Author’s response to reviews

Title: Retama monosperma n-hexane extract induces cell cycle arrest and extrinsic pathway-dependent apoptosis in Jurkat cells

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Author’s response to reviews: see over
Point-by-point Response to the Reviewers

Reviewer: Yung Hyun Choi

Reviewer's report:

This manuscript described the antitumor activity of Retama monosperma n-hexane extract in human leukemia cells. The studies are well-designed and the manuscript is well written. While the manuscript in general is well-written, they also need to address the following minor issues.

1. In Materials and methods, more detailed description on the devices used for mass spectrometry (GC/MS) and Gas chromatography and analysis is needed.

We have now included an extended explanation on the methods used for this analysis in the relevant section of the Methods (“Gas chromatography/mass spectrometry (GC/MS) analysis”).

2. There are no exact information about antibodies used in Western blots.

We now include reference numbers, commercial providers and appropriate dilutions of all antibodies used in the revised version of the manuscript (“Gel electrophoresis and immunoblotting”, Methods section).

3. It would be interesting to quantify cell toxicity by other techniques, such as MTT assay or LDH dehydrogenase method in order to clarify this point.

The Cell Viability assays performed in this study are, in essence, improved MTT assays optimized for luminometric detection. We now include an extended explanation on the characteristics of this assay in the relevant section of the Methods (“Cell Viability Assays”).

4. In Fig. 2C (pH2A.X) and 4B (Bax), more clear western blotting results had better be provided.

We have now substituted these images for much clearer ones in the modified version of the manuscript.

5. Other known markers of apoptosis such as DNA fragmentation, mitochondrial membrane potential or PARP degradation should be investigated.

We have used three different techniques to monitor apoptotic cell death in this study. On one hand, we have performed the flow cytometric-based “gold-standard” of apoptosis determination: Annexin V/Propidium iodide double staining. We have also used flow cytometry in fixed cells to quantitate the percentage of <2n cells (sub-G1), and finally have determined directly the increase in caspases 3/7 activity using a specific luminometric assay detection kit, which correlates with the detection of cleaved caspases shown by immunoblot. Altogether, we feel that we have clearly shown that
Rm-HE induces apoptotic cell death and that adding further methods that detect apoptosis by other means would result in similar, redundant, results but would not add insightful data to our manuscript.

6. Some paragraphs begin with title that is actually a kind of conclusion. It should be avoided because all conclusions can be done after results discussion based on obtained results.

We have now changed the titles, instead of summarizing the conclusions of each section they are now just a description of the work performed.

7. Authors stated that Rm-HE activates both extrinsic pathway. However, caspase-8 was also activated. Just observing the activation of caspase-9 and induction of Fas-L are not sufficient to support authors hypothesis.

We have not properly understood this precise question. However, throughout the manuscript we have tried to convey the impression that both pathways can participate in the apoptotic process engaged by Rm-HE.

Reviewer: Mahmoud ALHOSIN

Reviewer's report:

In this manuscript, the authors investigated the anti-cancer effects of (Rm-HE) (Retama monosperma hexane extract) on the Jurkat cells. They found that Rm-HE could inhibit the cell viability and cause cell cycle arrest in Jurkat cells. Sub G1 analysis also suggested that Rm-HE also induce apoptosis in Jurkat cells. Authors tried to study the molecular mechanism leading to Rm-HE-induced apoptosis in Jurkat cells.

Major Compulsory Revisions:

1. JNK is a key regulator of Fas-L expression, so a time-course effect on JNK phosphorylation in Jurkat cells should be performed to confirm the data shown in Figure 4D.

We have now analyzed the pattern of p-JNK in response to Rm-HE and have included it in the revised version of the manuscript (Figure 4F).

2. Figure 2D shows that the percentage of early apoptotic cells at 24 h was 2.5%. This data is not in agreement neither with Figure 2A showing that the percentage of SubG1 (apoptotic cells) was 33.05% at 24h or with Figure 3B showing that cell viability was reduced to 40% of control after 24 h of treatment with Rm-HE. The authors need to clarify these controversy findings.

We thank the reviewer for highlighting this apparent controversy, which has enabled us to detect an important mistake in Figure 2D. We had wrongly labelled the Annexin V/PI dot plots as 24h and 48h when the correct time points were 12h and 24h, respectively. We have now corrected this mistake in the revised version of the manuscript.
3. The authors mentioned that Rm-HE caused a time-dependent increase in Fas-Ligand expression. This is not clear in the Figure 4C. To confirm this conclusion, the authors should make a statistic analysis for Fas-L blot in Figure 4C (three different experiments).

We have now performed the suggested blot quantification and correspondent statistical analysis and include it as Figure 4D in the revised version of the manuscript. We agree that quantification of a representative number of blots strengthens this observation and thank the reviewer for this appreciation.

4. The authors mentioned in this study (in conclusion section) that this work clearly indicates that bioactive components of Rm-HE act either alone or in combination to promote cellular apoptosis. Effect of isolated major compounds (such as #-Linoleic acid, Stigmasterol, and Campesterol) on cell viability or apoptosis in Jurkat cells should be performed to show the anti-proliferative and pro-apoptotic effects of bioactive components of Rm-HE.

The aim of this report is to demonstrate that Rm-HE exerts cytotoxic activity against Jurkat cells, and provide a general characterization of its cellular effects. Furthermore, chemical analysis of Rm-HE has indicated the presence of a number of bioactive components. However, our conclusion that the indicated components (Linoleic acid, Stigmasterol, and Campesterol) might be responsible for the anticancer effects of Rm-HE is entirely speculative, although we believe it is a plausible hypothesis considering the cited reports that describe the antitumor effects of these compounds in various cell lines. We have never intended to convey the idea that these compounds are the bioactive components responsible for the effects of Rm-HE. Thus, we have now toned down our conclusions and simply state that “Our results suggest (instead of clearly indicate) that bioactive components of Rm-HE act either alone or in combination…”

Furthermore, if we were to prove this point (which is not our goal here), performing cytotoxicity assays with isolated compounds would not be sufficient to demonstrate that any of them, or all, are responsible for the effects of a complex extract containing at least 60 different components. We hope the reviewer will understand our point of view on this issue.

5. The authors should improve the quality of western data; some blot is not suitable for publication in the scientific journals (eg. Fig2C, Fig3C Caspase-3). Moreover, the same Tubulin blot has been used in all western blots in this study.

We thank the reviewer for highlighting these mistakes; they have been corrected in the revised version of the manuscript.

Minor comments

In the discussion section, the authors indicated the presence of a previous study about the anti-leukemic effect of RmHE and cited the reference number [28], this last (Reactive oxygen species contribute to cell killing and P-glycoprotein downregulation by salvicine in multidrug resistant K562/A02 cells. Cancer BiolTher.; 6(11), 1794-9) does not talk about the extract RmHE.
Again, we thank the review for detecting this mistake. We have now corrected it and cite the appropriate reference (25) in the revised version of the manuscript.