Re: **Manuscript NO: 38588**

Dear editors:

Thank you so much for receiving your email.

Many thanks for the valuable advice given by the editorial reviewers. We have finished the revision of the manuscript and answered these comments according to these kind advices and the suggestions from reviewers. Thank you very much for all your help. Please review my manuscript again and contact me if you have any questions. Thanks again!

Best wishes,

Yours sincerely,

Weiguo Dong

2018.4.3

Revisions made to the revised manuscript:

Response: Thanks for editor's comments.

1. Followed your requirements, we have added the "Running title", "Institutional review board statement", "Data sharing statement", "authors abbreviation names and manuscript title", "Audio core tip" and "ARTICLE HIGHLIGHTS" in our revised manuscript (***See Page 1; Page 2; Page 5; Page 16-19***). Meanwhile, we have added scale bars and label "●, ■, ▲" to the figures.

2. We have added the full terms of BSO and NAC as they are first mentioned there (***See Page 3 Line 13***). We have deleted the full terms ATP as it can be used directly (***See Page 8 Line 4***).

3. We have rectified a false description about $\mathbf{IC_{50}}$. (***See Page 10 Line 22-23***)

Response to the reviewer's comments

Thank you for the valuable comments on the manuscript.

Reviewer #1

1. The reviewer's comment: How's the cytotoxicity of α-Hederin on normal hepatic cells. Please discuss it.

Response: Thanks for reviewer's comment and suggestion. In our study, we have observed the effect of α-Hederin on hepatic function of nude mice. As shown in Table 1 below, we found that the α-Hederin with various doses (2.5, 5.0, 10 mg/kg) has no significant effect on the serum concentration of ALT, AST. These results suggested that, α-Hederin has no obvious cytotoxicity on normal hepatic cells.

Table I. Effect of α-Hederin on Hepatic and Renal Function

Group	ALT(U/L)	AST(U/L)	Urea(μmol/L)	Cr(μmol/L)
Normal control	32.38±5.67	136.42±15.67	8.25±1.12	12.87±2.98
α-Hederin 2.5 mg/kg	33.36±5.98	135.58±16.77	9.16±1.23	11.32±1.82
α-Hederin 5 mg/kg	35.42±7.63	138.32±17.85	8.98±0.99	12.56±1.66
α-Hederin 10 mg/kg	34.78±6.88	137.69±14.32	7.99±1.34	13.31±2.89

Data are presented as the mean ± standard deviation of the mean, with n=6 mice/group. No differences were observed in the ALT, AST, Urea and Cr levels among all groups ($P > 0.05$). ALT, alanine aminotransferase; AST, aspartate aminotransferase; Urea, blood urea nitrogen; Cr, creatinine.

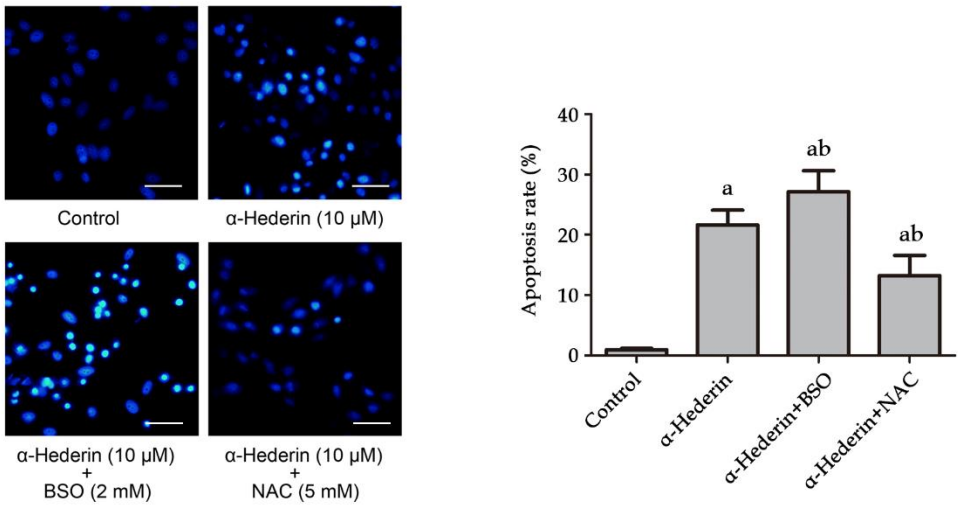
2. The reviewer's comment: What's the possible mechanism for α-Hederin in inducing ROS production in HCC cells. Please discuss it.

Response: Thanks for reviewer's comment and suggestion. It has been reported that cancer cells have increased ROS production compared to normal cells. ROS is generated through a variety of extracellular and intracellular actions. Severe accumulation of cellular ROS may induce lethal damage in cells. Glutathione (GSH) is one of the most common intracellular compounds that play a vital role in the cellular defense against ROS damage. GSH clears intracellular ROS by non-enzymatic and enzymatic catalysis. The non-enzymatic process is GSH acting directly. The enzyme catalyzed process is based on GSH as the substrate, and induces the clearance of ROS in cells under the catalysis of glutathione peroxidase (GSH-Px) or glutathione S transferase (GST) [1, 2]. During intracellular GSH synthesis, two ATP-dependent enzyme catalysis are required: glutamate cysteine ligase (GCL) and glutathione synthetase (GS)[3]. Our study shows α-Hederin significantly reduced cellular ATP levels. Therefore, a reduction in intracellular ATP contributes to a decrease in GSH, leading to ROS accumulation and cellular damage. We have added these discussion above into our revised manuscript ***(See Page 13-14)***.

3. The reviewer's comment: Is α-Hederin inducing apoptosis of HCC cells a direct or indirect effect? Please discuss it.

Response: Thanks for reviewer's comment and suggestion. In our study, we found that the N-acetylcysteine (NAC) can be partially alleviating the apoptosis promoting effect of α-hederin (10 μM) on SMMC-7721 cells, but DL-buthionine-*S,R*-sulfoximine (BSO) with the opposite effect ***(See Figure 2A)***. This result suggested that, α-Hederin inducing apoptosis of HCC cells in a indirect way which is closely related to GSH. However, α-hederin has been reported to have membrane permeabilizing activity, which can directly induce cell death[4]. So it's necessary to conduct an in-depth research to clarify specific mechanism in the future studies. We have added related discussion above into our revised manuscript ***(See Page 11)***.

Figure 2A



Reviewer #2

1. The reviewer's comment: Figure 1A: What is the unit of the x-axis in Fig. 1A (α-hederin 0 – 60)? The authors stated that α-hederin significantly reduced HCC cell viability in a dose- and time-dependent manner but there is no statistical comparison among the three time periods to support their statement. A "trend" does not mean statistical significance. The authors may use t-test, ANOVA, or other applicable statistical tool to verify their findings.

Response: Thanks for reviewer's comment and suggestion. The unit of the x-axis in Fig. 1A (α-hederin 0~60) is μM and we have added it into Figure 1 of revised manuscript. As your suggestion, we use the one-way ANOVA to analyze our data and found that, there are statistical significance among the IC50 value of three time periods. We have added these results into our revised manuscript ***(See Page 10-11)***.

2.The reviewer's comment: Figure 1B: In the Results, the authors stated that "α-hederin induced the apoptosis of HCC cells in a dose-dependent manner". However, the figure merely showed that the degree of apoptosis of each dosage of α-Hederin (i.e., 5, 10, and 20 μM) was significantly different from that of the control (marked by "a" above each bar). Despite a seemingly progressive increase with increasing dosage, it seems that there was no statistically significant difference among the three dosages. Therefore, the authors' claiming of a "dose-dependent manner" is incorrect unless proved otherwise. The authors may use "p for trend" to validate the dose-dependent relationship. The same argument also applies to Fig. 1C, D, and E. and Figure 4. Minor comments Please state the full term in figure captions as it first appeared (e.g., AIF) because each figure is stand-alone.

Response: Thanks for reviewer's comment and suggestion. As your suggestion, we have used "*p* for trend" to re-analyze the relationship between dose and other indicators in Fig. 1 B-E and Fig. 4 A-B. We found that there are dose-dependent effect in each group (*p* for trend $<$ 0.05). We have added these results into our revised manuscript ***(See Fig. 1 B-E and Fig. 4 A-B)*** .

REFERENCES

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3 Jiang Y, Tao R, Shen Z, Sun L, Zhu F, Yang S. Enzymatic Production of Glutathione by Bifunctional gamma-Glutamylcysteine Synthetase/Glutathione Synthetase Coupled with In Vitro Acetate Kinase-Based ATP Generation. APPL BIOCHEM BIOTECH 2016; 180(7): 1446-1455.

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