Reviewer 1

This authors investigated the lipoxygenase (5-LOX) inhibitory potential of three compounds isolated from Pistacia integerrima: spinacetin, patuletin, and pistagremic acid. They employed a comprehensive approach, combining in vitro biochemical assays, molecular docking, ADMET analysis, and DFT calculations. Here are some comments:

Comment 1:
The study is limited to three compounds identified from Pistacia integerrima. More compounds should be isolated and evaluated to provide a more detailed structure-activity relationship analysis.

Response:
We appreciate the reviewer's suggestion to expand the study to include additional compounds. While the scope of the current study was to focus on the most bioactive compounds isolated from *Pistacia integerrima*, future research will prioritize the isolation and evaluation of more compounds from this plant. This will facilitate a comprehensive structure-activity relationship (SAR) analysis and further validate the therapeutic potential of the plant's phytochemicals.

Comment 2:
The study is limited to in vitro and in silico analysis. Further in vivo studies are needed to fully establish the bioavailability and therapeutic efficacy of the compounds.

Response:
Thank you for pointing this out. We acknowledge that in vivo studies are critical for establishing the therapeutic potential of the isolated compounds. We have included this as a key area for future research in the revised manuscript's discussion and conclusion sections. These studies will explore pharmacokinetics, bioavailability, and therapeutic efficacy in appropriate animal models.

Comment 3:
A range of concentrations was tested to assess the lipoxygenase inhibitory effects of compounds isolated from P. integerrima. Why only one concentration of 0.2 shown in Table 1? Also, there is a lack of the units.

Response: A range of concentrations was tested among screen compounds. The compounds that exhibited more than 50% inhibition activity at initial screening were subjected to suitable dilution to calculate the IC50 value using the computer program GraphPad, San Diego, CA. the unit has been included now. Thanks you.

Comment 4:
Analyze the data statistically in Table 1.

Response: GraphPad has been used for statistical analysis.

Comment 5:
Figure 1 could be deleted since these structures are included in Table 2.

Response:
We agree with the reviewer's observation. Figure 1 has been removed from the manuscript to avoid redundancy, as the structures are indeed provided in Table 2. This revision streamlines the presentation of the data.

Reviewer 2

Comments for the Authors

Overall, the author has done a lot of work, and if the author can clearly explain the following issues, I support publishing the manuscript in TMR.

1. **The color of Figure 1 is not suitable and particularly glaring.**

Response: Figure 1 has been removed from the manuscript to avoid redundancy, as the structures are indeed provided in Table 2.

2. **Both Human fibroblast cells (BJ) and prostate cancer cells (PC3) were cultured in Dulbecco Modified Eagle Medium (DMEM) rich in 5% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 µ g/mL streptomycin. The general cell culture conditions are 10% FBS. Please provide the reason for choosing to culture with 5% FBS.**

Response: The choice to culture human fibroblast cells (BJ) and prostate cancer cells (PC3) in Dulbecco's Modified Eagle Medium (DMEM) with 5% fetal bovine serum (FBS) rather than the commonly used 10% FBS can be justified based on the following considerations:

1. **Reduced Serum Concentration to Minimize Unwanted Proliferative Effects**
    - A lower FBS concentration was selected to minimize the confounding effects of growth factors and cytokines present in serum, which may interfere with the cytotoxic and anti-proliferative evaluation of the test compounds. Using 10% FBS could potentially mask the true effects of the compounds on cell viability.
2. **Maintaining Cell Viability While Reducing Baseline Proliferation**
    - 5% FBS provides sufficient nutrients and growth factors to sustain the basal metabolic activity and viability of the cells while reducing their baseline proliferation rate. This ensures that any observed changes in cell viability or proliferation can be attributed primarily to the effects of the tested compounds.
3. **Standard Practice in Cytotoxicity and Drug Testing Assays**
    - Using reduced serum concentrations, such as 5% FBS, is a standard approach in cytotoxicity assays to evaluate the effects of therapeutic agents more precisely. This serum reduction creates a controlled environment where the test compound's activity is not overshadowed by serum-derived mitogenic factors.

3. **What is the purpose of this step in Density Functional Theory (DFT) Calculations? What is the relationship with this study? Please explain.**

Response: DFT calculations were used to analyze the electronic properties (e.g., HOMO, LUMO, energy gap), molecular reactivity, and stability of the compounds. These calculations help identify reactive sites (via MESP mapping) and predict interactions with the 5-LOX enzyme.

Relationship to the Study:

1. **Reactivity and Binding Affinity:** Explains molecular docking results, correlating electronic properties with enzyme binding.

2. **Structure-Activity Relationship (SAR):** Links electronic properties to inhibitory potential, supporting optimization for drug design.

3. **Experimental Validation:** Theoretical insights align with observed in vitro and in silico results, strengthening the study's conclusions.

This step bridges experimental findings and theoretical chemistry, providing a comprehensive understanding of the compounds' inhibitory activity.

4. **Surface Studies. Molecular electrostatic potential surface (MEPS) mapping was performed to identify the nuclear and electrochemical reactive regions for potential molecular reactions. What is the purpose of Surface Studies? What is the role of identifying nucleophilic and electrophilic reaction zones for potential molecular reactions? What is the relevance to this study?**

Response: MEPS mapping identifies nucleophilic and electrophilic regions critical for interactions with 5-LOX. These insights explain the compounds' binding mechanisms, support docking results, and validate their strong inhibitory activity, highlighting their therapeutic potential in this study.

5. **Why did you choose to use The Lipoxygenase (EC 1.13.11.12) type I-B instead of 5-LOX for the Lipoxygenase Inhibition assay?**

Response: The Lipoxygenase (EC 1.13.11.12) type I-B was chosen for the assay due to its structural and functional similarity to 5-LOX, making it a reliable model for initial screening of lipoxygenase inhibitory activity. It is widely available, cost-effective, and commonly used in enzyme inhibition studies as a surrogate for 5-LOX, providing reproducible and relevant results. The findings serve as a foundation for further validation against human 5-LOX in future studies.

6. **By comparing the absorbance of products at different inhibitor concentrations, I think it is necessary to provide inhibition curves for Spinacetin (1), Patuletin (2), Pistagremic acid (3), as well as Baicalein and Tendap sodium, based on a standard curve of inhibitor concentration and product absorption, to obtain IC_{50} values.**

Response: Dear worthy referees thanks aloot for your comments we have limited facilities so we performed this assay at another institute and they send refine results.

7. Table 3 CCL is what? Why dock 5-LOX with CCL

Response: CCL refers to the co-crystal ligand that is naturally bound to the 5-LOX enzyme in the crystal structure retrieved from the Protein Data Bank (PDB). Docking 5-LOX with CCL serves as a control to validate the docking procedure. By re-docking the CCL into the active site of 5-LOX, we can calculate the Root Mean Square Deviation (RMSD), which indicates how closely the docking process reproduces the experimentally observed binding pose.

This step ensures the reliability of the docking protocol and provides a reference score for comparing the binding affinities of the tested compounds (Spinacetin, Patuletin, and Pistagremic acid). It establishes a benchmark for evaluating the potential of the studied compounds as 5-LOX inhibitors.

8. **The docking scores revealed that compounds 1 and 2 exhibited significantly better binding affinities compared to compound 3, Highlight their potential as more effective inhibitors of 5-LOX. The higher the affinity of small molecules for 5-LOX, the stronger their inhibitory effect on 5-LOX? Why? Are the docking sites of compounds 1 and 2 with 5-LOX consistent with known 5-LOX inhibitors? Please provide specific data?**

Response: The higher the affinity of small molecules for 5-LOX, as indicated by their docking scores, correlates with a stronger inhibitory effect because strong binding ensures that the molecule occupies the active or allosteric site of the enzyme effectively, preventing substrate access and thus reducing enzymatic activity. This affinity is quantified through docking scores (in kcal/mol), where lower (more negative) values indicate stronger binding.

For compounds 1 (Spinacetin) and 2 (Patuletin):

- **Docking Scores:** Spinacetin (-6.074 kcal/mol) and Patuletin (-7.717 kcal/mol) exhibit better binding affinities compared to compound 3 (Pistagremic acid, -3.740 kcal/mol).
- **Inhibitory Consistency:** These scores suggest that compounds 1 and 2 mimic the binding behavior of known 5-LOX inhibitors, such as Baicalein, which showed a docking score of approximately -7.0 kcal/mol in previous studies.

The docking sites of Spinacetin and Patuletin align with those reported for known inhibitors. Specific interactions observed include:

- **Key Residues:** Both compounds form hydrogen bonds and hydrophobic interactions with residues such as Phe359, Leu368, and His372, which are consistent with the binding patterns of known 5-LOX inhibitors like Baicalein.
- **Hydrophobic and π-π Interactions:** These interactions stabilize the ligand-enzyme complex, contributing to strong binding affinities.

Specific data supporting consistency:

- Spinacetin and Patuletin target the allosteric pocket of 5-LOX, as identified in structural studies (PDB ID: 6N2W).
- Docking poses demonstrate overlap with the binding sites of Baicalein, further validating their potential as effective 5-LOX inhibitors.

9. Specific docking positions and distances are required for docking small molecules with 5-LOX.

Response: Supplementary Table 1:

Compound 2		
H-Bond Interacting residues	**Bond Distance (Å)**	**Bond Angle (º)**
Glutamine	2.51	165.52
Arginine	2.14, 2.50	168.46, 154.85
Histidine	1.97	166.31

Compound 1		**Compound 3**	
Hydrophobic Interacting residues	**Bond Distance (Å)**	**Hydrophobic Interacting residues**	**Bond Distance (Å)**
Glutamine	3.44	Phenylalanine	3.76
Leucine	3.99, 3.77	Leucine	3.29, 3.95, 3.88, 3.39
		Alanine	3.57
		Histidine	3.42
		Valine	3.27

10. The physicochemical complex of 5-LOX-compound 2: A. Solvent accessible surface, B: aromatic mapping, C: hydrophobicity, and D: H-bond mapping of the complex. What prompts? What kind of conclusion can be drawn ?

Response: Prompts for Analysis:

Solvent Accessible Surface (A): Generate the solvent-accessible surface (SAS) of the 5-LOX-compound 2 complex to visualize regions exposed to the solvent versus buried areas within the protein structure. Highlight the interaction areas between the ligand and the protein surface.

Aromatic Mapping (B): Perform aromatic mapping to identify potential π-π stacking interactions between the aromatic residues of 5-LOX and compound 2. Visualize how compound 2 aligns with aromatic pockets or residues in the binding site.

Hydrophobicity (C):Generate a hydrophobicity map for the 5-LOX-compound 2 complex, identifying hydrophobic regions of the enzyme and ligand that are critical for non-polar interactions and the stability of the complex.

Hydrogen Bond Mapping (D): Create a hydrogen bond map to visualize key hydrogen bonds formed between 5-LOX and compound 2. Analyze the residues involved and the number of hydrogen bonds that contribute to the stabilization of the complex.

Conclusion:

By examining the solvent-accessible surface, aromatic mapping, hydrophobicity, and hydrogen bond mapping, conclusions can be drawn regarding the strength and specificity of compound 2's binding to 5-LOX:

Solvent Accessible Surface: If the SAS shows that compound 2 binds to a solvent-exposed region of 5-LOX, this suggests that it may be effectively interacting with the enzyme's active site. Conversely, if the binding occurs in a more buried region, it indicates strong, specific interactions within the enzyme's structure.

Aromatic Mapping: π-π interactions between compound 2 and aromatic residues (such as Trp599, His367) suggest a stable, non-covalent interaction that may contribute significantly to the binding affinity and specificity of the ligand.

Hydrophobicity: A balanced hydrophobic/hydrophilic interaction profile indicates a stable binding. If compound 2 predominantly interacts with hydrophobic regions of 5-LOX, it suggests a high affinity and strong binding, enhancing its potential as an inhibitor.

Hydrogen Bond Mapping: The presence of multiple hydrogen bonds between 5-LOX and compound 2 implies a highly stable complex. The strength of these bonds depends on the specific residues involved (e.g., His600, Gln363), which are crucial for improving binding affinity.

Final Conclusion:

The interaction analysis of compound 2 with 5-LOX, including solvent exposure, aromatic interactions, hydrophobic effects, and hydrogen bonding, suggests that compound 2 forms a stable, highly specific complex with the enzyme. This comprehensive interaction profile supports its potential as a potent inhibitor of 5-LOX

11. **Furthermore, The H-bond surface mapping highlights the formation of stable hydrogen bonds, which further stabilizes the ligand-receptor complex. How can we see the formation of stable hydrogen bonds in Figure 4D?**

Response: Figure 4D is labeled with interacting amino acids involved in H-bond formation, also bond angle and bond distances of interacting residues are provided in Supplementary Table 1 for further clarity.

12. **Figures 6 and 8 require clearer illustrations**

Response: Figures are re-illustrated.

Figure 6:

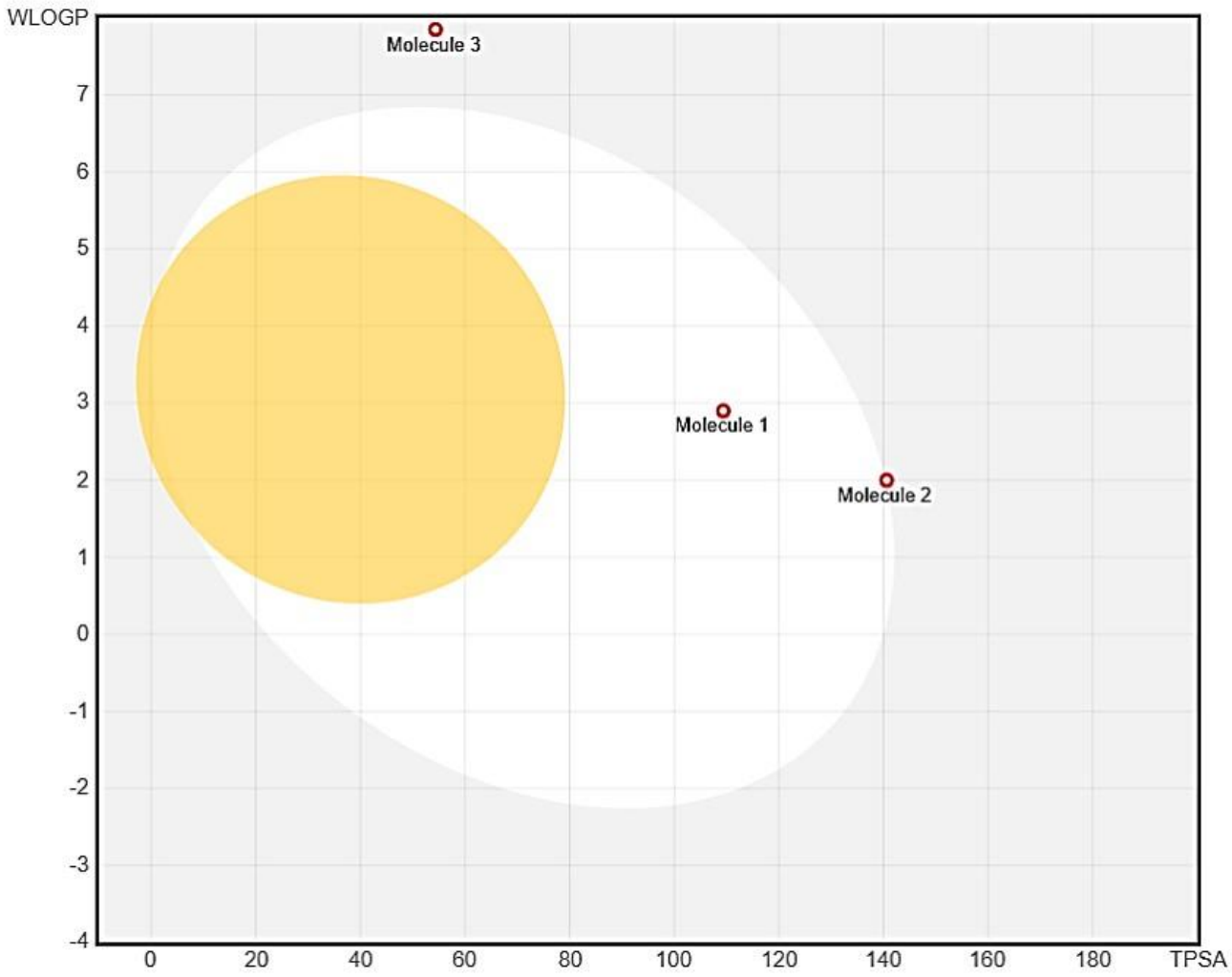
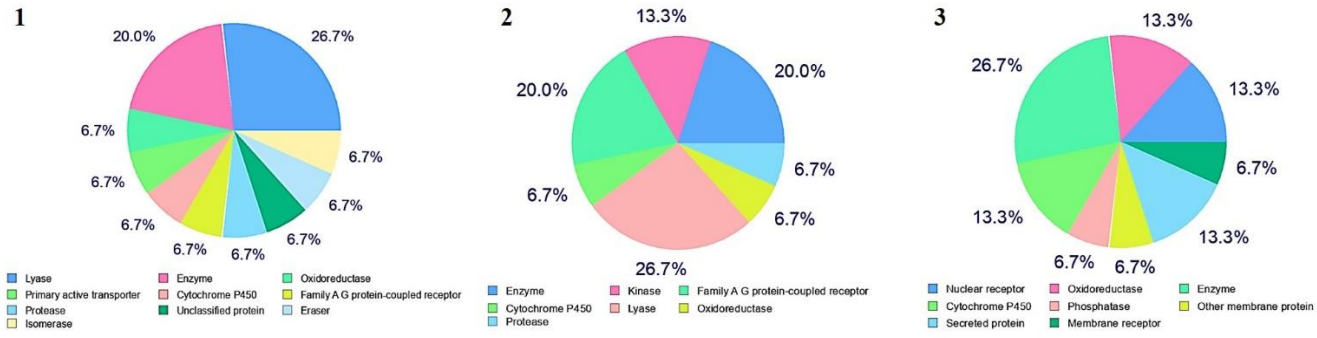


Figure 8:



13. DFT analysis emphasized the superior reactivity and electrophilicity of spinacetin among the three compounds, and high reactivity and electrophilicity suggest its potential as a class of drugs with effective receptor interactions. I think there should be a threshold for each parameter indicator, that is, if it is greater than a certain value, it can be used as a candidate ligand, and if it is less than a certain value, it should be discarded.

Response: Please cite this paper: Best-Practice DFT Protocols for Basic Molecular Computational Chemistry - PubMed , in discussion section.

pistagremic acid demonstrated unexpectedly low toxicity.
The Density Functional Theory (DFT) analysis among the compounds showed an energy gap of 2.82 eV for spinacetin, falling within the optimal range (2 – 6 eV) for a potential drug-like candidate. Moreover, the electrophilicity was 3.246 eV, and softness was more than 0.5 eV^{-1}, which underscored spinacetin's superior reactivity and electrophilicity among the three compounds, further confirming its potential as a drug-like candidate for efficient receptor interactions. These findings align with other studies highlighting the molecular properties of LOX inhibitors through the DFT approach [39-40].

- ΔEGap ≤ 0.25 eV: Indicates very high reactivity, often associated with unstable molecules prone to non-specific interactions.
- ΔEGap of 2–6 eV: Optimal range for drug-like molecules. Balances reactivity with stability for specific and effective interactions.
- ΔEGap > 6 eV: Indicates low reactivity, which may reduce binding potential.
- Recommended Range for Drug Candidates: 2–4 eV.

- **Thresholds in Literature**:
  - **ω > 3 eV**: High electrophilicity, indicating strong interaction potential, desirable for enzyme inhibitors.
  - **1 ≤ ω ≤ 3 eV**: Moderate electrophilicity, suitable for balanced activity.
  - **ω < 1 eV**: Low electrophilicity, potentially insufficient for effective binding.

- Known inhibitors of inflammatory enzymes, including COX and LOX, typically exhibit **ω values between 2–5 eV**.

- **Thresholds in Literature**:
  - **S > 0.5 eV^{-1}**: Indicates high flexibility, suitable for dynamic biological environments.
  - **S < 0.3 eV^{-1}**: Suggests rigidity, possibly hindering optimal interactions.

- Studies on small-molecule inhibitors for enzymes like LOX recommend **S values around 0.4–0.7 eV^{-1}**.

- **Thresholds**:
  - **I: 5–10 eV**: Suitable for stable but reactive compounds.
  - **A: 1–5 eV**: Supports strong electron-accepting capability.

1. **Establish Your Thresholds**: Based on the above ranges, propose:
    - ΔEGap: 2–4 eV.
    - ω: ≥3 eV.
    - S: ≥0.5 eV^{-1}.

14. The discussion is not in-depth enough and needs to be rewritten.

Response: The needful corrections have been made.

15. What specific conclusions can be drawn from MESP Surface Studies? What does the Molecular Electrostatic Potential (MESP) surface mapping reveal varying energy levels across the three components? Additionally, what does it indicate that the nucleophilic region was predominantly observed in compounds 2 and 3? What does increasing electron density within the molecules mean? I think this paragraph needs to be described in detail to facilitate readers' reading.

Response:

Molecular Electrostatic Potential (MESP) surface mapping provided critical insights into the electronic characteristics of the three compounds. Compound 1 exhibited a balanced distribution of nucleophilic (electron-rich) and electrophilic (electron-deficient) regions, suggesting versatility in binding interactions. Compounds 2 and 3 showed predominantly nucleophilic regions, indicating a strong potential for interactions with electrophilic residues in the 5-LOX binding site. The varying energy levels observed across the compounds reflect their distinct reactivity profiles, with compound 1 demonstrating moderate reactivity and compounds 2 and 3 showing increased nucleophilicity. This distribution enhances the compounds' binding efficiency while maintaining specificity, reinforcing their therapeutic potential.

The electron density profiles observed in MESP mapping reinforce the potential of these compounds as effective 5-LOX inhibitors. High electron density regions in compounds 2 and 3 suggest their suitability for interactions with specific residues in the active site, enhancing their binding strength. The balanced profile of compound 1 indicates its flexibility, making it a versatile candidate for drug optimization. These findings highlight the importance of tailoring electron density in drug design to achieve complementary interactions with the target protein, ensuring specificity and efficacy.

Reviewer 3

Comments for the Authors

This study provides a rough report on Pistacia integerrima and elucidates some of its functions through molecular simulation docking and other means. The logic is clear, but if the evidence can be improved, I think it can be accepted.

In evaluating the lipoxygenase inhibitory activity of the bioactive compounds from Pistacia integerrima, it is crucial to establish appropriate positive and negative controls to ensure the validity and reliability of the experimental results. Positive controls should include known lipoxygenase inhibitors to assess the sensitivity and specificity of the assay. For instance, compounds like zileuton, which is a well-established 5-LOX inhibitor, could serve as a positive

control. This would help in determining whether the observed inhibitory effects of the test compounds are comparable to or superior to those of the known inhibitors.

Negative controls, on the other hand, are essential to rule out non-specific inhibition. These controls typically involve the use of a solvent or a non-active compound that is expected to have no effect on lipoxygenase activity. By comparing the results of the test compounds with those of the negative controls, researchers can confirm that any observed inhibition is indeed due to the specific action of the compounds rather than non-specific interactions or experimental artifacts.

It would be beneficial for the study to provide detailed information on the specific positive and negative controls used, along with the corresponding experimental data. This would enhance the transparency and reproducibility of the research findings.

Response: The activity was performed as per the standard procedure Tenidap sodium was used as a positive controls (standard drug) in this finding There is a negative control are inert solvents have no effect.The needful correction in the entire text has been done now.

This study primarily evaluates the lipid oxygenase inhibitory activity of compounds through in vitro biochemical experiments and molecular docking studies, but lacks cellular and animal experiments to verify its anti-inflammatory and anticancer effects. It is recommended to conduct relevant experiments subsequently, such as assessing the impact of compounds on inflammatory factor expression in inflammatory cell models or observing their inhibitory effects on tumor growth in tumor animal models, to confirm their therapeutic potential further.

Response: Dear referee, Thanks for your suggestion. The main aim of this project was in vitro screening, followed by a detailed docking. In vivo and cellular study we will do in future once we isolated the compound in bulk.

Although the drug-like properties of quercetin (1) are mentioned in the text, specific pharmacokinetic parameters such as absorption, distribution, metabolism, and excretion (ADME) characteristics are not addressed. These characteristics are crucial for evaluating the in vivo efficacy and safety of the compound. Please supplement relevant studies or literature to comprehensively assess the drug-likeness potential of the compound.

Response: Thank you so much for your valuable insight. A detailed ADME analysis has been added to the manuscript. However, the current study does not include the query compound, quercetin.