Peer Review File

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Reviewer A

Comment 1： In all figures, the resolution of pictures is low. In particular, differences are invisible in the result of wound healing assay in Figure 3H.

Response： We improved the quality of the figures. It should be better than our previous submission. Thanks!

Comment 2： According to the manuscript (line 30-32 of page 11), the authors perform continuous administration of limonin from a week before the injection of MDA-MB-231 cells and confirm its anti-metastatic effect. However, they do not examine whether limonin suppresses metastasis outbreak after metastasis occurs. Considering that, is it appropriate to conclude that limonin has a "therapeutic" effect on breast cancer metastasis (line 5-6 of page 12)? Also, is it reasonable to employ the intraperitoneal injection method for drug investigation? If they want to utilize limonin as a dietary supplement, I suppose the oral administration method is better than the intraperitoneal injection method, or at least the authors should discuss it.

Response： Thanks for the reviewer's insightful questions and suggestions. We have conducted our studies according to published literature [(1)] and have modified "therapeutic" in the revised article (page12,20, line14,10). Additionally, intraperitoneal injection limonin is acceptable in published papers [(2)]. Considering that limonin, one of dietary supplements, oral administration method is indeed more reasonable than intraperitoneal injection method. We will employ this method for drug investigation in our future experimental design and have added a brief statement about the limitation of present study and the way to overcome it in the "Discussion" section of our revised manuscript (page15, line22-26). Thanks again.

Reference：

1. Fang, Y., et al., Inhibition of breast cancer metastases by a novel inhibitor of TGFbeta receptor 1. J Natl Cancer Inst, 2013. 105(1): p. 47-58.

2.Somasundaram, S., et al., Citrus limonin lacks the antichemotherapeutic effect in human models of breast cancer. J Nutrigenet Nutrigenomics, 2012. 5(2): p. 106-14.

Comment 3. Some papers relevant to the description in lane 27-31 of page 9 should be cited in the manuscript.

Response： We have updated two recent reference in the revised manuscript (Page 9, line 27-31 and Page 17, line33). Thanks!

Comment 4. Please provide some references for SHP-1 in lane 18 of page 10.

Response： We have added some recent references in the revised manuscript (Page 11, line 24 and Page 19, line1-6). Thanks!

Comment 5. Is the description "Limonin inhibits breast cancer metastasis via the STAT3 signaling pathway." (lane 10 of page 11) based on the results of Figure 3? Or are there any previous reports? If the description is based on Figure 3, it might be better to replace "metastasis" by "invasion". If the statement is based on previous studies, it might be appropriate to include references.

Response： We greatly appreciate the reviewer's suggestion. As shown in our studies, we have performed a number of experiments to demonstrate limonin inhibits breast cancer metastasis *via* the STAT3 signaling pathway shown in *vitro* Western Blot analyses（Fig. 3） and *in vivo* IHC results （Fig. 4E）. We observed that limonin suppressed the phosphorylation of IGFR1 (at tyrosine 1131) with or without IGF2 (100 ng/ml) and the activation of the downstream target STAT3 (at tyrosine 705), upregulated the expression of SHP-1, a key negative regulator of STAT3, at the protein level dose-dependently（Fig. 3B）. Moreover, we also demonstrated that limonin could inhibit *in vitro* breast cancer cell invasion and migration in Fig. 3E and H, and could prevent and suppress *in vivo* breast cancer metastasis through establishing two animal metastasis models widely used in present breast cancer studies（Fig.5 and 6）. Our result showed that limonin does inhibit breast cancer metastasis via the STAT3 signaling pathway. However, whether the target is STAT3 or not, we will do the experiments to verify it in future work. To address reviewer's concerns, we have replaced "metastasis" by "invasion", and we also have added some discussion in revised manuscript. Thanks.

Comment 6. In Figure 5, please state the number of mice used in the experiment. Also, the control mouse in the right side seems to have brain metastasis whereas limonin-treated mice have almost no brain metastasis. Does limonin have preventative

effects on brain metastasis as well as on lung metastasis?

Response: We greatly appreciate the reviewer's comments. As described in "Materials and Methods Section", Eight female BALB/c athymic nude mice in each group were given MDA-MB-231-luc cells (1×10^6) *via* tail vein injections or left ventricle injection assays, respectively. Additionally, as shown in Figure 4 and Figure 5, one week prior to tail vein injection, the development of lung metastases or brain metastasis nearly disappeared in mice that received pretreatment with 25 mg/kg/day limonin compared with that of the control group. Moreover, the photon flux in the lungs of mice treated at this dose was significantly reduced, indicating that limonin could preventative brain metastasis as well as on lung metastasis. We have briefly discussed it in our revised manuscript (Page 12).

Minor concerns:

Question 1: In line 26, line 28, and line 30 of page 1, please check the following: line 26: breast cancer cell -> breast cancer cells, line 28: HUVECs cell -> HUVECs cells, line; RNA isolation, -> RNA isolation, (no space).

Response: We are sorry for the mistakes. Each of them has been corrected accordingly. Thank you! (see Page 1, line28-31).

Question 2: In this manuscript, the words "in vivo" and "in vitro" are used in multiple times, but some are written in the normal font while the others are written in italic font. Personally, I would prefer to see them in a uniform font of characters.

Response: We thank the reviewer's suggestions. We have modified them in the revised manuscript (see Page 1, line30; Page 2, line2; Page 9, line26-27).

Question 3: In line 14-19 of page 8, please specify which cell lines are used for western blotting.

Response: Thanks! We have updated it in our revised manuscript (see Page 9, line 18).

Question 4: In line 10 of page 21, please check the following: Western blotting assay. -> Western blotting assay. (no space).

Response: The revised manuscript has been thoroughly and carefully edited. Therefore, we believe that we have significantly improved the quality of the

manuscript (see Page 21, line 33). Thank you!

Question 5: In line 4 of page 22, please check the following: determined -> determine.
Response: We have modified it in the revised manuscript (see Page 22, line 11).

Question 6: In line 3-4 of page 23, please change the following fonts: A -> (A), B -> (B).
Response: Thanks! We have modified them in the revised manuscript (see Page 22, line 19.20 and Page 22, line 22,24,26.).

Reviewer B

Comment1： Limonin is the agent focused on studying in the manuscript, however, more introduction about the agent need to be given. What is the purity of the compound used in the study? Which vehicle is used to dissolve the compound?
Response: We greatly appreciate the reviewer's suggestions. Limonin (purity > 99.37%) was purchased from Shanghai Winherb Medical Science. A 50 mmol/L stock solution was prepared in dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO), stored at -20°C and then diluted as needed in cell culture medium. We have added some information in the revised manuscript.(Page 3, line 3-5)

Comment2： Some figures especially western blotting and microscopic images are too small to see clearly (e.g., Figure 1E, 2, 3).
Response: We greatly appreciate the reviewer's suggestion. We have updated the figures in the revised manuscript.
Changes in the text： We have modified them in Figs. 1E, 2 and 3.

Comment 3: Figure 3E, the label is not clear. The ICC measured SHP-1? It showed the effectiveness of siRNA knocking down SHP1, but what are the effects of SHP1 siRNA on angiogenesis related proteins such as VEGF, VEGFR?
Response: We thank the reviewer's suggestions. We have modified it in the revised manuscript. Protein tyrosine phosphatase SHP-1 is considered a key negative regulator of STAT3, therefore, in our present study, we just focused on measuring the effect of limonin on SHP-1 and the effectiveness of siRNA knocking down SHP1 in breast cancer MDA-MB-231 cells by Western Blot and Transwell invasion assays. We

did not observe the expression of VEGF and VEGFR in siRNA knocking down SHP1 cells after limonin treatment. Due to the limited time, we were unable to complete the relevant experiments. However, we will continue to do them to verify them in the following study. Thanks again.

Comment 4: It is amazing to see that limonin almost completely inhibited/killed tumor after 45 days and 28 days (Figure 4 and 5). The MDA-MB-231 cells were injected in mice through tail vein and left ventricle. Why choose tail vein or left ventricle? What's the difference between those two routes? Why the MDA-MB-231 cells did not metastasize to other location of the body? Based on Figure 4A, most of the tumor cells are located in the chest. Figure 5A showed tumor metastasis. So only left ventricle injection had metastasis?

Response: We greatly appreciate the reviewer's comments. To evaluate the potential of limonin as a preventative and therapeutic agent, we next investigated whether limonin could prevent and inhibit breast cancer metastasis in two mammary tumor metastasis models: the mouse tail vein injection tumor metastasis model and the mouse left ventricle injection tumor metastasis model, which are extensively accepted models in present breast cancer studies [1, 2]. The most common sites of metastasis are mainly determined by the injection site and blood flow route. In the former model, the luciferase-labeled MDA-MB-231 mammary tumor cells were injected through the mouse tail vein, then directly spread in lung region through the blood circulation system. However, in the latter one, tumor cells were just injected through the mouse left ventricle, normally they will metastasize to lung, brain, bone, and regional lymph nodes, et al. As shown in Figure 5A and Figure 4A, either the mouse tail vein injection or the mouse left ventricle injection could occur tumor metastases, but where tumor cell would spread, depending on the injection method.

Reference:

1. Zhang T, Li J, He Y, et al. A small molecule targeting myoferlin exerts promising anti-tumor effects on breast cancer. Nat Commun. 2018;9(1):3726. Published 2018 Sep 13. doi:10.1038/s41467-018-06179-0

2. Fang, Y., et al., Inhibition of breast cancer metastases by a novel inhibitor of TGFbeta receptor 1. J Natl Cancer Inst, 2013. 105(1): p. 47-58.

Comment 5： Figure 4B, what's the significance of photon flux? How is it related to

tumor metastasis?

Response： Photon flux is widely used in the mouse models studies and can be applied to predict where and how tumor metastasis it is. The higher luminous intensity in mice is, the larger area of tumor metastasis in mice is.

Comment 6: Page 11, line 24, immunohistochemical = IHC.

Response: Thanks. We have modified it in the revised manuscript (page 10, line 20-24).

Comment 7： It is worthy to measure the expression of proteins (e.g., E-cadherin, cytokeratins, N-cadherin, vimentin, etc.) involved in Epithelial-to-mesenchymal transition, an important process: causing metastasis. Please see the reference: https://febs.onlinelibrary.wiley.com/doi/full/10.1002/1878-0261.12017,and https://pubmed.ncbi.nlm.nih.gov/32408199/,and https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2654352/

Response: We greatly appreciate the reviewer's insightful suggestions. However, due to the limited time, we were unable to complete the relevant experiments. However, we will continue to do them to verify the effects of limonin on EMT expression proteins in the following study. Thanks again.

Comment 8： A paper (https://pubmed.ncbi.nlm.nih.gov/22907263/) indicated that citrus limonin lacks the antichemotherapeutic effect in human models of breast cancer. This is not consistent with authors' conclusion. Can the authors provide some explanation?

Reply8： Through our careful reading of this paper, the author suggests that limonin could be beneficial for breast cancer patients undergoing chemotherapy. Therefore, the author's viewpoint is consistent with ours. Thanks a lot!