Peer Review File

Article information: https://dx.doi.org/10.21037/tcr-21-920

Reviewer A

Comment 1: The present study by Lin et al. follows a previous line of work of this group dealing with the likely pharmacological properties of a grape seed proanthocyanidin extract. The aim of the work is to further deepen in the mechanism of action of this proanthocyanidin extract and its ability to induce apoptosis in HL-60/ADR cells. Although such family of natural products has emerged as a potential source of interesting treatments in different areas (e.g., cancer, inflammation, antioxidant), and this field is yielding promising drugs, there are several aspects that deter me from acceptance of the manuscript in its actual form.

Reply 1: We thank reviewer A for the positive comments and valuable suggestions regarding our research. We have revised our manuscript according to reviewer A's advice and responded to the comments point-by-point as follows.

Major concerns:

Comment 2: English revision is needed. It is quite difficult to follow the narrative because of the poor English. It is highly recommended to edit the English grammar before acceptance. Please, use a 'native English speaker' to check the text.

Reply 2: We thank the reviewer for pointing out it. We invited Dr. Erik Matro, a native English speaker from America, to help improve this manuscript's English language quality.

Changes in the text: Please see manuscript.

Comment 3: Lack of detail from the proanthocyanidin extract. The authors do not provide an analysis of the components of the extract. They only report that the extract was purchased from Tianjin Jianfeng Natural Product R&D Co., Ltd. (Tianjin, China) with a purity exceeds 95% (¿?). A more detailed description of the extract is highly recommended (i.e., components and quantity/concentration).

Reply 3: We thank the reviewer for this valuable advice. We have added a detailed description of the extract in the first paragraph of the Discussion section.

Changes in the text: GSPE, a flavonoid polyphenolic compound extracted from grape seeds, has been reported to exhibit antitumor activity in the setting of malignancies such as bladder, breast and lung cancers (23,24). GSPE is composed of both monomeric and polymeric +catechin, -epicatechin gallate and -epigallocatechin linked together via either C4-C6 or C4-C8 bonds (13,25). According to the structural classification of proanthocyanidins, their commonest constitutive units can be divided into A-type and B-type procyanidins; GSPE is a B-type procyanidin (26).

Comment 4: Lack of detail from the total RNA extraction. How, when and where did

the authors obtain the RNA from the cell cultures? Only from attached cells, supernatants, or altogether?

Reply 4: We thank the reviewer for pointing this discrepancy out. We have added detailed information regarding total RNA extraction in the manuscript as follows.

Changes in the text: Cells were seeded in 6-well plates at 1.0×10^6 cells/well (2mL of cell suspension with a concentration of 1×10^6 cells/mL) and treated with different concentrations (0, 25, 50, 75, 100 μg/ml) of GSPE for 24 h. Total RNA of all treated cells, including attached cells as well as cells in the supernatant, was extracted using Trizol reagent and reverse transcribed into cDNA using the RT Reagent Kit.

Minor concerns:

Comment 5: I recommend not to include result comments in the figure legends.

Reply 5: We thank the reviewer for pointing this out. We have revised our figure legends according to the reviewer's advice.

Changes in the text:

Figure 1. GSPE directly inhibits HL-60 and HL-60/ADR cell proliferation.

(A) Cell validity of different GSPE concentrations. (B) Effects of different GSPE concentrations (0, 6.25, 12.5, 25.0, 50.0, 75.0, 100.0, 125.0, 150.0 and 200.0 μg/mL) on the survival rate of HL-60/ADR cells cultured for 24 h as compared to controls (GSPE 0 μg/mL); (C) Effects of different durations of cell culturing and different GSPE concentrations on HL-60/ADR cell proliferation. $^{***}P < 0.001$

Figure 2. Effects of different GSPE concentrations on HL-60/ADR cell count under trypan blue staining.

(A)-(E) Observations of apoptotic HL-60/ADR cells cultured with different GSPE concentrations for 24 h under microscopy after trypan blue staining: (A) 0 μg/mL; (B) 25 μg/mL; (C) 50 μg/mL; (D) 75 μg/mL; (E) 100 μg/mL; dark blue cells in each image were apoptotic HL-60/ADR cells. A size scale in the lower right corner of the image shows a 100 μm distance. (F) HL-60/ADR cells survival rates when cultured with different GSPE concentrations. $^{***}P< 0.001$ when *vs.* control

Figure 3. Effects of different GSPE concentrations on HL-60/ADR cell apoptosis were detected by Annexin V-FITC/PI double staining.

(A)-(E) Cell apoptosis in different GSPE concentrations: (A) 0 μg/mL; (B) 25 μg/mL; (C) 50 μg/mL; (D) 75 μg/mL; (E) 100 μg/mL; cells in the Q4 phase were considered to be in the early stage of apoptosis while cells in the Q2 phase were considered to be in the late stage of apoptosis. (F) Quantification of cell apoptosis in HL-60/ADR cells cultured with different GSPE concentrations. The proportion of apoptotic cells was calculated by the ratio of Q2 to Q4 quadrant cells. $^{***}P < 0.001$ *vs.* control

Figure 4. Effects of different GSPE concentrations on Bax and Bcl-2 mRNA expression in HL-60/ADR cells.

(A) Bax mRNA expression at different GSPE concentrations (0, 25, 50, 75, 100 μg/mL). (B) Bcl-2 mRNA expression at different GSPE concentrations. (C) The ratio of Bax/Bcl-2 at different GSPE concentrations. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, *vs.* control (GSPE 0 μg/mL).

Figure 5. Effects of different GSPE concentrations on the activity of caspases-3 and -9 in HL-60/ADR cells

(A) Effects of different GSPE concentrations (0, 50, 75, 100 μg/mL) on the activity of caspase-3; (B) Effects of different GSPE concentrations (0, 50, 75, 100 μg/mL) on the activity of caspase-9, after HL-60/ADR cells were treated with GSPE for 24, 48 and

72 h. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, *vs.* control (GSPE 0 µg/mL).

Comment 6: I think that Figure 1B (table?) is useless as long as the main text includes the same information.
Reply 6: We thank the reviewer for pointing this discrepancy out. We revised our Figure 1 and deleted Figure 1B from Figure 1.
Changes in the text: Please see Figure 1 text as above.

Comment 7: References 14 and 23 are duplicated.
Reply 7: We thank the reviewer for pointing this out. We have deleted reference 23 from the manuscript.
Changes in the text: Deleted reference 23.

Comment 8: The assumption stated in the conclusion section that "GSPE had a potential clinical application value, not only can be used as a combination of chemotherapy drugs to reduce drug resistance, but also can be directly used to inhibit the proliferation of tumor cells" is over-estimated, as long as an analysis in non-tumoral proliferating cells should also be done in order to test toxicity in 'normal' or 'control' cells before its clinical application.
Reply 8: We thank the reviewer for pointing this out. We have revised the conclusion of our study as follows:
Changes in the text:
The present study demonstrated that GSPE induces apoptosis of HL-60/ADR cells via the Bcl-2/Bax caspase-3/9 pathway. GSPE can not only be used as an adjunct in the setting of combination chemotherapy to reduce drug resistance, but also to directly inhibit tumor cell proliferation. A further toxicity study of GSPE in non-tumoral proliferating cells is thus warranted to evaluate its potential uses in clinic.

Reviewer B

Comment 1: The article describes the anti-cancer activity of grape seed proanthocyanidine extract against HL-60/ADR cells.
Reply 1: We thank the reviewer for the accurate summary of our study.
Changes in the text: N/A

Comment 2: Except for English corrections, the article is well written. The article requires a thorough English check.
Reply 2: We thank Reviewer B for pointing this out. We invited Dr. Erik Matro, a native English speaker from America, to help improve this manuscript's English quality.
Changes in the text: Please see manuscript.

Comment 3: How did the authors ascertained the authenticity and purity of the extract which has been commercially purchased for this study.

Reply 3: We thank the reviewer for pointing this out. The purity report of the extract was provided by the manufacturer. We have added this information in the Materials and Methods section as follows.

Changes in the text: GSPE with a purity exceeding 95% was purchased from Tianjin Jianfeng Natural Product R&D Co., Ltd. (Tianjin, China) (manufacturer's content report)

Comment 4: Similar articles with grape seed extract has been published earlier with the AML cells (10.1158/1078-0432.CCR-08-1447, 10.4103/jcrt.JCRT_766_19, 10.1016/j.biopha.2020.109885) how is this article adding additional/ novel information

Reply 4: We thank the reviewer for pointing this out.

The first article (10.1158/1078-0432.CCR-08-1447) entitled "Induction of apoptosis in human leukemia cells by grape seed extract occurs via activation of c-Jun NH2-terminal kinase", concluded that "GSE induces apoptosis in Jurkat cellsthrough a process that involves sustained JNK activation and Cip1/p21up-regulation, culminatingin caspase activation". The cell line used in the first article consisted of Jurkat cells. The cell line we studied was HL-60/ADR, a multidrug resistant human AML cell line. We added a brief introduction of the first article in the Discussion section.

The second article (10.4103/jcrt.JCRT_766_19) was not found via DOI.

The third article (10.1016/j.biopha.2020.109885) entitled "Grape seed proanthocyanidin extract reverses multidrug resistance in HL-60/ADR cells via inhibition of the PI3K/Akt signaling pathway" was a prior study conducted by our team. The relationship between the third article and the present manuscript was introduced and discussed throughout the Introduction and Discussion sections.

Changes in the text: Gao et al. previously reported that GSPE induces apoptosis in Jurkat cells via the c-Jun NH_2-terminal (JNK) pathway by sustained JNK activation and Cip1/p21 up-regulation that finally culminates in caspase activation (31).