Format for ANSWERING REVIEWERS

February 18, 2014

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: **7628-review.doc**).

Title: Inhibitory effects of dihydromyricetin on the migration and invasion in SK-Hep-1 and MHCC97L cells by down-regulation of matrix metalloproteinase-9 expression

Author: Qingyu Zhang, Ran Li, Guofang Zeng, Bin Liu, Jie Liu, Yang Shu, Zhongkao Liu, Zhidong Qiu,

Dongjun Wang, Huilai Miao, Mingyi Li, Runzhi Zhu

Name of Journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: 7628

The manuscript has been improved according to the suggestions of reviewers:
1 Format has been updated

2 Revision has been made according to the suggestions of the reviewer

Review1#

Inhibitory effects of dihydromyricetin on the migration and invasion in SK-Hep-1 and MHCC97L cells by down-regulation of matrix metalloproteinase-9 expression In this study, the authors found that Dihydromyricetin (DHM) induced the suppression of migration of HCC lines (SK-Hep1 and MHCC97L). DHM also inhibited production of MMP9 and the activation of MAPK pathways. These findings are novel and of interest, but several experiments are required for publication.

Comments:
1. In Figure5, they found that protein production of MMP9 is significantly down-regulated by the addition of DHM. However, the activities of these MMPs were regulated by the cleavage of themselves. Thus, it is important to analyze the activities of these MMPs regulated by DHM using gelatin-zymography or other methods.
Answer: We applied a kit provide by Merck millipore (Calbiochem, CBA003) to detect the MMP2/9 activity. The detailed process is described in the method part of manuscript. We found the MMP2/9 activity actually was decreased by DHM as a concentration-dependent manner and this result were presented in the result part (Fig. 5 C).

2. In Figure 5, ERK, p38MAPK, JNK, and PKC were regulated by DHM. However, there were no data about the correlation between these signal molecules and cell migration (or MMP production). I recommend that they do the experiment to inhibit these signal molecules using chemical inhibitors in cell migration assays and MMP expression assays.
Answer: we presented these proteins(Erk, p38MAPK, JNK, and PKC) level change after DHM exposure in my manuscript because many study have demonstrated that these signaling pathways were involved in the process of cancer cells invasion or migration by regulating expression of MMP2/9.In

our study we found that DHM actually affects the phosphorylation of these proteins so it is reasonable we propose that DHM inhibit cancer cells migration by decreasing of these migration-related signaling protein phosphorylation levels.

Review2#

Li et al. describe in their manuscript the "Inhibitory effects of dihydromyricetin on the migration and invasion in SK-Hep-1 and MHCC97L cells by down-regulation of matrix metalloproteinase-9 expression".

- Reference #1 is not appropriate; the authors comment on the prevalence of cancer, reference #1, however, is a review about "Curcumin and liver cancer". Although this review cites original sources for statistics of cancer prevalence, it is not an original source for statistics. Li et al. are advised to use original references, such as publications from the American Cancer Society or similar –

Answer: We amend another reference (reference#2) that provide original statistics about cancers on global.

English language and grammar have to be improved and corrected throughout the manuscript. - Some formatting is incorrect, e.g. degree centigrade.

Answer: We carefully corrected the grammar and language errors throughout the manuscript.

Material and Methods: Please give more detailed information on the cell lines used. From what type of hepatic cancer do they derive?

Answer: we give the detailed cell lines information in the "2.2. Cell line and culture "section.

- Results 3.1. The authors talk wrongly about the effects of MTT on cell proliferations; the authors should correctly talk about cell viability (as they have correctly indicated on the figure). In addition, the authors should state their rationale to choose the specific range of 5 – 100 µM. Even 5 µM appears to be quite high within the physiological context. More detail is needed.

Answer: we explain the reasons why we used these concentrations in this study. (We have tested the acute toxicity of DHM range from 150mg/kg(500µM) -1.5g/kg(5000µM) body weight in mice and cause no significant injury(body weight, tissue morphology). Because body fluid only takes 60% proportion of body weight and taking bioavaliablility into account we estimated the concentration of DHM at 100µM is a safe concentration. Secondly, our team have demonstrated that the DHM inhibited a liver cancer cells (HepG2) proliferation and induce its apoptosis at the concentration of 10-150µM. Therefore, we adopted concentrations from 5µM to 100µM in this study.)

- Fig. 2 C/D: It is unclear how exactly the actual cell numbers were obtained. The authors describe in the Material & Methods section that photographs were taken, but they did not state how cells were counted. It appears not to be feasible to count actual cell numbers in light microscopic pictures without any kind of stain, as single cells cannot be distinguished from each other once they grow in close proximity.

Answer: we analyzed the photographs by image analysis software (Image J) via a tool named "analyze particles". When cells grow in close proximity, the tool also can measure the total area and single cell area so that we could estimate the cells number migrated out.

- Discussion: Give references to both statements "DHM is the principal pharmacological components of Ampelopsis grossedentata, which is a traditional Chinese herb used to treat tinea corporis in South China"

Answer: two references were added.

- Discussion: The authors mention in the discussion ", we found DHM could not inhibit the viability of hepatic L02 cells, an immortalized nontumorigenic normal human hepatocyte cell line (data not

shown)". This information is quite relevant, and data should be included in the Result and Material & Methods section.
Answer: our lab found that DHM have no side effect on the nontumorigenic normal human hepatocyte cell line (L02) and this data was elucidated in one paper which has been submitted. On the other hand, we think this data was not necessary to support our standpoint. Based on these reasons, we deleted this sentence", we found DHM could not inhibit the viability of hepatic L02 cells, an immortalized nontumorigenic normal human hepatocyte cell line (data not shown)" and this section was rewritten carefully.

Review3#

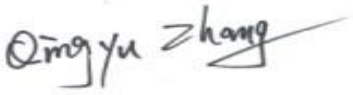
The author's review manuscript indicates that DHM can lead to suppression of HCC migration and invasion. DHM decreased the expression of MMP-9, p38 and JNK in a concentration-dependent manner. However, p38, JNK and ERK1/2 are widely expressed protein kinase intracellular signaling molecules that are involved in functions including the regulation of meiosis, mitosis in differentiated cells. Decreasing those signals will effect to cell proliferation. However, MTT assay result indicated that DHM did not effect HCC proliferation.
Answer: First, we found that DHM didn't affect the total protein level in HCC. Second, we detected phosphorylation site that were related with the regulating of MMPs and we demonstrated that these signaling protein phosphorylation level were down-regulation. Therefore, we propose that DHM may be has no effect on inhibition of cell growth but it could affect the cell migration by modulating of MMPS expression via decreasing the phosphorylation of p38, ERK1/2 and JNK.

The method of MTT assay did not follow general methods. However, author's manuscript is well organized, interesting and suitable for publication. Major comments In the material and methods, the method of MTT assay was not executed properly. After incubation of cells with MTT, the formazan crystals were formed in the culture dish. If the media is removed from the culture dish, MTT and formazan crystals are removed. Please check to experimental methods and compare with industrial or previous reported methods. Also, a negative control is needed, such as 0.5% BSA without FBS.
Answer: After incubation of cells with MTT, the formazan crystals were formed in the bottom of dish. We can remove the media by syringe (1ml) carefully and then DMSO was added in for dissolving of formazan crystals.

Minor comments Temperature symbol did not show in contents.
Answer: we correct these errors.

3 References and typesetting were corrected

Thank you again for publishing our manuscript in the *World Journal of Gastroenterology*.



Qingyu Zhang

Key Laboratory of Hepatic Disease, Department of Hepatobiliary Surgery, Affiliated Hospital of Guangdong Medical College.

Guangdong, 524001, P.R.China

E-mail: hepatolab@gmail.com

Tel: 86-759-2369036

Fax: 86-759-2387596