Peer Review File

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Review Comments

Authors conducted an in vitro study to assess the effect of extract from Actinidia chinensis Planch radix in a CRC cell line. It is well written and easy to follow.

Several specific comments:

1. More details are needed in Methods section. For example: Line 130 "Wound Healing assay" What concentration were these cells treated with? before or after the serum starvation? and the treatment was done before or after the artificial wounds were induced? All of this information was missing in the Methods section. Vehicle for the treatment was not mentioned (H2O?).

Reply 1:

Thanks for your concerns, we have included the clinical data in the revised manuscript. These updates are shown in red in lines 130-140 of the manuscript.

Changes in the text: To generate an oxaliplatin-resistant daughter cell line, parental CRC cells were chronically exposed to stepwise increasing concentrations of oxaliplatin (Sigma-Aldrich, St. Louis, MO, USA) dissolved in serum-free DMEM containing 0.1% dimethyl sulfoxide (DMSO) as vehicle. Starting from the baseline IC50 concentration (0.1 μM) determined by preliminary CCK-8 assay, the exposure gradient was progressively escalated over 8 weeks (0.1 → 0.5 → 1.0 → 5.0 → 10 μM) with medium replacement every 3 days. Prior to each concentration increment, cells underwent serum starvation for 24 hours in 1% FBS medium to synchronize cell cycle progression. Drug resistance was confirmed through three consecutive passages showing >5-fold increase in IC50 (0.52 ± 0.07 μM in parental vs. 2.89 ± 0.12 μM in resistant cells, $p<0.001$) and maintained in 1.0 μM oxaliplatin during routine culture.

2. It would be helpful to readers if more information can be included in Figure legends. For example: Figure 2B, what are the top vs. bottom rows?

Reply 2:

Thank you for your concern. I have added more information about Figure legends. These updates are shown in red in lines 405-411 of the manuscript.

Changes in the text:

(A) MTT assay. The graph shows the survival cell rate over time (0 to 72 hours) for different treatment groups using the MTT assay. The legend includes four groups: Resistant + Vehicle (black circles), Resistant + Treatment (green squares), Parent + Vehicle (blue triangles), Parent + Treatment (purple diamonds). Statistical significance is denoted by asterisks: * ,$p < 0.05$ and ***, $p < 0.001$ by one-way ANOVA at the same time point.(B) Wound healing assay.There are four columns corresponding to the four treatment groups:Parent + Vehicle, Resistant + Vehicle, Parent + Treatment, Resistant + Treatment. The MTT assay shows that resistant cells maintain higher survival rates over time, especially when treated with vehicle compared to treatment. The wound healing assay illustrates that resistant cells (both with vehicle and treatment)

exhibit faster and more extensive migration compared to parental cells, indicating enhanced migratory capacity.

3. Figure 3B. “Parent + treatment” showed lower transferrin than “Parent + vehicle”, suggesting that treatment had some effect on parental cells, although GPX4 level was not changed. Can authors discuss this?

Reply 3:

Thank you for your concern. I have added more information in the manuscript in lines 226-237.

Changes in the text:

As shown in Figure 3A and 3B, the expression of transferrin—a ferroptosis marker—was significantly elevated in oxaliplatin-resistant HT29 cells treated with the extract. Conversely, GPX4 expression was reduced in resistant cells supplemented with the extract (Figure 3A, 3C). Notably, the 'Parent + treatment' group exhibited lower transferrin levels compared to the 'Parent + vehicle' group (Figure 3B), suggesting that the extract may exert distinct regulatory effects on ferroptosis markers in parental versus resistant cells. While GPX4 levels remained unchanged in parental cells, the observed reduction in transferrin implies that the treatment could modulate iron metabolism or ferroptosis sensitivity in a cell type-specific manner. This differential response may reflect inherent variations in redox homeostasis or iron-handling pathways between parental and resistant cells, potentially linked to oxaliplatin resistance mechanisms. Further investigation into the interplay between transferrin regulation, GPX4-independent ferroptosis pathways, and drug-resistant phenotypes is warranted to elucidate these findings.

4. Figures 5A. is it possible that the label was not correct? “Resistant + vehicle” showed increase or decrease based on quantification data (5B-D), but it seems this is not the case in 5A gel images.

Reply 4:

Thank you for bringing this descriptive error to our attention. We have made this modification of the label.

5. Figure 5B, C, and D showed related protein expression quantitatively. It looks like it was quantified relatively to GADPH for each treatment and control, but not relative to “Parent + Vehicle” as the relative mRNA expression was shown in Figure 4. It would be good to consistently show the relative level for mRNA and protein in both figures 4 and 5.

Reply 5:

We sincerely appreciate the reviewer’s thoughtful suggestion to harmonize the normalization methods between mRNA and protein expression data. While we agree that methodological consistency is generally desirable, we respectfully maintain our original analytical approach for the following scientifically justified reasons:In Figure 4, mRNA levels were normalized to Parent + Vehicle to emphasize biological changes relative to untreated parental cells, which is

standard in comparative transcriptomics).In Figure 5, protein quantification was normalized to GAPDH as a technical control to account for lane-to-lane variability in Western blotting, per established guidelines. We thank the reviewer for prompting this important discussion about normalization strategies.

6. Line 110: how was the concentration of extract measured?

Reply 6:

Thanks for your concern. Changes in the text: These updates are shown in lines 121-123 of the manuscript.

Changes in the text:

The mixture was heated to 100 ℃ for 1 h under reflux condensation to prevent solvent loss, followed by filtration through a 0.22μm cellulose membrane (Millipore). The aqueous extract was freeze-dried using a Labconco FreeZone 2.5 L lyophilizer (−50 ℃, 48 h), yielding a dry powder.

7. Line 360: Student t test was used for statistical analysis. However, in Methods section Line 159, this was not mentioned.

Reply 7:

Thank you for your concern. I have added more information in Methods section in lines 183-185.

Changes in the text:

Data are presented as the mean ± standard deviation (SD). Differences between more than two groups were compared using Student's t-test (two-tailed, unpaired) to compare differences between two experimental groups.

8. Line 267 “A few others limitations to this study….”: Should it be “A few other limitations…..”?

Reply 8:

Thank you for bringing this descriptive error to our attention. We have made this modification in the manuscript, edited in red in line 295.

Changes in the text:

A few other limitations to this study should also be acknowledged.

9. Only one CRC cell line was assessed. It would be good to include another CRC line to see how consistent the observed effect is.

Reply 9:

We sincerely appreciate the reviewer’s insightful suggestion regarding the inclusion of additional colorectal cancer (CRC) cell lines to validate the consistency of our findings. We fully acknowledge that assessing multiple cell lines would strengthen the generalizability of

our conclusions. In the current study, we focused on the HT29 cell line due to its well-established role as a model for oxaliplatin resistance in CRC and its frequent use in ferroptosis-related research. While the observed effects on transferrin and GPX4 were robust in this model, we agree that extrapolating these results to other CRC subtypes requires caution. Unfortunately, due to time and resource constraints during the revision period, we were unable to incorporate additional cell lines. Thank you for highlighting this critical point, which undoubtedly enhances the translational relevance of our work.