plant materials and preparation of extract: Where is Terangganu? In Malaysia?

   Answer:

   The Terengganu is at the East Peninsular of Malaysia.

4. In Methods, Anti-hyaluronidase enzymatic assay (Morgan -Elson assay): Authors should delete this section. Otherwise, “Anti-hyaluronidase enzymatic assay” should be changed to “Hyaluronidase assay”. In this case, the detail of colorimetric assay is not necessary, and just mention “N-acetylglucosamine at the reducing terminal was determined by the method of Reissig, et al”.

   Answer:

   Since we had modified some part of the Reissig et al. method, we would like to retain the steps in this manuscript as we had explained in our answers for questions 1 in the Major compulsory revision.

5. In Methods, Cell cultures: “Normal human articular chondrocyte---The cells were passaged weekly.” is enough. Authors should delete the lower part of this section.

   Answer:

   Cell culture method has been simplified as suggested by the reviewer.
6. In Methods, Zymography: Citation number is not correct. Why is [41] here? [42], [43], too. Reference number should be checked through the text. Also, there is too much inappropriate capital letter notation through the text. For example, Anti-Hyaluronidase—(hyaluronidase), Normal Human Articular Chondrocyte—(Normal human articular chondrocyte), Reverse Transcription Polymerase Chain Reaction—(Reverse transcription-polymerase chain reaction), etc.

Answer:

Methods, Zymography – reference number and unnecessary capital letter notations throughout the text have been corrected.

7. In Methods, Western Blot: “The gel, from which --- the background becomes clear” should be deleted.

Answer:

Method, Western blot – “The gel from which....the background becomes clear” – have been removed.

8. Results and Discussion, Hyaluronidase Inhibitory activity: This section should be removed.

Answer:

We would like to retain the results & discussion of the screening results in this manuscript.

9. Results and Discussion, Effect of Payena dasyphylla methanolic extract---: Description of reference citation is not correct. [14, 15, 16] should be [14-15]. Authors should correct them all through the text.

Answer:

Results and discussion, Effect of Payena dasyphylla methanolic extract... – references citations have been corrected.

10. Results and Discussion, Effect of Payena dasyphylla methanolic extract---: Authors showed “Apigenin” in the legends of figure 1, 3, and 4. But there is no explanation about “Apigenin”. Therefore authors should explain it in this section.

Answer:

Brief explanation about Apigenin have been added in the results and discussion, Effect of Payena dasyphylla methanolic extract... section.
11. Abbreviations: “TEMED” could not be found in the text, and should be deleted.

*Answer:*

TEMED has been removed in the abbreviations section.

12. Tables: Table 1 should be removed.

*Answer:*

We would like to retain the anti-hyaluronidase screening results in Table 1 to be published in this manuscript. We believe, with the screening results alone, it will be difficult to be accepted for publication elsewhere.

13. Figures: Figure 2 should be removed.

*Answer:*

We also would like to retain the phenolic and flavonoid contents results of the *P. dasypylla* fractions in Table 2 to be published in this manuscript.

Thank you for your time and consideration.

Yours sincerely,

Syahida