Author's response to reviews

Title: Spica Prunellae Promotes Cancer Cell Apoptosis, Inhibits Cell Proliferation and Tumor Angiogenesis in A Mouse Model of Colorectal Cancer via Suppression of STAT3 Pathway

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

Thank you very much for your valuable comments and suggestion on our manuscript (MS: 7861677988933350). Here we made the point-to-point responses as following, and revised our manuscript accordingly.

Reviewer: Ping-Chung Leung:
1. The authors could include discussions on the use of prunella in traditional treatment of cancer in the old formulations.

We revised the manuscript as following:

Spica Prunellae is a well-known Chinese medicinal herb with multiple pharmacological applications including anti-tumor property. The anti-cancer activity of Spica Prunellae was recorded in several ancient Chinese medical books, including <Treasury of Words on the Materia Medica> (‘Ben Cao Hui Yan’ in Chinese) written in Ming Dynasty, and <A Newly Revised Materia Medica> (‘Ben Cao Cong Xin’ in Chinese) written in Qing Dynasty. Indeed, Spica Prunellae has long been used as a major component in several TCM formulas for cancer treatment [39,40].


2. The applicability of the in-vivo study results could be discussed.

We revised manuscript accordingly.
Reviewer: Yibin Feng:

Major concern

1. It is suggested the author should commit the pure compound Rosmarinic acid to compare the effect of crude extract with pure compound. This will help audience identify the evidence base of using EESP to treat CRC.

✓ The major objective of this manuscript was to evaluate the effect of *Spica Prunellae* on tumor growth and investigate the possible mechanism. Rosmarinic acid (RA) was used in this study as a standard control for HPLC analysis of EESP. Since we thought the material basis of bioactivity of *Spica Prunellae* was not one of the specific aims in this study, we did not include the data of RA’s activities here. However, as shown in below Figure, we did perform MTT assay indicating that RA could dose-dependently reduce the viability of HT-29 cells. Thank you very much for your valuable suggestion, we will determine the material basis of bioactivity of *Spica Prunellae* in other projects.

2. The other concern is that the author used only one dose of EESP in animal study. This is not fine for any pharmacological study since we could not see the dose-dependent effect of this investigational drug. But things will be different if the author could conduct in vitro study using CRC cell lines (at least two doses should be given to the CRC cells in this part) to confirm his in vivo observation.

As suggested by the reviewer, we added the in vitro data and revised the manuscript accordingly:

To evaluate the *in vitro* anti-tumor activity of EESP, we performed MTT assay to examine its effect on the viability of human colon carcinoma HT-29 cells. As shown in Figure 2D, treatment with 0-2 mg/ml of EESP 24 h respectively reduced cell viability by ?, compared to untreated control cells (P<0.01), suggesting that EESP inhibits CRC cell growth *in vitro* in dose-dependent manners.
Minor concern:
Fig. 2, the author should display the actual image of tumors which were dissected out from mice xenograft. H&E staining is required to view the structural changes of tumor.
√ Thank you very much for your valuable suggestions. However, we did not take pictures for tumor tissues when we dissected tumors out from xenograft mice. We will keep in mind in the future studies.

As suggested by the reviewer, we did histological examination by HE staining.

(Hematoxylin-eosin staining of tumor tissues×200)
Tumor cells were densely packed in tissues of the control group, but were sparse in tissues of EESP-treated group. There was marked cytonuclear pleomorphism, mitotic activity (red arrow). Large blood vessels and red blood cells could be observed in control group. In EESP group, portions of cells were necrotic and inflammatory cells were observed around the tumor tissue.
Fig. 3 TUNEL assay. I cannot see difference between the control and treatment group. PCNA assay, it will be better if the author use Ki67 as tumor proliferation marker.

√ As mentioned in Figure Legends, quantification of IHC assay for TUNEL was shown as averages with S.D. (error bars) from 9 individual mouse in each group. Taking your valuable suggestion, we selected another photograph for representative image.

![TUNEL assay](image)

Fig. 4, immunobloting should be conducted to examine the p-STAT3 level. Total STAT3 expression should be used as control.

√ Both Immunohistochemistry (IHC) and Western Blotting can be used to determine protein expression. Thank you very much for your suggestion, we determined the expression of total STAT3 using IHC and found there was no significant difference in protein expression of total STAT3 between control and treated groups.

![p-STAT3 assay](image)

Fig. 5&6 again, since the author did not have cell study, it is required that protein level determination should be conducted with immunoblotting. This help us to know the global level of protein changes.

√ We agree with the reviewer, it would be better to perform Western Bolt to confirm the IHC results. We will pay more attention in the future studies.

![STAT3 assay](image)
In Fig. 5-8, why the author marked A, B, C, D at the upper left of each figure? Level of interest: An article whose findings are important to those with closely

√ Fig. 5-8 was initially labeled as Fig. 5A-D in our original manuscript. However, when we uploaded Figures 5A, B, C, D individually, the system automatically changed the labels.