**Reviewer’s report**

**Title:** 2-Ethoxystypandrone, A Novel Small-Molecule STAT3 Signaling Inhibitor from Polygonum Cuspidatum, Inhibits Cell Growth and Induces Apoptosis of HCC cells and HCC Cancer Stem Cells

**Version:** 0  **Date:** 06 Aug 2018

**Reviewer:** Yin Quan Tang

**Reviewer's report:**

**General comments:**

The written manuscript should have been read and edited by a native English speaker with knowledge of the techniques.
Please define the first use of the abbreviation in the text.
Suggest to ensure all the citation is related to content.
Please state clearly which brand and model of machines/equipment used for this study.
Please choose and standardize the using either the "test sample", "compound" or "isolate" word.
Suggest not to bracket compound (1) and compounds (2-8) as it will be mistaken as part of citation.
Please choose and standardize the using either the "HCC cancer stem cells (CSCs)" or "HCC CSCs" word throughout the text.
Please choose and standardize the using either the "2-Ethoxystypandrone" or "cuspidatone" word.
Please recheck all figure are correctly labelled.
Please cite the source from where chemical compounds 1-8 was obtained.
Pyridone 6 as a control only was used for MTT? How about other assay?

**Abstract:**

Generally, the abstract is good but minor corrections are needed for clarity.
Please use italic for "in vitro"
Suggest to replace "anti-tumour" with "anticancer" as no animal experimentation is performed in this study.
Please define" EtOAc".
Please space after a full stop.
Incomplete sentence: "The phosphorylation .....western blot".
Please reconstruct, "In-vitro anti-cancer..... respectively".

**Background:**

Introduction is good and sufficient.
Please include references for stating juglone analogues exhibited many biological activities.
Please define" EtOAc".
Methods:
Please state the brand and model of machines/equipment used in this study.
Please define "TLC", "MeOH-H2O", "EtOAc", "PE-EtOAc", "EtOAc-MeOH"
May authors state identification of Dr. Peng? A qualified botanist?
Confusing sentence: "….using MeOH as eluting solvent to afford…"
Please correct the word "…to obtain resveratrol…"
Suggest to rewrite the whole section of "Extraction and isolation" as hard to follow and understand.
Please identify the cell types of "Huh-7", "Li-7" and "SK-HEP-1".

Luciferase reporter assay
o Please describe what are the test samples?
o May authors state which promega luciferase kit is used?
o May authors explain why cells stimulated with IL-6 for 5.5hr in luciferase reporter assay and 15 min in western blot?
o Confusing sentence: "……was controlled by equal seeding."

Cell Viability
o Please write full of MTT in the first time use.
o Please describe what are the "compounds"?
o Please state the reason for incubating the cells for 3hrs after treatment?

HCC Cancer Stem cells Culture
o Please re-check the citation for [17] as they not involving in stem cell culture selection.
o Author should explain why Huh-7 is selected for stem cell culture selection?
o Incomplete sentence, "…..the cells with the high-stemness….."

Tumorsphere passage and tumorsphere formation assay
o Please use italic for "in vitro"
o Please state the version of ImageJ is used.

Western Blot analysis
o Please state the range of different concentrations
o May authors explain why cells stimulated with IL-6 for 15 min in western blot but 5.5hr in luciferase reporter assay?
o Please state the percentage of gel used.
o Please state the secondary antibody is anti-mouse as all primary antibodies are from mouse.
o Suggest to include catalogue number and brand of antibodies used in this study.

Flow Cytometric analysis
o "…..of cuspidatone (1)….." Please state clearly whether compound 1 is 2-ethoxystypandrone or cuspidatone.
o Suggest to re-construct "The dots in…..respectively"
o There is no "+" and "-" superscript in Annexin V and PI.
o Please define "PI"
For analysis of apoptosis and cell cycle of HCC CSCs, no Annexin V/PI staining is required?

Statistical analysis
- Author did not mention which types of statistical tests were used for different experiments.

Results:

Isolation and structural elucidation
- Please write "Figure 1" in text instead of Fig.1.
- Please state the identification of compound 1 before the rest.
- Please cite the articles/website from where the chemical structure of compounds 1-8 obtained.

Inhibitory activity …..pathway.
- "HepG2/STAT3-luciferase cells……for 6.5h and…" But in Material section, authors stated cells were treated for 1h. Please check.
- Statement "HepG2/STAT3-luciferase cells……for 6.5h and MTT assay.." is unacceptable as in both assays, cells were treated with compounds for different times; 1hr (luciferase assay) and 72 hr (MTT assay).

2-Ethoxystypandrone……..apoptosis in HCC Cancer Stem Cells.
- Please use italic for "in vitro"
- Please choose and standardize the using either the "HCC cancer stem cells (CSCs)" or "HCC CSCs" word.
- Contradicting statement: Author stated no more than 20 passages of sphere will be used under the Material section. But in discussion section, tumorsphere were passaged more than 30 generations. Please check properly.
- Authors stated Huh-7 cells were treated with 6 different concentrations, but in Figure 4B, there were 7 different concentrations of 2-Ethoxystypandrone tested.
- Tumorsphere or spherogenesis?
- From Figure 4(C), IC50 is 2.42um? Please check.

Discussion:
- What does author mean "the complete SAR data will be published elsewhere"?
- Discussion is rather weak although with good data.
- Authors are keep repeating about the result without further/depth discussion of obtained result except the second paragraph.
- Please include more published articles to support the hypothesis.

Tables and Figures:
- No standard deviation in IC50 value in Table 2

Figure 2:
- (B): Misleading chart title and no x-axis title.
- (C) and (D): 2-Ethoxystypandrone or cuspidatone? Please check.
Figure 3:
- (A): Please correct "live" Y-axis and no standard deviation for IC50 values
- (B): Please add "Propidium iodide - PE"

Figure 4:
- (C): Please explain how authors determined IC50=2.42um from the graph?

Figure 5:
- (A) Please check the labelling because cells were treated with 0, 2, 4 and 8um as mentioned in text.

Are the methods appropriate and well described?
If not, please specify what is required in your comments to the authors.

Yes

Does the work include the necessary controls?
If not, please specify which controls are required in your comments to the authors.

No

Are the conclusions drawn adequately supported by the data shown?
If not, please explain in your comments to the authors.

Yes

Are you able to assess any statistics in the manuscript or would you recommend an additional statistical review?
If an additional statistical review is recommended, please specify what aspects require further assessment in your comments to the editors.

I am able to assess the statistics

Quality of written English
Please indicate the quality of language in the manuscript:

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