Gene, Cell and Tissue
Volume 8 · Issue 1 · e108995

OPEN PEER REVIEW

Anti-cancer Effects of Pomegranate Seed Oil on Esophageal Cancer Cell Line (KYSE-30)

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SUBMITTED	REVISED	ACCEPTED	DOI
31 Aug 2020	20 Oct 2020	26 Oct 2020	10.5812/gct.108995

About this report

This open peer review (OPR) report shows the public comments made on this article during peer review, by the reviewers, the associate editor and the editor-in-chief, for every revision round, together with the authors' replies. Comments appear only with the consent of the people who wrote them. Reviewers who chose to sign their report are named; the others are shown as "Referee". Comments are edited for language only, and confidential remarks to the editors are not included.

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1 Original submission

5 comments · 6 Sep 2020 – 21 Sep 2020

Referee 1 Reviewer

REJECT 6 Sep 2020

Hello, thanks for your interesting paper. Please include the following comments.

1. Please explain your reason for choosing HF2FF as the normal cell line. You can cite a previous paper.
2. The following quote is not acceptable: "Ethical Approval: The research was done in vitro and there was no need for ethical approval." You need ethical approval for in vitro studies too.
3. In line 7, the solution was sterilized with a 0.2 nm filter. Can this step affect the ingredients of your component?
4. In line 76, why did you use heat-inactivated fetal bovine serum? Is there any special reason?
5. In the keywords, please replace Wound healing with Wound healing assay.
6. Lines 121-126 indicate that your oil has high toxicity! Is there any statistical analysis that provides strong support for your result?
7. I think you need to comprehensively revise the data presentation in your manuscript; however, your results indicate that the effect of your oil on cancer cell death is not significant.

I apologize, but the data as presented are not acceptable.

Best Regards

Referee 2 Reviewer

MINOR REVISION 6 Sep 2020

Dear Editor,
There are several points about the manuscript entitled " Anticancer Effects of Pomegranate Seed Oil on Esophageal Cancer Cell Line" as follows:
Which type of medium was used for cancer cell culture?
At which passage were the cancer cells treated with pomegranate oil?
Why did the authors conduct the wound healing assay on cancer cells when they could have used flow cytometry?
How can the authors declare that the DMSO, which was mixed with pomegranate oil, did not affect cell viability?
Some grammatical errors and misspellings can be seen in the manuscript, so English editing is highly recommended.

Saeedeh Salimi Reviewer

MAJOR REVISION 19 Sep 2020

Dear editor
The manuscript is an in vitro analysis of the effects of Pomegranate Seed oil on an Esophageal Cancer Cell Line. Although the manuscript has some novel aspects, it has several problems, as follows:
1. The Background in the abstract is long and consists of redundant subjects. However, the methods and results sections need to be improved; for example, the authors should add the method and results for wound healing.
2. Please add the word “medium” after RPMI… in the abstract.
3. Please replace “ ELIZA” with “ ELISA” (page 3, line 88).

4. What is Chart 1? (page 3, line 114 and 116)
5. figure 1 has a serious problem. In all columns (each concentration), cell viability is higher at longer exposure times: 24< 48<72. We expect that longer exposure times would lead to lower viability for cancer cells.
6. Indeed, the effect of this component on the fibroblast cell line (control) is similar to (and sometimes greater than) its effect on the cancer cell line, which is not a favorable finding. This problem is present with chemotherapy agents too. The authors should discuss this problem in the discussion.
7. The authors should revise their conclusion in the abstract and at the end of the discussion using tentative wording.
8. Which parameter was measured for wound healing? And which test was used for comparisons between the case and control cell lines and among different concentrations, given that the authors have emphasized the effect of this component on migration?
9. There are no statistical results for the different comparisons. There is only one on page3, line 115.
10. There is no result for the IC50 assay.
11. There are several grammatical errors.

Associate Editor MAJOR REVISION 21 Sep 2020

Based on the reviewers' comments and my reading of the manuscript, this manuscript needs major revisions and a point-by-point reply to the comments.

Editor-in-Chief MAJOR REVISION 21 Sep 2020

Based on the reviewers' and AE's comments, this manuscript needs major revisions and a point-by-point reply to the comments.

2

Revision 1

AUTHORS' RESPONSE TO REVIEWERS

Authors' reply to reviewers

The next pages contain the authors' point-by-point reply to the comments of this revision round, as submitted with the revised manuscript. It is published as the authors wrote it.

Dear Editor in Chief

Thanks for considering and reviewing the manuscript "Anticancer effects of Pomegranate seed oil on Esophageal Cancer cell linev (KYSE-30)". The respectable reviewer made a total of **40 comments** about the manuscript. The comments of reviewers were replied and the manuscript was edited according to the precious comments of the respectable reviewers.

Reviewer 1:
Dear Editor:
There are several points about the manuscript entitled " Anticancer Effects of Pomegranate Seed Oil on Esophageal Cancer Cell Line" as follows:

1-Which type of medium was used for cancer cell culture?
Authors Response: The KYSE-30 cells were cultured in RPMI-1640/Ham's F12 medium. The HF2FF cells were seeded in RPMI-1640 medium. The necessary explanation was added in revised manuscript (Yellow highlighted, page 1, 2).

2- In which passage cancer cells were treated with pomegranate oil?
Authors Response: The cells were passaged for two times to adapt to the culture condition, and were treated at third passage. The necessary explanation was added in revised manuscript (Blue highlighted, page 3).

3- Why the authors conducted the wound healing assay on cancer cells while they could use flowcytometry?
Authors Response: In the present study, we also aimed to evaluate the effect of the pomegranate oil on cancer cell migration, and the wound healing assay was a cheap and simple technique for this purpose. According to several documents, the wound healing assay is a standard *in vitro* technique for monitoring the cell migration, especially for cell types that migrate as collective epithelial sheets. Moreover, it could be done without extra cost during cell culture treatment (references 34, 35).
However, according to the comment of respectable reviewer, flowcytometery technique could also be used. But considering the cost of experiment, the wound healing assay has been preferred. Related references were added in revised manuscript (Green highlighted, page 3).

*34- Grada A, Otero-Vinas M, Prieto-Castrillo F, Obagi Z, Falanga V. Research Techniques Made Simple: Analysis of Collective Cell Migration Using the Wound Healing Assay.The Journal of Investigative Dermatology. February 2017;**137** (2): e11–e16. doi:10.1016/j.jid.2016.11.020*

35- Jonkman JE, Cathcart JA, Xu F, et al. An introduction to the wound healing assay using live-cell microscopy. Cell Adh Migr. 2014;8(5):440-451. doi:10.4161/cam.36224

4- How authors can declare that the DMSO, which mixed to pomegranate oil did not affect cell viability?

Authors Response: DMSO is a widespread and universal solvent for pharmacological substances, therapeutic applications, and treats cultured cells. Considering its toxic effects on cell culture on high concentration, researchers suggest to use it at low concentration (lower than 0.1 or 0.5 or 1%). So, in this study the final concentration of DMSO in cell culture were used at 0.1%.

The extra explanation and related-reference (references 22-24) were added in revised manuscript (Green highlighted, page 2).

22. Jamalzadeh L, Ghafoori H, Sariri R, Rabuti H, Nasirzade J, Hasani H, et al. Cytotoxic Effects of Some Common Organic Solvents on MCF-7, RAW-264.7 and Human Umbilical Vein Endothelial Cells. *Avicenna J Med Biochem*. 2016;**4**(1):e33453.

23. Banerjee N, Talcott S, Safe S, Mertens–Talcott SU. Cytotoxicity of Pomegranate Polyphenolics in Breast Cancer Cells in Vitro and Vivo - Potential Role of miRNA-27a and miRNA-155 in Cell Survival and Inflammation. 2012;**136**(1):21-34.

24. Leporini M, Catinella G, Bruno M, Falco T, Tundis R, Loizzo MR. Investigating the Antiproliferative and Antioxidant Properties of Pancratium maritimum L. (Amaryllidaceae) Stems, Flowers, Bulbs, and Fruits Extracts. *Evid Based Complement Alternat Med*. 2018;**2018**(9301247).

5- Some grammatical points and misspelling can be seen in the manuscript, so English editing is highly recommended.

Authors Response: According to the comment of respectable reviewer the manuscript was completely edited for English writing. (Written in blue color)

Reviewer 2:

Hello, thanks for your interesting paper. Please include the following comments.

1. please explain your reason to choose HF2FF as the normal cell line, you can cite any previous paper.

Authors Response: The application of HF2FF cell line as normal cells was according to the previous studies, and also its availability for us. So, the related-references were added in revised manuscript (references 25, 29, 30) (Blue highlighted, page 2).

However, considering the epithelial-originated of KYSE-30, we agree that it was better to use epithelial cells as a normal cell line.

25. Fallah N, Ebrahimi S. The Anti-Cancer Effect of Watercress (Rorripa Nasturtium Aquaticum) Extract on Breast Cancer Cells. *Zahedan J Res Med Sci*. 2016;**18**(9):e2725 doi: .

29. Kiani S, Akhavan-Niaki H, Fattahi S, Kavoosian S, Babaian Jelodar N, Bagheri N, et al. Purified sulforaphane from broccoli (Brassica oleracea var. italica) leads to alterations of CDX1 and CDX2 expression and changes in miR-9 and miR-326 levels in human gastric cancer cells. *Gene*. 2018;**678**:115-23. doi:org/10.1016/j.gene.2018.08.026.
30. Moradi R, Ebrahimi S, Taravati A, Asrardel F, Khorasani HR, Aghajanpour-Mir SM, et al. Cytotoxic Effects of the Hydroalcoholic Extract of Rorippa Nasturtium Aquaticum on Hela Cell Line. *IBBJ*. 2017;**3**(2):73-9.

2. The following quota is not acceptable "Ethical Approval: The research was done in vitro and there was no need for ethical approval." you need ethical approve for in vitro studies too.
Authors Response: With respect to the right comment of reviewer, we should explain that there was not any human participate in the present study. Also there was no use of human samples such as blood, tissue, serum, plasma, urine or other fluids. Also, laboratory animals have not been used in the present study.

This study was done as an M.S thesis in Payame Noor University, and was registered and approved in Iranian Research Institute for Information Science and Technology (Iran Doc) with registered code of 8812222. (Yellow highlighted, page 6)

3. line 7., the solution was sterilized with 0.2 nm filter, can this step affect the ingredients of your component?
Authors Response: Sterilization through 0.2 nm filter is commonly used for plant-derived extracts and substances in literatures. The related-references were added in revised manuscript (references 25-28) (Pink highlighted, page 2)

25. Fallah N, Ebrahimi S. The Anti-Cancer Effect of Watercress (Rorripa Nasturtium Aquaticum) Extract on Breast Cancer Cells. *Zahedan J Res Med Sci*. 2016;**18**(9):e2725 doi: .
26. El-Sayed WM, Hussin WA, Mahmoud AA, AlFredan MA. The Conyza triloba Extracts with High Chlorophyll Content and Free Radical Scavenging Activity Had Anticancer Activity in Cell Lines. *Biomed Res Int* 2013;**2013**(945638).
27. Mohamadi T, Hoveizi E. Comparison of anti proliferative effect of cinnamon (Cinnamomumzeylanicum) hydroalcoholic extract with cyclophosphamide medicine on A459 Cancer Cells. *RaziJournal ofMedicalSciences*. 2018;**25**(167).
28. Khojasteh A, Metón I, Camino S, Cusido RM, Eibl R, Palazon J. In Vitro Study of the Anticancer Effects of Biotechnological Extracts of the Endangered Plant Species Satureja Khuzistanica. *Int J Mol Sci*. 2019;**20**(10):2400.

4. line 76, why did you use heat inactivated fetal bovine serum? is there any special reason?

Authors Response: Heat inactivation is used to inactivate complement system. It is important for cells that are used in cytotoxicity assays where complement proteins may have an unwanted influence.
For this, commercial companies like Biosera present heat inactivated FBS for use in cell culture systems
(Bio Sera product: Foetal Bovine Serum, Heat Inactivated Product Codes FB-1051H, FB-1350H, FB-1365H)
Related references were added in revised manuscript (references 31-33) (Yellow highlighted, page 3)

31. Bruinink A, Tobler U, Hälg M, Grünert J. Effects of serum and serum heat-inactivation on human bone derived osteoblast progenitor cells. *J Mater Sci Mater Med*. 2004;**15**(4):497-501. [PubMed:15332624].
32. Johnson M. Fetal Bovine Serum. *MATER METHODS*. 2012;**2**:117. doi:org/10.13070/mm.en.2.117.
33. Triglia R, Linscott W. Titers of nine complement components, conglutinin and C3b-inactivator in adult and fetal bovine sera. *Mol Immunol*. 1980;**17**:741-8
34. Grada A, Otero-Vinas M, Prieto-Castrillo F, Obagi Z, Falanga V. Research Techniques Made Simple: Analysis of Collective Cell Migration Using the Wound Healing Assay. *The Journal of Investigative Dermatology*. 2017;**137**(2):e11-e6. doi:10.1016/j.jid.2016.11.020.

5. in keywords, please substitute Wound healing by Wound healing assay.
Authors Response: According to the respectable reviewer comment, wound healing assay was added in key words (Green highlighted, page 1).

6. 121-126, indicates that your oil has a high toxicity! is there any statical analysis which indicates stron support for your result?
Authors Response: The results in this study were presented according to the mentioned statistical analysis. The tumor cell viability was decreased significantly in treated groups compared with the control group. The viability of normal cells were also decreased, however the decrease rate of normal cells were significantly lower compared to the tumor cells after 24h and 48 h of treatment. The definition was added in the revised manuscript (Grey highlighted, page 4).

7. i think you need comprehensive revise for your manuscript in data presentation, however your results indicate that the effete of your oil is not significant on cancer cell death.
i apologize, but the data in this way is not acceptable.
Authors Response: we would appreciate the comment of the respectable reviewer. According to the results, in 24 h the viability of KYSE-30 cells were decreased from 94% (concentration of 0.03125 mg/ml) to 12% (2 mg/ml). In 48 h, the viability of tumor cells was decreased from 90% (at the concentration of 0.0625 mg/ml) to 18% (2 mg/ml). The viability of tumor cells was decreased from 99% (at the concentration of 0.0625 mg/ml) to 29% (2 mg/ml).

Indeed, according to the statistical analysis, the cell viability were decreased **significantly** in treated groups compared with the control group ($p < 0.001$)

Best Regards

Reviewer 3:
Dear editor
The manuscript is an in vitro analysis on the effects Pomegranate Seed oil on Esophageal Cancer Cell Line. Although the manuscript has some nobilities, it has several problems as bellow:
1. The Background in the abstract is long and consists of redundant subjects. However, the methods and results sections need to improve, for example, the authors should add the method and the results of wound healing.
Authors Response: The Abstract were edited and revised according to the comment of respectable reviewer. The background of abstract were also summarized (Blue highlighted, page 1)

2. Please add the “medium” word after RPMI…. In abstract.
Authors Response: The mentioned word was added in abstract (Green highlighted, page 1)

3. Please replace the “ ELIZA” with “ ELISA”(page 3, line 88)
Authors Response: The mentioned word was corrected in the revised manuscript (Pink highlighted, page 3)

4. What is Chart 1? ? (page 3, line 114 and 116)
Authors Response: The mentioned word (chart 1) was corrected with Figure 1 in the revised manuscript (Yellow highlighted, page 4)

5. figure 1 has a serious problem? In all columns (each concentration), the cell viability is higher in longer times 24< 48<72. We expect that the higher times of exposure leads to lower viability for cancerous cells.
Authors Response: The presented results were just according to the outcome data of MTT test. Moreover, the similar results were found in several documents which show that the most cytotoxicity and cell death were happened at the shorter time of treatment (24 h or 48 h).
The extra definition and related references (references 39-42) were added in discussion section in revised manuscript (Blue highlighted, page 4, 5)

39. Aziz A, Zuraina MY, Zulkiflee AB. Concentration and Time Dependent Cytotoxic effect of Methanolic Crude Extracts of Pseuduvaria macrophylla on the Human Cancer Cell Line. *International Congress on Science & Technology*. 2016;**Bali, Indonesia**.
40. Fathi N, Tafvizi F, Mirzaie A. Antibacterial and anti-cancer activities of Artemisia turcomanicaextract on gastric cancer cell line (AGS) and its

interaction on cyclinD1 and cyckinE genes. *Journal of Medicinal Plants*. 2020;**19**(74):163-76.
41. Nabiuni M, Bahar Ara J, Amini E, Jalali H. Determination of Apoptotic Doses of Bee Venom on Human Promyelocytic Leukemia Cells *Journal of Cell & Tissue (JCT)* 2012;**2**(4):425-33.
42. Bajbouj K, Schulze-Luehrmann J, Diermeier S, Amin A, Schneider-Stock R. The anticancer effect of saffron in two p53 isogenic colorectal cancer cell lines. *BMC Complementary and Alternative Medicine* 2012;**12:69**.

6. Indeed, the effect of this component on fibroblast cell line(control) similar to cancerous cell line (some times higher) is not a favorite finding. This problem is present in the chemotherapy agents too. The authors should discuss about this problem in discussion.
Authors Response: with respect to the comment of reviewer, the viability of normal cells was significantly higher than tumor cells at 24 h and 48 h of treatment.

7. The authors should revise their conclusion in abstract and the end of the discussion with probable sentences.
Authors Response: According to the comment of respectable reviewer, the mentioned parts were corrected (Pink highlighted, page 1, and 6)

8. Which parameter was measured for wound healing? And which test was used for comparison between case and control cell lines and different concentrations? Because the authors have emphasized the effect of this component on migration.
Authors Response: Wound healing assay was used to evaluate the effect of the pomegranate oil on cancer cell migration. Migration ability of tumor cells is a critical characteristic for metastasis, and invasion to distant organs.
According to several documents, the wound healing assay is a standard *in vitro* technique for monitoring the cell migration, especially for cell types that migrate as collective epithelial sheets. (References 34, 35) (Green highlighted, page 3).

This assay is done by making a scratch on a cell monolayer and capturing microscopic images at regular intervals. The scratch produces a cell-free area and the cells with migration ability could migrate and close the gap.
According to the cell images in present study, the existing gap on cell monolayer was rather closed with emigrant cells in control sample. But the migration of tumor cells was decreased along with the concentration increasing of the pomegranate oil. So, the gap remained empty at the concentration of 2µg/ml which indicated the effect of pomegranate oil on migration ability of KYSE-30 cells.

*34- Grada A, Otero-Vinas M, Prieto-Castrillo F, Obagi Z, Falanga V. Research Techniques Made Simple: Analysis of Collective Cell Migration Using the Wound Healing Assay.The Journal of Investigative Dermatology. February 2017;**137** (2): e11–e16. doi:10.1016/j.jid.2016.11.020*

35- Jonkman JE, Cathcart JA, Xu F, et al. An introduction to the wound healing assay using live-cell microscopy. Cell Adh Migr. 2014;8(5):440-451. doi:10.4161/cam.36224

9. There are no statistical results about different comparisons. There is only one in the page3 line 115.
Authors Response: the significance level were added in abstract and result section (Grey highlighted page 1 and 4)

10. There is no result for IC50 assay.
Authors Response: According to the comment of respectable reviewer, the results of IC50 were added in results section in the revised manuscript (Red highlighted and table 1, page 4).
11. There are several grammatical errors.
Authors Response: According to the comment of respectable reviewer the manuscript was completely edited for English writing by a native English speaking colleague (written in blue color).

Reviewer 4:
Dear Author(s)
I read your manuscript. Due to the increasing prevalence of many diseases and gastrointestinal disorders, your research activity shows the role of complementary and alternative treatments well. I have several comments and suggestions to improve your manuscript.

1- In some places there are small grammatical errors that can be corrected with a review.
Authors Response: According to the comment of respectable reviewer the manuscript was completely edited for English writing by a native English speaking colleagues

You have implemented this protocol in a part of the text of materials and methods: "The cultured cells of KYSE-30 and the HF2FF were collected and counted. Then a total of cells per well were cultured in 96-well plates and treated at 75% confluence with different concentration of pomegranate oil (2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125 mg/ml) for three period of time (24, 48, 72 h). After these three times, the supernatant was removed and washed with PBS. Then, 50 µl of MTT solution (5 mg/ ml) was added to each well and stored for 3 hours at 37 °C." 2. How many times did you repeat this step?
Authors Response: Three replicate were considered for each concentration. It was mentioned in the manuscript as: All tests were carried out three times (Grey highlighted, page 4).

3. The protocol of this project has been approved by which research and ethics committee and in which institute or university this project has been carried out.
Authors Response: we should explain that there was not any human participate in the present study. Also there was no use of human samples such as blood, tissue, serum, plasma, urine or other fluids. Also, living laboratory animals have not been used in the present study.

This study was done as an MS thesis in Payame Noor University of Babol. The study was registered and approved in Iranian Research Institute for Information Science and Technology (Iran Doc) with registered code of 8812222. (Yellow highlighted, page 6)

4- The half maximal inhibitory concentration (IC50) is correct not the 50% mortality rate (IC50). How was IC50 calculated?
Authors Response: According to the comment of respectable reviewer, the IC50 definition was corrected and the related explanation about IC50 was added in the revised manuscript (Red highlighted, page 3). Also, the results of IC50 were added in results section in the revised manuscript (Red highlighted, page 4).

5- It seems that the results for concentrations 62.5 and 31.25 need more precision. Because the results presented should be the average of all data and can rarely reach 100%.
Authors Response: The presented results were just according to the outcome data of MTT test, without any correction. Similar results have been shown in the mentioned document. (Reference 42)

42. Bajbouj K, Schulze-Luehrmann J, Diermeier S, Amin A, Schneider-Stock R. The anticancer effect of saffron in two p53 isogenic colorectal cancer cell lines. *BMC Complementary and Alternative Medicine* 2012;**12:69**.

6- It seems that the results of Figures 2 and 3 do not show a significant difference between the two cell lines.
Authors Response: The viability of tumor cells were significantly lower than normal cells at 24 h and 48 h ($p<0.05$). However at 72 h, the viability decrease of normal cells were higher compared with the tumor cells.

7- This sentence needs a reference: Golestan province in Iran has been long 138 known as one of the areas with a high incidence of gastrointestinal cancers.
Authors Response: According to the comment of respectable reviewer, two references were added for the mentioned sentence. (Pink highlighted, page 4)

2 Revision 1

4 comments · 3 Oct 2020 – 7 Oct 2020

Azam Asemi Rad Reviewer — ACCEPT 3 Oct 2020

Considering that the manuscript entitled " Anticancer Effects of Pomegranate Seed Oil on Esophageal Cancer Cell Line (KYSE-30) " underwent peer review in the previous version, I reviewed the sections modified by the author in response to the reviewers. It should be pointed out that this version is suitable for acceptance.
Sincerely,

Referee 1 Reviewer — MAJOR REVISION 5 Oct 2020

It is obvious that the authors attempted to improve the manuscript.
However, the authors have declined to address some comments or have addressed them only partially.

As you know, ethical approval is not limited to human studies. I am not sure that the registered code can be used instead of an ethical approval code!

Please mention the appropriate reference for your claim "Heat inactivation is used to inactivate complement system. It is important for cells that are used in cytotoxicity assays where complement proteins may have an unwanted influence."

Your data presentation is still poor! And this is the main problem in your manuscript.

Associate Editor — MAJOR REVISION 6 Oct 2020

Based on the reviewers' and AE's comments, this manuscript needs major revisions. The authors must revise it precisely and reply to the comments point by point.

Editor-in-Chief — MAJOR REVISION 7 Oct 2020

Based on the reviewers' and AE's comments, this manuscript needs major revisions. The authors must revise it precisely and reply to the comments point by point.

3

Revision 2

AUTHORS' RESPONSE TO REVIEWERS

Authors' reply to reviewers

The next pages contain the authors' point-by-point reply to the comments of this revision round, as submitted with the revised manuscript. It is published as the authors wrote it.

Dear Editor in Chief

Thanks for reconsidering and reviewing the manuscript "Anticancer effects of Pomegranate seed oil on Esophageal Cancer cell line (KYSE-30)".

The comments of reviewers were replied and the manuscript was edited according to the comments of the respectable reviewers.

Reviewers' Comments:
Reviewer 1:

Dear Author(s)
Thanks a lot for correcting the comments. However, I noticed that some of my comments went unanswered.

1- How was IC50 calculated?
Response: For IC50 calculation, the graph of concentration against the growth inhibition (x-y graph) was plotted and the linear equation was obtained. The value of 50 was used for Y in the linear equation of the graph to obtain the IC50 concentration.

With respect to the comment of respectable reviewer, the definition was mentioned in the revised manuscript, in Materials and Methods, section Statistical Analysis. (yellow highlighted, page 3)

The related image for IC50 at 24 h was also added in the revised manuscript. (Figure 2) (yellow highlighted in page 4)

2- In addition, I suggest that the images provided be changed, in other words, the current images do not look very scientific.
Response: According to the comment of respectable reviewer, the images have been modified in the revised manuscript.

3- The question also arises that despite the very comprehensive and good studies in this regard, what was the need for your study?
For example:
Molecular targets of pomegranate (Punica granatum) in preventing cancer metastasis
Iran J Basic Med Sci. 2019 Sep; 22(9): 977–988.
doi: 10.22038/ijbms.2019.34653.8217

Pomegranate for Prevention and Treatment of Cancer: An Update
Molecules. 2017 Jan; 22(1): 177.
Published online 2017 Jan 24. doi: 10.3390/molecules22010177

Please explain the importance of the subject in the text of the manuscript.
Response: With respect to the right comment of the reviewer, there are several researches about the anticancer properties of the pomegranate. However, as far as we searched, there was no study regarding the effects of pomegranate oil on **esophageal cancer**. Actually, the

effect of pomegranate on esophageal cancer was not mentioned in the above perfect review references as well.
The first reference was added in the revised manuscript.(Ahmadiankia, 2019) (reference 49).

So, considering the different underlying carcinogenesis mechanism in various cancers, and the high prevalence of esophageal cancer in Iran, our study was carried out to evaluate the effects of pomegranate oil on this significant cancer.
The related definition was added in the introduction section in revised manuscript (yellow highlighted, page 2)

Reviewer 2:

Considering that the manuscript entitled" Anticancer Effects of Pomegranate Seed Oil on Esophageal Cancer Cell Line (KYSE-30)" had a peer review in the previous version, I reviewed the sections modified by the author in response to the reviewers. It should point out that this version is suitable for accepting.

Sincerely,

Reviewer 3:

It is obvious that authors attempted to improve the manuscript.

However some comments are declined by the authors or partially updated.

1- as you know Ethical Approval is not limited to human studies. I am not sure that registered code can be used instead of Ethical Approval code!
Authors Response: With respect to the comment of respectable reviewer, this study has been done and finished as an M.S thesis in Payame Noor University, and was registered and approved in **Iranian Research Institute for Information Science and Technology (Iran Doc) with registered code of** 8812222. (Yellow highlighted, page 6).

So, considering the ending of the study, it is not possible to request for an ethical approval code.
Indeed, our recently published paper and accepted paper (in journals of university of medical sciences) which were similarly carried out on cancer cell lines used the same code of Iran Doc.

2- Please mention the appropriate reference for your claim "Heat inactivation is used to inactivate complement system. It is important for cells that are used in cytotoxicity assays where complement proteins may have an unwanted influence."

Response: According the comment of respectable reviewer, three references were mentioned in the last revision of manuscript. (31-33)

Furthermore, the related description is available at the ATCC (American Type Culture Collection).
webpagehttps://www.google.com/url?sa=t&rct=j&q=&esrc=s&source=web&cd=&cad=rja&uact=8&ved=2ahUKEwjFqIeg0qXsAhVmQxUIHWmyAqcQFjABegQIChAC&url=https%3A%2F%2Fwww.atcc.org%2Fsupport%2Ffaqs%2Fff985%2FPurpose%2Bof%2Bheat-inactivating%2Bserum-154.aspx&usg=AOvVaw3TTjQTjDGieRCt_exJJ5BC

Also, another main related reference was added in current revision of manuscript (Thomas et al., 2015). (reference 34) (blue highlighted in pages 3 and 8)

3- your data presentation is still poor! and this is the main problem in your manuscript.
Response:
Authors Response: The presented data were just according to the outcome results of the study which were performed in triplicate and repeated for three times. The best result of cytotoxicity on esophageal cancer cells was observed at 24 h of treatment. Also, the viability of tumor cells were significantly lower than normal cells at 24 h and 48 h ($p<0.05$).
Moreover, the similar results were found in several documents which show that the most cytotoxicity and cell death were happened at the shorter time of treatment (24 h or 48 h). (references 40 -43)
The extra definition and related references (references 40-43) were added in discussion section in revised manuscript (Blue highlighted, page 4, 5)

40. Aziz A, Zuraina MY, Zulkiflee AB. Concentration and Time Dependent Cytotoxic effect of Methanolic Crude Extracts of Pseuduvaria macrophylla on the Human Cancer Cell Line. *International Congress on Science & Technology*. 2016;**Bali, Indonesia**.
41. Fathi N, Tafvizi F, Mirzaie A. Antibacterial and anti-cancer activities of Artemisia turcomanicaextract on gastric cancer cell line (AGS) and its interaction on cyclinD1 and cyckinE genes. *Journal of Medicinal Plants*. 2020;**19**(74):163-76.
42. Nabiuni M, Bahar Ara J, Amini E, Jalali H. Determination of Apoptotic Doses of Bee Venom on Human Promyelocytic Leukemia Cells *Journal of Cell & Tissue (JCT)* 2012;**2**(4):425-33.
43. Bajbouj K, Schulze-Luehrmann J, Diermeier S, Amin A, Schneider-Stock R. The anticancer effect of saffron in two p53 isogenic colorectal cancer cell lines. *BMC Complementary and Alternative Medicine* 2012;**12:69**.

Additionally, the results of the wound healing assay indicated a decrease in the mobility and migration of esophageal tumor cells after treatment with different concentration of pomegranate seed oil.

3 Revision 2

4 comments · 10 Oct 2020 – 12 Oct 2020

Referee 1 Reviewer

REJECT 10 Oct 2020

Dear editor in chief,

Unfortunately, the authors' comments are not satisfactory to me.
The data presentation is poor, and the authors' responses do not support the accuracy of the methods and results.

Saeedeh Salimi Reviewer

MINOR REVISION 11 Oct 2020

Dear editor
The authors have corrected the manuscript according to previous comments. I recommend that the authors add complete information about the materials, for example, Gibco, ... and others.
Best regards

Associate Editor

MINOR REVISION 12 Oct 2020

Based on the reviewers' comments and my reading, revisions have been made. However, some revisions are still needed, and a response to the comments is required.

Editor-in-Chief

MINOR REVISION 12 Oct 2020

Based on the reviewers' comments and my reading, revisions have been made. However, some revisions are still needed, and a response to the comments is required.

4

Revision 3

AUTHORS' RESPONSE TO REVIEWERS

Authors' reply to reviewers

The next pages contain the authors' point-by-point reply to the comments of this revision round, as submitted with the revised manuscript. It is published as the authors wrote it.

Dear Editor in Chief

Thanks for reconsidering and reviewing the manuscript "Anticancer effects of Pomegranate seed oil on Esophageal Cancer cell line (KYSE-30)".
The comments of reviewers were replied and the manuscript was edited according to the comments of the respectable reviewers.

Reviewers' Comments:

Reviewer 1:

Dear editor

The authors have corrected the manuscript according to previous comments. I recommend the authors to add complete information for materials, for example, Gibco,..... and others. Best regards

Response: According to the comment of respectable reviewer, the complete information for materials was added in the revised manuscript. (Yellow highlighted, pages 2 and 3)

Reviewer 2:
Dear editor in chief:

Unfortunately the authors comments are not satisfying for me.
The dated presentation is poor and the authors responses do not support the accuracy of the methods and results

Authors Response: we respect the opinion of the reviewer.
We did our best to comprehensively answer the comments of the respectable reviewer, and we referred to 20 more new references in the last two revision of the manuscript.

However as we mentioned previously, the presented data were just according to the outcome results of the study, without any artificial correction.

Moreover, it is the first report about the effects of pomegranate seed oil on esophageal cancer which is so prevalent in Iran. Our results showed the cytotoxic effects of pomegranate oil on esophageal cancer and the best result was observed at 24 h of treatment.

Reviewer 3:

It can be accepted.

4 Revision 3

3 comments · 25 Oct 2020 – 26 Oct 2020

Saeedeh Salimi Reviewer — ACCEPT 25 Oct 2020

Dear editor
The manuscript is acceptable.
Best

Associate Editor — ACCEPT 26 Oct 2020

Based on the reviewers' comments and my reading, the revisions were done well. Thus, this manuscript can be accepted for publication in GCT.

Editor-in-Chief — ACCEPT 26 Oct 2020

The revisions based on the reviewers' and AE's comments were completed well. Thus, this manuscript can be accepted for publication in GCT.