Author’s response to reviews

Title: Coix lacryma-jobi var. ma-yuen Stapf sprout extract has anti-metastatic activity in colon cancer cells in vitro

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Reviewer reports:

Xiangnan Zhang (Reviewer 1): This investigation studied the anti-metastasis effects of CLSE on colon cancer. The topic is interesting, and several models are employed in this study. But the conclusions raised by the authors were not well supported by the current evidences, in particular the signaling part. The details are listed below,

1. Pg.5 ln.76, what is 'annual plant'?
Response: The term ‘annual plant’ has been removed because of its ambiguity.

2. The background should be rewritten, since it was not closely related to the topic of this paper. It is not clear why the authors aimed to study the anti-metastasis effects of CLSE?
Response: As suggested, we have modified the background section so that it better explains our aim in studying the anti-metastasis effects of CLSE as follows:

Abstract/ Background: Coix lacryma-jobi var. ma-yuen (Rom.Caill.) Stapf has been used in China as an herbal medicine. Many studies of this plant have reported anti-proliferative and apoptotic activities on human cancer cell lines. Therefore, this study of the anti-metastatic effect
of Coix lacryma-jobi var. ma-yuen Stapf sprout extract (CLSE) in colorectal cancer cells may provide a scientific basis for exploring anti-cancer effects of edible crops (Abstract/Background section, pg.3 line 31-35).

Background section: Previous studies have reported that Coix extract has anti-proliferative and apoptotic activities on human lung cancer, histolytic lymphoma, and colon cancer cells, as well as chemopreventive effects on lung cancer in vivo (Liu et al., 2014) (Kuo CC et al., 2001) (Lee MY et al., 2008) (Chang HC et al., 2003). Although a few studies have reported that Coix has anti-cancer effects in terms of regulating the proliferation and cell cycle of cancer cells, the effects of Coix lacryma-jobi var. ma-yuen Stapf sprout extract (CLSE) on cancer metastasis are unknown. Therefore, this study aimed to explore the anti-cancer effects of CLSE in colorectal cancer cells. (Background section, pg.5 line 84-90).

3. In figure 1, it should be stated as 'Cell Viability' rather than 'Cell growth'.

Response: Figure 1 has been changed to read ‘Cell Viability’.

4. In figure 2 and figure 3, the authors used the DFO to mimic hypoxia, however it is not clear whether the CLSE has direct chemical reaction with DFO and subsequently neutralizes its effects. To clarify this, the authors should have treated the cells with hypoxic gas. Alternatively, the authors should demonstrate that DFO still lead to hypoxia in the presence of CLSE.

Response: DFO is commonly used to mimic hypoxic conditions (Wu and Yotnda, 2011). In Figure 2, we used DFO to confirm the inhibitory effect of CLSE on migration of HCT116 cells under hypoxia as shown in Figure 2C and 2D. However, as you pointed out, we performed the scratch-wound healing assay under hypoxic conditions and also observed that CLSE inhibited migration of HCT116 cells by 96 % under hypoxia (revised on Figure 4D and E).

In Figure 3C, the adhesion assay was performed to determine whether CLSE-treated cells under hypoxic conditions adhere to Matrigel-coated wells. In this assay, we used DFO to maintain the hypoxic state of cells after subculture, and to allow opening of the culture dish without affecting the hypoxic conditions.

5. In figure 4, why the signaling proteins were determined after 72 h of incubation? If the anti-metastasis effects can be observed with 24 h of incubation, these signaling should had been activated at an earlier stage.

Response: We thank the reviewer for this suggestion. We have added figures indicating the regulation of signaling proteins by CLSE treatment in HCT116 cells at an earlier stage. As in the case with 72 h of incubation, phosphorylation of ERK1/2 and AKT were reduced in CLSE-treated cells after 8 h of incubation. Furthermore, when activators of ERK1/2 and AKT were co-treated with CLSE, the inhibitory effect of CLSE was compromised (revised on Figure 4B). However, activation of ERK1/2 and AKT under hypoxic conditions (revised on Figure 4B, lane
1 and 2) is weaker at 8 h of incubation than at 72 h of incubation. Activation of other signaling proteins (p38, p65, STAT3, JNK) was not affected by CLSE treatment at an earlier stage (revised on Figure 4C).

6. In figure 4 B and C, the design is not related with the thesis of this paper. The author claimed that 'CLSE… through inactivation of the ERK1/2 and AKT pathway…'. To this end, they should reverse these signaling by activators and further observe whether the migration can be compromised with the presence of BOTH CLSE and signaling activators.

Response: Please refer to our response to your point 5 above, in reference to Figs. 4B and 4C. Regarding your suggested experimental design, we carried out the following experiments.

Firstly, we used PMA and SC79 to activate the ERK1/2 and AKT pathways, respectively, and conducted western blot analysis followed by the scratch-wound healing assay using these activators. The resulting western blot showed that PMA (5 ng/mL) and SC79 (5 μg/mL) both compromise the inhibitory effect of CLSE on the activation of the ERK1/2 and AKT pathways under hypoxic conditions (revised on Figure 4B).

In the scratch-wound healing assay, the migratory ability of HCT116 cells decreased with CLSE treatment, and recovered in the presence of PMA (10 ng/mL) or SC79 (2.5 μg/mL) under hypoxia (revised Figure 4D and 4E). Therefore, we conclude that CLSE decreases migration of HCT116 cells through inactivation of the ERK1/2 and AKT pathways under hypoxic conditions.

7. For the reasons aforementioned, the current evidences cannot support the conclusions.

Response: We addressed several issues that you pointed out by performing additional experiments using activators of ERK1/2 and AKT, and the results support that CLSE suppresses migration of colon cancer cells by impairing ERK1/2 and AKT pathways under hypoxia.

8. The manuscript should be proof-read by some English native speakers.

Response: English in this manuscript has been checked by at least two professional editors, both of whom are native speakers of English. For certification, please refer to the following website:

http://www.textcheck.com/certificate/Xs9UUn

Sasitorn Chusri (Reviewer 2):

1. Please clarify why the anti-metastatic activity was selected to study? How did this activity correlate with the traditional use?
Response: Previous studies have reported that Coix seed has anti-proliferative and apoptotic activities on human cancer cell lines (lung cancer, lymphoma, colon cancer) as well as chemopreventive effects on lung cancer in vivo (Kuo CC et al., 2001) (Lee MY et al., 2008) (Chang HC et al., 2003) (Background section, pg. 5 line 84-86). Accordingly, we endeavored to further examine the anti-cancer effects of the CLSE in terms of cancer metastasis. Based on our results, we conclude that Coix sprout extract has anti-metastatic effects on colon cancer cells.

2. Where did the authors select to study the sprout extract of Coix lacryma-jobi var. ma-yuen (Rom.Caill.) Stapf? Which part has been used as a traditional medicine in China?

Response: We prepared the Coix sprout extract in the herbarium of the Herbal Crop Research Institute (Eumsung, Republic of Korea) and harvested young barley leaves from Coix sprouts for this research. Chinese traditional medicine uses all parts of Coix.

3. How did the authors identify the scientific name of the herbal component, please indicate the botanist name and where the voucher specimens of the herbal components are deposit and what are the voucher specimen numbers?

Response: The scientific name is Coix lacryma-jobi and its botanical name is Coix lacryma-jobi L. var. ma-yuen (Rom. Caill.) Stapf. The voucher specimen was deposited in the herbarium of Herbal Crop Research Institute (Eumsung, Republic of Korea) under specimen number HPR-208 (Methods section, pg. 6 line 111-112).

4. Chemical profiling of the extract should be included.

Response: The primary purpose of this research is to investigate the anti-cancer effect of Coix sprout extract as a whole and thus, chemical profiling of the extract is not essential at this time. We will investigate the chemical profile in the future.

5. The safety of the plant should be include in this study both in vivio and in normal cell line.

Response: The safety of the plant has been included in the Background section as follows (Background section, pg. 4 line 76-pg. 5 line 83):

Coix lacryma-jobi var. ma-yuen (Rom.Caill.) Stapf, which is an important cereal crop for many indigenous groups in upland areas, is characterized by having a similar appearance and taste to rice, with a standing crop comparable with corn. This plant is utilized as a rice alternative, health-promoting staple crop, and as an alternative livelihood and income source through value-added products. An increase in the number of health-conscious individuals has also contributed to the popularity of Coix, with the market currently growing due to increased acceptance of this product. Coix is largely consumed for household food security as a rice alternative or used to make porridge, champorado, and other recipes.
Additionally, we have added data showing the viability of normal cells treated with CLSE to Figure 1B. For normal cells, we used human colon cells, CCD-18Co. The results show that CLSE does not have much effect on the viability of normal cells compared to cancer cells.

Yongxiang Chen, Ph.D. (Reviewer 3): Please include all comments for the authors in this box rather than uploading your report as an attachment. Please only upload as attachments annotated versions of manuscripts, graphs, supporting materials or other aspects of your report which cannot be included in a text format.

Please overwrite this text when adding your comments to the authors.

Coix lacryma-jobi var. ma-yuen (Rom.Caill.) Stapf has been used in China as an herbal medicine. Although this plant has been used as a diuretic, stomachic, analgesic, anti-arthritis, and anti-spasmodic agent, and may reduce liver fat accumulation and protect cells from tumor-stimulating compounds, it is unknown if Coix lacryma-jobi var. ma-yuen Stapf sprout extract (CLSE) has an anti-metastatic effect in colon cancer. The authors determined the anti-metastatic effect of CLSE on colon cancer cells. Based on the results, the authors concluded that CLSE could inhibit migration, invasion, and adhesion of colon cancer cells and tube formation by HUVECs via repression of the ERK1/2 and AKT pathways under hypoxic conditions. Therefore, CLSE may be used to treat patients with colon cancer.

Specific comments:

1. Coix cultivars were obtained from the National Institute of Crop Science (Miryang, Republic of Korea). Coix lacryma-jobi var. ma-yuen (Rom.Caill.) Stapf sprout extract (CLSE) was manufactured by the Rural Development Administration (Chungbuk, Republic of Korea). How to make a quality control of the herbal source for the study?

Response: We did not normalize the extracts using specific chemical components because our primary focus of the research was to investigate the anti-cancer effects of CLSE as a whole, and therefore we have not undertaken the chemical profiling process. However, we established a standard for the yield of the extract from crushed plant materials (200 g each) of at least 25% by weight.

2. The methods and references for the identification, collection and extraction of CLSE preparation should be provided in more detail.

Response: We described the methods of CLSE preparation in more detail in the “Methods” section as follows (Methods section, pg. 6 line 106-112):

We used a water extraction method because most traditional Oriental herbal materials are decocted with boiling water. In addition, we found CLSE to be more soluble in water than in
organic solvents. Crushed plant material (200 g each) was extracted three times under reflux with distilled water. The water extracts were combined and lyophilized. The yield was 25% (wt/wt) of dried Coix sprout. Extracts were stored at –20°C until usage. A voucher specimen (HPR-208) was deposited in the herbarium of Herbal Crop Research Institute (Eumseong, Republic of Korea).

3. In additional to colon cancer cells, will CLSE cause any cytotoxicity to "normal" cells?

Response: We have added data showing the viability of normal cells treated with CLSE to Figure 1B. For normal cells, we used human colon cells, CCD-18Co. The results show that CLSE does not have a meaningful level of toxicity to normal cells compared to colon cancer cells.

4. Why DFO was used to induce cancer cell migration?

Response: We opted to use DFO to induce cancer cell migration because DFO is an agent commonly used to mimic hypoxic condition, which is conducive to cancer cell migration (Wu and Yotnda, 2011).

5. Phospho-p65 rather than phosphor-NF-kB should be labelled for Figure 4.

Response: We changed the phosphor-NF-kB to phosphor-p65 in Figure 4A, as suggested.

6. The possible major compounds and mechanisms on the anti-cancer effects of CLSE should be discussed.

Response: Recently, many studies have tried to identify the active components in Coix and determine their mechanism of action. Specifically, neutral lipid isolated from endosperm of Coix inhibits the growth of pancreatic cancer cells (Bao et al., 2005), and the ethyl acetate fraction from the ethanolic extract of adlay testa has an inhibitory effect on the allergic response (Chen et al., 2011). In addition, five compounds (coixspirolactam A, coixspirolactam B, coixspirolactam C, coixlactam, methyl dioxindole-3-acetate) isolated from Coix bran exhibit anti-proliferative effect on lung and colon cancer cells (Lee et al., 2008). Because CLSE is obtained from a young sprout from Coix, we believe it to share a similar chemical composition with the mature plant. As such, it is highly likely that CLSE also contains coixspirolactams and methyl dioxindole-3-acetate, which may contribute to the anti-metastatic effects of CLSE (Discussion section, pg. 14 line 271-280).

Our investigation showed that CLSE has anti-metastatic effect in colon cancer cells. Migration, invasion, and adhesion of cancer cells result from the loss of epithelial markers and the degradation of basement membrane. Therefore, we expect that CLSE may have anti-metastatic effects via regulation of E-cadherin, vimentin, MMP-2, and MMP-9 in colon cancer cells. We
plan to undertake further experiments to address these topics in the future (Discussion section, pg. 15 line 287-290).

7. Are there any potential side effects of herbal-based approach? Please discuss the safety of these herbal preparations.

Response: We discussed the side effects of herbal-based approach and the safety of CLSE preparation in the “Discussion” section as follows:

It has been reported through some animal research that Coix consumption might cause embryotoxicity and enhance uterine contractility during pregnancy (Tzeng et al., 2005). Furthermore, some herbal medicines are reported to interfere with the efficacy and safety of conventional medicines (Alsanad SM et al., 2016). Therefore, more research on the stability and efficacy of herbal supplements such as Coix is needed (Discussion section, pg. 15 line 299-303). Accordingly, we plan to further investigate the anti-tumor effects of bioactive compounds of CLSE and endeavor to elucidate the mechanism of anti-metastatic effects of CLSE in the future.

8. The authors should discuss the severe limitations of their approach and give future directions for research in the field of anti-cancer research with traditional medicine.

Response: Our primary focus in this research was to explore the anti-cancer effects of CLSE as a whole, and we have been successful in this regard. Further studies on this subject will not only reinforce our findings, but will also contribute greatly to the field of oncology. We recommend the following: detailed chemical profiling of CLSE and mechanistic studies of each component, as well as in vivo experiments using CLSE (Discussion section, pg. 15 line 303 - pg. 16 line 307).

References


