Author’s response to reviews

Title: Inhibitory effects of ChondroT and its constituent herbs on RANKL-induced osteoclastogenesis

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Author’s response to reviews:

Dear Sir.

This is to resubmit a revised version of the manuscript entitled “Inhibitory effects of ChondroT and its constituent herbs on RANKL-induced osteoclastogenesis”. We thank editors very much for giving us the chance of revision. We changed our manuscript as reviewer’s recommendation and the critique will be answered in this response letter. We highlighted the changes we made in the manuscript using colored text. This manuscript has not been published or presented elsewhere in part or in entirety and is not under consideration by another journal. We have read and understood your journal’s policies, and we believe that neither the manuscript nor the study violates any of these. There are no conflicts of interest to declare.

Thank you for your consideration. We are looking forward to hearing from you.

Sincerely,

Reviewer reports:

NORAZLINA BINTI MOHAMED (Reviewer 1): 1. Generally well-written. There are a few typo and grammatical errors. Avoid adding discussion or references in Results section.
→ Thank you for your comments. Our manuscript has received English language editing service by the ‘HARRISCO’ group. We have corrected the typo and grammatical errors. As suggested, we removed the references in Results section.

2. Methodology: should include description on the different concentrations used for all the herbs. Describe the preparations of the different concentration of the herbs.

→ Thank you for your comments. We added the preparation of different concentrations of the herbs in Methods Section as “Stock solutions (100 mg/mL) of all the herbs were diluted using phosphate buffered saline (PBS), filter-sterilized, and then diluted with PBS for the working concentration”.

3. For some of the analysis, dose used was 0.3mg/mL however this selection was not explained anywhere.

→ Thank you for your comments. In this study, ChondroT and its five constituent herbs exhibited the antiosteoclastogenic effect in a dose-dependent manner. We did the statistical analysis using ANOVA, and found although 0.5 mg/ml concentration showed the better inhibitory effects, there are no significant compared with 0.3 mg/mL concentration. Therefore, we choose the concentration of 0.3 mg/mL. In order not to make the figure complicated, we did not show it in the figure.

4. Statistical analysis: t test was used, which compare between two groups. I suggest to compare among the tested herbs by using Anova or its equivalents.

→ Thank you for your comments. Statistical differences were evaluated using ANOVA according to your suggestion.

5. Results: A few times authors mentioned that some herbs gave better inhibitory effects than others. Without the statistical evidence, this statement is meaningless.

→ Thank you. As suggested, we used ANOVA for statistical analysis to compare among the tested herbs.

6. Discussion very brief. Please elaborate on the different doses, different herbs. In results section, there were mentions of some herbs to be superior to the other. But in discussion, this was not mentioned at all.

→ Thank you for your comments. We have added the contents of comparison of different herbs on osteoclastogenesis in Discussion Section.
7. Figure legends: please revise. No need to repeat those which have been mentioned in Methods.

→ Thank you. As suggested, we have revised the Figure Legends to make it simpler and clearer.

8. Figures, where applicable, please provide label or arrow showing important structures.

→ Thank you for your comments. We have added the arrow in the Figures showing the important structures.

9. Check the label indicators. Must tally with figure descriptions.

→ Thank you for your comments. We have checked the label indicators.

10. Figure 4 c: Why did the Bay group had higher level of NFATc1 compared to RANKL group?

→ Thank you for your comments. In RANKL/RANK signaling pathway, NFATc1 could be regulated by many downstream signal pathways, such as NF-κB and MAPKs (Reference 1. Punicalagin inhibits RANKL-induced osteoclastogenesis by suppressing NF-κB and MAPK signaling pathways, Int J Clin Exp Med, 2018; Reference 2. DJ-1 controls bone homeostasis through the regulation of osteoclast differentiation, Nature Communications, 2017). Bay 11-7082 is a NF-κB inhibitor, it may inhibit the NFATc1 expression or may not inhibit NFATc1 expression. In our Figure 4, the Bay group had higher level of NFATc1 compared to RANKL group, which indicated Bay 11-7082 could inhibit the upstream NF-κB signal pathway, but did not affect the NFATc1 protein expression.

Yuankun Zhai, Ph.D. (Reviewer 2): The present study demonstrates that ChondroT and its constituent herbs shown inhibitory effects on RANKL-induced osteoclastogenesis. The experiments are well conducted using appropriate designs and methodology as well as adequate statistical analysis. The interpretations are in accord with the results obtained. However, there are still some revisions are required before this paper can be accepted.

(1) Please draw a cartoon to show the main constituent and mechanisms of Chondro T, including inhibit/stimulate specific proteins, how to effects on NF-κB and MAPKs pathways, this will make the research more clear.

→ Thank you for your comments. In order to make the research clearer, we have drawn a cartoon to illustrate the constituent and mechanisms of Chondro T, and shows it as Fig. 7.

(2) In the reagents part, please indicate the catalog number of all antibodies.
→ Thank you. As suggested, we have indicated the catalog number of all antibodies in Reagent Part.

(3) Generally, the detection of the cell viability always use the absorbance at 490 nm if the formazan crystals were dissolved by DMSO [reference: 1. Controlled growth and differentiation of MSCs on grooved films assembled from monodisperse biological nanofibers with genetically tunable surface chemistries, Biomaterials. 2011; 2. Expression and role of SDF-1α-CXCR4 axis in Human Dental Pulp, J Endod. 2008], and at 570 nm if the formazan dissolved in DMF/SDS (pH 4.7), [reference: 3. Measurement of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction activity and lactate dehydrogenase release using MTT, Neuroscience Research, 2000], please explain why you choose the test wavelength at 570 nm, and whether use a reference wavelength in your calibration.

→ Thank you for your comments. We bought MTT powder from Sigma which indicated the blue crystals are solubilized with acidified isopropanol and the intensity is measured colorimetrically at 570 nm. In addition, DMSO was reported to be the best solvent for dissolving the formazan product, the response curves for the plate reader at 550 and 600 nm are parallel, the optical density being ~1.7 times as high at 550 nm as at 600 nm (Reference: 1. A study of some variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity, Br. J. Cancer (1987), 56, 279-285). Moreover, many studies detected the cell proliferation by cytotoxicity with MTT using the absorbance at 570 nm [Reference: 2. Test for chemotherapeutic sensitivity of cerebral gliomas: use of colorimetric MTT assay. J Neurooncol. 1992; Reference: 3. Evaluation of anti-cancer effects of DPP-4 inhibitors in colon cancer- an in vitro study, Biomed Res Int. 2015; Reference: 4. Propugnating effect of Bark of Rhizophora mucronata against different toxicants viz carbon tetrachloride, ethanol and paracetamol on HepG2 cell lines, J Pharmacopuncture. 2019].

(4) In the Figure 1 and Figure 2, it looks Phellodendron amurense Rupr. (P) even have stronger inhibition effects on osteoblasts, it means Only Phellodendron amurense Rupr. (P) can inhibit osteoclastogenesis already. If you can decrease the concentration of P, it should have good inhibition effects and also lower cytotoxic effects. Can the authors give some supplementary data show the cytotoxic effects at lower concentration?

→ Thank you for your comments. We decreased the concentration of P (0, 12.5, 25, 50µg/mL) to show the cytotoxic effect at lower concentration, and determined the effect of P on osteoclastogenesis at lower concentration. MTT assay and TRAP staining showed Phellodendron amurense Rupr. (P) showed the significant inhibition on TRAP activity without any cytotoxicity at the concentration of 12.5, 25, 50 µg/mL.

(5) Please add the scale bar in Figure 1, 2 and 3.

→ Thank you for your comments. We have added the scale bar in Figure 1, 2, 3 and 5.
(6) The current manuscript only show inhibition effects on osteoclasts, and lack data of animal experiment, if the author can show the osteoprotective effects in vivo, it will make data more solid, at least the authors need give some discussion about the osteoprotective effects in vivo and predict its ability which been developed into a new drug for osteoporosis therapy.

→ Thank you for your comments. We are sorry that we did not show the in vivo experiments. As far as we know, almost no relevant animal experiments of the five constituent herbs. Only ultrafine Angelica gigas was reported to have antiosteoporosis properties in Ovariectomized rats [Reference: 1. Ultrafine Angelica gigas powder normalizes ovarian hormone levels and has antiosteoporosis properties in ovariectomized rats: particle size effect, J. Med. Food 15 (2012) 863–872]. Decursin, the major active compound of Angelica gigas NAKAI root, exhibited inhibitory effects on LPS-induced bone erosion in vivo [(Reference: 2. Decursin inhibits osteoclastogenesis by downregulating NFATc1 and blocking fusion of pre-osteoclasts, Bone 81 (2015) 208–216)]. In our manuscript, ChondroT showed the significant inhibitory effect on osteoclastogenesis which was better than Angelica gigas NAKAI. These results suggest it may have the anti-osteoprotective effects in vivo. We have added this content in Discussion Section.

Wan Iryani Wan Ismail, PhD (Reviewer 3): Good manuscript. Just spotted a few typos.

→ Thank you. Our manuscript has received English language editing service by the ‘HARRISCO’ group. We have corrected the typo and grammatical errors.