Author's response to reviews

Title: Potential anti-osteoporotic effects of herbal extracts on osteoclasts, osteoblasts and chondrocytes in vitro

Authors:

Yoshiki Mukudai (mukudai@dent.showa-u.ac.jp)
Seiji Kondo (seikondo12@dent.showa-u.ac.jp)
Tomoyuki Koyama (tskoyama@kaiyodai.ac.jp)
Chunnan Li (haruo1968@hotmail.com)
Seika Banka (seika.banka@gmail.com)
Akiko Kogure (akkoquitar@yahoo.co.jp)
Kazunaga Yazawa (yazawa@kaiyodai.ac.jp)
Satoru Shintani (shintani@dent.showa-u.ac.jp)

Version: 4 Date: 31 August 2013

Author's response to reviews: see over
Tom Rowles  
Executive Editor, *BMC Complementary and Alternative Medicine*

Dear Dr. Rowles:

We thank the Referees for their careful reading of our manuscript and for their fruitful comments. In response to the Referees’ comments, we have revised our manuscript (MS: 1787132277990851) entitled “Potential anti-osteoporotic effects of herbal extracts on osteoclasts, osteoblasts and chondrocytes in vitro” by Yoshiki Mukudai, Seiji Kondo, Tomoyuki Koyama, Chunnan Li, Seika Banka, Akiko Kogure, Kazunaga Yazawa and Satoru Shintani.

Following the Referees’ kind instructions, we have addressed all of the comments as indicated on the attached pages, and we hope that our explanations and revisions are satisfactory.

We hope to have our manuscript published in *BMC Complementary and Alternative Medicine* as an original article. We look forward to hearing from you at your earliest convenience.

Sincerely yours,

Yoshiki Mukudai, PhD, DDS  
Assistant Professor  
Department of Oral and Maxillofacial Surgery, School of Dentistry  
Showa University  
2-1-1 Kitasenzoku, Ota-ku, Tokyo 145-8515, Japan  
Phone: 81-3-3787-1151; Fax: 81-3-5498-1543  
E-mail: mukudai@dent.showa-u.ac.jp
Responses to the Reviewers

To Reviewer #1:
We are grateful to the reviewer for the useful suggestions that have helped us to improve our manuscript. As indicated in the responses that follow, we have taken the suggestions into account in the revised manuscript.

Comment #1
Statistical differences are missing in all figures of the manuscript, which does not allow a precise interpretation of the data. Moreover, a semiquantitative analysis should be performed when demonstrating the cytochemical figures.

Response
The data of all biochemical and molecular assays have been subjected to Student’s *t-test* in Fig. 1, 3, 6, 8 and 9. If \( p<0.05 \), the graph has been asterisked.

Comment #2
Authors claim that the herbal extracts inhibit proliferation of osteoclasts (see abstract, results and discussion sections). This conclusion was drawn from the fact that the number of cells stained by Crystal violet or formazan were decreased after treatment with the extracts. But there are some inconsistencies in Figures 1 and 2: while treatment with 1 and 10 \( \mu \)g/ml of atratum and azedarach produced less Crystal violet-stained cells than the control condition, 100 \( \mu \)g/ml seemed to produce more stained cells than 10 \( \mu \)g/ml. This behavior does not correlate with the MTT assay results, which showed that those extracts induce loss of viability in a dose-dependent manner. On the other hand, treatment with the 3 concentrations of turtschaninovii seemed to keep the number of cells constant, compared with the control condition (Figure 2), but the MTT result showed that all 3 concentrations decreased cell viability, similarly to the AD treatment. But the AD treatment, in the Crystal violet assay, produced a decreased number of stained cells. Therefore, authors should discuss these data and distinguish growth inhibition from loss of cell viability. To be sure that there is growth inhibition, the number of cells should be plotted (for control, AD, and extracts) versus time (day 0, 1, 2 and 3).

Comment #5
Regarding osteoblasts, the same comment about growth inhibition mediated by
AD: how to distinguish growth inhibition of cell viability loss (Figure 5)? Also, an increased ALP staining (Figure 5) is not so convincing. As commented above, this staining should be semi-quantitated. The differentiation status is much more evident in the activity assay (Figure 6).

Comment #6
Regarding the chondrocytes: the ALP staining does not convince (Figure 7).

Combined Response
All of the microphotographs in Fig. 2, 5, 7 and 9 have been replaced with the ones in higher magnification, and the focus, contrast and brightness have been adjusted, in order to represent more consistent results with the biochemical assays. Furthermore, the discrepancy between histochemistry and biochemical assays has been discussed in the main manuscript (Page 15 line 7-8, and Page 22 line 1-5).

Comment #3
The fact that several apoptosis markers are increased after the extracts treatments corroborates that the mechanism of less Crystal violet-staining is due to cell death (and not growth inhibition).

Response
The discrepancy between Western blotting for apoptosis-related proteins and histochemistry has been more discussed in the Results and Discussion sections (Page 15 line 13-15, and Page 22 line 9-10).

Comment #4
Section Results, Assessment of optimal dosage of alendronate used as an apoptosis-inducible control: The sentence These results indicated that a concentration greater than 10 uM induces not only apoptosis, but also necrosis due to cytotoxicity should be reinterpreted. Concentrations above 10 uM decreased viability but did not activated caspases (Figure 1).

Comment #8
Section Results, Assessment of optimal dosage of alendronate used as an apoptosis-inducible control: a concentration of 10 uM AD should be used instead of a dose of 10 uM...

Combined Response
Several sentences in the Result section have been revised (Page 13 line 10-14).

Comment #9
Several misspelling are found along the manuscript. Therefore, an orthographic review is needed along the whole text.

Response
The authors have carefully checked the manuscript for typographical errors, and the revised manuscript was proofread by a native English reviewer.

Comment #10
Table 1: it is advisable to mention the accession number of the gene.

Response
The GenBank accession numbers have been added in Table 1.
To Reviewer #2:
We are grateful to the reviewer for the useful suggestions that have helped us to improve our manuscript. As indicated in the responses that follow, we have taken the suggestions into account in the revised manuscript.

Comment
The main criticism is about the quality of microscopy images, which are impossible to evaluate or make any consideration due to the much reduced size. Additionally, more information must be extracted further analyzing the morphological changes, and then confronting with RT-PCRq assays results. Osteoblast differentiation and osteoclast behavior are poorly observed in the images. In the same way, bone extracellular matrix (ECM) is a relevant issue not quite mentioned or further commented in the discussion as being part of the turnover or/and recovering after herbal extracts treatment. Is it a consequence or stimuli triggered by the root extracts? Is there any relationship Cell-ECM? My major revision request is to give more attention for microscopy results, which in the present form are inappropriate. Thus, enlarging the images is critical. As necessary, it might be discard some redundant images of doses or time of exposition, and I would not see it as a problem.

Response
All of the microphotographs in Fig. 2, 5, 7 and 9 have been replaced with the ones in higher magnification, and the focus, contrast and brightness have been adjusted. Furthermore, in Fig. 9, the number of positive cells were counted, and represented as a bar graph (panel B). As well, the discrepancy between histochemistry and biochemical assays has been discussed in the main manuscript (Page 15 line 7-8, and Page 22 line 1-5).