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OPEN PEER REVIEW

Regulation of Apoptosis, Autophagy, and Metastasis by Luteolin in Human Bladder Cancer EJ138 Cells: An Experimental Study

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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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Contents

1 **Original submission** Referee 1, Referee 2 2
2 **Revision 1** Referee 2, Associate Editor 4
Authors' reply to reviewers 5
Reviewer and editor comments 16
3 **Revision 2** Referee 2, Associate Editor 17
Authors' reply to reviewers 18
Reviewer and editor comments 20

1 Original submission

2 comments · 10 Sep 2024 – 11 Sep 2024

Referee 1 Reviewer

MINOR REVISION 10 Sep 2024

Please note the provided comments and ensure that they are carefully considered and that any necessary corrections are made to the relevant sections of the manuscript.

Background

1. The background section should give a thorough overview of the topic, including relevant studies and highlighting any existing knowledge gaps. This will help to demonstrate the importance and originality of the research, especially in the final paragraph. You should emphasize the significance and novelty of the research in the last paragraph.

Results

2. The quantity of cells utilized at each phase of the study has not been documented. It is imperative to provide this information.

Discussion

3. It is advisable to enhance the discussion section by incorporating pertinent literature for comparative analysis, delineating the implications of the study's findings, addressing potential constraints, and proposing prospects for future research.

4. The writing in the discussion section lacks clarity and coherence and could benefit from improvement.

5. It is essential to acknowledge the limitations of your study. However, refrain from reiterating the results in the discussion section.

Conclusion

6. The conclusion should be succinct yet impactful, encapsulating the significant outcomes of the study and emphasizing the relevance and applications of the research findings to the scientific community.

7. It is advisable to refrain from referencing the BC patient in your work, as your research was conducted solely on cell lines and not on patients or through in vivo studies.

Referee 2 Reviewer

MAJOR REVISION 11 Sep 2024

The paper examines how Luteolin regulates apoptosis, autophagy, and metastasis in human bladder cancer EJ138 cells. Luteolin caused cell death in human bladder cancer cells by increasing the expression of P53, ULK1, and ATG12, while decreasing the expression of BCL2 mRNA. I really liked evaluating this manuscript. Nevertheless, I have pinpointed these significant problems that need to be addressed:

Q1. In the " Material and methods" section, page 3, line 90, why did the authors choose Luteolin as a treatment at 17.92 µM for 48 hours? Please clarify.

Q2. In the " Material and methods" section, page 3, line 94, please explain why the authors did not conduct the Western Blot test and instead concentrated on the RT-PCR findings.

Q3. In the " Material and methods" section, page 3, line 105, please identify which test was analyzed using One-way ANOVA and the T test.

Q4. In the " Results" section, page 4, line 111, how did lutein restrain cell growth in a dose-dependent manner? Please explain.

Q5. In the " Discussion " section, page 5, line 160, could the lack of change in gene expression related to metastasis be attributed to the presence of confounding factors despite the presence of lutein? Please explain.

Q6. In the " conclusion " section, page 5, line 160, according to the study findings, is lutein a possible anti-cancer medication or can it be further studied as a supplemental treatment alongside chemotherapy?
Q7. It is more appropriate to avoid using the term "proved" and its synonyms when referring to observations or reports in a scientific context. Please correct this in the manuscript.

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.

27 September 2024

Dear Editor-in-Chief

Manuscript ID: https://ijpr.brieflands.com/en/document/details/153408

On behalf of my co-authors, I submit the responses to the reviewer's comments and the revised manuscript entitled "Regulation of Apoptosis, Autophagy, and Metastasis by Luteolin in Human Bladder Cancer EJ138 Cells".

Please find in the next pages the responses to the reviewer's comments. We have addressed all of the comments and revised the manuscript accordingly. The revised sections have been highlighted in the text. We are appreciative of the reviewer for their important input. We believe the suggested changes have helped to substantially improve the quality of this paper over the previous version.

We want to extend our appreciation for the given opportunity to have this work considered for publication in the journal of "IJ Pharmaceutical Research". Please do not hesitate to contact me should you have any further questions.

Yours faithfully

Mohammad Vakili Ojarood

Cancer Immunology and Immunotherapy Research Center, Ardabil University of Medical Sciences, Ardabil, Iran

On behalf of my co-authors

Reviewer's comments:

Reviewer #1:

Please noted and ensure that the provided comments are carefully considered and that any necessary corrections are made to the relevant sections of the manuscript.

Q1. The background section should give a thorough overview of the topic, including relevant studies and highlighting any existing knowledge gaps. This will help to demonstrate the importance and originality of the research, especially in the final paragraph. You should emphasize the significance and novelty of the research in the last paragraph.

R: Thanks for your consideration. The background was checked and the suggested item has been added (P 2, Line 66-71).

Q2. The quantity of cells utilized at each phase of the study has not been documented. It is imperative to provide this information.

R: Thank you. The suggested quantity of cells was highlighted in manuscript. P 3, Line 95 / P3, Line 102.

Q3. It is advisable to enhance the discussion section by incorporating pertinent literature for comparative analysis, delineating the implications of the study's findings, addressing potential constraints, and proposing prospects for future research.

R: Thanks for your constructive comment.

Q4. The writing in the discussion section lacks clarity and coherence, and could benefit from improvement.

R: Thanks for help us to clarify this issue.

Q5. It is essential to acknowledge the limitations of your study. However, refrain from reiterating the results in the discussion section.

R: Thanks for your constructive comment. The suggested issue has been explained in discussion. P 6, Line 186-190.

Q6. The conclusion should be succinct yet impactful, encapsulating the significant outcomes of the study and emphasizing the relevance and applications of the research findings to the scientific community.

R: Thanks for your constructive comment. The conclusion have been checked and luteolin's capacity has been disscused.

Q7. It is advisable to refrain from referencing the BC patient in your work, as your research was conducted solely on cell lines and not on patients or through in vivo studies.

R: Thanks for your constructive comment. All the references have been checked again and in vivo studies have been deleted (references 21 and 28).

Reviewer #2:

The paper examines how Luteolin regulates apoptosis, autophagy, and metastasis in Human Bladder Cancer EJ138 Cells. Luteolin caused cell death in Human Bladder Cancer cells by increasing the expression of P53, ULK1, and ATG12, while decreasing the expression of BCL2 mRNA. I really liked evaluating this manuscript. Nevertheless, I have pinpointed these significant problems that need to be addressed:

Q1. In the " Material and methods" section, page3, line 90, why did the authors choose Luteolin as a treatment with a value of 17.92µM for 48 hours? Please clarify.

R: Thanks for your consideration. This value was utilized due to IC50 value of the impact of luteolin on the EJ138 BC cell line was 17.92µM for 48 h. P 3, Line 102-103.

Q2. In the " Material and methods" section, page3, line 94, please explain why the authors did not conduct the Western Blot test and instead concentrated on the RT-PCR findings.

R: Thanks for your consideration. focusing on the RT-PCR findings instead of the Western Blot was due to several reasons. RT-PCR is faster and less technically complex than Western blotting, which requires additional steps like protein extraction and antibody probing. Western blotting requires specific antibodies for each protein of interest, which may not have been affordable or available. On the other hand, RT-PCR only needs gene-specific primers, which are easier to obtain and less expensive.

Q3. In the " Material and methods" section, page 3, line 105, Please identify which test was analyzed by the One-way ANOVA and the T test.

R: Thank you for being very careful in our manuscript. Student's t-test was used to compare the means of two groups to determine if there is a significant difference between them. P 4, Line 119.

Q4. In the " Results" section, page 4, line 111, how did lutein restrain cell growth in a dose-dependent manner? Please explain

R: Thank you for your comment. Based on figure 1, cell viability has been reduced by increasing luteolin dose from 10µM to 50µM. Therefore, luteolin restrain cell growth in a dose-dependent manner. P 4, Line 124-125.

Q5. In the " Discussion " section, page 5, line 160, Could the lack of change in gene expression related to metastasis be attributed to the presence of confounding factors despite the presence of lutein? Please explain

R: Thanks for your careful reading of our manuscript. The lack of observed changes in metastasis-related gene expression despite the presence of luteolin could be attributed to several confounding factors, including the timing of analysis, complexity of metastasis regulation, suboptimal luteolin dosage, post-transcriptional regulation, cellular heterogeneity, pathway cross-talk, and environmental factors. recognizing these confounders is essential for designing more precise experiments that can fully capture luteolin's impact on metastasis, potentially involving a combination of techniques (e.g., Western blot for protein analysis, invasion assays, or migration assays) to complement RT-PCR findings.

Q6. In the " conclusion " section, page 5, line 160, According to the study findings, is lutein a possible anti-cancer medication or can it be further studied as a supplemental treatment alongside chemotherapy?

R: Thanks for your consideration. While luteolin demonstrates considerable potential as an anti-cancer agent, it is more likely to be studied as an adjuvant treatment or complementary alongside chemotherapy due to its multi-targeted effects on cancer cells, its ability to overcome resistance, and its possible role in minimizing side effects. Further research, including in vivo studies and clinical trials, is necessary to thoroughly assess its therapeutic potential and to determine the best strategies for incorporating it into cancer treatment regimens. P 6, Line 192-199.

Q7. It is more appropriate to avoid using the term "proved" and its synonyms when referring to observations or reports in a scientific context. Please correct in the manuscript.

R: Thank you, the suggested item has been replaced.

Reviewer Assistant Comments:

These comments were generated by AI based on your manuscript. Please review them and consider incorporating the suggestions to enhance your article.

Based on the provided study titled "Regulation of Apoptosis, Autophagy, and Metastasis by Luteolin in Human Bladder Cancer EJ138 Cells," here is a comprehensive review using the relevant checklists for observational studies, specifically the STROBE cross-sectional checklist.

1. ***Title**: The title should indicate the study's design. The current title is descriptive but does not explicitly state the study design (e.g., "cross-sectional study").*

R: Thank you for your comment, the proposed issue has been corrected.

*2. **Abstract**: The abstract provides a summary of what was done and what was found. It is structured but could benefit from explicitly mentioning the study design.*

R: Thanks for your constructive comment. It was added.

Introduction

3. ***Background/Rationale****: The background is well-explained, highlighting the challenges in BC therapy and the potential role of luteolin.*

R: Thank you for your comment.

4. ***Objectives****: The objectives are clearly stated, focusing on the regulation of mRNA expression of specific genes involved in apoptosis, autophagy, and metastasis.*

R: R: Thank you for your comment.

Methods

5. ***Study Design****: The study design is not explicitly mentioned early in the paper. It should be clearly stated that this is a cross-sectional study.*

R: Thanks for your careful reading of our manuscript, the proposed issue has been corrected. P3, Line82

6. ***Setting****: The setting, including locations and relevant dates, is not described. This information should be included.*

R: Not related.

7. ***Eligibility Criteria****: The criteria for selecting EJ138 BC cells should be detailed.*

R: Thank you. EJ138 BC cells were selected for this study based on their established use as a model for BC research. These cells are widely utilized in experiments exploring cancer biology, treatment response, and gene expression analysis because of their malignant characteristics and relevance to human BC. No further specific selection criteria, such as genetic mutations or prior treatments, were applied in selecting these cells for the study.

*8. **Variables**: The study clearly defines the outcomes (mRNA expression of BCL2, P53, ULK1, ATG12, MMP2, and MMP9) and the exposure (luteolin treatment).*

R: Thank you for your comment.

*9. **Data Sources/Measurement**: The methods of assessment (MTT assay, flow cytometry, RT-PCR) are described, but the comparability of these methods is not discussed.*

R: Thank you for your comment. In terms of comparability, these methods are complementary rather than interchangeable. MTT provides a broad overview of cell viability, flow cytometry gives specific details on the mechanisms of cell death. RT-PCR, on the other hand, offers molecular insights into gene regulation. Although they measure different aspects of cellular response, combination of these methods enables a thorough analysis of how treatments like luteolin affect cancer cells. Still, caution is necessary when interpreting data across these assays, as each method has its own sensitivity and may respond differently to various experimental conditions.

*10. **Bias**: Potential sources of bias are not addressed. This should be discussed, including how the study controls for confounding factors.*

R: Not related.

*11. **Study Size**: The rationale for the chosen sample size is not provided. This should be explained.*

R: Not related.

*12. **Quantitative Variables**: The handling of quantitative variables is described, but the rationale for chosen groupings (e.g., different concentrations of luteolin) should be included.*

R: thanks for your comment. Different concentrations of luteolin are utilized to determine the dose-response relationship to establish the optimal concentration at which luteolin effectively

induces apoptosis, modulates autophagy and inhibits metastasis in BC cells, and ensure safety and efficacy

13. ***Statistical Methods****: The statistical methods used are not described in detail. Information on how data were analyzed and how confounding was controlled should be included.*

R: It was discibed in method section.

Results

14. ***Participants****: The number of cells used at each stage of the study is not reported. This information should be provided.*

R: Thank you. The suggested quantity of cells was highlighted in manuscript.

15. ***Descriptive Data****: Characteristics of the cells and conditions (e.g., luteolin concentrations) should be described.*

R: thanks for your comment. Different concentrations of luteolin (10-50 µM) were utilized.

16. ***Outcome Data****: The results are presented, but more detailed statistical analysis (e.g., confidence intervals) should be included.*

R: It was discibed in method section.

17. ***Main Results****: The main results are summarized, but unadjusted and adjusted estimates should be provided.*

R: The main result disscused in conclusion section.

18. ***Other Analyses****: Any additional analyses (e.g., subgroup analyses) should be reported.*

R: Not related.

Discussion

*19. **Key Results**: The key results are summarized with reference to the study objectives.*

R: Thanks for your comment.

*20. **Limitations**: The limitations of the study are not discussed. This should include potential sources of bias and imprecision.*

R: Thanks for your constructive comment. The suggested issue has been explained in discussion. P 6, Line 186.

*21. **Interpretation**: The interpretation of the results is provided, but it should be more cautious, considering the study's limitations.*

R: Thanks for your constructive comment. The suggested issue has been explained in discussion. P 6, Line 186

*22. **Generalisability**: The generalisability of the study results is not discussed. This should be included.*

R: Not related.

Other Information

*23. **Funding**: The source of funding and the role of the funders are not mentioned. This information should be provided.*

R:It was added.

Conclusion

The study provides valuable insights into the effects of luteolin on apoptosis, autophagy, and metastasis in bladder cancer cells. However, it would benefit from a more detailed description of the study design, setting, statistical methods, and discussion of limitations and generalisability. Addressing these points would enhance the transparency and reproducibility of the research.

R: Thanks for your constructive comments.

2 Revision 1

2 comments · 4 Oct 2024 – 4 Oct 2024

Referee 2 Reviewer

MINOR REVISION 4 Oct 2024

I thoroughly enjoyed reviewing this manuscript and only have some minor requests for revision.
Q1. It is better to suggest further related western blot studies as future research to better understand the mechanism of the anti-metastatic action of lutein. Please add this suggestion to the discussion section.
Q2. It is more appropriate to avoid using the term "proved" and its synonyms when referring to your observations or the reports of other studies in a scientific context. In the revised manuscript, "proved" was utilized on pages 1, 2 and 5. Please replace "proved" throughout the manuscript with other appropriate words, such as observed or reported.

Associate Editor

MINOR REVISION 4 Oct 2024

Review for IJPR-153408 R1
Reviewer 1:
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Q1. It is better to suggest further related western blot studies as future research to better understand the mechanism of the anti-metastatic action of lutein. Please add this suggestion to the discussion section.
Q2. It is more appropriate to avoid using the term "proved" and its synonyms when referring to your observations or the reports of other studies in a scientific context. In the revised manuscript, "proved" was utilized on pages 1, 2 and 5. Please replace "proved" throughout the manuscript with other appropriate words, such as observed or reported.
Decision:
Minor revision

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.

6 October 2024

Dear Editor-in-Chief

Manuscript ID: https://ijpr.brieflands.com/en/document/details/153408

On behalf of my co-authors, I submit the responses to the reviewer's comments and the revised manuscript entitled "Regulation of Apoptosis, Autophagy, and Metastasis by Luteolin in Human Bladder Cancer EJ138 Cells".

Please find in the next pages the responses to the reviewer's comments. We have addressed all of the comments and revised the manuscript accordingly. The revised sections have been highlighted in the text. We are appreciative of the reviewer for their important input. We believe the suggested changes have helped to substantially improve the quality of this paper over the previous version.

We want to extend our appreciation for the given opportunity to have this work considered for publication in the journal of "IJ Pharmaceutical Research". Please do not hesitate to contact me should you have any further questions.

Yours faithfully

Mohammad Vakili Ojarood

Cancer Immunology and Immunotherapy Research Center, Ardabil University of Medical Sciences, Ardabil, Iran

On behalf of my co-authors

Reviewer's comments:

Reviewer 1:

I thoroughly enjoyed reviewing this manuscript and only have some minor requests for revision.

Q1. It is better to suggest further related western blot studies for future studies to better understand the mechanism of anti-metastatic action of lutein. Please add this suggestion to discussion section.

R: Thanks for your constructive comment. We added this comment in discussion section (P5, Line 177-178).

Q2. It is more appropriate to avoid using the term "proved" and its synonyms when referring to your observations or the reports of other studies in a scientific context. In the revised manuscript "proved" was utilized in pages 1, 2 and 5. Please replace "proved" in whole the manuscript by another appropriate words like observed, reported.

R: Thanks for your constructive comment. We replaced "proved" by another appropriate words, (P1, Line 24), (P2, Line 54), (P5, Line 153), (P5, Line 155), (P6, Line 186).

3 Revision 2

2 comments · 13 Oct 2024 – 16 Oct 2024

Referee 2 Reviewer

ACCEPT 13 Oct 2024

Dear Authors; Congratulations and thank you for submitting the revised manuscript.
My recommendation is to accept the manuscript.

Associate Editor

ACCEPT 16 Oct 2024

The revised manuscript is acceptable for publication in IJ Pharmaceutical Sciences.