Author's response to reviews

Title: Ethanol extract of propolis protects macrophages from oxidized low density lipoprotein-induced apoptosis by inhibiting endoplasmic reticulum stress-C/EBP homologous protein pathway

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

On behalf of my co-authors, we thank you very much for giving us an opportunity to revise our manuscript, and we appreciate reviewers very much for their positive and constructive comments and suggestions on our manuscript entitled "Ethanol extract of propolis protects macrophages from oxidized low density lipoprotein-induced apoptosis by inhibiting endoplasmic reticulum stress-C/EBP homologous protein pathway " (1651053095158727).

We have studied reviewers’ comments carefully and have tried our best to revise our manuscript according to the comments. Please find the attached revised version for your kind reconsideration.

We would like to express our great appreciation to you and reviewers for comments to our manuscript. We look forward to hearing from you.

Best regards.
Yours sincerely

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List of Responses

Reviewer 1

Tian H et al tried to explore the protective effect of ethanol extract from propolis on ox-LDL induced macrophage apoptosis through ER-stress mediated pathway.

I only have the following comments.

Minor Essential Revisions:

1. Authors should raise their scientific hypothesis in the end of introduction.
   
   Thank you very much for your advice. We have added following scientific hypothesis in the end of introduction. “we hypothesize that EEP may protect macrophages from ox-LDL-induced apoptosis through suppressing ER stress-CHOP signalling pathway.” (Line 23, Page 4)

2. Do authors mean “4 degree” (Line 15, Page 7) and “37 degree” (Line 4, Page 8)?
   
   Yes. They are 4 °C and 37 °C.

3. Where were caspase-3 assay kit and TUNEL staining kit purchased from? (Page 7 and 8)
   
   Caspase-3 assay kit and TUNEL staining kit were purchased from Calbiochem (San Diego, CA, USA) and Roche (Mannheim, Germany), respectively, which have been shown in the Reagents (Materials and methods) (Line 24, Page 5).

4. Where was CD36 antibody purchased from?
   
   Anti-CD36 antibody was purchased from Abcam (Cambridge, MA, USA), which has been shown in the Reagents (Materials and methods) (Line 15, Page 5).

5. Authors should state why RAW264.7 cells treated with ox-LDL at 100 mg/L and for 24 h (Line 19-20, Page 10).

6. Why were RAW264.7 cells treated with EEP for 24 h?
   

7. Total PERK should be test by western blot (figure 4, 5).

8. Total eIF2α should be test by western blot (figure 4, 5).
   
   According to your advice, total PERK and eIF2α have been presented in figure 4, 5.
9. The molecular weight of protein should be added on the right side of western blot in figures.

   According to your advice, the molecular weight of protein have been added on the right side of western blot in figures.

Level of interest: An article of importance in its field

Quality of written English: Acceptable

Statistical review: Yes, and I have assessed the statistics in my report.

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

Reviewer 2

Comments to Tian et al. paper,

(General comments)

In this paper, Tian et al. showed that treatment with Ethanol extract of propolis (EEP) suppress ox-LDL-induced apoptosis as ER stress-inhibitor PBA in macrophages. They also showed that EEP treatment suppress induction of ER stress-CHOP pathway through suppression of ox-LDL intake and the up-regulation of CD36 induced by ox-LDL in macrophages.

The data shown here are clear and meaningful. However, there are some questions concerning their conclusions. In Fig. 2&5, the authors show EEP suppress TM-induced ER stress pathway. If EEP suppress ox-LDL-induced apoptosis through suppression of ox-LDL intake and the up-regulation of CD36 induced by ox-LDL, the mechanisms how EEP suppresses TM-induced ER stress pathway remains unknown. The authors should elucidate those mechanisms.

Thank you very much. The main purpose of this present study was to explore the protective effect of EEP on ox-LDL-induced cytotoxicity in RAW264.7 macrophages and specifically the ER stress-CHOP pathway-mediated apoptosis. TM, which induces ER stress by inhibiting protein glycosylation, is widely used to develop ER stress model. The results of inhibitory effect of EEP on TM-induced ER stress response and apoptosis in macrophages further indicates that EEP is able to attenuate CHOP-mediated cell apoptosis, which may support the conclusion that EEP protects macrophages from ox-LDL-induced apoptosis through suppressing ER stress-CHOP pathway.

(Specific comments)
1. In Fig2C, the author should show the LDH activity in the case of total cell death as the positive control.

LDH, which leaks from cells to the media after plasma membrane injury, is used to evaluate cell injury. As shown in Figure 2C, LDH activity in media increased significantly after cells were incubated with ox-LDL compared with control group. However, pretreatment with EEP dramatically reduced the LDH release compared with ox-LDL group, indicating that EEP is able to attenuate ox-LDL-induced macrophage injury.

2. If the mechanisms of the suppression of ER stress pathway are different between EEP and PBA, treatment with both EEP and PBA show synergetic effect?

Thank you very much for your advice. As mentioned above, the main purpose of this present study was to explore the protective effect of EEP on ER stress-CHOP pathway-mediated macrophage apoptosis induced by ox-LDL. PBA, an ER stress inhibitor, was used as a positive control in the experiment to ensure that a reduction in ER stress can prevent ox-LDL-induced apoptosis. Whether there would be other different mechanisms between EEP and PBA and whether there may be synergetic effect when treating with both EEP and PBA will be considered in our future studies.

3. In Fig. 4A, GRP78 show double bands, but, in Fig. 5A, GRP78 show single band. The authors should mention about this point.

The cause of the result of GRP78 may be as following. We used two polyclonal GRP78 antibodies (sc-13968 and sc-1050), and the results of Western blot in Fig 4A and Fig. 5A were visualized using ChemiQ4800mini chemiluminescence imaging system (Bioshine, Shanghai, China) and chemiluminescence film in dark room, respectively, which have different sensitivity.

4. In Fig. 4A, induction of CHOP is barely suppressed by the treatment with PBA. However, in this condition, apoptosis is effectively suppressed in Fig. 3A. The authors should mention about this point.

Flow cytometry analysis of apoptosis and Western blot analysis of CHOP are two different experimental methods. It is possible that the change tendency of the two different parameters are not completely consistent. The expression level of CHOP is normalized to β-actin level. As seen in Fig. 4 A, ox-LDL-induced upregulation of CHOP was attenuated by PBA ($P<0.05$). Additionally, CHOP is one of the key molecules (CHOP, caspase-12, JNK, et al) that mediate ER stress-induced apoptosis, so it is acceptable that the suppressing degree of PBA on apoptosis is stronger than its
suppression of CHOP.

5. In Fig. 4A&5A, the authors should show also the results of total PERK and eIF2α.

   According to your advice, total PERK and eIF2α have been shown in Fig. 4A&5A.

6. To show the mechanisms of the suppression of ER stress pathway, the authors should show the results of other ER stress-specific molecules such as IRE1, ATF6, XBP1.

   Thanks a lot for your kindly advice. This has been in our next plan to continue this study and we try to publish another paper focusing on the other ER stress-specific molecules including caspase-12.

**Level of interest:** An article whose findings are important to those with closely related research interests

**Quality of written English:** Acceptable

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

**Declaration of competing interests:** Nothing to declare.