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OPEN PEER REVIEW

Combinatorial Effects of Chrysin with Doxorubicin, 5-Fluorouracil, and Cyclophosphamide on Triple-Negative Breast Cancer Cell Line

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1 Original submission

2 comments · 13 Nov 2024 – 15 Nov 2024

Referee 1 Reviewer

MAJOR REVISION 13 Nov 2024

Method Section: Specify the concentration range used for each material.

FIX Calculation: Clarify the method for calculating the FIX and other indications.

Substance Addition Method: Describe the process of adding substances, with one drug at a fixed concentration and another at a range of concentrations added to the media.

Toxicity Chart: Present the toxicity chart as a curve, rather than a columnar chart.

Hemolytic Test Purpose: Provide the rationale behind conducting the hemolytic test.

Data Reporting in Table 1: Include the amount of standard deviation (SD) or standard error of the mean (SEM) in Table 1.

Flow Cytometry Quadrants: Explain the criteria for selecting quadrants in the flow cytometry diagrams.

Figure Quality: Improve the quality of Figures 3 and 4, using better resolution and distinctive colors.

Figure 5: Create a table to display the results of the multiple t-test for Figure 5.

Referee 2 Reviewer

MAJOR REVISION 15 Nov 2024

The authors present a manuscript entitled "Combinatorial effects of Chrysin with Doxorubicin, 5-Fluorouracil, and Cyclophosphamide on triple-negative breast cancer cell line". After carefully reading the manuscript, I believe that major revisions are needed before it is suitable for publication in IJPR.
Comments:

The authors should use a normal cell line for comparison with these results.
The authors should report the apoptosis and cell cycle results in a table. (average ± SD)
Apoptosis with the CP+CH and DOX+CH complexes does not differ significantly from that with CH.
DOX increased the G2/M phase compared with the control. Is this true?
What was the basis for choosing the drug concentrations in the apoptosis, cell cycle, and migration analyses?

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.

Manuscript ID: 157446

Manuscript Title: *Combinatorial effects of Chrysin with Doxorubicin, 5-Fluorouracil, and Cyclophosphamide on triple-negative breast cancer cell line*

Dear Dr Elham Rezaee

Editor in Chief

IJ Pharmaceutical Research

We thank the reviewers for carefully evaluating the manuscript and for their constructive critiques. We have revised the manuscript according to the reviewers' comments. Below is our point-by-point response to the reviewers' critiques/suggestions:

Modified and added items in the article are highlighted in yellow.

Reviewer #1:

1 - Method Section: Specify the concentration range used for each material.

Author's Response: Thank you for your comment. In the Method section (Cell proliferation assay), we specified the concentration ranges for each material as follows: [Chrysin: 13-800 µM], [5-fluorouracil: 1.4-92 µM], [Doxorubicin: 0.09-6 µM], and [Cyclophosphamide: 25-1600 µM]. These ranges were selected based on preliminary studies and relevant literature to ensure reproducibility and validity of the results. In other sections, the concentrations used are stated, and changes were applied if they were not mentioned. **(Page 4, Line 79-81. Page 5, Line 104)**

2 - FIX Calculation: Clarify the method for calculating the FIX and other indications.

Author's Response: Thank you for your suggestion. The following texts were added to the method section of the manuscript in the checkerboard method.

Drug interactions were analyzed through fractional inhibitory concentrations (FIC), calculating FIC_A and FIC_B based on combined versus individual IC_{50} values. FIC_A represents the IC_{50A} in combination divided by the IC_{50A} alone, and FIC_B denotes the IC_{50B} in combination divided by the $IC50_B$ alone. After determining the IC_{50} of each drug, a linear dilution corresponding to the concentration range of $0.2IC_{50}$ to $2IC_{50}$ of each drug was used by making a 1:1 mixture of two drugs for calculating the FIC of each drug. A fractional inhibitory index (FIX) was derived from $FIC_A + FIC_B$, where a FIX below 1 indicates a

synergistic effect. A FIX value greater than 1 indicates a non-additive (antagonistic) effect (24). **(Page 4, Line 89-93). Reference 24 in the main manuscript.**

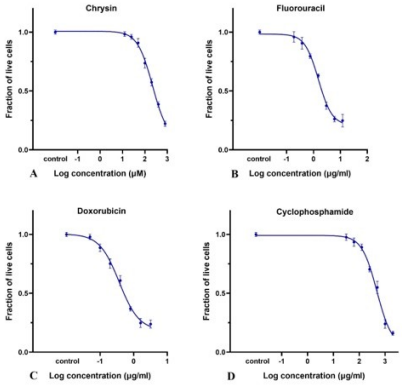
3 - Substance Addition Method: Describe the process of adding substances, with one drug at a fixed concentration and another with a variable range added to the media.

Author's Response: Thank you very much for your suggestion. In the evaluation of apoptosis, Cell cycle analysis, and Cell migration *assay*, a fixed concentration of each drug equal to IC_{25} was used. In the modified Checkerboard method, according to reference 24, after determining the IC_{50} of each drug, a linear dilution corresponding to the concentration range of $0.2IC_{50}$ to $2IC_{50}$ of each drug was used by making a 1:1 mixture of two drugs for calculating the FIC of each drug.

References: 24. Cokol-Cakmak M, Bakan F, Cetiner S, Cokol M. Diagonal method to measure synergy among any number of drugs. J Vis Exp. 2018(136):e57713. https://doi.org/10.3791/57713.

4 - Toxicity Chart: Present the toxicity chart as a curve rather than a columnar chart.

Author's Response: Thank you for your insightful suggestion regarding the presentation of the toxicity data. We agree that presenting the data as a curve can provide a clearer visualization of trends and relationships. We revised the chart accordingly and replaced the columnar chart with a curve representation in the updated manuscript. **(Page 15, Line 368)**



5 - Hemolytic Test Purpose: Provide the rationale behind conducting the hemolytic test.

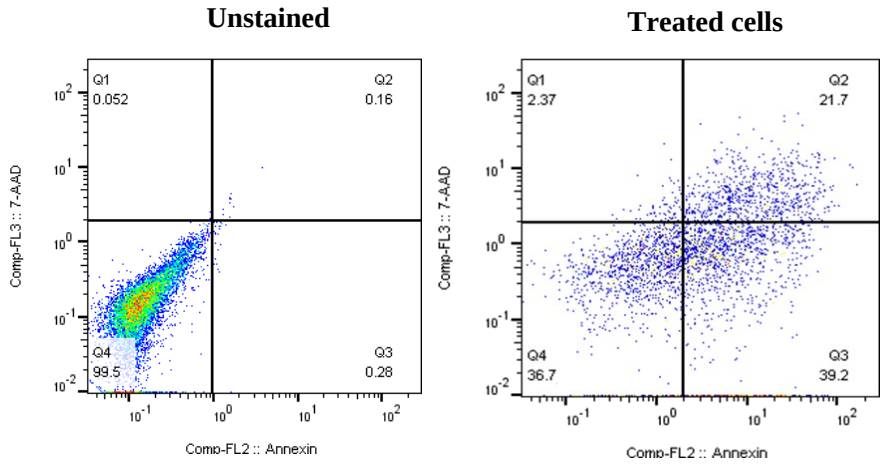
Author's Response**:** Thank you for your valuable comment. The rationale behind conducting the hemolytic test was to evaluate the biocompatibility of the tested substances, specifically their potential to cause red blood cell lysis. This test is a critical step in assessing the safety profile of the substances for biomedical applications, ensuring that they do not induce significant hemolysis at the effective concentrations used. Because chrysin is a natural compound that has not been clinically used, the hemolytic test was used to assess its effect on hemolysis.

6 - Data Reporting in Table 1: Include the amount of standard deviation (SD) or standard error of the mean (SEM) in Table 1.

Author's Response: Thank you for your thoughtful suggestion. We understand the importance of including measures of variability, such as standard deviation (SD) or standard error of the mean (SEM), to provide a clearer representation of the data. Your suggestion has been implemented. **(Page 13-14, Line 360-366)**

7 - Flow Cytometry Quadrants: Explain the criteria for selecting quadrants in the flow cytometry diagrams.

Author's Response: The cells were gated according to the unstained control group. Subsequent gating strategy was based on the gating viable cells (Q4: double negative Annexin, 7-AAD), early apoptotic cells (Q3: single positive with Annexin), necrotic cells (Q1: single positive with 7-AAD), and late apoptotic cells plus necrotic cells (Q2: double-positive cells). Representative data is shown below:

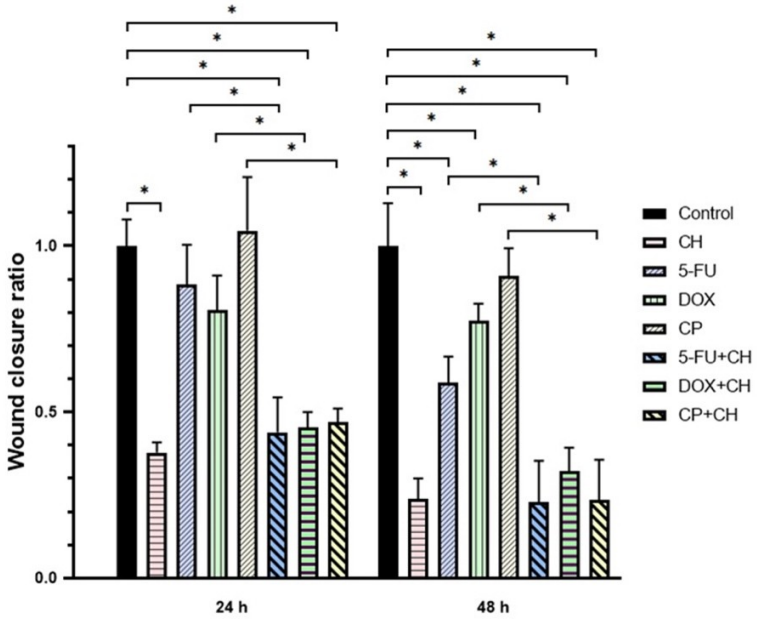


8 - Figure Quality: Improve the quality of Figures 3 and 4 using better resolution and distinctive colors.

Author's Response: Thank you for your suggestion. Your suggestion has been implemented. **(Page 17-18, Line 375-381)**

9 - Figure 5: Create a table to display the results of the multiple t-test for Figure 5.

Author's Response: Thank you for your suggestion. We created a table summarizing the statistical results based on multiple t-Test for Figure 5 and included it in the revised manuscript. **(Page 19, Line 387-391)**



Treatments								
Time (h)	**Control**	**CH**	**5-FU**	**DOX**	**CP**	**5-FU+CH**	**DOX+CH**	**CP+CH**
24	1.00 ± 0.08^{a}	0.38 ± 0.03^{b}	$0.88 \pm 0.12^{a,c}$	$0.81 \pm 0.11^{a,c}$	$1.05 \pm 0.16^{a,c}$	$0.44 \pm 0.10^{b,d}$	$0.45 \pm 0.05^{b,d,e}$	$0.47 \pm 0.04^{b,e}$
48	1.00 ± 0.13^{a}	0.24 ± 0.06^{b}	0.59 ± 0.08^{c}	$0.77 \pm 0.05^{c,d}$	$0.91 \pm 0.08^{a,d}$	$0.23 \pm 0.12^{b,e}$	$0.32 \pm 0.07^{b,e,f}$	$0.24 \pm 0.12^{b,e,f}$

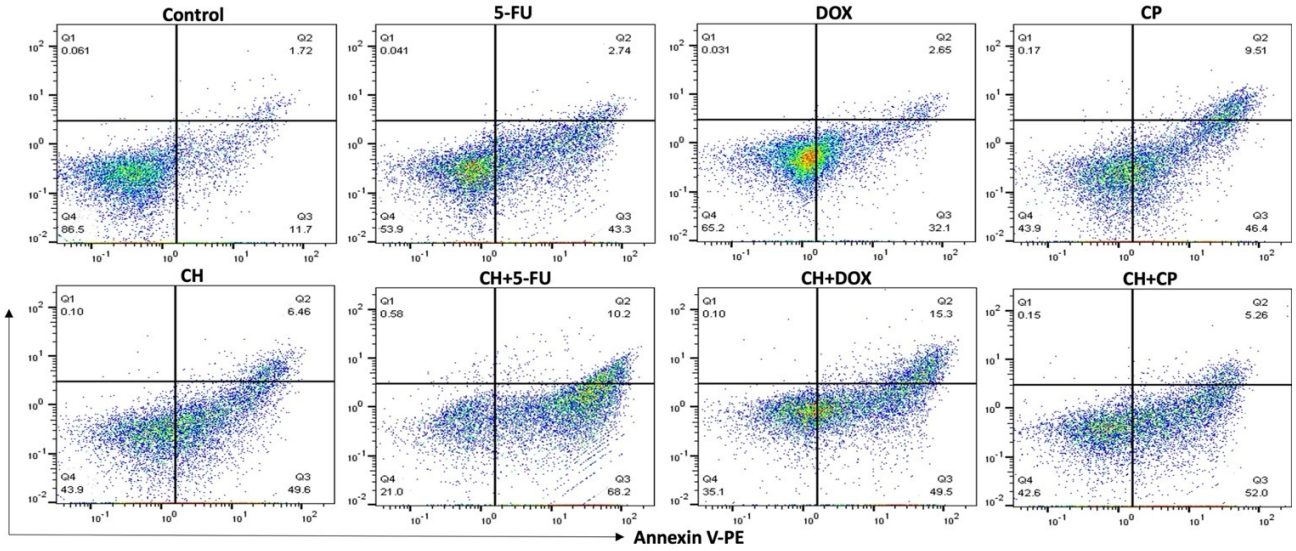
Reviewer #2:

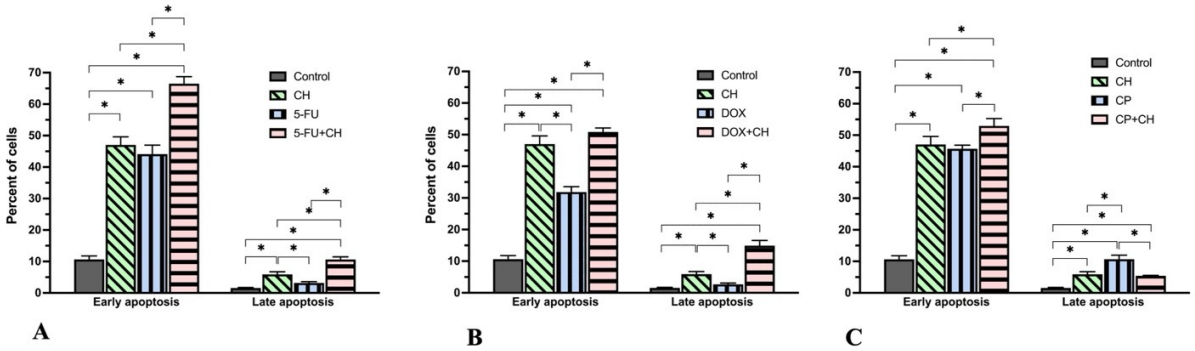
1 - The author should be used a normal cell line for comparison of this result.

Author's Response: Thank you very much for your constructive suggestion. One of the main reasons why normal cells were not used as a treatment control in the current study was the low toxicity of CH by a growing body of studies. Furthermore, this study aimed to investigate the antitumor impacts of CH with chemotherapeutic agents without consideration of other effects. However, this can also be considered as one of the limitations of the present study that was added to the manuscript. **(Page 9, Line 210-211)** Nevertheless, we acknowledge that including a normal cell line would have enriched the study and will consider this in future research.

2 - The author should be reported apoptosis and cell cycle results at table. (average ± SD)

Author's Response: Thank you for your valuable comment. We understand the importance of presenting the apoptosis and cell cycle data in a clear and comprehensive format. In response to your suggestion, we revised the manuscript to include a table that summarizes the results as the mean ± SD for each group. **(Page 17-18, Line 375 and 381)**

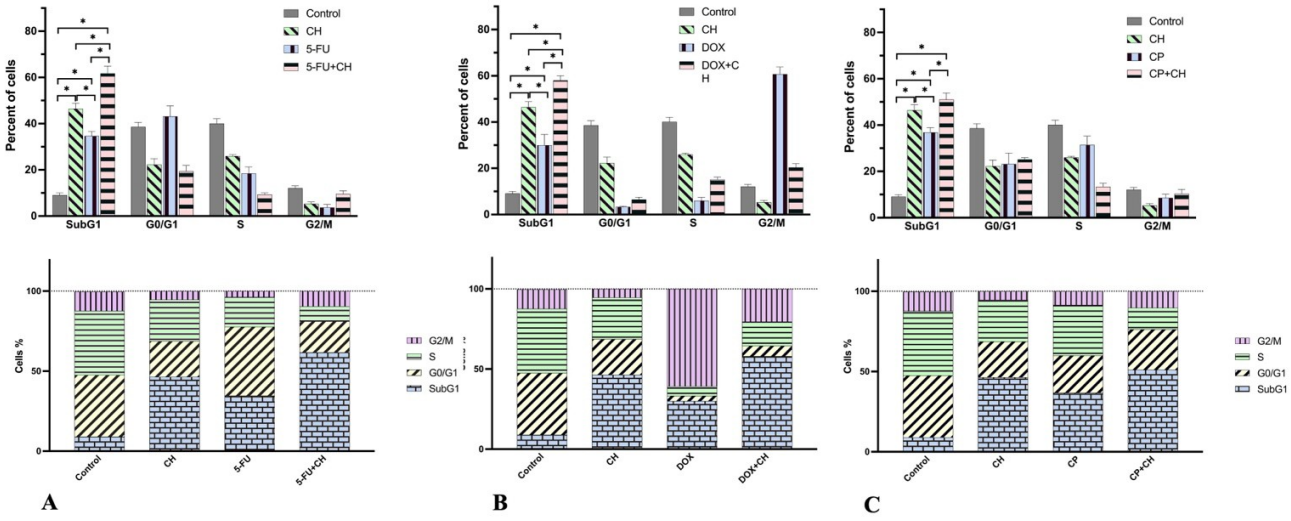




Percent of cells in treatments				
Apoptosis	**Control**	**CH**	**5-FU**	**5-FU + CH**
Early	10.7 ± 1.10	47.0 ± 2.5	44.1 ± 2.9	66.5 ± 2.2
Late	1.8 ± 0.2	5.9 ± 0.8	3.2 ± 0.4	10.6 ± 0.9

Percent of cells in treatments				
Apoptosis	**Control**	**CH**	**DOX**	**DOX + CH**
Early	10.7 ± 1.10	47.0 ± 2.5	31.2 ± 1.7	50.9 ± 1.3
Late	1.8 ± 0.2	5.9 ± 0.8	2.7 ± 0.4	14.9 ± 1.6

Percent of cells in treatments				
Apoptosis	**Control**	**CH**	**CP**	**CP + CH**
Early	10.7 ± 1.10	47.0 ± 2.5	45.7 ± 1.1	52.9 ± 2.3
Late	1.8 ± 0.2	5.9 ± 0.8	10.7 ± 1.3	5.4 ± 0.2



Percent of cells in treatments				
Cell cycle	**Control**	**CH**	**CP**	**CP + CH**
Sub G1	9.0 ± 0.9	46.4 ± 2.4	36.8 ± 2.1	51.1 ± 2.8
G1/G0	38.6 ± 2.0	22.3 ± 2.6	23.2 ± 4.7	25.2 ± 0.7
S	40.0 ± 2.0	26.0 ± 0.6	31.4 ± 3.8	13.4 ± 1.5
G2/M	12.0 ± 1.0	5.3 ± 0.8	8.5 ± 1.6	10.4 ± 1.9

Percent of cells in treatments				
Cell cycle	**Control**	**CH**	**5-FU**	**5-FU + CH**
Sub G1	9.0 ± 0.9	46.4 ± 2.4	34.7 ± 1.9	61.7 ± 3.2
G1/G0	38.6 ± 2.0	22.3 ± 2.6	43.1 ± 4.5	19.4 ± 2.5
S	40.0 ± 2.0	26.0 ± 0.6	18.5 ± 2.8	9.3 ± 0.7
G2/M	12.0 ± 1.0	5.3 ± 0.8	3.7 ± 1.3	9.5 ± 1.4

Percent of cells in treatments				
Cell cycle	**Control**	**CH**	**DOX**	**DOX + CH**
Sub G1	9.0 ± 0.9	46.4 ± 2.4	30.0 ± 4.7	58 ± 2.1
G1/G0	38.6 ± 2.0	22.3 ± 2.6	3.3 ± 0.4	6.5 ± 1.0
S	40.0 ± 2.0	26.0 ± 0.6	6.0 ± 1.4	15.1 ± 1.1
G2/M	12.0 ± 1.0	5.3 ± 0.8	60.7 ± 3.1	20.4 ± 1.6

3 - The apoptosis of CP+CH and DOX+CH complexes don't have a significant difference with CH.

Author's Response: Thank you for your observation. However, our data analysis showed that the apoptosis levels of CP+CH and DOX+CH complexes were statistically different from CH alone. We believe this might not have been presented in the original manuscript, and we revised the text to ensure this result is properly highlighted. Regarding the CP+CH complex, our data indicate that while CP+CH

showed a significant increase in early apoptosis compared to either drug used alone ($p< 0.05$), it did not exhibit a significant difference in late apoptosis. **(Page 7, Line 146-148)**

However, for the DOX+CH complex, we observed significantly synergistic effects in late apoptosis compared to DOX and CH alone ($p< 0.05$). **(Page 6-7, Line 145-146)**

4 - DOX increased G2/M phase compare to control, Is this true?

Author's Response: Thank you for your insightful comment. Yes, our data indicate that DOX treatment led to an increase in the G2/M phase compared to the control. This observation aligns with the well-documented effect of DOX, which is known to interfere with DNA replication and repair mechanisms, causing cell cycle arrest in the G2/M phase. **(Page 9, Line 198-202)**

More references:

Bar-On O, Shapira M, Hershko DD. Differential effects of doxorubicin treatment on cell cycle arrest and Skp2 expression in breast cancer cells. Anticancer Drugs. 2007;18(10):1113-21. [PubMed ID:17893511]. https://doi.org/10.1097/CAD.0b013e3282ef4571.

5- What was the basis for choosing the concentration of drugs in apoptosis, cell cycle and migration analysis?

Author's Response: Thank you for your insightful question. The concentrations of the drugs used in the apoptosis, cell cycle, and migration analyses were chosen based on the Preliminary Dose-Response Experiments: We conducted preliminary dose-response experiments to determine the effective concentration ranges that induce measurable effects without causing excessive cytotoxicity. So, we used IC_{25} of the desired drugs in apoptosis, cell cycle, and migration analysis studies.

Checklist Evaluation by AI

1- Statistical Analysis: Include details on how sample sizes were determined, statistical methods used for analysis, and how confounding factors were controlled.

In the context of cell culture studies, determining a traditional sample size is not applicable, as experiments are typically performed using biological or technical replicates. In our study, all experiments were conducted with three independent replicates to ensure reproducibility and reliability. The results are presented as the mean ± standard deviation (SD).

For statistical analysis, we employed appropriate statistical tests based on the nature of the data and the comparisons being made. The specific tests used for each analysis are detailed in the figure legends and the statistics.

Regarding potential confounding factors, strict experimental protocols were followed to minimize variability, including using identical cell passages, consistent culture conditions, and standardized treatment durations. These measures were implemented to ensure the validity and robustness of the result.

2- Data Presentation: Provide specific numerical results, including p-values and confidence intervals, to support the claims made in the results section.

We would like to clarify that the results have been presented with specific numerical values, including the mean ± standard deviation (SD), throughout the results section.

Additionally, p-values have been reported for all statistical analyses to indicate the significance of the findings. Where applicable, the exact p-values and statistical comparisons are provided in the text and figure legends.

3- Flow Diagram: Consider including a flow diagram to illustrate the experimental process and participant flow, if applicable.

While we appreciate the value of flow diagrams in clarifying experimental processes, we would like to note that this type of study, which is based on in vitro cell culture experiments, does not involve participant flow or a process that lends itself to a flow diagram.

Instead, we have provided a detailed description of the experimental procedures in the Methods section to ensure clarity and reproducibility. If you believe additional visual aids would enhance the manuscript, we would be happy to explore alternative options.

4- Discussion of Limitations: Discuss potential limitations of the study, including any biases that may have affected the results.

The potential limitations of the study have been addressed at the end of the Discussion section.

We hope that we have satisfactorily addressed the reviewers' critiques/suggestions and that our revised manuscript now merits publication in *IJ Pharmaceutical Research*.

Sincerely,

Correspond, author,

2 Revision 1

1 comment · 19 Jan 2025

Referee 2 Reviewer

ACCEPT 19 Jan 2025

After the revision, this manuscript is suitable for publication in this journal.