Peer Review File

Article information: https://dx.doi.org/10.21037/jgo-23-842

Reviewer A

The source of used chemicals must be identifeid and the purity of samples and molecular doking studies must be done to explain the obtained biological results.

Response： Thank you for your comment. According to your suggestion, we have indicated the source of chemicals, details such as supplier information, catalog numbers, purity and any relevant specification employed in our experiments in the revised manuscript (Page 4-5, line 114-123) as follows:

"*The chemical reagents used in our study including Acetylshikonin (lot number: A832580, 99% purity), Shikonin (lot number: S914687, 98% purity), Deoxyshikonin (lot number: D861265, 98% purity), β,β-dimethylacrylshikonin (lot number: D799242, 98% purity), Lithospermidin E, Shikonofuran A (lot number: C11637623, 99% purity) were all purchased from MACKLIN (Shanghai, China). Cell counting kit-8 (CCK-8) assay kit, DNA content assay kit (Solibao, Beijing, China) were obtained form Solibao (Beijing, China). Annexin V-FITC/PI double staining assay kit, caspase 3 enzyme activity kit, JC-1 kit wer purchased from Beyotime (Shanghai, China). Other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.*"

As for the molecular docking study, we appreciate your insightful comments regarding the need for molecular docking experiments in our study. However, in the present study, we have demonstrated the acetylshikonin's role as a tubulin polymerization inhibitor, exerting its effect in anti-tumor mitosis. We have not delved into a detailed discussion of its specific binding sites on tubulin. Another possibility, as reported in the literature (*J Biol Chem* **2019**, 294: 8161-8170; *Mol Pharmacol* **2019**, 96: 711-719), is that the compound may exhibit a range of physiological activities by promoting the degradation of tubulin. Therefore, in our further study, we will focus on investigating whether the compound acts by binding to amino acid residues of tubulin or by promoting the degradation of tubulin to inhibit its aggregation. Consequently, the results of relevant molecular docking analyses will be presented in the next manuscript. Thank you for your understanding and consideration.

Reviewer B

1- I recommend the future evaluation of antiproliferation effect of acetylshikonin on different cancer cell lines

Response : Thank you for your valuable feedback. We appreciate your thorough review of our work. In the present study, we assessed the toxicity of acetylshikonin on cancer cells including the epithelial carcinoma lung cell line, epidermoid cervical cancer cell line, MHCC-metastatic hepatocellular carcinoma cell line, prostate carcinoma cell line, adenocarcinomal cell line, and observed that acetylshikonin exhibited relatively higher activity against hepatocellular carcinoma cell lines. Consequently, in this study, we selected hepatocellular carcinoma cell lines for further investigation. According to your suggestion, in the future study, more cancer cell lines derived from different tissues will be selected for the antiproliferative activity evaluation.

Changes in the text: No changes in the text.

2- I recommend the assessment of different proteins mediating the molecular mechanism of antiproliferation effect of acetylshikonin.

Response : Thank you for your valuable comment. Your recommendation to assess different proteins mediating the molecular mechanism of the antiproliferative effect of acetylshikonin is noted and appreciated. In response to your suggestion, we plan to expand our experimental design in the further studies to delve deeper into the molecular mechanisms of acetylshikonin's impact on cell proliferation. Specifically, we will conduct assessments of various proteins to elucidate the compound's specific molecular targets and pathways contributing to its antiproliferative activity. This additional analysis will enhance the comprehensiveness of our research and provide a more detailed understanding of the therapeutic potential of acetylshikonin. Thank you for your valuable input.

Changes in the text: No changes in the text.

Reviewer C

The paper titled “Identification of acetylshikonin as a novel tubulin polymerization inhibitor with antitumor activity in human hepatocellular carcinoma cells” is interesting. In this study, acetylshikonin was identified as a microtubule-targeting agent (MTA) against hepatocellular carcinoma and can serve as an effective lead compound for further anti-cancer drug development. However, there are several minor issues that if addressed would significantly improve the manuscript.

1) The abstract is not sufficient and needs further modification. The research background did not indicate the clinical needs of the research focus.

Response : Thank you for your suggestion, and the abstract was revised as follows (Pages 1-2, Line 31-59):

Background: Microtubules are attractive targets for anticancer drugs. However, the microtubule target agets (MTAs) currently in clinical use exhibit inevitable drug resistance. Therefore, there is an urgent need to discover novel MTAs for the clinical treatment of cancer.

Methods: Bioactive compounds extracted from *Lithospermum erythrorhizon* were assessed for in vitro anti-proliferative activities against a panel of human cancer cell lines using cell counting

kit-8 (CCK-8) assay. Tubulin polymerization inhibition assay, colchicine competitive binding site assay, and immunofluorescence were used to validate the tubulin inhibition effect of acetylshikonin. Flow cytometry, Hoechst staining, and caspase-3 activity evaluation were performed to assess cell cycle arrest and cell apoptosis. 5,5',6,6'-tetrachloro-1,1',3,3'-tetramethylbenzimidazolylcarbocyanine iodide (JC-1) staining and dichloro-dihydro-fluorescein diacetate (DCFH-DA) staining were used to evaluate mitochondrial membrane potential (MMP) and reactive oxygen species (ROS), respectively.
Results: Acetylshikonin exhibited potent anti-proliferative activities against a panel of human cancer cell lines (IC_{50} values: 1.09-7.26 μM) and displayed comparable cytotoxicity against several drug-resistant cell lines. Further mechanism studies revealed that acetylshikonin induced cell cycle arrest of MHCC-97H cells at G_2/M phase, and significantly promoted apoptosis marked by a collapse of MMP and abnormal ROS accumulation.
Conclusions: In this study, acetylshikonin was identified as MTA against hepatocellular carcinoma and can serve as a promising lead compound for further development of anti-cancer drug, underscoring its potential clinical significance.

2) What is the potential application value of acetylshikonin in clinical practice? What is the basis for selecting the concentration of acetylshikonin in this study? Is the dosage safe in clinical practice? Please provide literature support.

Response : Thank you for your valuable suggestion. The potential application value of acetylshikonin in clinical practice stems from its demonstrated potent anti-proliferative activities against various human cancer cell lines, particularly in hepatocellular carcinoma (HCC). The findings suggest that acetylshikonin could serve as a promising lead compound for further development as an anticancer drug. Lead compound for drug Development should undergo additional preclinical and clinical investigations to assess its safety and efficacy for potential use in human patients. While these findings are promising, it's important to note that the translation of compounds from preclinical studies to clinical practice involves thorough evaluation of safety, pharmacokinetics, and efficacy in humans. Additional research and clinical trials will be necessary to establish the full potential and safety profile of acetylshikonin for clinical applications.

The selection of acetylshikonin concentration in this study was based on its effectiveness in inhibiting cell proliferation, as evidenced by half-maximal drug inhibitory concentration (IC_{50}) values ranging from 1.09 to 7.26 μM across various cancer cell lines. The IC50 of acetylshikonin towards MHCC-97H cell line was 1.09±0.27 μM. We selected three concentrations, low (0.75 μM), medium (1.5 μM), and high (3.0 μM), which induced approximately 20%, 50%, and 80% cell death in cancer cells, respectively.

The safe dosage of acetylshikonin in clinical practice is unknown. The determination of the safety of acetylshikonin dosage in clinical practice involves a comprehensive and progressive assessment across multiple stages of research. The initial cell-level studies explore different concentrations to evaluate the compound's biological effects while minimizing cytotoxicity. Subsequent phases include animal models to assess in vivo effects and potential toxicity. Preclinical studies investigate metabolism, pharmacokinetics, and toxicity in the whole

biological system. Clinical trials further refine the dosage for safety and efficacy in humans, beginning with early safety trials and advancing to later-stage efficacy assessments. The dosage used in clinical practice is ultimately determined through a meticulous process that integrates findings from these various stages, ensuring a balance between safety and effectiveness. Therefore, the dosage of acetylshikonin is subjected to rigorous evaluation to establish its safety in clinical application.

Changes in the text: No changes in the text.

3) What are your future work plans? What is the biggest problem faced if used in clinical applications?

Response : Thank you for your valuable feedback. Our future work plans mainly include: a) Further Elucidation of Mechanisms: We plan to conduct more in-depth studies to elucidate the molecular mechanisms underlying the anti-proliferative effects of acetylshikonin, particularly focusing on its interaction with specific cellular pathways and targets. b) Preclinical Studies: Our future work will involve comprehensive preclinical studies, including animal models and toxicity assessments, to gather essential data on the compound's safety profile and pharmacokinetics.

Biggest Problem Faced in Clinical Applications:

One of the significant challenges faced in the clinical application of acetylshikonin is the translation from preclinical efficacy to successful human outcomes. The following aspects pose potential challenges:

Safety Profile: Ensuring the compound's safety in humans is critical. Unexpected side effects or toxicity may arise, necessitating careful monitoring and adjustment of dosage.

Bioavailability: Achieving optimal bioavailability is crucial for the effectiveness of the compound. Addressing issues related to absorption, distribution, metabolism, and excretion will be essential for successful clinical use.

Specificity and Selectivity: While acetylshikonin shows promise against certain cancer cell lines, ensuring its specificity for cancer cells without harming normal, healthy cells is a key concern.

Addressing these challenges will be integral to the successful clinical translation of acetylshikonin, and our future work aims to systematically address these aspects to enhance its potential as a clinical therapeutic agent.

Changes in the text: No changes in the text.

4) There are still some weak points in this paper. It is suggested that the author increase the research of signaling pathway. This is more conducive to support the conclusions of this study.

Response : Thank you for your constructive suggestion. The investigation of signaling pathways indeed further support the conclusions of our study. In response to your

recommendation, we plan to expand our research to include a more in-depth exploration of the signaling pathways associated with the anti-proliferative effects of acetylshikonin. We will focus on identifying key signaling molecules and pathways affected by acetylshikonin, emphasizing their relevance to cell cycle regulation, apoptosis, and other cellular processes implicated in the anti-cancer effects observed in our futher study.

Changes in the text: No changes in the text.

5) The introduction part of this paper is not comprehensive enough, and the similar papers have not been cited, such as "Identification of potential prognostic biomarkers for hepatocellular carcinoma, J Gastrointest Oncol, PMID: 35557563". It is recommended to quote the article.

Response : Thank your for your comment. According to your suggestion, the revelant literatures have been cited in our revised manuscript on Page 5, Line 129.

6) It is recommended to increase in vivo experiments, which may be more meaningful.

Response : Thank your for your comment. The in vivo anti-tumor acitivity evaluation will be performed in our futher study.

Changes in the text: No changes in the text.

7) Why did this study not conduct research on target genes? If you increase the study of target genes, the results may be more convincing and meaningful.

Response : Thank your for your insightful comment. We appreciate your suggestion regarding the study of target genes, and we recognize the potential value in enhancing the depth and significance of our results. In response to your recommendation, we plan to expand our research to include an investigation of target genes associated with the observed effects of acetylshikonin. By delving into the molecular mechanisms at the gene level, we aim to provide a more comprehensive understanding of how acetylshikonin exerts its anti-proliferative activities.

Our focus will be on identifying and analyzing specific target genes related to cell cycle regulation, apoptosis, and other relevant cellular processes. These studies will be peformed in the futher study.

Changes in the text: No changes in the text.

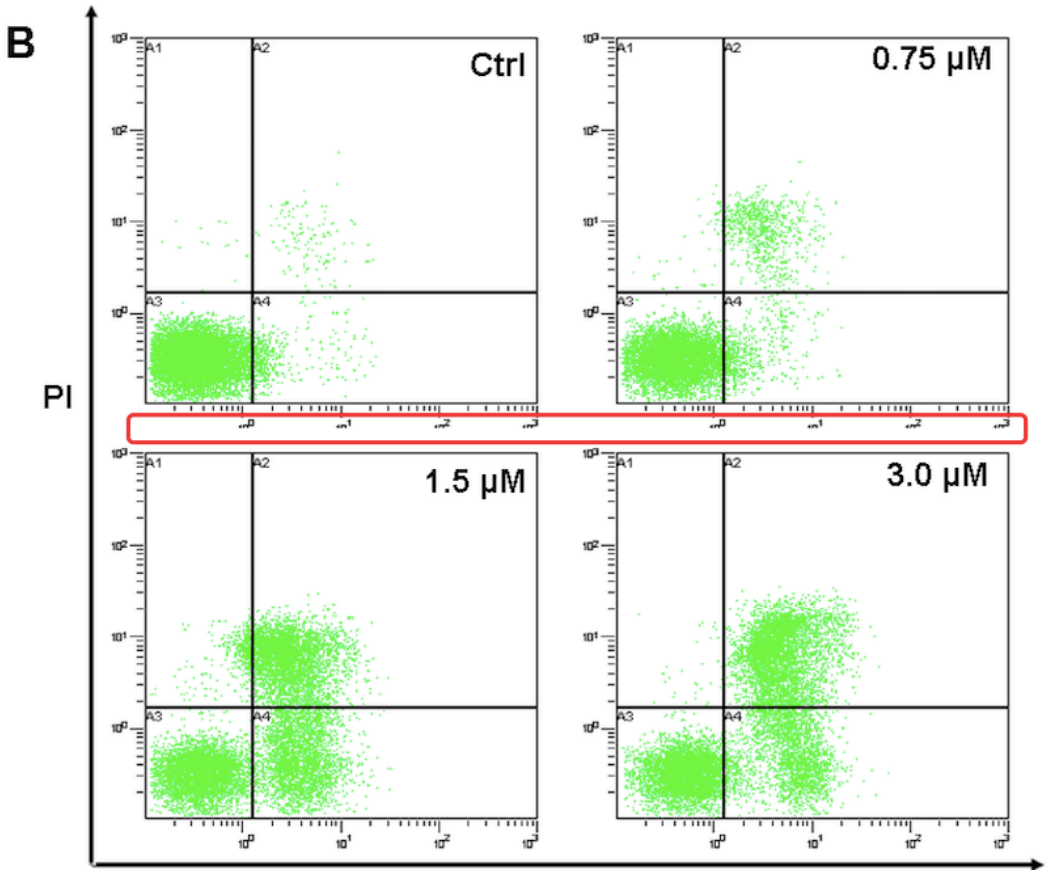
Reviewer D

1. The authors mentioned "studies...", while only one reference was cited. Change "Studies" to "A study" or add more citations. Please revise. Please number references consecutively in the order in which they are first mentioned in the text.

Mechanistic studies have elucidated that MTAs bind to specific sites on microtubules or tubulin subunits, affecting their polymerization and stability (18).

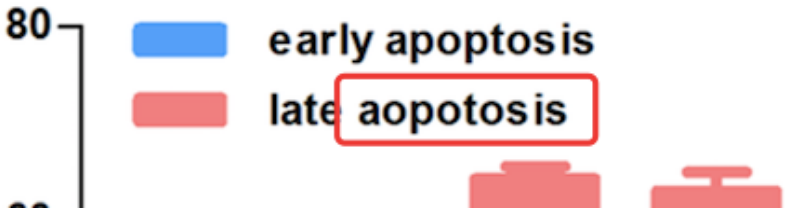
Reply: More citations have been added in the revised manuscript.
Changes in the text: Page 4, Line 101.

2. Figure 5B is incomplete as some words are covered. Please revise.



Reply: We have replaced Figure 5B in the Figure 5- revised.

3. Figure 5C: Please check and revise this typo.



Reply: The typo error has been corrected in the Figure 5-revised.

4. Please explain the meaning of “d” in the legend.

677 Table 3 Cytotoxicity of acetylshikonin towards drug-resistant cancer cell lines

Cell lines	IC_{50}, mean ± SE (μM)	Resistance index[a]
MHCC-97H	1.09±0.27	0.78
MHCC-97H/CDDP[b]	0.85±0.01	
PC-3	3.52±0.36	1.20
PC-3/ENZR[c]	4.22±0.17	
HCT-8	7.26±0.14	0.90
HCT-8/VCR[d]	6.51±0.62	

678 [a]Resistance index = (IC_{50} of drug-resistance cell lines)/(IC50 of corresponding cancer
679 cell lines). [b]A549/CDDP, A549 cell line resistant to cisplatin; [c]PC-3/ENZR, PC-3 cell
680 line resistant to enzalutamide.

Reply: The explanation of "d" in the legend has been added in Table 3.