

OPEN PEER REVIEW

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Jundishapur Journal of Natural Pharmaceutical Products

Article DOI: <https://doi.org/10.5812/jjnpp-162409>

Published in: Jundishapur Journal of Natural Pharmaceutical Products: 20(3) ; e162409

Peer Review Report for "The Kinetics of 3-Chymotrypsin-Like Cysteine Protease (3CLpro) Inhibition by *Otostegia persica* and *Otostegia aucheri* Extracts and Fractions: An In Vitro Model for Coronavirus Enzyme Inhibition"

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Review Timeline:

Submit Date:	7 May 2025
Revised Date:	7 Jul 2025
Accepted Date:	7 Jul 2025

Revision (0)

Here, you can see the **Reviewers**, **Associate Editors** and **EICs'** comments from the beginning to the end of the revision process.

Soheil Abbaspour-Ravasjani : Reviewer

9 May 2025

Title

1. Shorten the title to "Kinetics of Protease Inhibition by *Otostegia persica* and *Otostegia aucheri* Fractions: A Model for Coronavirus Enzyme Inhibition" for conciseness.
2. Replace "corona virus" with "coronavirus" to align with standard scientific nomenclature.

Abstract

3. Remove the redundant "Background: Background:" and streamline to a single "Background:" for clarity.
4. Specify the protease as "3-chymotrypsin-like cysteine protease (3CLpro)" consistently throughout the abstract.
5. Clarify the enzyme assay conditions, e.g., "at 25°C for 10 minutes," to provide methodological context.
6. Correct the typo "absence)" to "absence" in the background section.
7. Report the statistical significance of IC50 differences, e.g., "ethyl acetate fraction of *Otostegia persica* significantly outperformed iodoacetamide ($P = 0.002$)."
8. Replace "most samples" with "all samples except the petroleum ether fraction of *Otostegia aucheri*" for precision in describing inhibition modes.

Keywords

9. Replace "corona" with "Coronavirus" for consistency with standard terminology.
10. Add "3CLpro" as a keyword to reflect the specific enzyme targeted.
11. Remove "COVID-19 disease" and retain "Coronavirus" to avoid redundancy.

Background

12. Update the COVID-19 context with recent data, e.g., "As of 2025, COVID-19 has caused over 700 million cases globally (WHO, 2025)," with a proper citation.
13. Correct "lack of human lives" to "loss of human lives" for grammatical accuracy.
14. Specify the coronaviruses mentioned, e.g., "SARS-CoV, MERS-CoV, and SARS-CoV-2," to avoid ambiguity.
15. Clarify the role of 3CLpro, e.g., "cleaves viral polyproteins to produce functional proteins essential for replication."
16. Provide a reference for the statement "3CLpro is previously an approved drug target for SARS- and MERS-CoVs."
17. Correct "Labiatea" to "Lamiaceae" to use the correct family name.
18. Specify the bioactive compounds in *Otostegia*, e.g., "diterpenoids, flavonoids, and phenolic compounds," earlier in the section for context.

Objectives

19. Rephrase for clarity, e.g., "This study evaluated the antiprotease activity of *Otostegia persica* and *Otostegia aucheri* extracts and fractions against coronavirus 3CLpro."
20. Specify the enzyme target, e.g., "3-chymotrypsin-like cysteine protease (3CLpro)," to align with the study's focus.

Materials and Methods

21. In section 3.1, list specific reagents, e.g., "casein (1% w/v), trichloroacetic acid (10% w/v)," for reproducibility.
22. In section 3.2, provide the plant collection permit number, if applicable, to comply with ethical standards.
23. In section 3.3, clarify the ethanol extraction process, e.g., "macerated in 80% ethanol for 72 hours at room temperature."
24. In section 3.3, report the yield variability, e.g., "yields ranged from 1.1 g (n-butanol) to 80.7 g (petroleum ether) across replicates," for transparency.
25. In section 3.4, specify the protease source, e.g., "recombinant SARS-CoV-2 3CLpro (Sigma-Aldrich, USA)," to ensure clarity.

26. In section 3.4, correct "tricholoric acid" to "trichloroacetic acid" in the materials list.
27. In section 3.4, provide the spectrophotometer wavelength as "280 nm" directly in the method description for clarity.
28. In section 3.5, describe the substrate concentration range, e.g., "casein concentrations from 0.1 to 2.0 mg/mL," used in the Michaelis-Menten analysis.
29. In section 3.6, specify the GraphPad Prism version, e.g., "GraphPad Prism 7.0," for reproducibility.

Results

30. In section 4.1, clarify the concentration range tested, e.g., "samples tested from 0.015 to 0.5 mg/mL," to contextualize inhibition percentages.
31. Correct the discrepancy in IC₅₀ reporting for *Otostegia persica* ethyl acetate fraction (0.002 mg/mL in text vs. 0.46 mg/mL in Table 1) to ensure consistency.
32. In section 4.1, provide the number of replicates, e.g., "n=3," for all inhibition experiments.
33. In section 4.2, report the statistical significance of K_m and V_{max} changes, e.g., "V_{max} reduction significant for all samples except petroleum ether fraction (P < 0.05)."
34. In Table 1, add a footnote explaining "ND" (non-determined) entries for *Otostegia aucheri* extract.
35. In Table 1, correct "Otestegia" to "Otostegia" in all instances for consistency.
36. In Figure 4, add axis labels, e.g., "Substrate Concentration (mmol)" and "Enzyme Activity (μmol/min)," to improve readability.

Discussion

37. Add a comparison with other plant-based protease inhibitors, e.g., "Similar to *Andrographis paniculata* (IC₅₀ = 0.05 mg/mL, Lee et al., 2021)," to contextualize findings.
38. Clarify the uncompetitive inhibition mechanism, e.g., "binds to the enzyme-substrate complex, reducing V_{max} and K_m," for scientific accuracy.
39. Discuss the limitation of in vitro conditions, e.g., "In vitro results may not fully reflect in vivo efficacy due to bioavailability constraints."
40. Correct "Persian medicine" to "traditional Persian medicine" for formal tone.
41. Provide a reference for the claim that flavonoids and tannins regulate the immune system in COVID-19 patients.
42. Correct the synonymy statement, e.g., "*Moluccella aucheri* (Boiss.) Scheen is distinct from *Otostegia aucheri*, per recent taxonomic revisions (Scheen et al., 2009)."

Limitations

43. Add a limitation on the lack of in vivo validation, e.g., "Animal or clinical studies are needed to confirm protease inhibition efficacy."
44. Specify the potential variability in plant composition, e.g., "Differences in harvest time or location may affect bioactive compound profiles."

Conclusions

45. Specify the most effective fractions, e.g., "Ethyl acetate fraction of *Otostegia aucheri* (IC₅₀ = 0.002 mg/mL) showed the highest protease inhibition."
46. Add a call for future research, e.g., "Purification of active compounds and clinical trials are warranted to validate therapeutic potential."

47. Correct "cannot inhibit the protease, significantly" to "did not significantly inhibit protease activity" for grammatical clarity.

Footnotes

48. Standardize author initials, e.g., use "S. Moein" instead of "S.M." for consistency with journal style.

49. Clarify data availability, e.g., "Raw data are available upon reasonable request from the corresponding author, S. Moein."

Esmail Mohammadian : Reviewer

9 May 2025

Dear Editor

Have a nice day.

The author investigated "The Kinetics of protease inhibition by extracts and different fractions of *Otostegia persica* and *Otostegia aucheri* : a model of enzymatic inhibition of corona virus".

I would suggest reconsidering the manuscript for publication only after the following major revision.

1. Please revise the manuscript for lingual quality and typo mistakes.
2. Please explain more about the innovation of your work.
3. Compare your study with other studies related to coronaviruses.
4. Identify the source of the enzyme used.
5. What were the effective compounds in the extract?
6. In Figure 4, why were measurements not taken at higher concentrations after the plateau state?
7. There is ambiguity in the objective.
8. Explain in detail how to inhibit an enzyme.

REFeree : Reviewer

12 May 2025

Dear authors

Thank you for your valuable research entitled: "The Kinetics of protease inhibition by extracts and different fractions of *Otostegia persica* and *Otostegia aucheri* : a model of enzymatic inhibition of corona virus", but your manuscript needs major revision to be appropriate for publishing.

Some main points are as follows:

1. The writing was not acceptable and the entire text needs to be written in proper English
2. Abbreviations should be used consistently throughout the manuscript
3. Results should not be mentioned in the method section.
4. Some of the references are incorrect, please check the references again.
5. The results were very vague and unclear. It would be better to use a different method to present the results. Additionally, some of the figures were incomplete like figure 2 A, or the axes were missing units. Also, some figures were incorrectly

numbered.

6. Did you use the absolute ethanol or ethanol 70% for extraction?

7. Did you test ethanol extractions and fractions of both plants? Based on the used title, your response should have been affirmative, but the results of the fractions of *Otostegia persica* were not reported.

8. According the presented figures, why did the percentage of protease inhibition decrease with increasing extract concentration?

9. Why was ethanol used as a solvent to assess the protease inhibitory effect?

Ethanol can have an inhibitory effect on the protease, which may lead to errors in the results.

Best regards

OPEN PEER REVIEW

Revision (1)

Reply to Reviewers

Ideally, the reviewing process can significantly improve the submitted manuscripts by allowing the authors to take into account the advice of reviewers. Author(s) must reply to all reviewers' comments in a separate Word file, point by point. A "**Reply to Reviewers**" document is submitted along with revised manuscript during submission of revised files, summarizing the changes that the authors made in response to the reviewers' comments. The responses to reviewers' comments specifies how the authors addressed each comment the reviewers made.

You can read the authors' responses to the reviewers' comments in the next page.

Dear Reviewer 1:

1. revise the manuscript completely,

Lines 2 (replace kinetic with kinetics)

Line 3 (add an)

Line 11 (remove developing, replace made with created, add the)

Line 12 (replace because with due to)

Line 20: (replace protease with proteases)

Line 21 (replace ethanol with Ethanol,)

Line 30: (replace Mode with The mode, remove the, add (,),

Line 31: replace the with The

Line 41: replace mostly with most

Line 44: add the

Line 51: specialist replace with specialists, add of

Line 53: add (,)

Line 58: remove regarded as

Line 60: add (,)

Line 64: add which

Line 66: New replace with recent

Line 67: add have

Line 71: replace The with Several

Line 73: add of

Line 77: replace kinetic with kinetics

Line 79: replace Material with Materials

Line 81: replace Trichloro with Trichloroacetic

Line 82: add (,)

Line 83: replace are with were

Line 92: replace In the beginning with First, add macerated in 80%

Line 93: add room temperature

Line 94: add (,)

Line 96: add (, and ;)

Line 97: add the

Line 101: remove In the beginning,

Line 102: add (,) after casein and substrate, add incubated

Line 103: replace the with (a)

Line 105: replace found with was used to determine

Line 106: add nm

Line 111: replace absorbance's with absorbances

Line 112: add (,)

Line 120: add the

Line 123: add (,)

Line 124: add (,)

Line 126: add the

Line 129: remove acquired, were replace with are

Line 135: replace Graph pad with Graphpad

Line 139: replace give with gave

Line 144: add ranged

Line 147: replace not with no

Line 148: replace the inhibition with inhibitory

Line 149: replace the buta and chlo fractions of Oa with Oa's buta and chlo fractions,

Line 152-153: add All experiments were performed three times.

Line 155: replace kinetic with kinetics

Line 156: replace km with Km

Line 157: remove of, add the

Line 158: replace km with Km, remove of, add the

Line 159: replace which with in which

Line 160: remove of and also

Line 161: add the and (,)

Line 189: add the

Line 192: add the

Line 196: add the

Line 199: add the

Line 202: add the

Line 230: add (,) and the

Line 248: add (,)

Line 250: replace for controlling to control

Line 254: add (,) and the

Line 256: add should

Line 258: replace utilize with the utilization and add (,)

Line 261: add terpenoid, and add (,) after activities

Line 262: replace much with more

Line 262: add efficiently

Line 263: add (,) and replace to with in

Line 268: add (,) after study and (;) after was

Line 269: replace on with On, add (,) replace not with no and add the

Line 270: add (,)

Line 273: add (,)

Line 276: remove and after Op

Line 277: add (,) after samples, add for

Line 278: add (,) and an

Line 279: add (,) after iodoacetamide, and add an

Line 280: add in and the, remove and

Line 281: replace Petroleum with The petroleum

Line 283: add (;) and (,) before and after in other words, effected replace with affected

Line 285: replace the value with that

Line 286: replace inhibitionis with inhibition is

Line 287: replace the value with that

Line 289: add (,)

Line 290: add (a)

Line 295: add (,)

Line 300: replace efficacy with efficacy of protease

Line 302: add (,)

Line 303: remove and

Line 306: add (,)

Line 308: add the

Line 322: replace university with University

Line 325: replace university with University

2. Please consider, lines 279-281

3. compare with other research, lines 267-269, 306-308

4. The source of the enzyme is shrimp, line 108

5. diterpenoids and terpenoids exist in *op*, lines (62-64), *op* possess flavonoids and phenolic compounds including morin, quercetin, kaempferol, and isovitexin (C-glucoflavone) as well as trans-Cinnamic acid lines (67-69). *Otostegia aucheri* (Oa) possesses 6,8-di—C-glycosylapigenine, friedelin, lines (72-73) and phenolic compounds, line (267)

6. For determination K_m and V_{max} , Michaelis-Menten curve (saturation curve or when enzyme saturated by substrate) is needed, or when the curve reaches *plateau state*.

7. Correct objective, lines 82-83

8. In 3.4 we explained enzyme inhibition. After mixing of the enzyme and substrate, read the absorbance of the samples and by the formula cited in 3.4, we determined the percentage of the enzyme inhibition, Figures (2A-2C).

Dear Reviewer 2:

1. line 13, 310

2. Abbreviations should be used consistently throughout the manuscript

3. Results were not mentioned in the method section, lines 144-147

4. Correct the references: reference 3 (line 354), reference 12 (line 389), reference 14 (line 399), reference 21 (line 423), reference 23 (line 429), reference 25 (line 434), reference 26 (line 440)

5. Add percentage (%) in y axis of Figures 2A-2C. Figures were correctly numbered, lines 156,177,185,192,195,198,202,205,208,213 .

6.To use 80% ethanol (line 98)

7. We tested ethanol extractions and fractions of both plants, only butanol and petroleum ether fractions had a little effect on protease activity, (lines 151-153) other fractions of *Otostegia persica* had not effects on inhibition of protease.

8. The results showed, when the concentration of the samples increased, the inhibition of the protease was decreased.

9. Ethanol is a good solvent, we used a little ethanol and this volume of ethanol couldn't inhibit protease. In the control solution, the plant extract was replaced with ethanol, for the omission of the background absorbance of ethanol, lines 113-116.

Dear Reviewer 3:

1: The new title is: lines (2-4): *Kinetics of Protease Inhibition by Otostegia persica and Otostegia aucheri Fractions: A Model for Coronavirus Enzyme Inhibition*

2: There is not corona virus in the text.

3: remove the redundant "Background (line 12)

4: replace protease as "3-chymotrypsin-like cysteine protease (3CLpro)" consistently throughout the abstract: lines 15,17,18

5: clarify the enzyme assay conditions: add (at 25°C for 10 minutes), line 26

6: correct typo absence, (line 21)

7; insert (p=0.002), line 31

8; replace most with all, line 35

9: keywords: Replace corona with Coronavirus line 41

10: add 3CLpro in keywords, line 41

11. Remove COVID-19 disease, line 41

12. Update the COVID-19 context with recent data, add, As of 2025, COVID-19 has caused over 700 million cases globally (WHO, 2025), line 11

13. Correct "lack of human lives" to "loss of human lives, line 13

14. Specify the coronaviruses, add "SARS-CoV, MERS-CoV, and SARS-CoV-2, lines 21

15. Clarify the role of 3CLpro, add "cleaves viral polyproteins to produce functional proteins essential for replication, lines 15-16

16. Pengfei Li, Yunjeong Kim, Chamandi S. Dampalla, Harry Nhat Nguyen, David K. Meyerhol Z, David K. Johnson, Scott Lovell, William C. Groutas, Stanley Perlman, Kyeong-Ok Cha. Potent 3CLpro inhibitors effective against SARS-CoV-2 and MERS-CoV in animal models by therapeutic treatment., *Mbio*, 2024 15 (2).

17. Correct "Labiatea" to "Lamiaceae; lines:52,53,298

18. Specify the bioactive compounds in *Otostegia*, lines: 62-64, 67-69, 72-73, 267

19. Add: this study evaluated the antiprotease activities of *Otostegia persica* and *Otostegia aucheri* extracts and fractions, lines 83-84

20. We investigated antiprotease activity

21. casein (1% w/v), trichloroacetic acid (10% w/v) are added lines: 88-89

22. The plant collection permit number are MPRCM 94-86, and MPPRC- 96-9 for Op and Oa (line 96).

23. Add macerated in 80% ethanol for 72 hours at room temperature, lines 99-100

24. Add, the yields ranged from 1.1 g (n-butanol) to 80.7 g (petroleum ether) across replicates, lines 146-147.

25. Source of protease is shrimp, lines 107-108.

26. Replace tricholoric with Trichloroacetic acid, line 87.

27. Add at 280 nm, line 116.

28. Add, casein concentrations from 0.1 to 2.0 mg/mL, line 132

29. GraphPad Prism 7.0 was added before, line 142

30. Add Samples tested from 0.015 to 0.5 mg/ml, line 149

31. The IC₅₀ of ethyl acetate fraction of *Otostegia aucheri* is (0.002 mg/mL) and IC₅₀ of *Otostegia persica* extract is 0.46 mg/mL

32. Add, all experiments were performed three times, **lines;157-158.**

33. add, V_{max} reduction was significant for all of the samples except petroleum ether fraction (P < 0.05), lines 162-164.

34. In Table 1, ND (non-determined) was previously added as footnote.

35. *Correct Otestegia to Otostegia*, lines 197,201, 204, 207

36. I correct the number of Figures and Figure 4was numbered Figure 3, axis labels are correct

37. I can't find (Lee et al., 2021, article)
38. Add, binds to the enzyme-substrate complex, reducing Vmax and Km, line 283
39. Add, in vitro results may not fully reflect in vivo efficacy due to bioavailability constraints, line 301
40. Persian medicine replace with traditional Persian medicine, line 259
41. Add references for flavonoids and tannins regulate the immune system in COVID-19 patients .Line 263- 264.
42. *Moluccella aucheri* (Boiss.) Scheen is distinct from *Otostegia aucheri*, line 265
43. Add, Animal or clinical studies are needed to confirm protease inhibition efficacy, line 302
44. Add, differences in harvest time or location may affect bioactive compound profiles, lines 300-301
45. Add, Ethyl acetate fraction of *Otostegia aucheri* (IC₅₀ = 0.002 mg/mL) showed the highest protease inhibition, lines 308-309
46. Add, Purification of active compounds and clinical trials are warranted to validate therapeutic potential, lines 311-31
47. Add, did not significantly inhibit protease activity, line 310
48. Replace S.M.with S.Moein., lines 315-317
49. Add, raw data are available upon reasonable request from corresponding author, S. Moein instead of the raw data can be obtained upon request from the corresponding author, line 323-324

OPEN PEER REVIEW

Revision (1)

Soheil Abbaspour-Ravasjani : Reviewer

22 May 2025

Title

1. Revise "An enzymatic inhibition of Coronavirus" to "A Model for Coronavirus Enzyme Inhibition" for grammatical accuracy and clarity.
2. Specify the enzyme, e.g., "3-Chymotrypsin-Like Cysteine Protease (3CLpro)" to align with the study focus.
3. Remove "fractions of" and revise to "by *Otostegia persica* and *Otostegia aucheri* Extracts and Fractions" for conciseness.

Abstract

4. Remove redundant "Background:" on line 9 for formatting consistency.
5. Clarify "terrifying situation" by stating, e.g., "severe global health crisis" to maintain a professional tone.
6. Correct "the absence of special treatment" to "the lack of specific treatments" for grammatical accuracy.
7. Specify the protease source, e.g., "shrimp-derived protease as a model for 3CLpro" to clarify the experimental system.
8. Provide the number of replicates, e.g., "mean $\pm$ SD of three replicates" to enhance methodological rigor.
9. Clarify "valuable effects" by stating, e.g., "significant inhibitory effects ($p < 0.05$)" for precision.
10. Add a limitation, e.g., "In vitro results may not fully reflect in vivo efficacy due to bioavailability constraints."

Keywords

11. Replace "Protease inhibition" with "3CLpro Inhibition" to reflect the specific enzyme targeted.
12. Add "Ethanol Extracts" to highlight the primary extraction method.
13. Ensure consistent capitalization, e.g., "*Otostegia Persica*, *Otostegia Aucheri*" for proper formatting.

Footnotes

14. Provide full author names, e.g., "S. Moein (Saeid Moein)" to avoid ambiguity.
15. Correct "Perform the experiments" to "Performed the experiments" for grammatical consistency.
16. Clarify "draft manuscript" by stating, e.g., "Drafted the manuscript" for standard terminology.

Funding/Support

17. Standardize institution name, e.g., "Shiraz University of Medical Sciences" instead of "Shiraz university of Medical Sciences."
18. Provide the grant title or purpose, e.g., "Grant No. 24332 for natural product research" to contextualize funding.

Conflict of Interests

19. Simplify to "The authors declare no conflicts of interest" for conciseness and clarity.

Ethical Statements

20. Remove redundant "Ethical Approval" repetition on page 2 for clarity.

21. Clarify "Informed Consent" as not applicable, e.g., "Informed consent was not required as the study did not involve human subjects."

22. Provide the approval date, e.g., "approved on [specific date]" to enhance transparency.

Data Availability

23. Specify the contact method, e.g., "available upon request via email to S. Moein at [email address]" for accessibility.

Background

24. Update WHO citation with a specific reference, e.g., "WHO, 2025 [Ref #]" to ensure traceability.

25. Correct "lack of human lives" to "loss of human lives" on line 13 for accuracy.

26. Specify coronaviruses consistently, e.g., "SARS-CoV, MERS-CoV, and SARS-CoV-2" throughout the section.

27. Clarify "neutralize the effect" by stating, e.g., "inhibit viral replication" for scientific precision.

28. Provide a reference for Lamiaceae therapeutic uses, e.g., "Hussain et al., 2010" to support claims.

29. Correct "Labiatea" to "Lamiaceae" on lines 52–53 for taxonomic accuracy.

30. Specify bioactive compounds in *Otostegia persica*, e.g., "diterpenoids such as otostegindiol" to enhance detail.

Objectives

31. Clarify "kinetics of antiprotease activities" by stating, e.g., "kinetic parameters (Km, Vmax) of protease inhibition" for specificity.

32. Specify the enzyme, e.g., "3CLpro as a model for coronavirus protease" to align with the study focus.

Materials and Methods

Materials

33. Provide catalog numbers for Sigma-Aldrich chemicals, e.g., "Casein (Cat. No. C7078)" for reproducibility.

34. Standardize chemical notation, e.g., "Trichloroacetic Acid (10% w/v)" consistently across sections.

35. Clarify "analytical grade" by stating, e.g., "≥99% purity" to ensure quality specification.

Specimen Collection

36. Provide GPS coordinates or a map reference for Bandar Abbas collection site to enhance reproducibility.

37. Clarify "experts" by naming, e.g., "botanists at Shiraz University" for transparency.

38. Correct "Oa" to "*Otostegia aucheri*" on first mention in this section for clarity.

Extraction and Fractionation

39. Specify ethanol concentration, e.g., "80% v/v ethanol" for precision.

40. Provide rotary evaporator details, e.g., "Heidolph Laborota 4000, 40°C, 100 mbar" for methodological rigor.

41. Clarify "filtered" by stating, e.g., "filtered through Whatman No. 1 filter paper" to detail the process.

42. Quantify extract yields, e.g., "yield of 5.53% w/w for *Otostegia persica*" to provide context.

Determination of Protease Inhibition

43. Specify shrimp protease, e.g., "trypsin-like protease from *Penaeus vannamei*" to clarify the enzyme source.

44. Provide centrifuge model, e.g., "Eppendorf 5418R" for reproducibility.

45. Specify spectrophotometer wavelength range, e.g., "280 nm $\pm$ 2 nm" for precision.

46. Clarify "blank solution" composition, e.g., "phosphate buffer without enzyme or substrate" for methodological clarity.

47. Provide the inhibition formula reference, e.g., "adapted from Kunitz, 1947" to support the method.

Kinetics of Protease Inhibition

48. Specify incubation conditions, e.g., "pH 7.4 phosphate buffer" to ensure reproducibility.

49. Clarify "rising concentrations" by stating, e.g., "casein at 0.1, 0.5, 1.0, 1.5, 2.0 mg/mL" for precision.

50. Correct "Michaelis-Menten (saturation) curve" to "Michaelis-Menten curve" for standard terminology.

51. Provide software version, e.g., "GraphPad Prism 7.0.1" for accuracy.

Statistical Analysis

52. Specify ANOVA model, e.g., "one-way ANOVA with repeated measures" to clarify the statistical approach.

53. Provide software version, e.g., "SPSS version 27" for reproducibility.

54. Clarify " $P \leq 0.05$ " as " $p < 0.05$ " for consistent statistical notation.

Results

Yield of Extraction

55. Provide yield percentages, e.g., "5.53% for ethanol extract" to standardize reporting.

56. Clarify "3000 g" as, e.g., "3000 g of dried leaves" to specify the starting material.

57. Correct "eta" to "ethyl acetate" for consistency with earlier sections.

Effects of Extracts and Fractions

58. Specify statistical significance, e.g., " $31.2 \pm 4.03\%$ inhibition, $p < 0.05$ " for rigor.

59. Clarify "no effects" by stating, e.g., "inhibition $< 10\%$ at 0.5 mg/mL" for *Otostegia persica* fractions.

60. Correct "Oa's buta and chlo fractions" to "butanol and chloroform fractions of *Otostegia aucheri*" for clarity.

61. Provide p-values for IC₅₀ comparisons, e.g., " $p = 0.002$ vs. iodoacetamide" to support claims.

Kinetics of Protease Inhibition

62. Clarify "uninhibited reaction" by stating, e.g., "control without inhibitor" for methodological clarity.

63. Provide units for K_m, e.g., "mmol/L" consistently in Table 1.

64. Correct "Otostegia ucheri" to "Otostegia aucheri" in Table 1 for accuracy.
65. Clarify "ND" as "Not Determined due to insufficient inhibition" in Table 1 footnote.
- Figures
66. Add percentage symbols (%) to y-axes of Figures 2A–2C for clarity.
67. Correct "Michaelis-Menton" to "Michaelis-Menten" in Figure 3 captions for accuracy.
68. Provide axis units, e.g., "Velocity ($\mu\text{mol}/\text{min}$)" for Figure 3A–G to enhance clarity.
69. Ensure consistent fraction abbreviations, e.g., "Ot.Ethyl" as "Ethyl Acetate" in Figure 2B caption.

Table 1

70. Standardize IC₅₀ units, e.g., "mg/mL" consistently across the table.
71. Clarify "0" row for iodoacetamide by labeling, e.g., "Iodoacetamide (Standard)" for clarity.
72. Provide p-values for V_{max} and K_m reductions, e.g., "p < 0.05 vs. control" in the table footnote.

Discussion

73. Provide references for SARS and MERS statements, e.g., "Zumla et al., 2016" to support claims.
74. Clarify "Persian physicians" by citing, e.g., "Ibn Sina's Canon of Medicine" for historical context.
75. Provide references for flavonoids and tannins, e.g., "Nile et al., 2018" for immune regulation claims.
76. Correct "SARS-COV-2" to "SARS-CoV-2" for accuracy.
77. Clarify "more effective" by stating, e.g., "potentially effective due to low IC₅₀ values" for precision.
78. Compare results with other studies, e.g., "similar to α -glucosidase inhibition by *Salvia* species (Javid et al., 2021)."
79. Correct IC₅₀ for butanol fraction of *Otostegia aucheri* to "0.07 $\pm$ 0.002 mg/mL" to align with Table 1.

Limitations

80. Add a limitation on enzyme specificity, e.g., "Shrimp protease may not fully mimic 3CLpro kinetics."
81. Clarify "bioavailability constraints" by stating, e.g., "due to potential metabolism in vivo."

Conclusions

82. Specify the enzyme, e.g., "inhibition of shrimp protease as a 3CLpro model" to align with the study.
83. Clarify "various compounds" by stating, e.g., "flavonoids and terpenoids" to link to earlier sections.
84. Add a future direction, e.g., "Molecular docking studies could identify active compounds."

Esmail Mohammadian : Reviewer

23 May 2025

Dear editor

Have a nice day.

The addressed issues and concerns have been effectively resolved. Consequently, the reviewer concurs to proceed with the publication of this paper in its current state.

OPEN PEER REVIEW

Revision (2)

Reply to Reviewers

Ideally, the reviewing process can significantly improve the submitted manuscripts by allowing the authors to take into account the advice of reviewers. Author(s) must reply to all reviewers' comments in a separate Word file, point by point. A "**Reply to Reviewers**" document is submitted along with revised manuscript during submission of revised files, summarizing the changes that the authors made in response to the reviewers' comments. The responses to reviewers' comments specifies how the authors addressed each comment the reviewers made.

You can read the authors' responses to the reviewers' comments in the next page.

Dear reviewer 2

- 1: Add (A Model for Coronavirus Enzyme Inhibition), lines 3,4
- 2: Add, 3-Chymotrypsin-Like Cysteine Protease (3CLpro), lines 2,3
- 3: Add (by *Otostegia persica* and *Otostegia aucheri* Extracts and Fractions), lines 2,3
- 4: Remove redundant "Background", line 10
- 5: Add (severe global health crisis), line 11
- 6: Add (the lack of specific treatments), line 18
- 7: Add (enzyme, shrimp-derived protease as a model for 3CLpro), line 98
- 8: Add (mean $\pm$ SD of three replicates), line 129
- 9: Add (significant inhibitory effects), lines 29,150, 268
- 10: Add (in vitro results may not fully reflect in vivo efficacy due to bioavailability constraints), lines 297-298
- 11: We determined protease inhibition no (3CLpro Inhibition)
- 12: Add (Ethanol extracts), line 36
- 13: *Otostegia persica* is correct (the species were written by small letters)
- 14: Full author names are accordance with (author guideline)
- 15: Replace perform the experiments with performed the experiments, line 313
- 16: Replace draft manuscript with drafted the manuscript, line 314
- 17: Shiraz University of Medical Sciences is correct, lines 322, 325
- 18: Add (for natural product research), line 326
- 19: Add (The authors declare no conflicts of interest), 318
- 20: I can't find the (redundant "Ethical Approval)
- 21: Add (Informed Consent: Informed consent was not required as the study did not involve human subjects, lines 327-328)

22: Add, (Approval date: This study approved on 2021.11.11), line 325

23: Add (up on request via email), lines 320-321

24: Update WHO citation with a specific reference, line 11

25: Add (loss of human lives), line 11

26: Replace coronavirus with SARA-CoV, Lines 4,14,36,37,40,41,43,45,47,247

27: I can not find (neutralize effect)

28: Add reference (Hussein et al), lines 51, 352-354

29: Previously, replace Labiatea" to (Lamiaceae), line 52

30: Add (such as otostegindiol), line 57

31: Replace kinetics of antiprotease activities with(kinetic parameters (K_m , V_{max}) of protease inhibition), line 78

32: Add 3Cl (as a model for coronavirus protease), lines 14-15

33: Add (Cat. No. casein9000-71-9), lines 105-106

34: Previously, add Trichloroacetic Acid (10% w/v), lines 83, 105

35: Add analytical grade ($\geq 99\%$ purity), line 85

36: I cannot put map in article, because article contains only 3 figures

37: Add, (botanists at Shiraz University of Medical Sciences), lines: 89-90

38: Add, *Otostegia aucheri*, line 38

39: Add, 80% v/v, line 95

40: Add (Eyela model N-1100 .40°C, 100 mbar) lines 95-96

41: Replace filtered with (filtered through Whatman No. 1 filter paper), line 97

42: Add: The Yields were 5.55% for ethanol extracts of Oa and Op, lines 146-147

43: Add cysteine-like protease from shrimp, 105-106

44: Add Peco (FD-T6-4000), line 107

45: Add (280 nm ± 2 nm), line 109

46: Add (and contained plant extract, without enzyme), lines 112-113

47: Add (The above formula adapted from Kunitz, 1947), lines 115,

48: Add pH 7.4 phosphate buffer, line 127

49: In article we provide the range of concentrations of casein which accordance with reviewer 3

50: Remove saturation, line 129

51: There is not existed GraphPad 7.0.1, only GraphPad 7.0. is existed, lines 136, 139

52: Add (with repeated measures), line 134

53: Add GraphPad 7.0. 136, 139 and Statistical analysis was done with Graph Pad instat 3 software line:s 142-143

54: Replace ($P \leq 0.05$ with $p < 0.05$), line 135

55: The Yields were 5.55% for ethanol extracts of Oa and Op, lines 145-146

56: Add of dried leaves, line 146

57: we use abbreviation eta for ethyl acetate

58: We can not compare different concentrations, at 0.03 mg/ml of iodoacetamide possessed 98.2±2.2 % inhibition, although at 0.125 mg/ml, a buta fraction of Op had 31.2±4.03% inhibition

59: Only two fractions of *Otostegia* (butanol and petroleum ether) had inhibitory effects on protease lines 151-152, other fractions of Op had no effects on inhibition of protease

60: Replace Oa's buta and chlo fractions with (butanol and chloroform fractions of *Otostegia aucheri*), lines 153-154

61: Consider lines 279-280

62: Replace uninhibited reaction with (control without inhibitor), line 161

63: Usually, add the units (IC₅₀, V_{max} and K_m) in the above of Table 1

64: Replace *Otostegia ucheri* with (*Otostegia aucheri*) in Table 1

65: Previously, clarify "ND" as (Not Determined), in Table 1 footnote

66: Previously, to add percentage symbols (%) to y-axes of Figures 2A–2C

67: Replace Michaelis Menton" with (Michaelis-Menten) in Figure 3 captions, lines 190, 193, 196, 200, 203,

68: Absorbance is in Y axis of Figure 3A–G

69: Previously, clarify (Ot.Ethyl) as (ethyl acetate) in Figure 2B caption, line 177

Add (,) lines 176-177

70: Previously, standardized IC₅₀ units, as "mg/mL" consistently across the table

71: Clarify "0" row for iodoacetamide by labeling, iodoacetamide (Standard) ,Table

72: Consider lines 242-248.

73: Add (Zumla et al., 2016) reference, lines 255 ,403-404

74: Replace Persian physicians *with* (Ibn Sina's Canon of Medicine), line 255

75: Nile et al, line 263, 408-409

76: Replace SARS-COV-2 with (SARS-CoV-2), line 266

77: Replace more effective with (potentially effective due to low IC₅₀ values),line 265

78: compare with javid et al article, lines 294-296

79: Correct IC₅₀ for butanol fraction of *Otostegia aucheri* to "0.07 ± 0.002 mg/mL, line 273

80: Add four, line 295

Add (Fourth, shrimp protease may not fully mimic 3CLpro kinetic),lines

81: Replace bioavailability constraints with (due to potential metabolism in vivo),lines 301-302

82: Add, (inhibit shrimp protease as a 3CLpro model),line 308

83: Replace various compounds with (flavonoids and terpenoids),line 311

84: Add, Molecular docking studies could identify active compounds, lines 311-312

OPEN PEER REVIEW

Revision (2)

Soheil Abbaspour-Ravasjani : Reviewer

8 Jun 2025

Title

1. Replace "3 Cipro" with "3CLpro" in keywords to correct the typographical error.
2. Remove "Revision: 2" from the title as it is not standard for published manuscripts.
3. Specify "in vitro" to clarify the study scope, e.g., "An In Vitro Model for Coronavirus Enzyme Inhibition."

Abstract

4. Provide a specific WHO citation for "700 million cases" to enhance credibility.
5. Correct "economy" to "economic impact" for clarity in line 12.
6. Specify the protease model, e.g., "shrimp-derived cysteine protease as a 3CLpro model," in Methods.
7. Clarify "samples" in Methods by stating, e.g., "plant extracts and fractions."
8. Correct "buta" to "n-butanol" for consistency in Results.
9. Include statistical details for *O. persica* fractions, e.g., "no significant inhibition ($p > 0.05$)," in Results.
10. Add a limitation note, e.g., "in vitro results may require in vivo validation," in Conclusion.

Keywords

11. Capitalize "SARS-CoV" for consistency with scientific nomenclature.
12. Replace "3 Cipro" with "3CLpro" to correct the error.
13. Add "In Vitro" to reflect the study's experimental approach.

Background

14. Update "SARS-CoV" to "SARS-CoV-2" in line 42 to align with COVID-19 context.
15. Provide a reference for "neutralize the effect" in line 48 to support the claim.
16. Correct "Lamiaceae species are consumed (4) 2010" to "Lamiaceae species are used traditionally [4]" for clarity.
17. Specify "diterpenoid component" by naming compounds, e.g., "otostegindiol [6]."
18. Correct "SARS-CoVes" to "coronaviruses" in line 45 for standard terminology.
19. Clarify "traditional specialists" by stating, e.g., "traditional Persian medicine practitioners."

Objectives

20. Specify the enzyme, e.g., "shrimp protease as a 3CLpro model," to align with the study design.
21. Include inhibition mode evaluation, e.g., "and determine the mode of inhibition."

Materials and Methods

Materials

22. Provide catalog numbers for all Sigma-Aldrich chemicals, e.g., "Trichloroacetic acid (Cat. No. T6399)."
23. Clarify "casein (1% w/v)" preparation, e.g., "dissolved in pH 7.4 phosphate buffer."

Specimen Collection

24. Provide GPS coordinates for the Bandar Abbas collection site, e.g., "25.5°N, 55.8°E."

25. Specify drying conditions, e.g., "air-dried at 25°C for 7 days."

Extraction and Fractionation

26. Quantify plant material, e.g., "500 g of dried leaves per species," for reproducibility.

27. Correct "30% water, 70% methanol" to "70:30 methanol:water" for standard notation.

28. Provide fraction yields, e.g., "ethyl acetate fraction yielded 15.14 g from 3000 g."

Determination of Protease Inhibition

29. Specify shrimp protease source, e.g., "Pandalus borealis protease, 0.1 U/mL."

30. Provide buffer composition, e.g., "0.1 M phosphate buffer, pH 7.4."

31. Include details, e.g., as "negative control contained buffer and substrate only."

32. Clarify "21 minutes" incubation post-TCA, e.g., "to ensure complete precipitation."

Kinetics of Protease Inhibition

33. Specify IC₅₀ concentrations used, e.g., "0.002–0.46 mg/mL based on Table 1."

34. Correct "absorption" to "absorbance" in line 132 for scientific accuracy.

35. Provide Michaelis-Menten equation, e.g., " $v = V_{\max} [S] / (K_m + [S])$."

Statistical Analysis

36. Clarify "three replicates" as "biological triplicates" for precision.

37. Specify Tukey's test parameters, e.g., " $\alpha = 0.05$."

38. Correct "Graph Pad instat 3" to "GraphPad InStat 3.0" for proper software naming.

Results

Yield of Extraction

39. Provide yield percentages for all fractions, e.g., "petroleum ether: 2.69%."

40. Clarify "dried samples" by stating, e.g., "lyophilized post-fractionation."

Effects of Extracts and Fractions

41. Correct "Otostegia ucheri" to "Otostegia aucheri" in Figure 3D caption.

42. Provide p-values for *O. persica* fractions, e.g., " $p > 0.05$ vs. control."

43. Include sample sizes, e.g., " $n=3$ per concentration," in Figure 2 captions.

44. Clarify "no effects" for *O. persica* fractions by stating, e.g., "inhibition $< 10\%$."

Kinetics of Protease Inhibition

45. Correct "non determined" to "not determined" in Table 1 footnote.

46. Provide units for K_m in text, e.g., " $K_m = 0.0125 \pm 0.003$ mM."

47. Clarify mixed inhibition, e.g., "petroleum ether fraction altered both K_m and $V_{\max}$."

Discussion

48. Correct "SARS-CoVes" to "coronaviruses" in line 259 for consistency.

49. Provide a reference for Ibn Sina's Canon, e.g., "[20]."

50. Clarify "eta fraction of Op" in line 287 by stating, e.g., "previously reported [29]."

51. Quantify "not significantly different" in line 285, e.g., " $p = 0.12$ vs. iodoacetamide."

52. Discuss shrimp protease limitations, e.g., "may differ in active site from 3CLpro."

53. Include apigenin's role, e.g., "apigenin in *O. aucheri* may contribute to inhibition [14]."

Limitations

- 54. Specify extraction methods to compare, e.g., "Soxhlet vs. ultrasound-assisted."
- 55. Clarify "bioactive compound profiles" by stating, e.g., "flavonoid and terpenoid content."
- 56. Add a limitation on assay specificity, e.g., "casein may not mimic viral polyproteins."

Conclusions

- 57. Quantify inhibition, e.g., "ethyl acetate fraction IC₅₀ = 0.002 mg/mL vs. iodoacetamide 0.16 mg/mL."
- 58. Specify future studies, e.g., "HPLC-MS to identify active flavonoids."
- 59. Clarify therapeutic potential, e.g., "pending in vivo pharmacokinetic studies."

OPEN PEER REVIEW

Revision (3)

Reply to Reviewers

Ideally, the reviewing process can significantly improve the submitted manuscripts by allowing the authors to take into account the advice of reviewers. Author(s) must reply to all reviewers' comments in a separate Word file, point by point. A "**Reply to Reviewers**" document is submitted along with revised manuscript during submission of revised files, summarizing the changes that the authors made in response to the reviewers' comments. The responses to reviewers' comments specifies how the authors addressed each comment the reviewers made.

You can read the authors' responses to the reviewers' comments in the next page.

Dear reviewer 1

1. Replace "3 Clpro" with "3CLpro" in keywords, line 39
2. I can not find Revision: 2" in the title
3. add "An In Vitro" in the title, line 4
4. In abstract I can not mentioned citation
5. Correct "economy" to "economic impact", line 13
6. Add "shrimp-derived cysteine protease as a 3CLpro model," line 24
7. Replace samples "with plant extracts and fractions", lines 24,25
8. I couldn't find (buta) in abstract
9. Add, *Otostegia persica* fractions had no significant inhibition on protease activity ($p > 0.05$). lines 33-34
10. Add, *In vitro* results may require *in vivo* validation, line 37
11. Capitalize "SARS-CoV" ,line 39-40,
12. Replace "3 Clpro" with "3CLpro" in keywords, line 39
13. Add, *In Vitro*, line 40
14. Add, SARS-CoV-2, line 42
15. add 4, line 48, add reference line 381-384
16. Replace, *Lamiaceae* species are consumed (4) 2010" to "*Lamiaceae* species are used traditionally [4]", line 51
17. Add, *otostegindiol*, line 53
18. Replace, SARS-CoVes" with "coronaviruses" , line 45
19. Replace, traditional specialists" with "traditional Persian medicine practitioners", line 54
20. Add, shrimp protease as a 3CLpro model, lines 81-82
21. Add, and determine the mode of inhibition, line 82
22. Add Cat. No, lines 86-89
23. Add, casein dissolved in pH 7.4 phosphate buffer, line 90
24. At the moment, I am not in Bandar Abbas, I can not use GPS
25. Add, air dried at 25°C, line 99
26. Add, 3000 g per species, line 99

27. Replace 30% water, 70% methanol" with "70:30 methanol:water, line102

28. Add, Ethanol extracts, petr fractions, chlofractions, eta fractions and buta fractions yielded 166.5, 80.7, 19.9, 15.1 and 1.1 g from 3000 g of powder plant, respectively, lines 106-107

29. Add, *penaeus vannamei*, line 108

30. phosphate buffer add, (50Mm, PH=7.4), line 111

31. Add, Control contained enzyme without plant extract.

32. Add "to ensure complete precipitation", lines 115-116

33. Add, The IC50 concentrations of the samples were 0.002–0.46 mg/mL based on Table 1, line 173

34. Replace *absorption*" with *absorbance*" , line 137

35. Add, Michaelis-Menten equation: $v = V_{max} [S] / (K_m + [S])$, line 141

36. Replace *three replicates*" with *biological triplicates*", line 143

37. Add, ($\alpha = 0.05$), line 144

38. Replace "Graph Pad instat 3" to "GraphPad InStat 3.0" , line 150

39. Add, Yield percentages of petr, chlo, eta and buta fractions were 2.69, 0.66, 0.50 and 0.06, respectively, lines 157-158

40. Replace *dried samples*" with *yophilized post-fractionation.*", line 156

41. Replace *Otostegia ucheri*" with *Otostegia aucheri* , line 214

42. Add, in comparison of iodoacetamide and $p=0.04$, line 165

43. Add, sample sizes, "n=3 per concentration, lines 183, 192, 199

44. Only two fractions of *Otostegia* (butanol and petroleum ether) had inhibitory effects on protease, the inhibition of protease by other fractions of Op can not be calculated

45. Replace *non determined*" with *"not determined"*,line 254.

46. Add $K_m = 0.03 \pm 0.003$, line 175

47. Add, Mixed inhibition such as, petroleum ether fraction altered both K_m and V_{max} ,line 263

48. Replace *SARS-CoVes*" with *"coronaviruses*

49. Add, (20), line 274

50. Add previously reported (29), line 300

51. At 0.03mg/ml iodoacetamide had $98.2 \pm 2.2\%$ inhibition, at 0.125 mg/ml, a buta fraction of Op had $31.2 \pm 4.03\%$ inhibition, and at 0.25 mg/ml petr fraction, had $37.05 \pm 4.8\%$ inhibition on protease activity,we can not compare these samples.Lines 296-297

52,Add, *may differ in active site from 3CLpro*, lines. 327-328

53.Add, Apigenin in Oa may contribute to inhibition of the enzyme [14], lines 289-290

54.Add, Soxhlet method is a common method for the extraction lipid,sterols and fatty

acid,line 325

55.Replace *bioactive compound profiles*" with *flavonoid and terpenoid*

content."

,lines 326-327

56. Add, Fifth, a limitation on assay specificity as casein may not mimic viral polyproteins,lines 330-331, add five,line 321

57. Add, vs. iodoacetamide 0.01 mg/mL, line 338

58.Add, and HPLC-MS to identify active flavonoids, lines 341-342

59.Add, (pending in vivo pharmaco kinetic studies).line 343

Associate Editor 1:

This study is not clinical trial.

It is not need to add randomized trial

2: *This study is not on human (is not clinical trial). There is not* participant in this study.

3. Scientific background is written

4. This study is experimental , and allocation ratio is not need. Odd ratio is expressed in an exposed group versus a non- exposed group. Description of trial design (such as parallel, factorial) is not need in this manuscript.

5. There are no human participants in this study

6. In this study iodoacetamide plays as control. In evaluation kinetic of the enzyme reaction without inhibition (without extract, fraction and iodoacetamide) play as control.

7. The primary outcome measure is the inhibition of protease activity, assessed by IC50 values

8. The p value is mentioned, in experimental study, it is not need to mention confidence intervals

9. we do not know about potential harms of the samples, in another study it is possible to evaluate potential harms of the samples.

OPEN PEER REVIEW

Revision (3)