Bukti Korespondensi 1

Bukti Korespondensi Penulis (an Hasnah Natsir, Unhas) dengan Journal Pharmacia melalui email *hasnahnatsir@unhas.ac.id* dapat diuraikan dalam tabel berikut:

No	Tanggal	Uraian (Komunikasi Penulis dengan editor Journal Pharmacia)
1	8 November 2021	submitting the manuscript #77740
2	18 Desember 2021	penulis menanyakan kabar manuscript
3	24 Januari 2022	Informasi Major revision pada manuscript #77740 dan diberi waktu revisi 10 hari (paling lambat 3 Februari 2022), namun diberi kesempatan meminta perpanjangan waktu dalam hal penambahan data analisis dan revisi manuscript
4	3 Februari 2022	penulis mengusulkan perpanjangan waktu untuk penambahan data dan revisi manuscript sebagai tanggapan atas permintaan dan saran reviewer
5	4 Februari 2022,	Pharmacia menyetujui permintaan penulis, dan menanyakan sampai tanggal berapa perpanjangan waktu yang dibutuhkan
6	7 Februari 2022	Penulis meminta perpanjangan waktu revisi hingga 5 Maret 2022
7	13 Februari 2022	Pharmacia mengingatkan kembali waktu revisi
8	5 Maret 2022 10 Maret 2022,	Manuscript #77740: Submitted Manuscript #77740: Accepted
9	7 April 2022	Manuscript #77740: 1st PDF Proof Uploaded
10	11 April 2022	Manuscript #77740: 2nd PDF Proof Uploaded
11	12 April 2022	Manuscript #77740: 3rd PDF Proof Uploaded
12	14 April 2022	Manuscript #77740: Published

1. **Artikel submitted tertanggal 8 November 2021**

Pharmacia pharmacia@pensoft.net Nov 8, 2021, 11:33 AM

Dear Hasnah Natsir:
Thank you for submitting the manuscript #77740 "Inhibitory of Moringa oleifera L leaf extract on xanthine oxidase activity from bovine milk" to Pharmacia. You can keep track of its progress through the manuscript's URL: https://goto.arphahub.com/c3BHad4cWd2B
You can also view all manuscripts with which you are associated either as submitting author, or reviewer, or editor by clicking on the My tasks button on the journal's homepage, after login.
Thank you for considering Pharmacia as the venue for your work.

Pharmacia Editorial office

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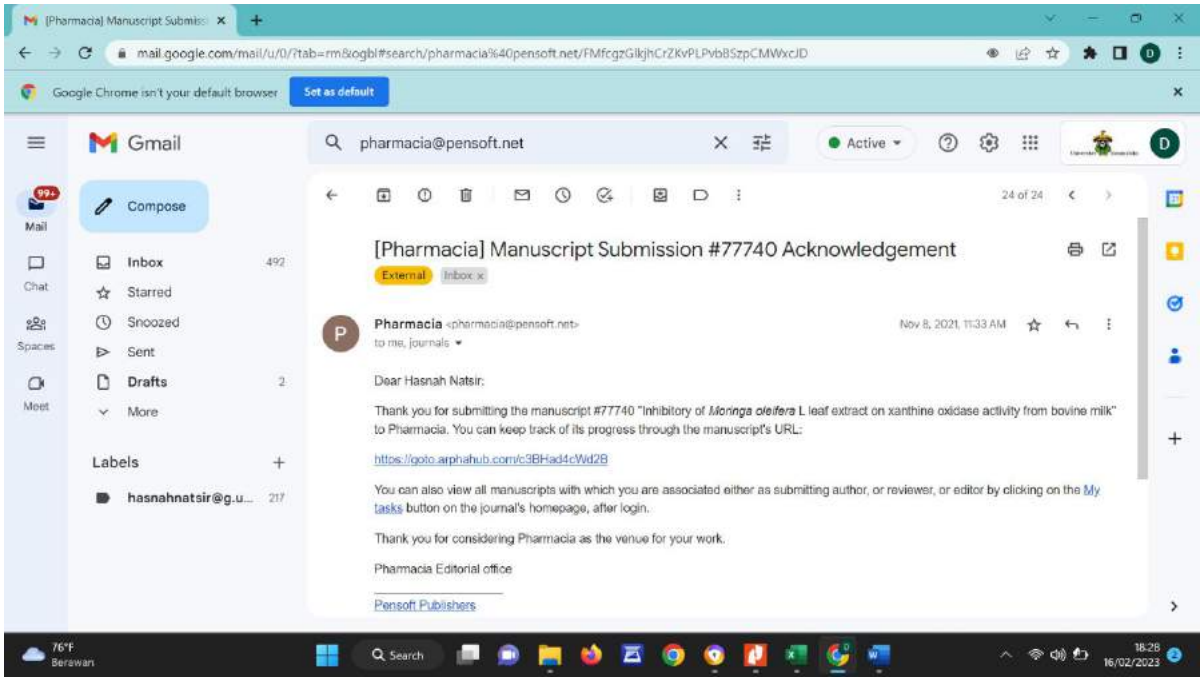


2. Penulis menanyakan kabar artikel ke J. Pharmacia, tgl 18 Des 2021

Sat, Dec 18, 2021, 11:18 AM

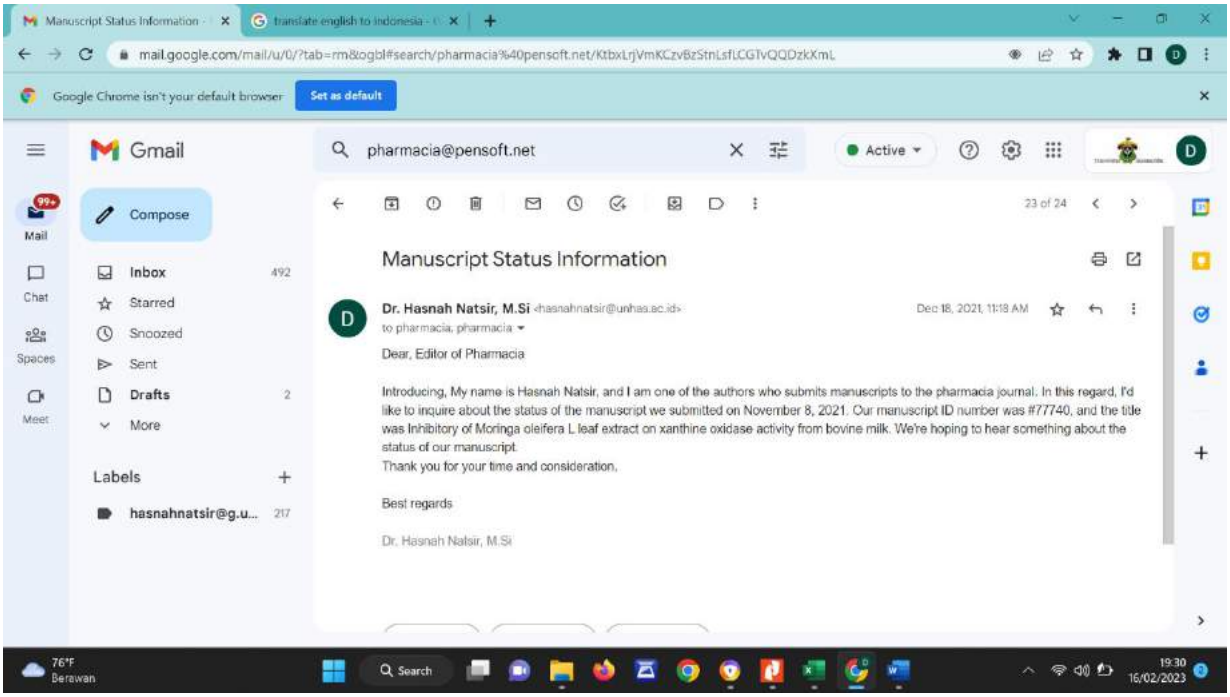
Dear, Editor of Pharmacia

Introducing, My name is Hasnah Natsir, and I am one of the authors who submits manuscripts to the pharmacia journal. In this regard, I'd like to inquire about the status of the manuscript we submitted on November 8, 2021. Our manuscript ID number was #77740, and the title was Inhibitory of Moringa oleifera L leaf extract on xanthine oxidase activity from bovine milk. We're hoping to hear something about the status of our manuscript.

Thank you for your time and consideration.

Best regards

Dr. Hasnah Natsir, M.Si



3. Tanggal 24 Januari 2022, Info **Major revision pada manuscript #77740**
   (diberi waktu hanya 10 hari, dan paling lambat 03/02/2022)

Dear Hasnah Natsir:

We are pleased to inform you that the review process for your manuscript #77740 "Inhibitory of Moringa oleifera L leaf extract on xanthine oxidase activity from bovine milk" has been completed and the editorial decision is: **Major revision.**
The revised version should be submitted as manuscript text in DOC, DOCS, ODF or RTF formats corrected by using Track Changes tools. Additional or supplementary files, figures and references can also be added or replaced at this point.
Authors must address all critiques of the referees in a response letter to the editor and submit it along with the revised manuscript through the online editorial system. Please copy all comments into one document and add your response behind each critique using different formatting to allow easy following of your response. In case a response letter is not submitted by the authors, the editor has the right to reject the manuscript without further evaluation.
We expect your revised version within 10 days, by 03/02/2022, if not sooner. Please kindly inform us if you will need more time to submit the revised version.
Once again, thank you for choosing Pharmacia as the venue for your work
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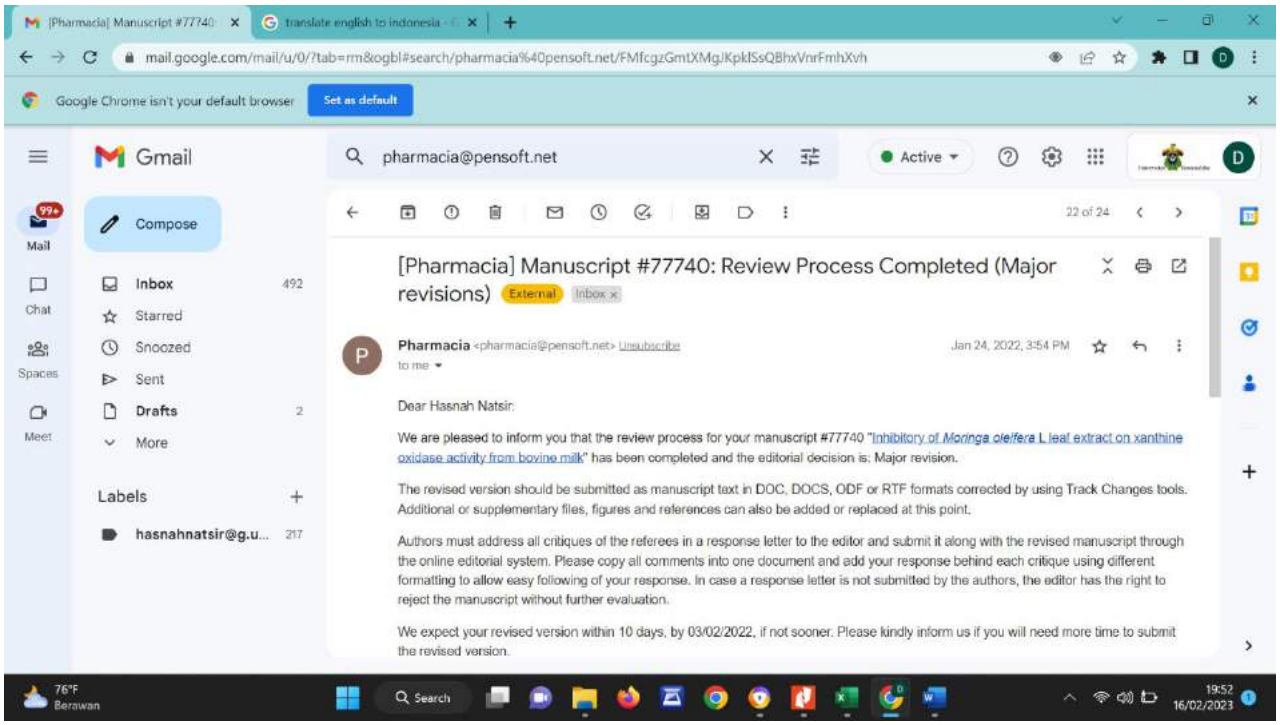


4. Tanggal 3 Februari 2022 penulis mengusulkan perpanjangan waktu untuk memperbaiki naskah sebagai tanggapan atas permintaan dan saran reviewer.

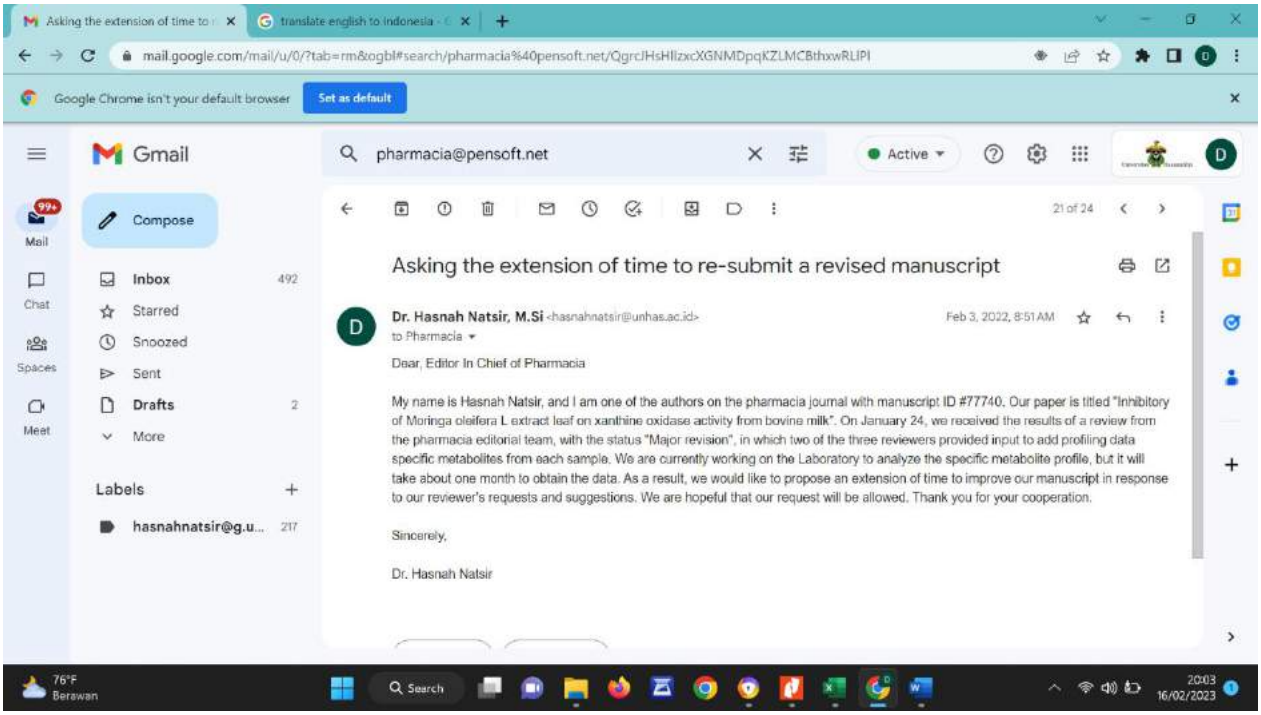
Feb 3, 2022, 8:51 AM

Dear, Editor In Chief of Pharmacia

My name is Hasnah Natsir, and I am one of the authors on the pharmacia journal with manuscript ID #77740. Our paper is titled "Inhibitory of Moringa oleifera L extract leaf on xanthine oxidase activity from bovine milk". On January 24, we received the results of a review from the pharmacia editorial team, with the status "Major revision", in which two of the three reviewers provided input to add profiling data specific metabolites from each sample. We are currently working on the Laboratory to analyze the specific metabolite profile, but it will take about one month to obtain the data. As a result, we would like to propose an extension of time to improve our manuscript in response to our reviewer's requests and suggestions. We are hopeful that our request will be allowed. Thank you for your cooperation.

Sincerely,

Dr. Hasnah Natsir



5. Tanggal 4 Februari 2022, Pharmacia menyetujui permintaan penulis, dan menanyakan sampai tanggal berapa perpanjangan waktu yang dibutuhkan.

Fri, Feb 4, 2022, 9:06 AM

Dear Hasnah Natsir:

We wish to remind you again about the delayed submission of the revised version of your manuscript #77740 "Inhibitory of Moringa oleifera L leaf extract on xanthine oxidase activity from bovine milk".

Can you please inform us about the estimated date when you will be ready to submit, so that we can change the due date accordingly. Moreover, if you require any technical guidance on the revised files and/or response letter's submission, please do not hesitate to contact us and we will gladly provide it. You can also refer to the respective Guidelines section. Please make sure to upload the revised files via the above link, and NOT by initiating a separate submission.

Once again, thank you for choosing Pharmacia as the venue for your work.

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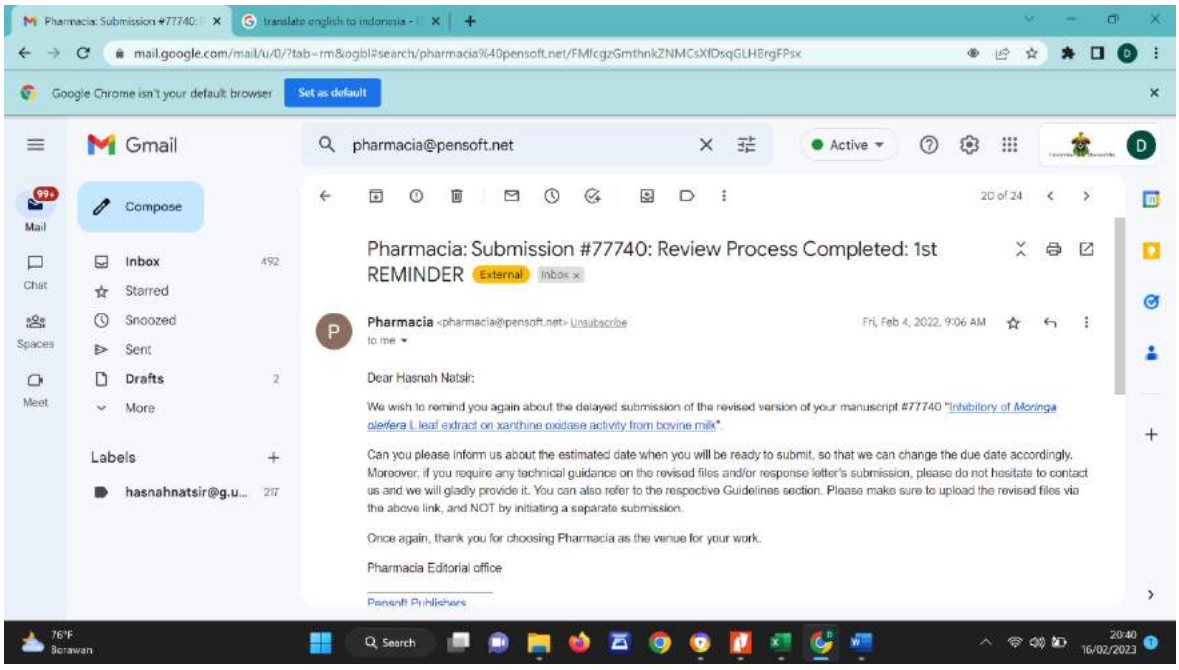


6. Tanggal 7 Februari 2022, penulis meminta perpanjangan waktu revisi hingga tgl 5 Maret 2022.

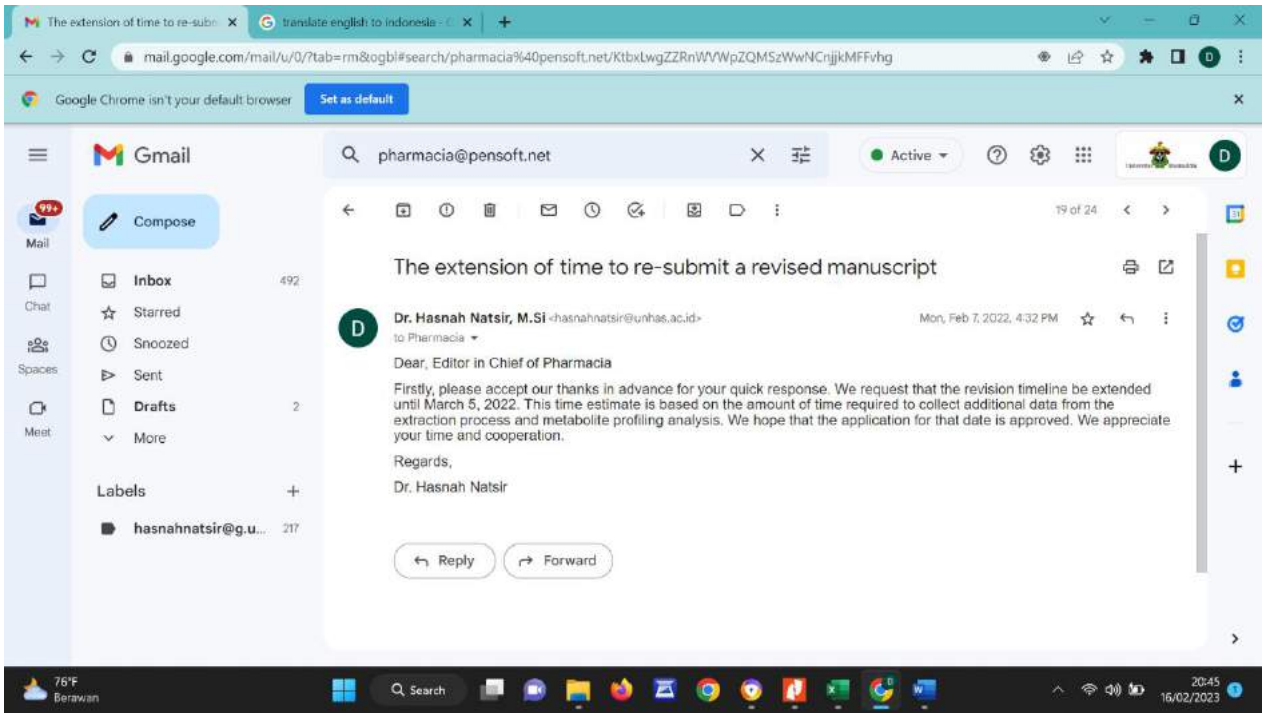
Mon, Feb 7, 2022, 4:32 PM

Dear, Editor in Chief of Pharmacia

Firstly, please accept our thanks in advance for your quick response. We request that the revision timeline be extended until March 5, 2022. This time estimate is based on the amount of time required to collect additional data from the extraction process and metabolite profiling analysis. We hope that the application for that date is approved. We appreciate your time and cooperation.

Regards,

Dr. Hasnah Natsir



7. Tanggal 13 Februari 2022, Pharmacia mengingatkan kembali waktu revisi

Sun, Feb 13, 2022, 9:06 AM

Dear Hasnah Natsir:

We wish to remind you for the third time about the delayed submission of the revised version of your manuscript #77740 "Inhibitory of Moringa oleifera L leaf extract on xanthine oxidase activity from bovine milk".

Can you please inform us about the estimated date when you will be ready to submit, so that we can change the due date accordingly. Moreover, if you require any technical guidance on the revised files and/or response letter's submission, please do not hesitate to contact us and we will gladly provide it. You can also refer to the respective Guidelines section. Please make sure to upload the revised files via the above link, and NOT by initiating a separate submission.

Once again, thank you for choosing Pharmacia as the venue for your work.

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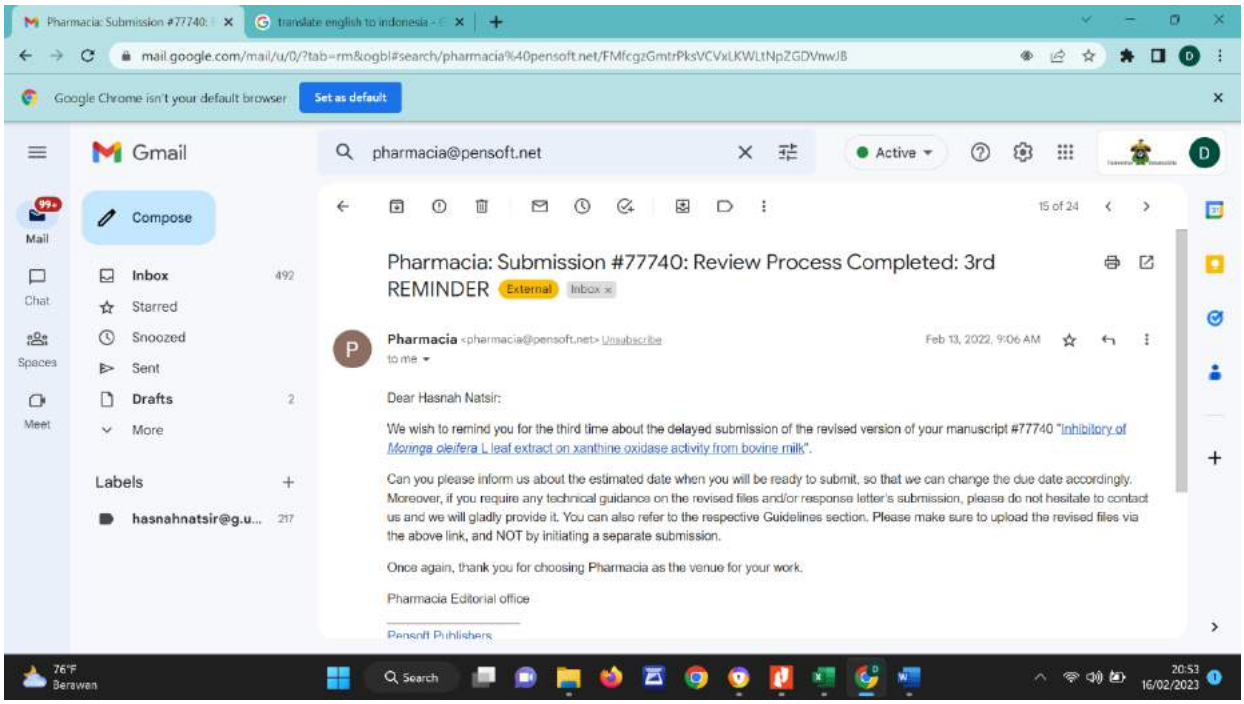

8. Tanggal 5 Maret Submitted, 10 Maret 2022, **Manuscript #77740: Accepted**

   Pharmacia: Manuscript #77740: Accepted , Thu, Mar 10, 2022, 8:57 PM

Dear Hasnah Natsir:

We are pleased to inform you that the review process of your manuscript #77740 "Inhibitory of *Moringa oleifera* L leaf extract on xanthine oxidase activity from bovine milk" has been completed and it was accepted for publication.
We expect that, even in cases when the revised version is accepted in the form in which it was submitted, there may be some small last-minute changes required or recommended. Please note that reviewers and editors might also have made comments and Track/Change corrections in your manuscript, which you should also check and
consider https://arphahub.com/manual#Handlemanuscriptsunderpeerreview
We shall expect your final version within 3 days, by 13/03/2022, if not sooner. Additional or supplementary files can also be replaced at this point with corrected versions.
We suggest that you help us increase the visibility of your study and thereby boost its citations and impact by sharing it on social media (i.e. Twitter, Facebook, Mendeley etc.), ideally using both your own and your institution's channels. Information and suggestions on how to promote your work to the international scientific audience and wider public can be found on our website.

Once again, thank you for choosing Pharmacia as the venue for your work!
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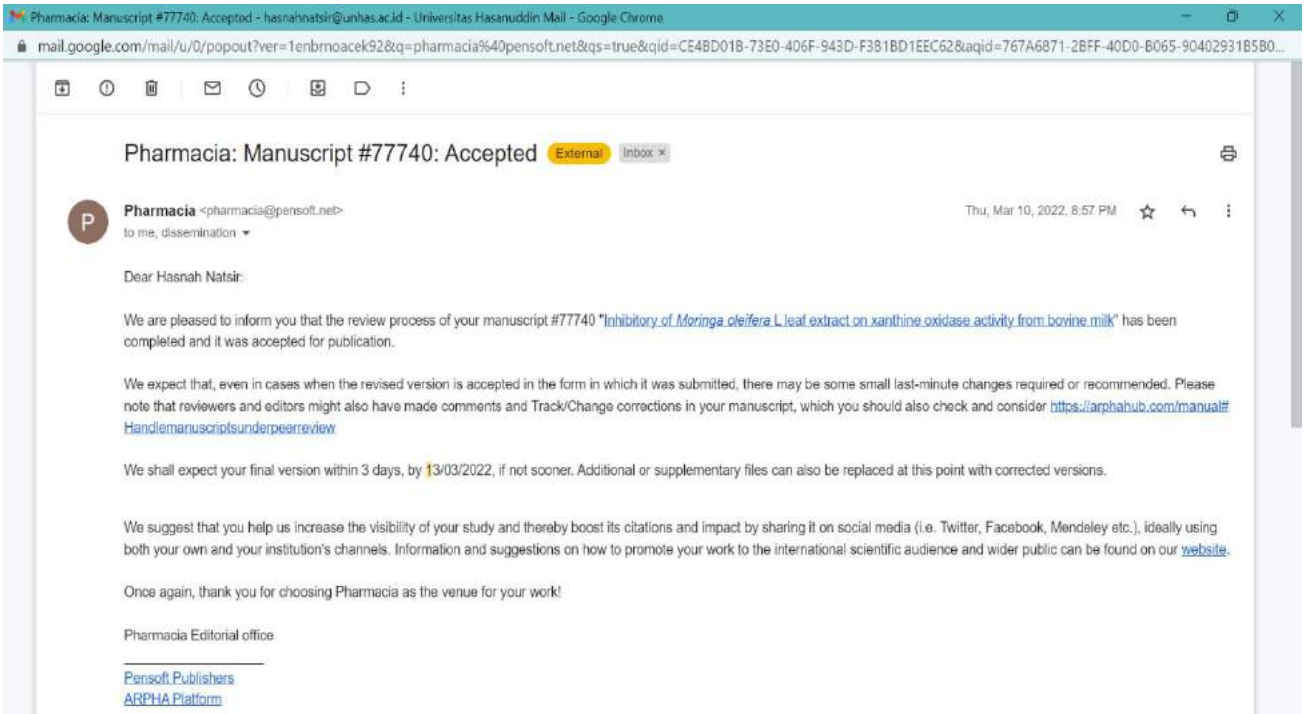

9. **The proof (in PDF format) of your manuscript #77740**

Pharmacia: Submission #77740: 1st PDF Proof Uploaded External Inbox

Apr 7, 2022, 11:51 PM

Dear Hasnah Natsir:

The proof (in PDF format) of your manuscript #77740 "Inhibitory of Moringa oleifera L leaf extract on xanthine oxidase activity from bovine milk" has been uploaded on the Pharmacia website for checking.

Please read through the proof carefully and mark all errors by using Sticky Note and Text Edit tools of Acrobat. Alternatively, you could upload a list of your corrections in a text file (e.g. DOC or RTF), specifying the exact page, paragraph and line of each correction and suggested text for replacement. Sending a list of corrections through email is possible, but not encouraged as it may cause unnecessary delay in processing your manuscript.

We expect an upload of your corrections on the journal's website within 3 days, by 10/04/2022, if not sooner. Please kindly inform us if you need more time for checking the proofs.

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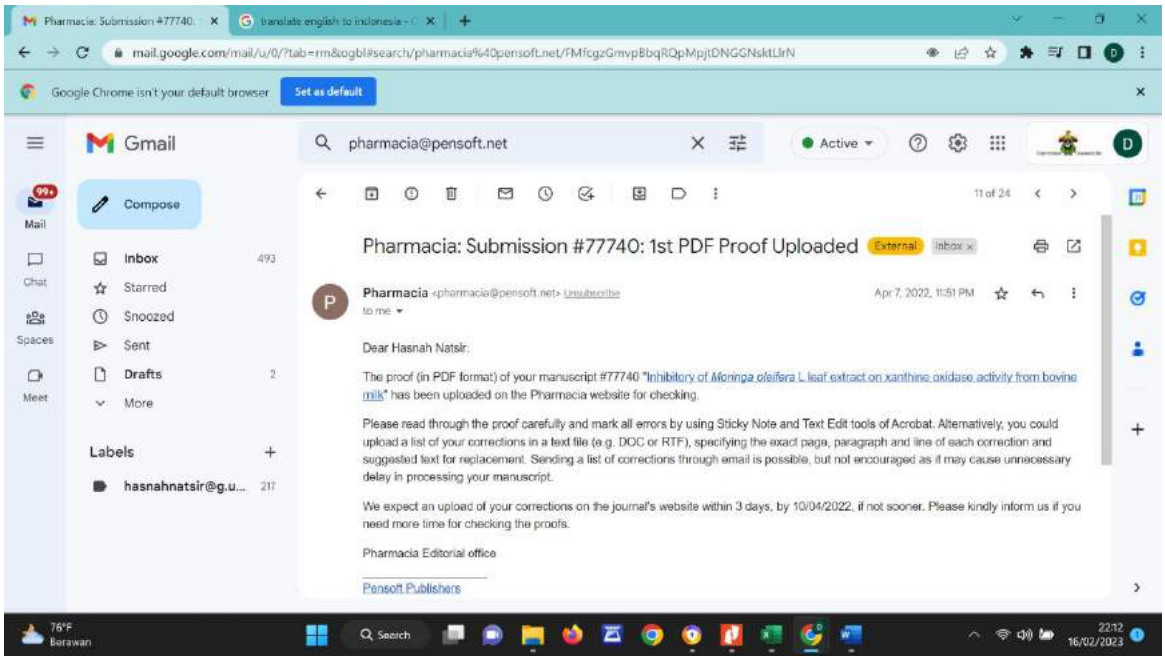
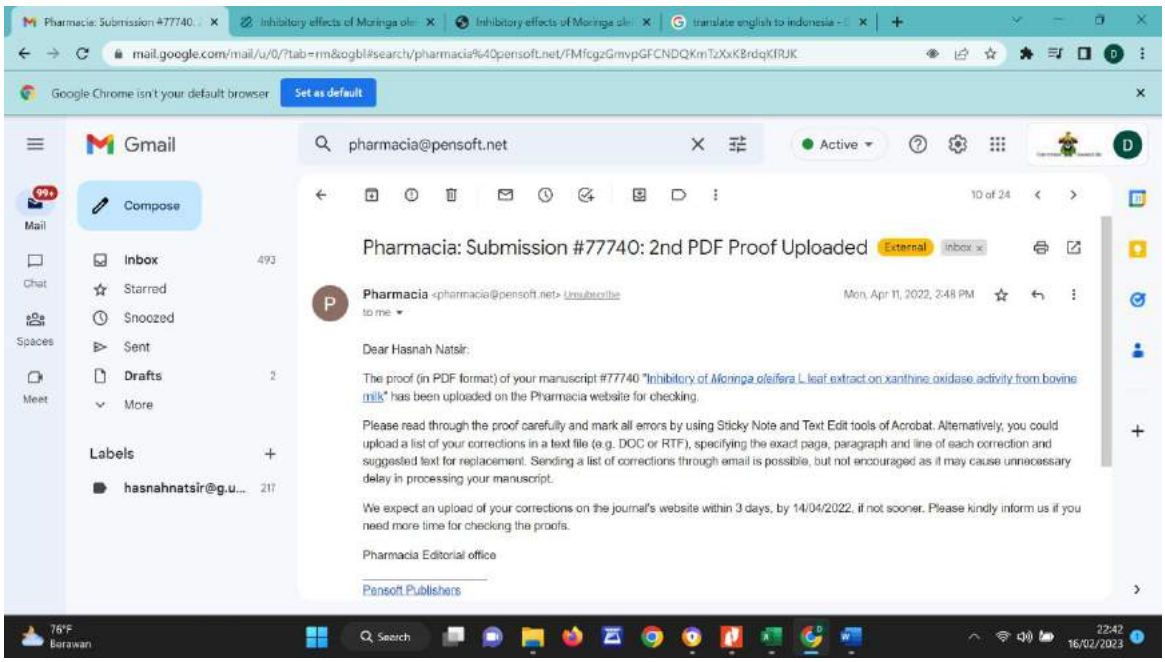


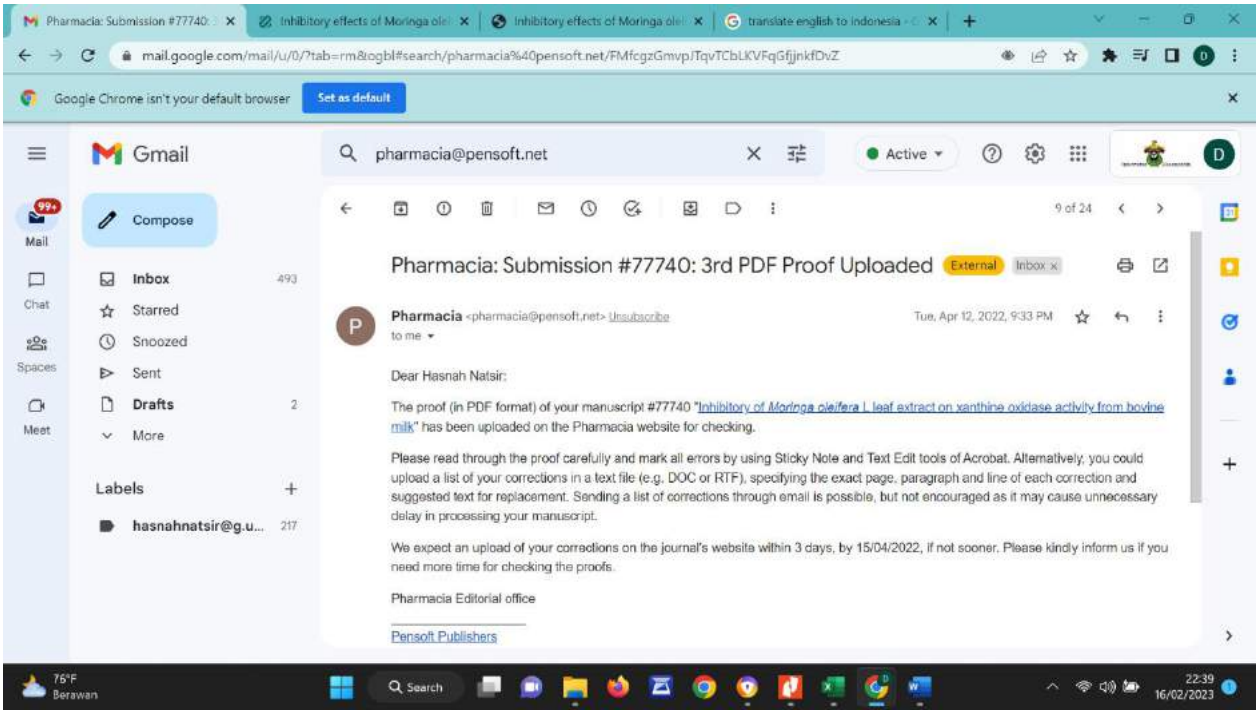
10. Tanggal 11 April 2022, Submission #77740: 2nd PDF Proof Uploaded



11. Submission #77740: 3rd PDF Proof Uploaded, Tue, Apr 12, 2022, 9:33 PM

Dear Hasnah Natsir:

The proof (in PDF format) of your manuscript #77740 "Inhibitory of Moringa oleifera L leaf extract on xanthine oxidase activity from bovine milk" has been uploaded on the Pharmacia website for checking.



12. **Pharmacia: Submission #77740: Manuscript Published**

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Thu, Apr 14, 2022, 10:09 PM

Dear Hasnah Natsir:
We are pleased to inform you that your paper #77740 "Inhibitory effects of *Moringa oleifera* leaves extract on xanthine oxidase activity from bovine milk" was published in Pharmacia, doi: 10.3897/pharmacia.69.e77740. Thank you for choosing Pharmacia as a publication venue for your work!
We suggest that you help us increase the visibility of your study and thereby boost its citations and impact by sharing it on social media (i.e. Twitter, Facebook, Mendeley etc.), ideally using both your own and your institution's channels. Information and suggestions on how to promote your work to the international scientific audience and wider public can be found on our website.
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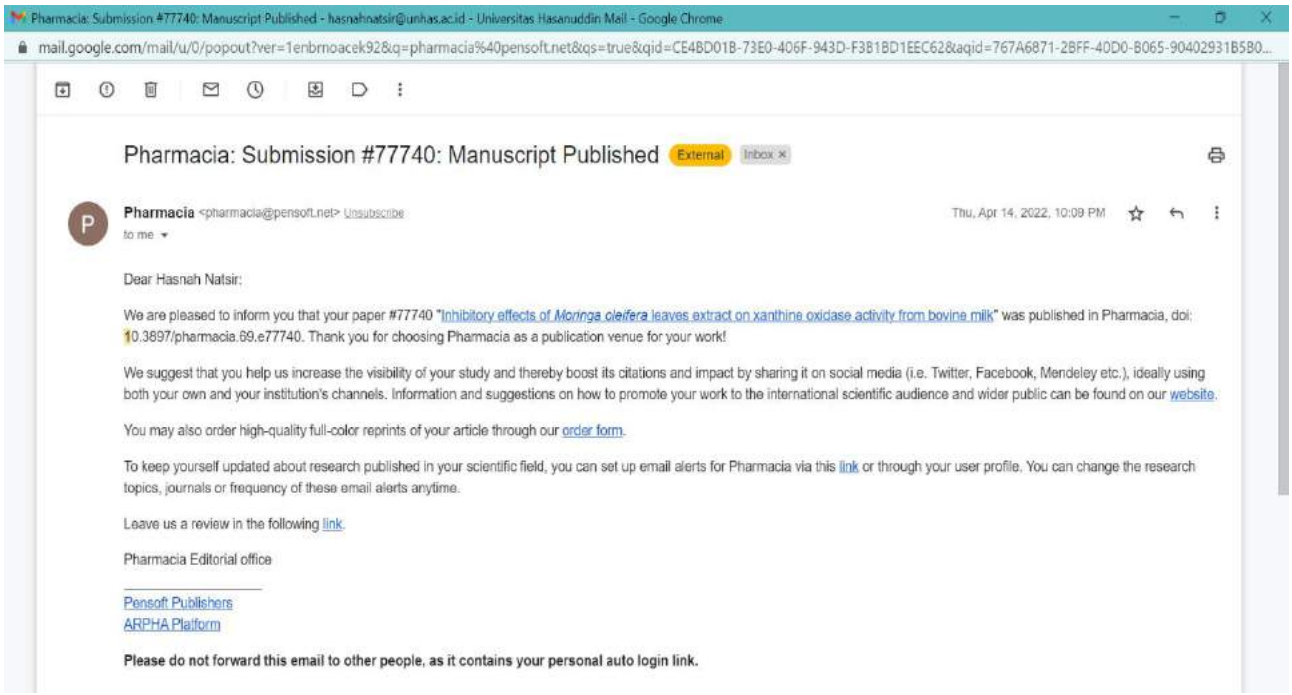
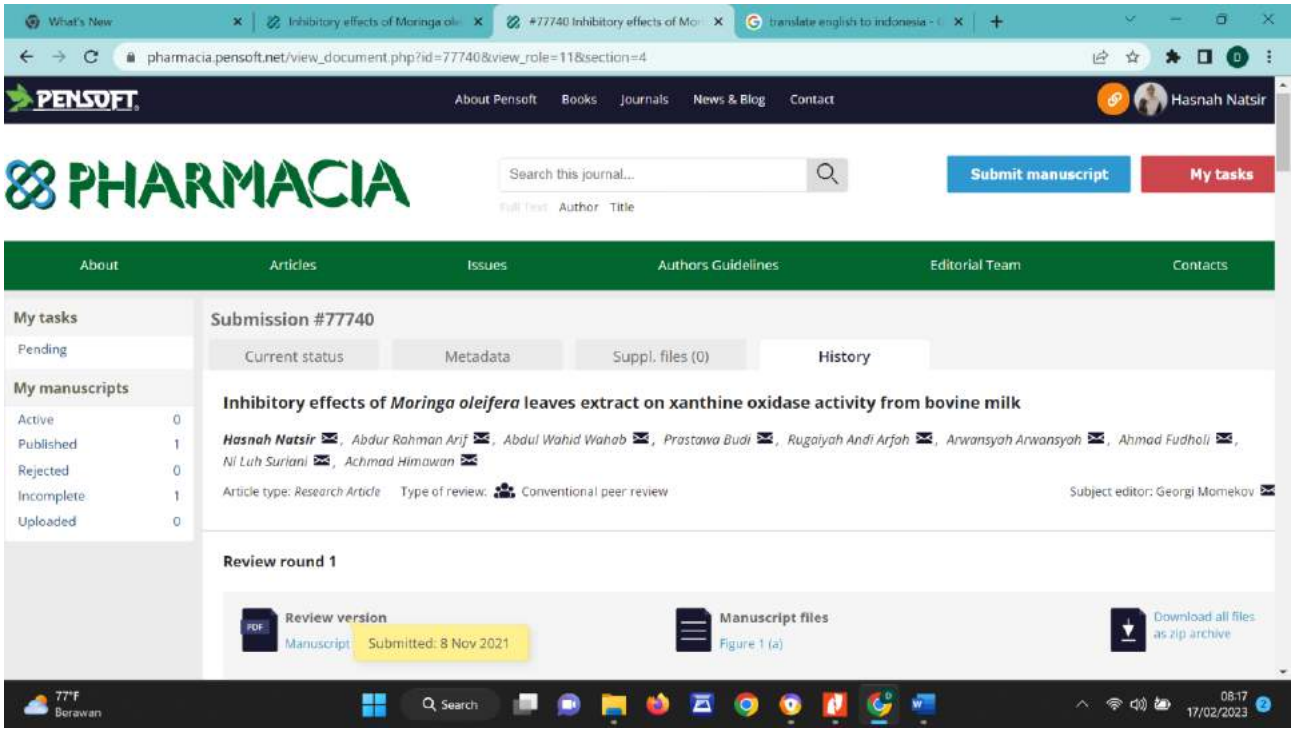


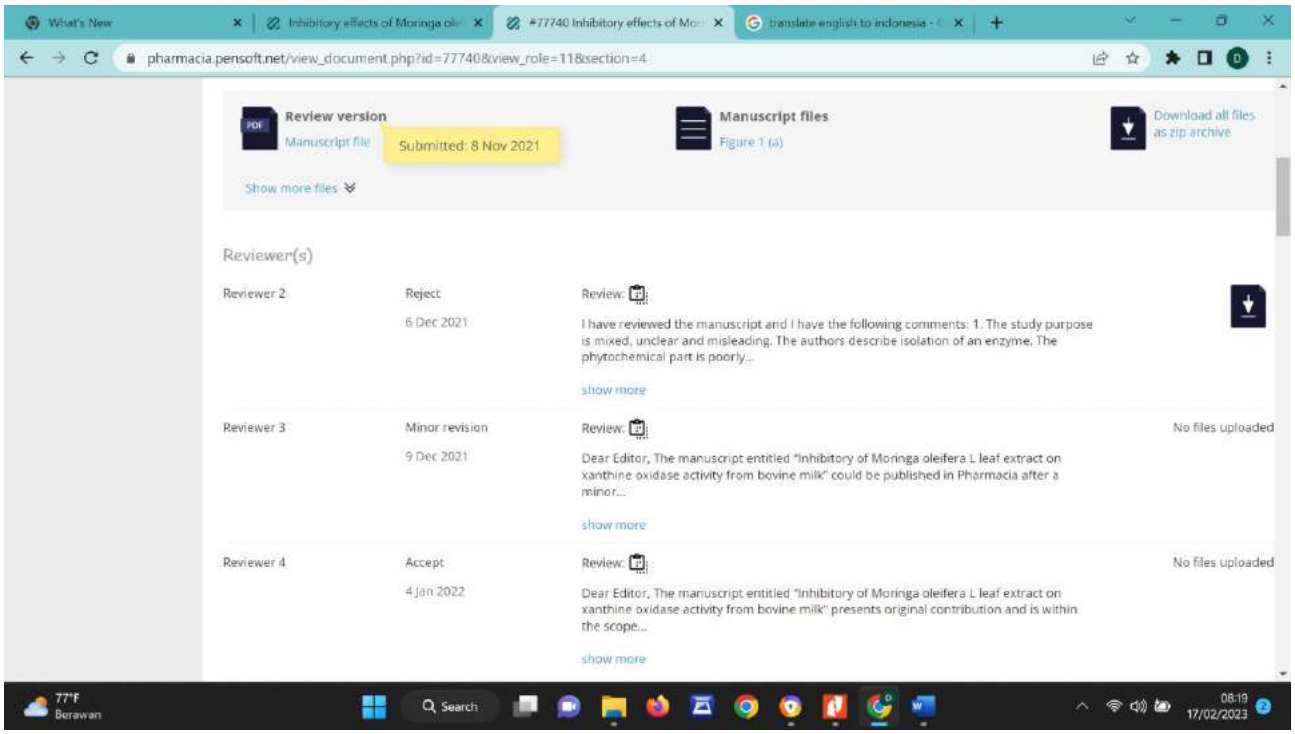
Bukti Korespondensi 2

Proses Revisi Manuscript dari Web Journal Pharmacia

No	Tanggal	HISTORY J. Pharmacia
1	8 Nov 2021	submitting the manuscript #77740
2	6 Dec 2021	Reviewer 2 Reject
3	9 Dec 2021	Reviewer 3 Minor Revision
4	4 Jan 2022	Reviewer 4 Accept
5	24 Jan 2022	Editorial decision (Georgi Momekov): Major Revision
6	5 Maret 2022	Manuscript #77740: Submitted
7	10 Maret 2022,	Manuscript #77740: Accepted
8	13 Maret 2022 25 Maret 2022	Copy editing 1 Copy editing 2
9	4 April 2022 7 April 2022	Layout editor 1, Submitted Layout editor , 1st PDF Proof Uploaded
10	10 April 2022 11 April 2022	Layout editor 1, Submitted Layout editor , 2 nd PDF Proof Uploaded
9	12 April 2022 12 April 2022	Layout editor 1, Submitted Manuscript #77740: 3rd PDF Proof Uploaded
10	14 April 2022	Manuscript #77740: Published

1. History : **Submitted, 8 Nov 2021**





Review round 1

Reviewer(s): Reviewer 2 , Reject, 6 Dec 2021

I have reviewed the manuscript and I have the following comments:

1. The study purpose is mixed, unclear and misleading. The authors describe isolation of an enzyme. The phytochemical part is poorly written, there is no analysis of these extracts, to prove what king of compounds are present to act like XO inhibitors.

2. On tha basis of a literature review (and not on phytochemical analysis) the manuscript deals with in silico analysis a=of the previously reported compounds from the plant. This is speculative - without an analysis of the extract, this study is separate and with no connection with the present extracts. The authors cannot prove the prescence of these compounds in the extract, so to imply XO docking and inhibition.

3. A detailed phytochemical analysis of the real extrats is needed if the manuscript is to stay in this form. Otherwise, the scientific novelty is relying only on the docking, which is based on the principal components in the literature.

4. I have further comments in the attached PDF.

5. The identity of the authors is visible, although the Journal's requrements imply annonimous files.

Reviewer 3, Minor revision, 9 Dec 2021

Dear Editor,
The manuscript entitled “Inhibitory of Moringa oleifera L leaf extract on xanthine oxidase activity from bovine milk” could be published in Pharmacia after a minor revision.

Comments

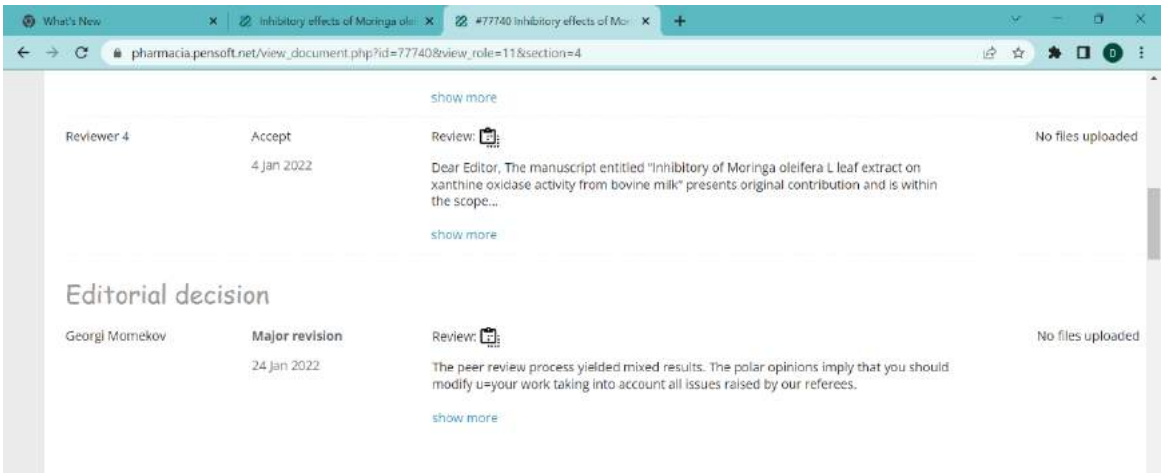
In the Introduction section the authors could provide more details on Moringa phytochemistry. Xanthine oxidase inhibitory activity of Moringa oleifera L leaf extract was previously investigated (Tian et al., Journal of Ethnopharmacology 270 (2021) 113808). The authors could clearly described the novelty and the aim of their work and to compare their results with previously published data.
Phytochemical profiling concerning the specialized metabolites exactly in the tested methanol and water extract is needed. Moreover, the used Molecular Docking should be based on a real phytochemical data. Differences between secondary metabolites of methanol and water extract could be discussed, analyzing differences in the activity of both extracts.

Reviewer 4, Accept, 4 Jan 2022

Dear Editor, The manuscript entitled “Inhibitory of Moringa oleifera L leaf extract on xanthine oxidase activity from bovine milk” presents original contribution and is within the scope...

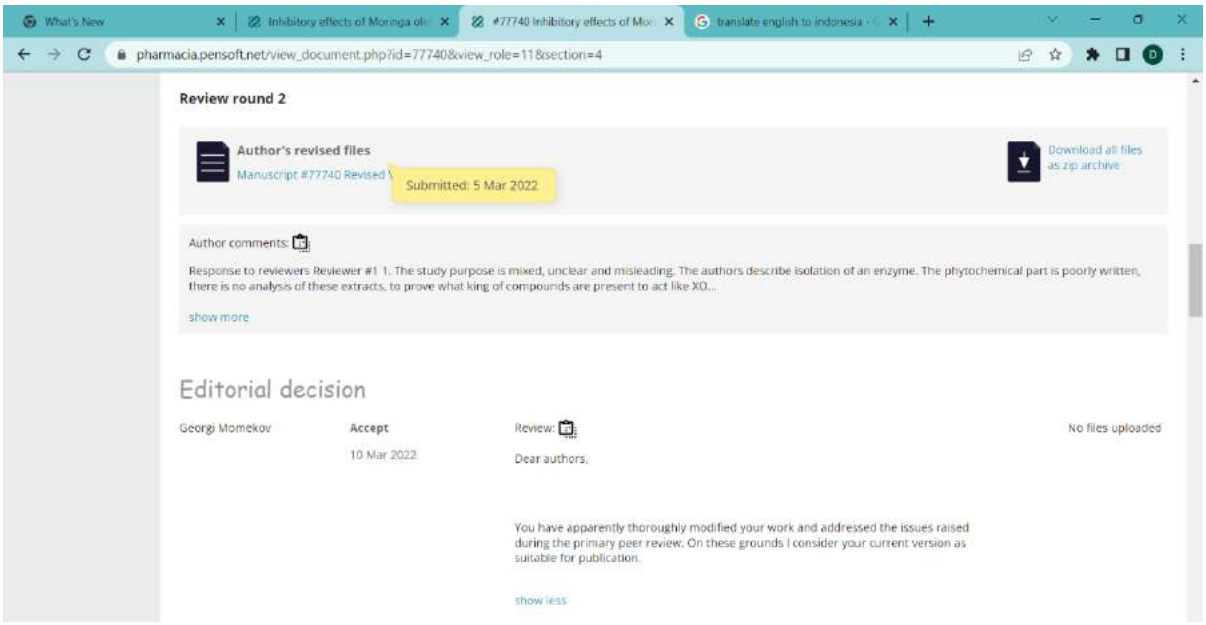
Editorial decision, Georgi Momekov, Major revision, 24 Jan 2022

The peer review process yielded mixed results. The polar opinions imply that you should modify u=your work taking into account all issues raised by our referees.



Review round 2

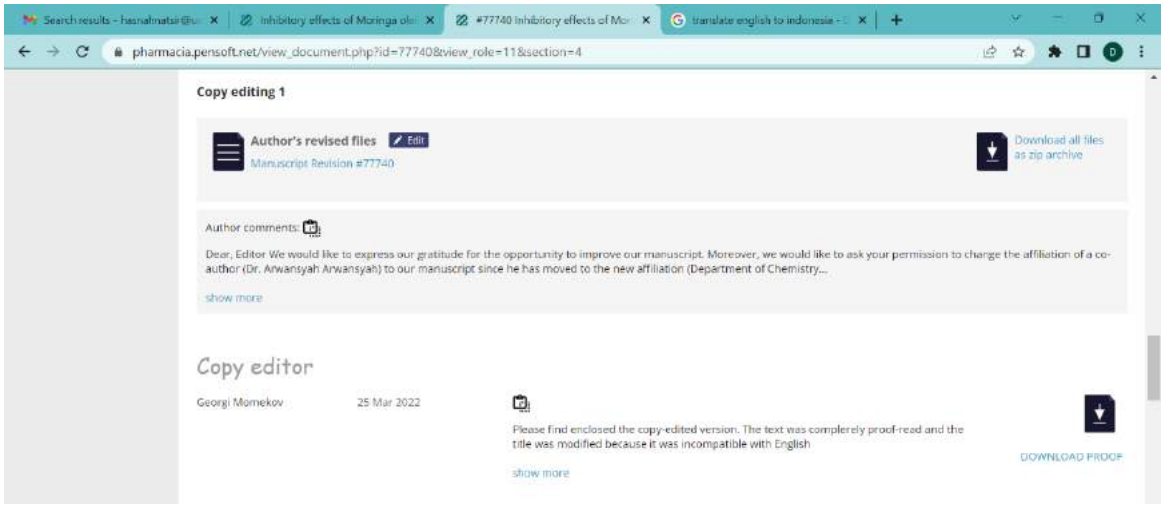
Author’s revised files, Submittes 5 Maret 2022

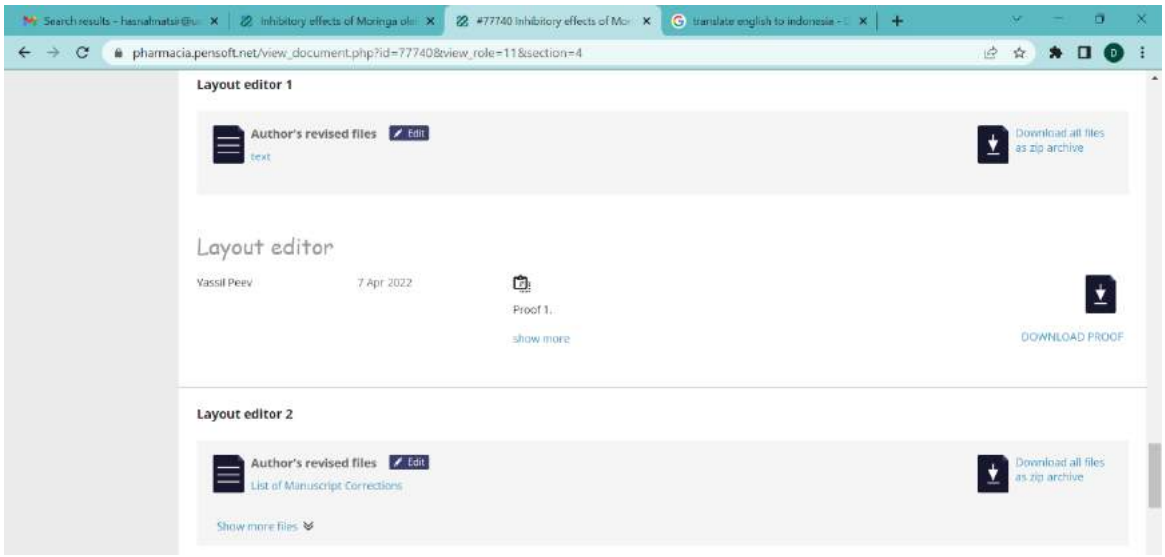


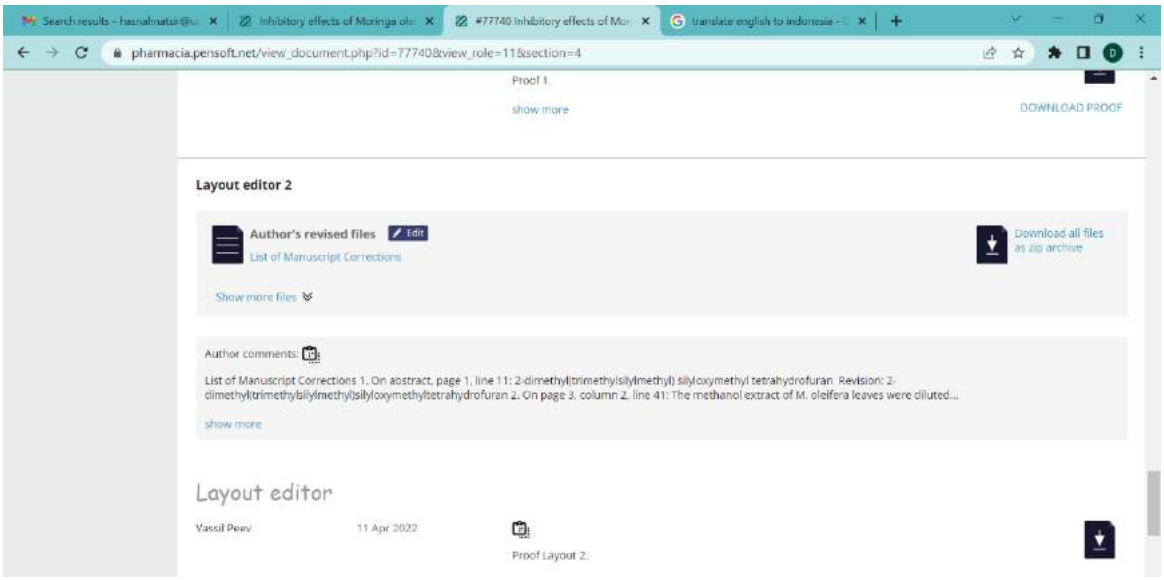
Editorial decision, Georgi Momeko**,** **Accept, 10 Mar 2022**

Dear authors,

You have apparently thoroughly modified your work and addressed the issues raised during the primary peer review. On these grounds I consider your current version as suitable for publication.



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List of Manuscript Corrections 1. On abstract, page 1, line 11: 2-dimethyl(trimethylsilylmethyl) silyloxymethyl tetrahydrofuran. Revision: 2-dimethyl(trimethylsilylmethyl)silyloxymethyltetrahydrofuran 2. On page 3, column 2, line 41: The methanol extract of M. oleifera leaves were diluted...
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Vassil Peev
11 Apr 2022
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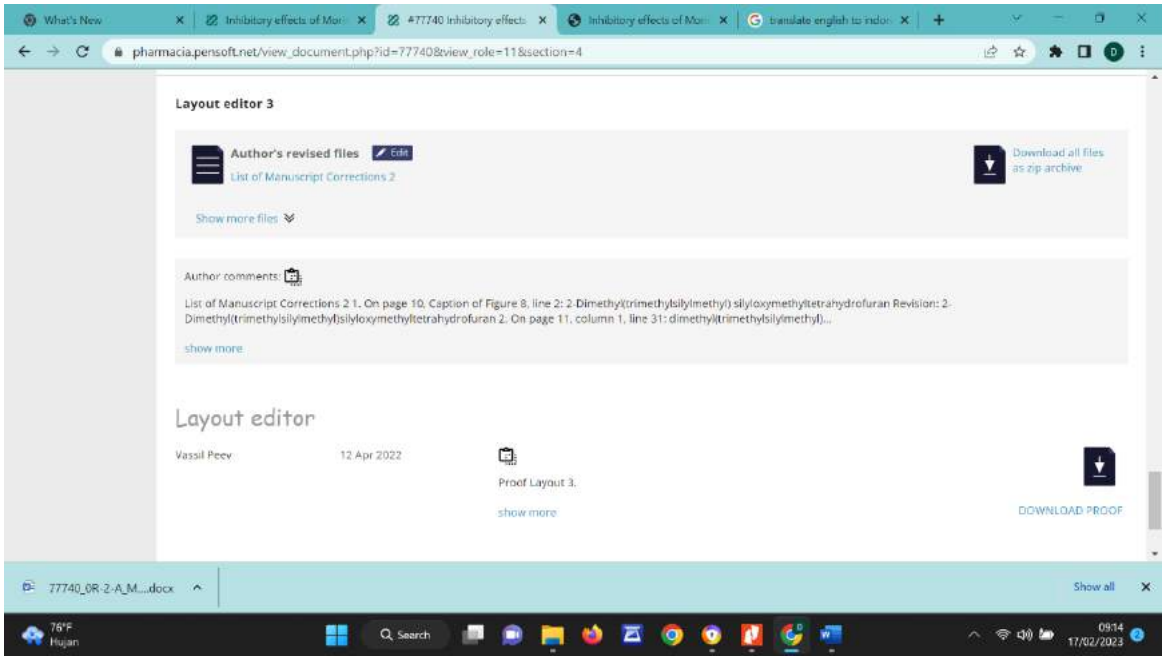
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List of Manuscript Corrections 2 1. On page 10, Caption of Figure 8, line 2: 2-Dimethyl(trimethylsilylmethyl) silyloxymethyltetrahydrofuran Revision: 2-Dimethyl(trimethylsilylmethyl)silyloxymethyltetrahydrofuran 2. On page 11, column 1, line 31: dimethyl(trimethylsilylmethyl)...
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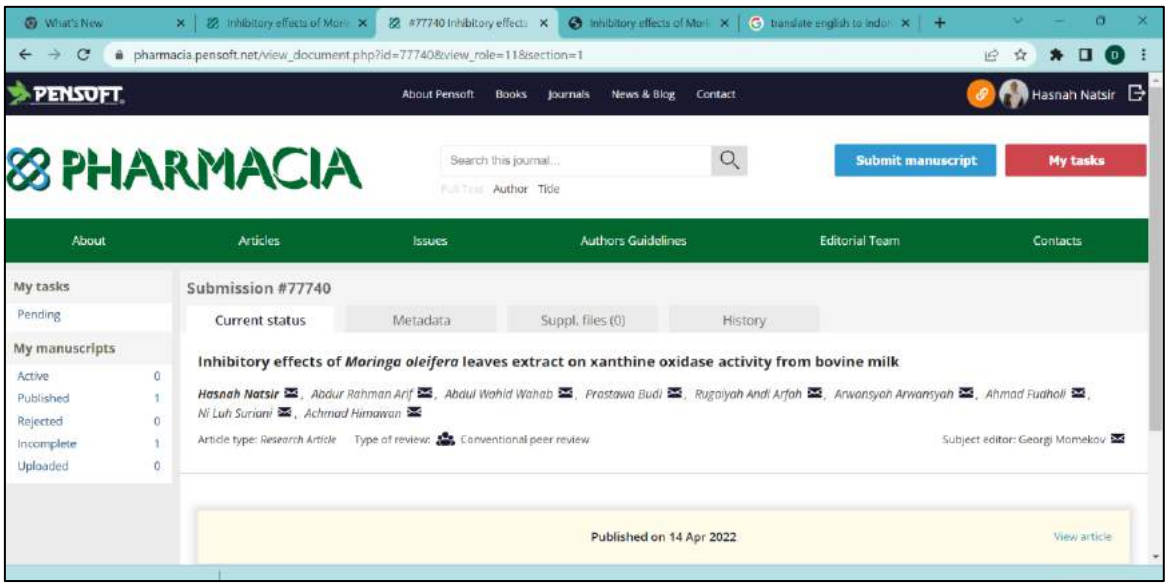
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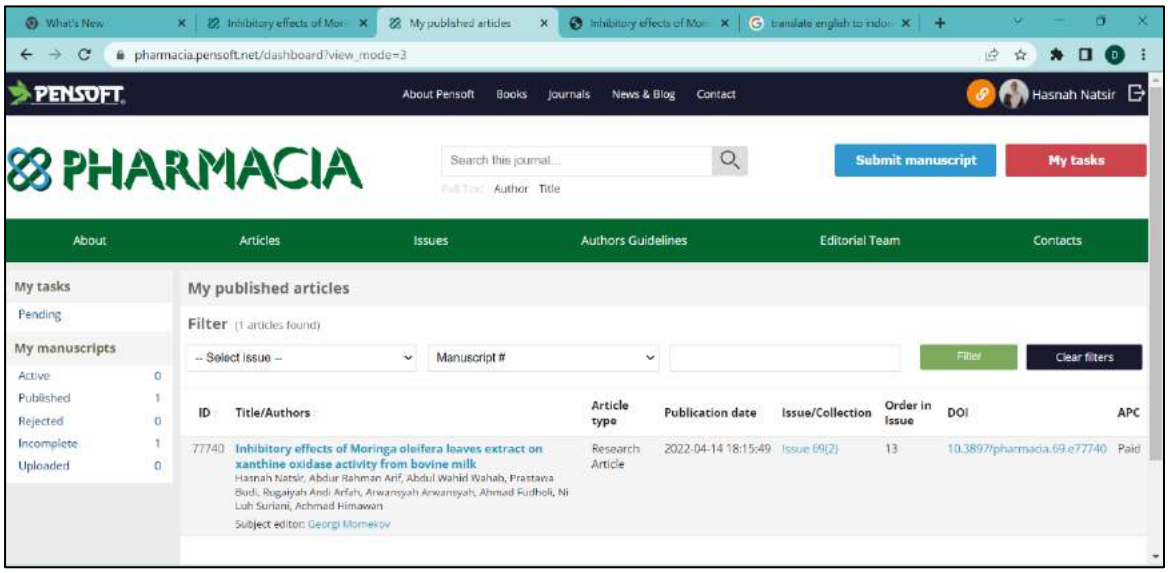
Rejected 0
Incomplete 1
Uploaded 0
Submission #77740
Current status
Metadata
Suppl. files (0)
History
Inhibitory effects of Moringa oleifera leaves extract on xanthine oxidase activity from bovine milk
Hasnah Natsir, Abdur Rahman Arif, Abdul Wahid Wahab, Prastawa Budi, Rugaiyah Andi Arfah, Arwansyah Arwansyah, Ahmad Fudholi, Ni Luh Suriani, Achmad Himawan
Article type: Research Article
Type of review: Conventional peer review
Subject editor: Georgi Momekov
Published on 14 Apr 2022
View article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77740
Inhibitory effects of Moringa oleifera leaves extract on xanthine oxidase activity from bovine milk
Hasnah Natsir, Abdur Rahman Arif, Abdul Wahid Wahab, Prastawa Budi, Rugaiyah Andi Arfah, Arwansyah Arwansyah, Ahmad Fudholi, Ni Luh Suriani, Achmad Himawan
Subject editor: Georgi Momekov
Research Article
2022-04-14 18:15:49
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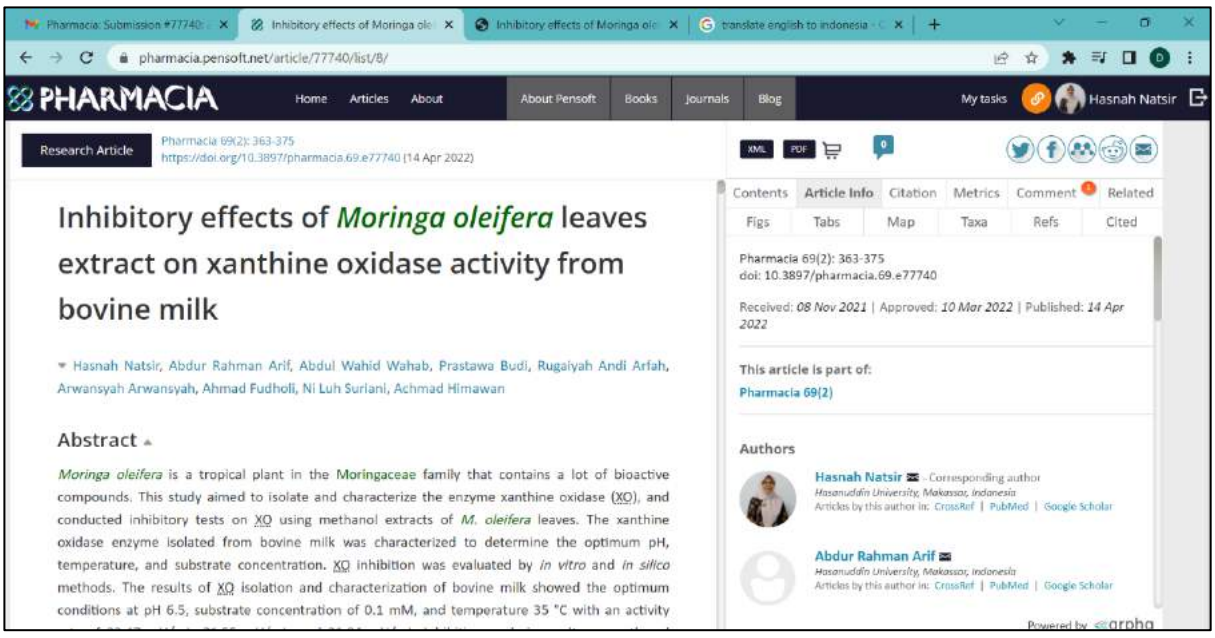
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Hasnah Natsir
Research Article
Pharmacia 69(2): 363-375
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Inhibitory effects of Moringa oleifera leaves extract on xanthine oxidase activity from bovine milk
Hasnah Natsir, Abdur Rahman Arif, Abdul Wahid Wahab, Prastawa Budi, Rugaiyah Andi Arfah, Arwansyah Arwansyah, Ahmad Fudholi, Ni Luh Suriani, Achmad Himawan
Abstract
Moringa oleifera is a tropical plant in the Moringaceae family that contains a lot of bioactive compounds. This study aimed to isolate and characterize the enzyme xanthine oxidase (XO), and conducted inhibitory tests on XO using methanol extracts of M. oleifera leaves. The xanthine oxidase enzyme isolated from bovine milk was characterized to determine the optimum pH, temperature, and substrate concentration. XO inhibition was evaluated by in vitro and in silico methods. The results of XO isolation and characterization of bovine milk showed the optimum conditions at pH 6.5, substrate concentration of 0.1 mM, and temperature 35 °C with an activity
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Inhibitory of *Moringa oleifera* L leaf extract on xanthine oxidase activity from bovine milk

Hasnah Natsir , *Abdur Rahman Arif* , *Abdul Wahid Wahab* , *Prastawa Budi* , *Rugaiyah Andi Arfah* , *Arv*
Ahmad Fudholi , *Ni Luh Suriani* , *Achmad Himawan*

Article type: *Research Article* Type of review: Conventional peer review Subjec

Review round 1

Review version

Manuscript file

Manuscript files

Figure 1 (a)

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Reviewer(s)

Reviewer 2

Reject

6 Dec 2021

Review:

I have reviewed the manuscript and I have the following comments:

1. The study purpose is mixed, unclear and misleading. The authors describ isolation of an enzyme. The phytochemical part is poorly written, there is n analysis of these extracts, to prove what king of compounds are present to act like XO inhibitors.

2. On tha basis of a literature review (and not on phytochemical analysis) th manuscript deals with in silico analysis a=of the previously reported compounds from the plant. This is speculative - without an analysis of the extract, this study is separate and with no connection with the present

extracts. The authors cannot prove the prescence of these compounds in tl extract, so to imply XO docking and inhibition.

3. A detailed phytochemical analysis of the real extrats is needed if the manuscript is to stay in this form. Otherwise, the scientific novelty is relying only on the docking, which is based on the principal components in the literature.

4. I have further comments in the attached PDF.

5. The identity of the authors is visible, although the Journal's requrements imply annonimous files.

show less

Reviewer 3	Minor revision 9 Dec 2021	Review: Dear Editor, The manuscript entitled "Inhibitory of *Moringa oleifera* L leaf extract on xanthine oxidase activity from bovine milk" could be published in Pharmaci after a minor revision. Comments In the Introduction section the authors could provide more details on *Morir* phytochemistry. Xanthine oxidase inhibitory activity of *Moringa oleifera* L leaf extract was previously investigated (Tian et al., Journal of Ethnopharmacology 270 (202 113808). The authors could clearly described the novelty and the aim of the work and to compare their results with previously published data. Phytochemical profiling concerning the specialized metabolites exactly in th tested methanol and water extract is needed. Moreover, the used Molecula Docking should be based on a real phytochemical data. Differences betwee secondary metabolites of methanol and water extract could be discussed, analyzing differences in the activity of both extracts. show less
Reviewer 4	Accept 4 Jan 2022	Review: Dear Editor, The manuscript entitled "Inhibitory of *Moringa oleifera* L leaf extract on xanthine oxidase activity from bovine milk" presents original contribution a is within the scope of the journal Pharmacia. I would like to recommend th article before being accepted. No remarks. show less

Editorial decision

Georgi Momekov	**Major revision** 24 Jan 2022	Review: The peer review process yielded mixed results. The polar opinions imply th you should modify u=your work taking into account all issues raised by our referees. show less

Review round 2

Author's revised files

Manuscript #77740 Revised Version

Author comments:

Response to reviewers

Reviewer #1

1. The study purpose is mixed, unclear and misleading. The authors describe isolation of an enzyme. The phytochemical part is p
analysis of these extracts, to prove what king of compounds are present to act like XO inhibitors.

Response: We appreciate the reviewer's encouraging comments on our manuscript and the kind suggestions. We have revised c
Based on the suggestions of the reviewers, we have separated and adjusted the research objectives for the inhibition of Xanthin
methanol extract of *M. oleifera* leaves and the isolation of XO enzyme. Phytochemical analysis has been improved, particularly th
method, which has been combined with FTIR and GCMS analysis to identify the main compounds in the methanol extract of *M. o*

2. On tha basis of a literature review (and not on phytochemical analysis) the manuscript deals with in silico analysis a=of the pr
compounds from the plant. This is speculative - without an analysis of the extract, this study is separate and with no connection
extracts. The authors cannot prove the prescence of these compounds in the extract, so to imply XO docking and inhibition.

Response: We thank the reviewer for the comments. For the phytochemical analysis, we have added experimental data regardir
profile of the methanol extract of Moringa leaves that we used. The addition of phytochemical analysis includes FTIR analysis to
groups of compounds in the extract. We used GCMS for the specific identification of the major constituents in the sample, and v
major constituents: 5-O-acetyl-thio-octyl-beta-l-rhamno furanoside, quinic acid, and 2-Dimethyl (trimethyl silylmethyl) silyloxy-m
use these three compounds as ligands in the docking process with XO receptors.

3. A detailed phytochemical analysis of the real extrats is needed if the manuscript is to stay in this form. Otherwise, the scientifi
the docking, which is based on the principal components in the literature.

Response: We thank the reviewer for the comments. Phytochemical profiling experimental data includes functional group analy
identification using GCMS with chromatogram output that becomes specific data for determining the concentration of each com
of Moringa leaves. The results of the chromatogram are combined with mass spectroscopic data in order to obtain detailed info
compounds investigated.

4. I have further comments in the attached PDF.

Response: We thank the reviewer for the comments. All of the comments and suggestions founded in the pdf manuscript file ha
corrected. Each improvement is identified with a yellow watermark to make it easier for reviewers to identify suggestions that h
completed.

5. justify this concentration why was this used? "The methanol extract of *M. oleifera* leaves were diluted to a 20 µg/mL concentra
phosphate buffer solution pH 7.5"

Response: We thank the reviewer for the comments. The dilution of the sample to 20 g/mL is intended to keep the concentratio
becoming excessively concentrated. A sample solution that is extremely concentrated will have an effect on the measurement r
analysis that will be performed using UV-Vis to determine the inhibitory activity. The concentration of 20 g/mL was chosen as th
concentration's midpoint.

6. The identity of the authors is visible, although the Journal's requrements imply annonimous files.

Response: Thank the reviewer's kindly suggestion. The identity of the author has been removed according to the comments fror

Reviewer #2

The manuscript entitled "Inhibitory of *Moringa oleifera* L leaf extract on xanthine oxidase activity from bovine milk" could be pub
minor revision.

Comments

1. In the Introduction section the authors could provide more details on *Moringa* phytochemistry.

Response: We thank the reviewer for the suggestion. We have described suggestions from reviewers regarding M. oleifera phyto
introduction, especially the leaf phytochemistry profile. We combine this information with the biological activity of each compon
which includes roots, leaves, drumstick, flowers and seeds of M. oleifera.

2. Xanthine oxidase inhibitory activity of *Moringa oleifera* L leaf extract was previously investigated (Tian et al., Journal of Ethnop
113808). The authors could clearly described the novelty and the aim of their work and to compare their results with previously

Response: Thank the reviewer's kindly suggestion. The reviewer's suggestions about novelty, objectives and comparisons with th
done have been improved and described in our manuscript. The novelty that we offer is related to the characteristics of the phy
Moringa leaves that we obtained from the topoyo regency, west sulawesi province. So far, exploration of moringa from this area
so it is very interesting to know its phytochemical composition. In addition, insilico analysis carried out by docking becomes a m
provides information regarding the interaction mechanism of the main compound in the methanol extract of *M. oleifera* leaves t

3. Phytochemical profiling concerning the specialized metabolites exactly in the tested methanol and water extract is needed. M
Molecular Docking should be based on a real phytochemical data. Differences between secondary metabolites of methanol and
discussed, analyzing differences in the activity of both extracts.

Response: We thank the reviewer for the suggestion. We have made improvements in the manuscript. We have completed the p
experimental data from the FTIR and GCMS tests, to identify specific compounds in the methanol extract of *M. oelifera* leaves. W
identified major compounds using molecular docking in order to obtain relation between phytochemical profiles, in vitro and in

show less

Editorial decision

Georgi Momekov

Accept

10 Mar 2022

Review:

Dear authors,

You have apparently thoroughly modified your work and addressed the issues raised during the primary peer review. On these grounds I consider your current version as suitable for publication.

show less

Inhibitory of *Moringa oleifera* L leaf extract on xanthine oxidase activity from bovine milk.

Hasnah Natsir[1], Abdur Rahman Arif[1], Abdul Wahid Wahab[1], Prastawa Budi[1], Rugaiyah A Arfah[1], Arwansyah[2], Ahmad Fudholi[3,4], Ni Luh Suriani[5], Achmad Himawan[6]

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Abstract

Moringa oleifera (*Moringa*) is a tropical plant from the Moringaceae family which is abundant in bioactive compounds**.** This study aimed to isolate and characterize the enzyme xanthine oxidase (XO), and conducted inhibitory tests on XO using water and methanol extracts of *Moringa oleifera* leaves. The xanthine oxidase enzyme isolated from bovine milk was characterized to determine the optimum pH, temperature, and substrate concentration. XO inhibition was evaluated by *in vitro* and *in silico* methods. The results of XO isolation and characterization of bovine milk showed the optimum conditions at pH 6.5, substrate concentration of 0.1 mM, and temperature 35 °C with an activity rate of 32.47 mU/mL; 21.55 U/mL, and 21.94 mU/mL. Inhibition analysis results on water and methanol extract of *Moringa* leaves showed the highest activity decrease at the extract concentration of 160 ppm, with a relative inhibition value of 35.42% for water extract, and 21.35% for methanol extract, while allopurinol as a positive control has a relative value inhibition of 61.21%. Relative value inhibition indicated the potential of *Moringa* leaves as a source of medicinal plants for gout sufferers. Additionally, a computational analysis was performed to observe the molecular interaction between the primary compounds of *Moringa* leaves, i.e., Astragalin, Isoquercetin, and Cryptochologenic acid, and XO using the molecular docking method. The finding implied that these compounds are bound to the catalytic sites of XO by hydrogen bonds and hydrophobic interactions, indicating the primary compounds of *Moringa* leaves could become XO inhibitors to treat gout disease.

Keywords

Moringa oleifera L**,** inhibition analysis, molecular docking, xanthine oxidase, bovine milk.

Introduction

Moringa oleifera is a medicinal plant that is widely cultivated in many tropical and subtropical countries (Boopathi & Raveendran, 2021). *Moringa* is a beneficial plant because practically every component of it can be used for food or other purposes, making it highly versatile. These include its usage as a water purifier, a functional food, an oil extraction agent in biofuel production, and health-related applications (Sagona, Chirwa, & Sajidu, 2020). *Moringa*'s high nutritional value and its numerous health advantages have sparked a lot of studies on the plant. Researchers have discovered that *Moringa oleifera's* leaves possess multiple pharmacological properties, including antiulcer, antipyretic, anti-inflammatory, antihypertensive, antiepileptic, antidiabetic, hepatoprotective, antibacterial, and anti-fungal properties as well as cholesterol-lowering abilities (Padayachee & Baijnath, 2020). On the other hand, *Moringa's* other pharmacological properties include its capacity to block enzymes that act as receptors for particular diseases (A. K. Singh et al., 2020).

XO is an enzyme that plays a role in catalyzing the oxidation of hypoxanthine to

xanthine, which becomes uric acid. XO is derived from the enzyme class molybdenum iron-sulfur flavin hydroxylase, mainly found in the liver, kidneys, brain, gastrointestinal tract (Maiuolo, Oppedisano, Gratteri, Muscoli, & Mollace, 2016). The enzyme is also present in the entire cardiovascular system. Inhibition of XO can suppress the biosynthesis of gout, which is one of the therapeutic approaches for treating gout, neuropathy, and kidney stones, which leads to hyperuricemia (Gliozzi, Malara, Muscoli, & Mollace, 2016; White, 2018).

Suppressing XO activity is the primary approach in treating hyperuricemia and gout in clinical settings because XO has an essential role in the formation of uric acid. Some XO inhibitors, such as allopurinol, can inhibit uric acid formation by regulating the action of XO. Allopurinol is a drug used clinically for the treatment of gout (Tămaş et al., 2021). This drug is a type of synthetic drug used by most people to inhibit uric acid synthesis (Seth, Asr, Buchbinder, Bombardier, & Cj, 2014). However, excessive use of allopurinol can cause nephropathy, hepatitis, digestive disorders, reduced white blood cells, allergies, and liver damage (Zeng et al., 2018). With these undesirable effects, alternative therapies derived from medicinal plants are expected to reduce the side effects and have a similar or more effective in eliminating XO (Mohos et al., 2019).

One of the plants that indicated to have the ability to inhibit XO activity is *Moringa oleifera* ~~L~~). *Moringa* leaves contain ~~quite a variety of secondary metabolite compounds~~ (Lu[illegible]ragoon et al., 2020). Based on our previous study, *Moringa* leaves contain secondary metabolites such as flavonoids, alkaloids, tannins, and saponins with antioxidant activity and α-glucosidase activity inhibitor (H. Natsir et al., 2019; H. Natsir, Wahab, Laga, & Arif, 2018). This study aimed to isolate and characterize XO from bovine and analyze XO inhibition using water and methanol extracts from *Moringa* leaves. Moreover, the molecular docking method was employed to investigate insight into the molecular recognition of the primary compounds of *Moringa oleifera* L leaves in binding to the moiety of XO (Blaney & Dixon, 1993; Kitchen, Decornez, Furr, & Bajorath, 2004).

Materials and Methods

Chemicals and Instruments

The materials used in this study included: bovine milk obtained from cattle farmers in Enrekang Regency, South Sulawesi, Moringa oleifera leaf from Topoyo Subdistrict, West Sulawesi Province (Latitude: 2°02'17.21"S and Longitude: 114°15'30.36"E), $CH_3OH_{(pa)}$, NaCl, $(NH_4)_2SO_4$, $NaOH_{(pa)}$, HCl, xanthine substrate (Sigma Aldrich) and allopurinol were purchase from Merck. The instruments used in this study were autoclave, centrifuge, rotary evaporator, vortex, stirrer magnetic, UV-Vis 1800 (Shimadzu)~~, and other instruments in the method~~.

Isolation of XO

The XO isolation process is a modified method from Bou-Salah (Bou-Salah, Benarous, Linani, Bombarda, & Yousfi, 2020), in which 500 mL fresh bovine milk was heated to a temperature of 30 ◦C, combined with 178.5 g of NaCl, then centrifuged at a speed of 3000 rpm for 30 minutes. The supernatant was fractionated with ammonium sulfate at 4 ◦C using an ice bath, then centrifuged at 8000 rpm at 4 ◦C for 20 minutes. The precipitate was dissolved in 0.05 M potassium phosphate buffer pH 7.5 to 250 mL.

Preparation of XO Solution

Xanthine substrate of about 15.21 mg was added to the measuring flask and then added with five drops of 1 M NaOH, shaken until dissolved. The solution was diluted with CO_2-free demineralized water to 100.0 mL (1 mM concentration). The xanthine substrate solution was prepared by diluting the mother liquor to obtain a standard solution, with a concentration of 0.05; 0.1;

0.15; 0.2, and 0.25 mM (Kostić et al., 2015).

Allopurinol Solution

The stock solution was made by weighing 10 mg of allopurinol and then put into a 10 mL volumetric flask. A few drops of 1 M NaOH were used to dissolve allopurinol and then diluted with CO_2-free demineralized water in a measuring flask. The volume of the solution was sufficient to the limit mark in the measuring flask to obtain 1000 µg/mL final concentration. The standard allopurinol solution was prepared by diluting the stock solution to get a series of allopurinol standard solutions, with a concentration of 0.1; 0.2; 0.5; 1.0 and 2.0 µg/mL (Gong, Shao, Guo, Pan, & Fan, 2020).

XO Crude Extract

The crude XO extract was weighed ± 22.17 mg using a 25 mL weighing bottle, then the extract was added into a volumetric flask and diluted with phosphate buffer solution. The volume was diluted to the limit mark to obtain an XO solution of 0.1 unit/mL (Kostić et al., 2015).

XO Characterization

The crude extract of the enzyme was characterized to determine the optimum conditions of the enzyme, such as pH, substrate concentration, and temperature effect (Kostić et al., 2015; Hasnah Natsir et al., 2002). The optimum conditions were determined by analyzing the optimum activity of the enzyme. It was calculated by Equation 1:

$$\text{Ea} = \frac{(\text{Ab} - \text{Ac})\ \text{V} \times \text{df}}{12.2 \times 0{,}1}, \qquad (1)$$

Where Ea is enzyme activity (mU/mL); Ab is the absorption of blank; Ac is the absorption of control; V is total volume assay (mL); df is dilution factor; 12.2 is uric acid eccentric coefficient at 290 nm (mM); and 0.1 is the volume of XO used U/mL of the enzyme.

Optimum pH

Phosphate buffer solutions of 0.2 M (3.9 mL) with a pH variation of 6; 6.5; 7; 7.5 and 8 were added 2 mL of 0.15 mM xanthine substrate solution, then pre-incubated for 10 minutes at 25 °C. 0.1 mL of XO was added to the mixtures and then incubated for 30 minutes at 25 C. The absorption of the sample was measured at λmax 232 nm using a UV-Vis spectrophotometer (Kostić et al., 2015; Hasnah Natsir et al., 2002; Sharma, Thakur, Thakur, Savitri, & Bhalla, 2016).

Optimum Substrate Concentration

The optimum substrate concentration was determined by adding 2 mL of phosphate buffer solution at the optimum pH, with a xanthine substrate concentration of 0.05 mM; 0.10 mM; 0.15 mM; 0.20 mM, and 0.25 mM. After pre-incubation, 0.1 mL of XO was added to the solution, and the mixture was incubated at 25 °C for 30 minutes. A similar procedure was applied for control by replacing the crude enzyme extract using 0.1 mL of distilled water (Kostić et al., 2015; Hasnah Natsir et al., 2002).

Optimum Temperature

Phosphate buffer solution 0.2 M (3.9 mL) of optimum pH was added to 2 mL of xanthine substrate with optimum concentrations, then pre-incubated for 10 minutes. The enzyme XO (0.1 mL) was added, incubated for 30 minutes at 20 °C; 25 °C; 30 °C; 35 °C, and 40 °C. After the incubation process, the absorption was measured at λmax 232 nm using a UV-Vis spectrophotometer (Sharma, Thakur, Bhalla, & others, 2019)/(Hasnah Natsir, Patong, Suhartono, & Ahmad, 2010).

Preparation and Extraction Moringa oleifera Leaves

Moringa leaves are harvested from the tree by manually collecting the 3rd to 5th petiole leaves. The leaves were then washed with water and then air-dried at room temperature for 7-10 days. After drying, the leaves were then processed into

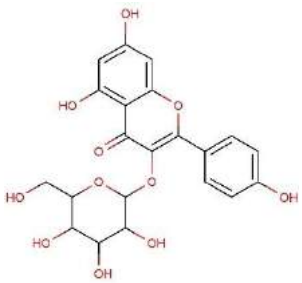
a fine powder using a grinding machine. The extraction process was carried out with methanol in a 1:20 ratio between dried leaves powder and the extraction liquid. The extraction process was conducted at 45 °C for 20 minutes with constant stirring using a magnetic stirrer. The extract obtained was filtered and then evaporated using a rotary evaporator to obtain a thick methanol extract. A similar extraction process is carried out with distilled water to obtain Moringa oleifera leaves water extract (H. Natsir et al., 2019, 2018; Rocchetti et al., 2019).

Inhibition Activity of Water and Methanol Extract of Moringa Oleifera Leaves Against XO

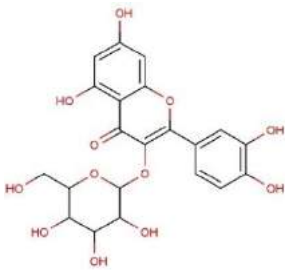
The water and methanol extract of Moringa leaves were diluted to a 20 µg/mL concentration with 0.05 mM phosphate buffer solution pH 7.5. An aliquot of 3 mL extract solution was added to a reaction tube, followed by 2 mL of 0.15 mM xanthine and 0.2 mL of XO, and then incubated at room temperature for 45 minutes. After incubation, 1 mL HCl (0.58 M) was added to the mixtures to stop the enzymatic reaction. Water was used as the control solution for the negative control, and allopurinol as a positive control. The absorption of the solution was measured using a UV-Vis spectrophotometer at λ_{max} 232 nm. Calculation of inhibition ability was obtained from the linear equation of the time versus concentration of the XO curve (Fachriyah, Ghifari, & Anam, 2018).

Molecular Docking

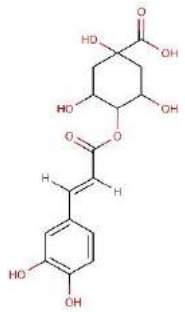
Molecular docking was performed using the AutoDock Vina package developed by Trott and co-workers to determine the ligand's binding site into the receptor's catalytic site (Trott & Olson, 2010). In this study, the Moringa oleifera leaves extracts were indicated to treat gout disease induced by hyperuricemia. A study reported that Astragalin, Isoquercetin, and Cryptochologenic were identified as the primary compounds of *Moringa oleifera* and therefore used as the ligand molecules for the docking (Vongsak, Sithisarn, & Gritsanapan, 2014). The chemical structures of those ligands were retrieved from the PubChem database, as shown in Figure 1. All ligands were downloaded and saved as sdf extensions. Open Babel 2.4.1 program packages were applied to convert sdf files to pdbqt extension (O'Boyle et al., 2011). As for the target molecule, XO was assigned as the receptor since this enzyme is related to gout disease. The tertiary structure of the receptor was taken from a protein data bank (PDB ID: 1v97) with a resolution of 1.94 Å, as shown in Figure 2 (Okamoto et al., 2004). The polar hydrogen and Kollman's united atom charges were added to the receptor using AutoDock Tools 1.5.6 created by Morris and co-workers (Morris et al., 2009). Afterwards, the XO was saved in pdbqt format.



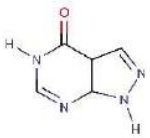
(**a**)



(**b**)



(**c**)



(**d**)

Figure 1. The chemical structure of the compounds of (**a**) Astragalin (PubChem ID: 5282102), (**b**) Isoquercetin (PubChem ID: 5280804), (**c**) Cryptochologenic acid (PubChem ID: 97986668), and (**d**) Alluporinol (PubChem ID: 135401907) as the positive control.

In performing molecular docking, a grid box parameter is required to decide the positional and rotational of the ligand into the moiety of the receptor (A Arwansyah et al., 2021). The grid box was placed on 24 × 24 × 26 points with a grid spacing of 1.00 Å. Meanwhile, the grid box centre was set at the coordinates x=148.649, y=43.411, and z= 26.399. The exhaustiveness was computed at 100. Other parameters were assigned as the default of AutoDock Vina. The Broyden-Fletcher-Goldfarb-Shanno (BFGS) algorithm was used as a search parameter to obtain the binding pose and conformation of the ligand into the receptor site. The docking protocols were set according to a similar procedure with our previous study provided in Ref. (Arwansyah Arwansyah, Arif, Syahputra, Sukarti, & Kurniawan, 2021; Sumaryada, Roslia, Ambarsari, Kartono, & others, 2016).

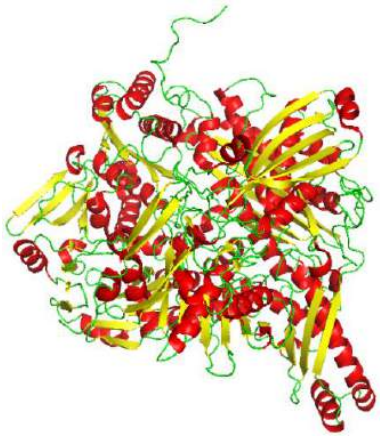


Figure 2. The tertiary structure of the created model of XO (PDB: 1v97) (Okamoto et al., 2004). The structures of α-helix, β-sheet, and turn are presented by cartoon models' red, yellow, and green colours.

Result and Discussion

Isolation results from bovine milk produced 340 mL of crude extract of XO enzyme. The enzymes used for the characterization and inhibition tests were stored at 4°C to maintain stability and avoid denaturation.

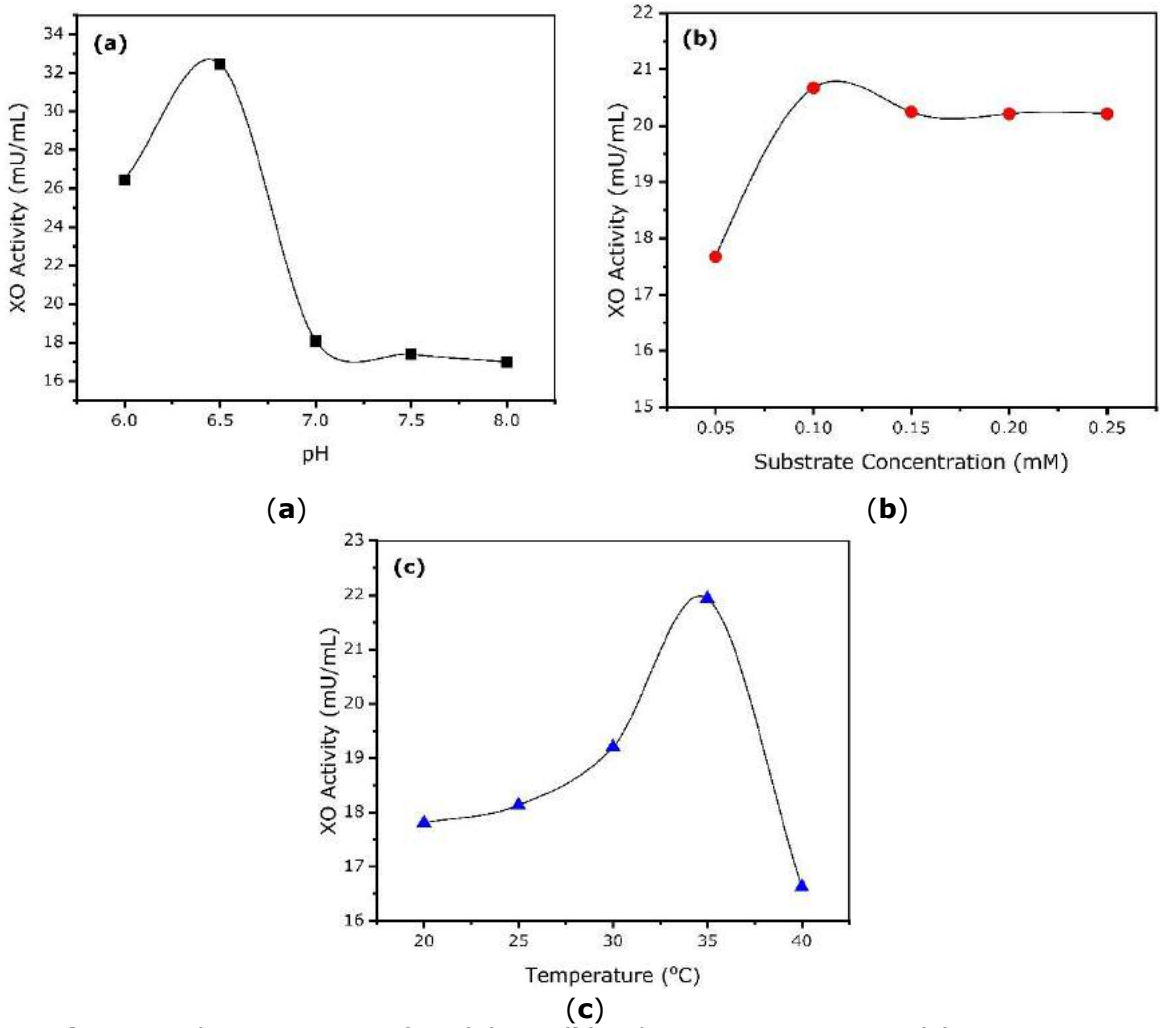
Characterization of XO

Determination of the optimum pH of the enzyme was carried out by conditioning the enzyme at a certain pH in the reaction between the enzyme and the substrate, as shown in Figure 3(**a**). Each type of enzyme has an optimum pH range, where the enzyme offers maximum activity in high stability. Generally, enzymes are amphiphilic, which means they can be acidic and base due to their active ability to provide functional groups of specific amino acid residues that are donor and acceptor proton (N. Singh, Kumar, Miravet, Ulijn, & Escuder, 2017). The XO activity showed that the optimum pH was at 6.5 with the activity of 32.47 mU/mL, and after pH increased at pH 7.0, XO activity decreased to 18.10 mU/mL. It was unveiled that the increase of enzyme activity at the optimum pH can be related to changes in ionization of the enzyme ionic group on the active site. Thus the conformation of the active site becomes more effective in binding and changing the substrate during the catalysis process (Huang et al., 2017).

The effect of substrate concentration was assessed to determine the optimum substrate concentration suitable for the enzyme. The substrate concentration used was 0.05; 0.1; 0.15; 0.2; 0.25 mM. The

results obtained are shown in Figure 3. The results demonstrated that higher enzyme activity was achieved at elevated substrate concentration. However, when after it reached the optimum substrate concentration, the activity tended to decrease. We reported that the highest enzyme activity was at 0.1 mM substrate concentration, with an activity of 21.55 U/mL. The increase in substrate concentration is directly related to the reaction rate until it reaches a maximum value of V_{max}. If the substrate concentration is increased, there will be no increase in the reaction rate because the substrate has saturated the enzyme's active site (Sharma et al., 2016).

Temperature is critical in enzymatic reactions because enzymes are proteins that are easily denatured against changing environmental conditions. The change in environmental temperature will affect enzyme activity (Claaßen, Gerlach, & Rother, 2019). The enzyme will show optimal catalytic activity at a specific temperature and denatured when exposed to extreme temperatures (Roche & Royer, 2018). When the temperature increases to optimal, the reaction rate would be accelerated because kinetic energy increases (Marañón, Lorenzo, Cermeño, & Mouriño-Carballido, 2018). Increased kinetic energy will accelerate the motion of vibration, translation, and rotation of both enzymes and substrates. It will increase the frequency of collisions between enzymes and substrates (Zhang et al., 2016). In this study, the determination of the optimum temperature of the XO enzyme used a variation of incubation temperature in the range of 20; 25; 30; 35 and 40 °C. The results obtained are shown in Figure 3. From the graph, it is clearly seen that the optimum temperature was reached at 35 °C with an activity of 21.94 mU/mL.



(**a**) (**b**) (**c**)

Figure 3. Characterization of XO (**a**) pH; (**b**) Substrate concentration; (**c**) Temperature.

Inhibition of Water and Methanol Extract of Moringa Leaves Against XO Activity

We reported that the water and methanol extracts of *Moringa* leaves demonstrated *in vitro* XO inhibition activity, and the results are presented in Table 1. The analysis inhibition showed that the effectiveness of inhibition was directly proportional to the increase in extract concentration. Water extract at a concentration of 10 mg/mL showed inhibition values of 21.84%, while at a 160 mg/mL concentration, the inhibition value was 35%. A similar activity showed by the methanol extract, where at a concentration of 10 mg/mL, the inhibition value was 5.73%, while at a concentration of 160 mg/mL, the value was 21.35%. The inhibition test results demonstrated that the water extract of *Moringa* leaves could inhibit XO by observing changes in activity, with the addition of inhibitors and without inhibitors. However, comparing the inhibition effectiveness between the two extracts verified that the water extract was more effective than the methanolic extract (Fachriyah et al., 2018; Kostić et al., 2015).

Table 1. The inhibition value of water and methanol extract of *Moringa oleifera* leaves against XO.

Sample concentrations (mg/mL)	**Methanol extract inhibition** (%)	**Water extract inhibition** (%)
10	5.73	21.84
20	7.04	24.03
40	8.83	28.63
80	10.02	30.33
160	21.35	35.42
Negative control	0	0
Allopurinol (positive control)	62.11	62.11

The secondary metabolite compounds in *Moringa* leaves are highly diverse such as flavonoids, alkaloids, tannins, saponins, and phenolic compounds (Ma, Ahmad, Zhang, Khan, & Muhammad, 2020). According to Vongsak et al. (Vongsak et al., 2014), the main compounds contained in *Moringa* leaves are astragalin and isoquercetin, which belong to the flavonoid group (Survay et al., 2011) and cryptochlorogenic acid, which is a polyphenol group in both fresh and dry leaves (Vongsak et al., 2014). The secondary metabolite compounds that have the potential as XO inhibitors are flavonoids and polyphenols (Altemimi, Lakhssassi, Baharlouei, Watson, & Lightfoot, 2017; Ewert, Glück, Strasdeit, Fischer, & Stressler, 2018). The properties of flavonoids and polyphenols tend to be polar, causing these compounds more distributed in water extracts than methanol. Water extract of *Moringa* leaves contains flavonoids and polyphenols, which can inhibit the activity of the enzyme XO, making it a potential inhibitor of XO. The ability of flavonoids to inhibit XO activity is through competitive inhibition mechanisms. Flavonoid compounds have similar structures to xanthine. It causes competition between xanthine and flavonoids bound to the active site of the enzyme (Fachriyah et al., 2018; Kostić et al., 2015; Mohos et al., 2019).

Molecular Docking Analysis

To analyze the molecular recognition in relation to the inhibitory activity, an advanced experimental investigation by X-ray analysis is required to obtain insight into the molecular interaction, including binding energy between the extraction of *Moringa oleifera* leaves and the tested enzyme (XO). However, computational analysis using the molecular docking method can currently investigate the structural and conformational changes of ligand-receptor complex (Qashqoosh, Manea, Alahdal, & Naqvi, 2019). Therefore, this method is employed to find the viewpoints of physical and chemical

properties regarding the binding action of *Moringa oleifera* into the moiety of XO.

Table 2. The binding energy of ligands in a complex with a receptor (XO) is obtained by molecular docking.

No.	Compound	Binding Energy (Kcal/mol)
1	Astragalin	-9.3
2	Isoquercetin	-8.2
3	Cryptochologenic acid	-10.6
4	Allopurinol	-6.6

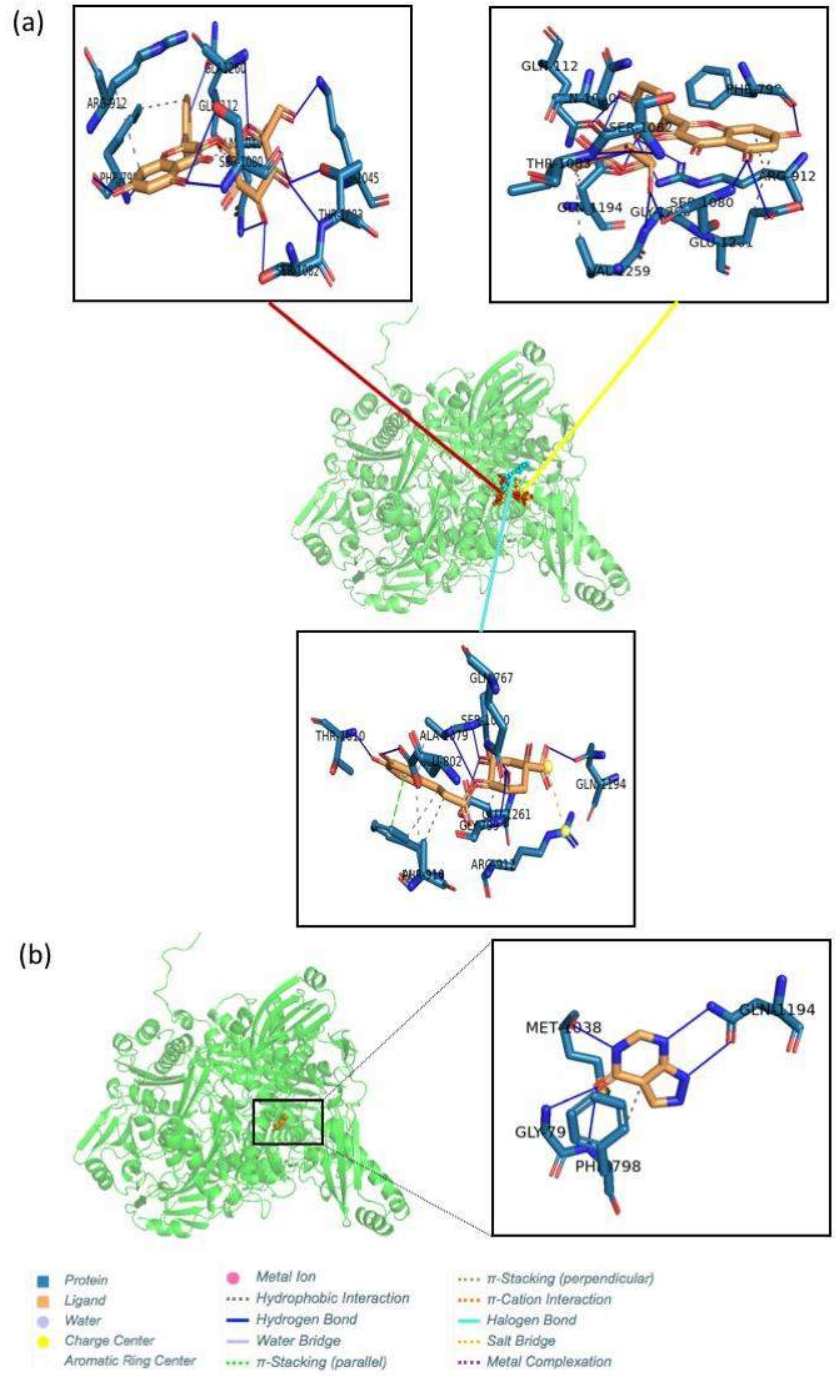


Figure 4. Binding pose of ligand in complex with a receptor (XO). Complex 1 consisted of mixed compounds where the red, yellow, and cyan lines refer to astragalin, isoquercetin, and cryptochologenic acid compounds, respectively, (b) complex 2 denoted to allopurinol (control). The conformation pose of each complex is visualized by the PLIP program (Salentin, Schreiber, Haupt, Adasme, & Schroeder, 2015) combined with Pymol v 2.3 program packages (DeLano, 2002).

In order to perform molecular docking, protein target (receptor) and promising drugs (ligand) are required to be prepared. As for receptor molecules, the crystal structure of XO was retrieved from the protein database (PDB: 1v97) (Okamoto et al., 2004). Meanwhile, the extracted *Moringa oleifera* was employed as the ligand molecules. From our docking simulations, the binding energies and the binding pose between ligands and receptors were obtained and presented in Table 2 and Figure 4, respectively. Further, the details of molecular interaction consisting of hydrogen bonds and hydrophobic interactions between the ligands/receptor complexes were provided in Table 3 and Figure 5, respectively.

Table 3. The hydrogen bonds of ligan in complex with receptor.

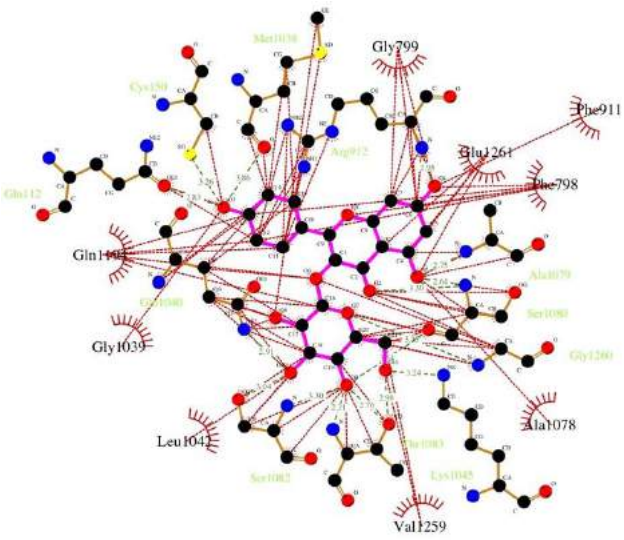
Compound	Residue	AA	Distance H-A	Distance D-A	Donor Angle	Donor Atom	Acceptor Atom
Astragalin	112	GLN	1.92	2.83	155.9	12344 [O3]	1032 [O2]
	798	PHE	3.58	4.03	109.14	7215 [N]	12335 [O2]
	798	PHE	2.7	3.05	101.95	12346 [O3]	7219 [O2]
	1040	GLN	1.96	2.91	154.67	9569 [N]	12361 [O3]
	1045	LYS	2.41	3.24	138.17	9617 [N3+]	12364 [O3]
	1080	SER	1.98	2.64	120.51	9925 [N]	12348 [O3]
	1080	SER	2.72	3.41	129.31	12348 [O3]	9931 [O3]
	1080	SER	2.91	3.54	124	12359 [O3]	9929 [O2]
	1082	SER	2.69	3.04	102.14	9947 [O3]	12361 [O3]
	1082	SER	2.28	3.04	133.82	12361 [O3]	9947 [O3]
	1083	THR	1.82	2.76	169.7	9955 [O3]	12359 [O3]
	1083	THR	1.75	2.71	156.45	9949 [N]	12359 [O3]
	1260	GLY	2.96	3.35	103.47	11634 [N]	12351 [O3]
Isoquercetin	112	GLN	2.16	2.66	110.05	12344 [O3]	1032 [O2]
	798	PHE	2.46	3.15	128.42	12350 [O3]	7219 [O2]
	912	ARG	2.61	3.18	115.08	8327 [N]	12346 [O3]
	912	ARG	2.35	2.96	117.67	8330 [N]	12346 [O3]
	1080	SER	1.67	2.47	131.9	9925 [N]	12348 [O3]
	1080	SER	2.25	3	133.63	12360 [O3]	9929 [O2]
	1082	SER	2.8	3.31	111.33	9941 [N]	12364 [O3]
	1083	THR	2.75	3.69	173.78	9955 [O3]	12364 [O3]
	1083	THR	2.54	3.45	147.98	9949 [N]	12364 [O3]
	1083	THR	2.85	3.69	145.13	12364 [O3]	9955 [O3]
	1194	GLN	2.99	3.88	146.19	11022 [N]	12344 [O3]
	1194	GLN	2.76	3.5	133.56	12346 [O3]	11021 [O2]
	1260	GLY	2.32	3.08	129.89	11634 [N]	12360 [O3]
	1261	GLU	2.19	3.09	153.65	12348 [O3]	11647 [O2]
Cryptochologenic acid	767	GLN	2.54	3.13	116.01	6926 [N]	12341 [O3]
	767	GLN	3.01	3.81	140.49	12341 [O3]	6925 [O2]
	799	GLY	2.77	3.22	107.05	7227 [N]	12341 [O3]
	802	GLU	1.94	2.89	164.34	12354 [O3]	7258 [O3]
	1010	THR	3.02	3.91	146.23	9302 [N]	12356 [O3]
	1079	ALA	3.14	4.06	150.05	9919 [N]	12343 [O3]
	1080	SER	2.01	2.68	120.83	9925 [N]	12333 [O3]
	1194	GLN	2.19	3.13	160.61	12337 [O.co2]	11021 [O2]
	1261	GLU	3.32	3.7	106.67	11648 [O3]	12345 [O2]

	1261	GLU	2.88	3.58	129.19	12333 [O3]	11648 [O3]
Control	797	GLY	1.87	2.8	150.6	7210 [N]	12327 [O2]
	798	PHE	2.22	3.1	143.5	7215 [N]	12327 [O2]
	1038	MET	2.29	2.88	115.99	12337 [N]	9550 [O2]
	1194	GLN	1.95	2.96	172.11	11022 [N]	12335 [N2]
	1194	GLN	2.04	2.96	149.52	12332 [Npl]	11021 [O2]

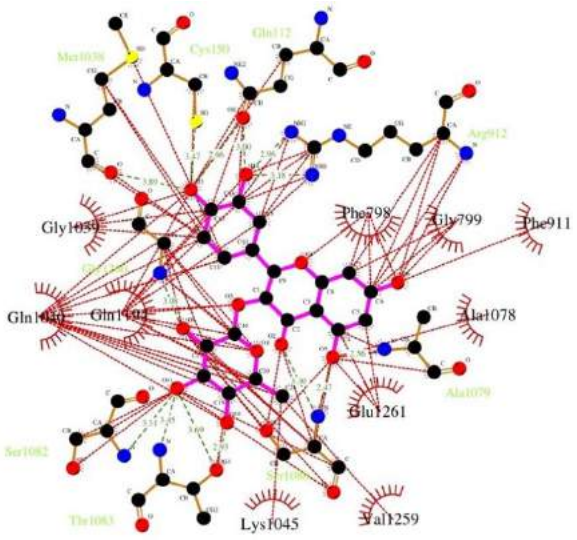
In this research, only the primary compounds of *Moringa oleifera*, i.e., Astragalin, Isoquercetin, and Cryptochologenic acid, were selected to become the ligand molecules (Vongsak et al., 2014). It was not easy to simulate three ligands simultaneously in docking protocols because ligands may overlap on each atom that causes structural changes from the initial configuration. Molecular docking was performed separately for each ligand, and overall results were combined to create one configuration because of the similar receptor (XO) to overcome this issue.

Three ligands, i.e., Astragalin (PubChem ID: 5282102), Isoquercetin (PubChem ID: 5280804), and Cryptochologenic acid (PubChem ID: 97986668) against XO are extracted from the PubChem database. The possibility of ligand binding to the receptor site is achieved when the binding energy of the ligand-receptor complex is a negative value. From our finding, three complexes were found with various binding energies, as listed in Table 2.

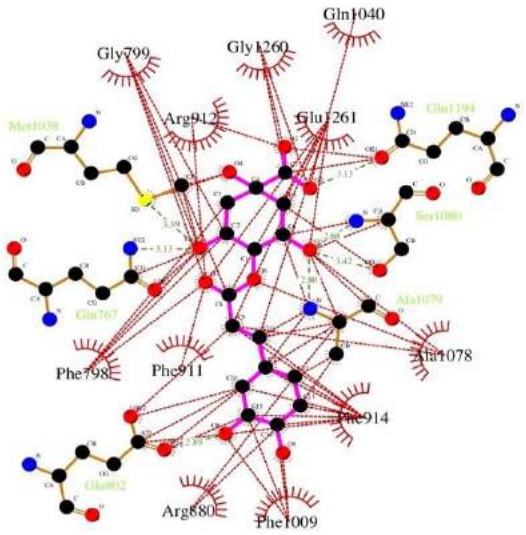
All ligands showed negative values binding energy which are -9.3 kcal/mol, -8.2 kcal/mol, and -10.6 kcal/mol for astragalin, isoquercetin and cryptochologenic acid consecutively, indicated that the ligands could bind to the receptor, forming a ligan-receptor complex. In addition to that, the ligands of all complexes have higher binding energies than Alluporinol (a positive control), with the binding energy of -6.6 kcal/mol. This finding revealed that those 3 ligands found in the extract of *Moringa oleifera* leaves probably hold the potential as inhibitors for XO. Even though the inhibition activity of allopurinol is higher compared to the extract, the results still demonstrate that the *Moringa oleifera* leaves extracts containing those primary compounds could inhibit the XO enzyme. Thus, to identify the molecular interactions, including hydrogen bond and hydrophobic interaction between ligand and receptor, the snapshot structure of those ligands was analyzed using PLIP server (Salentin et al., 2015) and LigPlot v.4.5.3 program packages (Wallace, Laskowski, & Thornton, 1995)



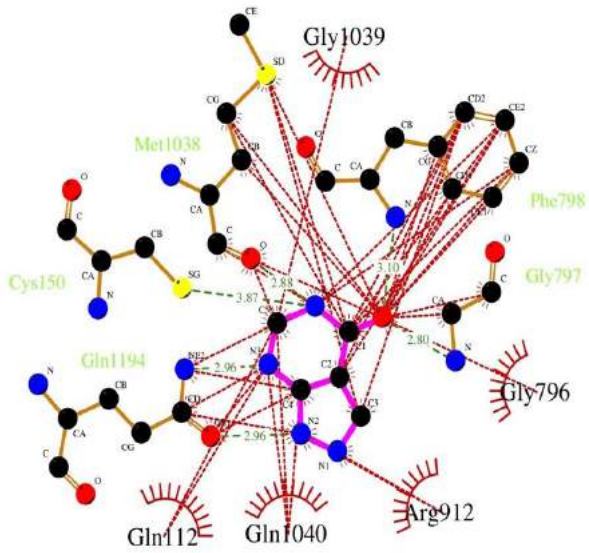
(a)



(b)



(c)



(d)

Figure 5. Hydrophobic interaction of ligand in complex with the receptor. (**a**) Astragalin, (**b**) Isoquercetin, (**c**) Cryptochologenic acid, (**d**) Alluporinol (control). The ligand refers to the stick model in magenta color. Redline is represented hydrophobic interaction between ligand and residues of the receptor.

Figure 5 illustrates the binding site and geometrical pocket of the ligands in complex with the receptor. The result of complex 1 was visualized from multiple dockings of the several ligands, then combined into one configuration. The lines in red, yellow, and colours correspond to the compounds of Astragalin, Isoquercetin, and Cryptochologenic acid, respectively. Meanwhile, complex 2 was denoted from the positive control into the site of XO. Of this figure, hydrogen bond and hydrophobic interactions contributed to the binding of ligand into the receptor. The detailed hydrogen bonds for those complexes are listed in Table 3. It was found that Astragalin of complex 1 participated in hydrogen bonds with residues GLN112, PHE798, GLN1040, LYS1045, SER1080, SER1082, THR1083, and GLY1260 of the receptor. In Isoquercetin, the hydrogen bonds were made in the residues of GLN112, PHE798, ARG912, SER1080, THR1083, GLN1194, GLY1260, GLU126. For Cryptochologenic acid, the hydrogen bonds were formed with the residues of GLN767, GLY799, GLU802, THR1010, ALA1079, SER1080, GLN1194, and GLU1261. Meanwhile, Alluporinol shown in complex 2 participated in hydrogen bonds with residues GLY797, PHE798, MET1038, and GLN1194. On the other hand, the hydrophobic interactions between ligand and receptor are presented in Figure 6. Astragalin of complex 1 formed the hydrophobic interaction with residues of the receptor, including MET1038, ARG912, GLY799, GLU1261, PHE911, PHE798, ALA1079, SER1080, GLY1260, ALA1078, THR1083, LYS1045, VAL1259, SER1082, LEU1042, GLY1039GLN1040, GLN1194, GLN112, AND CYS150. For Isoquercetin, the hydrophobic interaction was presented by the ligand's interaction with residues of the receptor such as MET1038, CYS150, GLN112, ARG912, PHE798, GLY799, PHE911, ALA1078, ALA1079, GLU1261, SER1080, VAL1259, LYS104, THR1083, SER1082, GLN1010, GLN1194, and GLY1039. Cryptochologenic acid participated in hydrophobic interaction with residues of GLY799, ARG912, GLY1260, GLN1040, GLU1261, GLN1194, SER1080, ALA1079, ALA1078, PHE914, PHE1009, ARG880, GLU802, PHE798, GLN767, AND MET1038. Meanwhile, for allopurinol, the hydrophobic interaction was observed between ligand and residues, including GLY1039, PHE798, GLY797, GLY796, ARG912, GLN1040, GLN112, GLN1194,

CYS150, MET1038. From these results, all primary ligands in the extracted *Moringa oleifera* leaves may become stable structures since the hydrogen bonds are coordinated between ligand and receptor. Also, the compounds of Astragalin, Isoquercetin, and Cryptochologenic acid bound to the similar residues of Alluporinol, indicated that those compounds have similar activity as an inhibitor for XO. Furthermore, in the paper presented by Okamoto and co-workers (Okamoto et al., 2004), the catalytic site of XO provided and visualized in the UniProt database (https://www.ebi.ac.uk/pdbe/entry/pdb/1v97/bound/MTE), which includes residues GLY797, MET1038, PHE798, ARG912, GLU1261, ALA1079, ALA1078, SER1080, GLN1040, VAL1081, SER1082, GLY1039, GLN1194, CYS150, and GLN112, is a crucial target for inhibiting the XO. Thus, the ligand that can bind to one of these residues is assumed to have a potential drug for treating goat disease. From our simulations, each ligand is bound to those residues by hydrogen bond and hydrophobic interaction, indicating those compounds can become inhibitor XO.

Conclusions

The results of enzyme characterization showed that the optimum activity of the enzyme isolated from bovine milk is at pH 6.5, substrate concentration of 0.1 mM, and reaction temperature of 35 ◦C. For XO enzyme inhibition, the increase in extract concentration linearly augmented the percentage of inhibition. Water extract of 160 mg/mL showed the highest inhibition value of 35.42%, while the methanol extract at the same concentration was 21.35%. These results indicated that the water extract of *Moringa oleifera* leaves was more effective in inhibiting XO than the methanol counterpart. Furthermore, computational analysis was performed to gain insight into the molecular interaction between the primary compounds of *Moringa oleifera* leaves, including Astragalin, Isoquercetin, and Cryptochologenic acid with XO using the molecular docking method. Our finding demonstrated that these compounds were bound to the catalytic sites of XO by hydrogen bonds and hydrophobic interaction, suggesting these primary compounds of *Moringa oleifera* leaves have pharmacology activities for inhibiting the XO.

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Conflicts of Interest

The authors have no conflict of interest.

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Inhibitory of *Moringa oleifera* L leaf extract on xanthine oxidase activity from bovine milk.

Abstract

Moringa oleifera is a tropical plant in the Moringaceae family that contains a lot of bioactive compounds. This study aimed to isolate and characterize the enzyme xanthine oxidase (XO), and conducted inhibitory tests on XO using methanol extracts of *M. oleifera* leaves. The xanthine oxidase enzyme isolated from bovine milk was characterized to determine the optimum pH, temperature, and substrate concentration. XO inhibition was evaluated by *in vitro* and *in silico* methods. The results of XO isolation and characterization of bovine milk showed the optimum conditions at pH 6.5, substrate concentration of 0.1 mM, and temperature 35 ^{o}C with an activity rate of 32.47 mU/mL; 21.55 U/mL, and 21.94 mU/mL. Inhibition analysis results on methanol extract of *M. oleifera* leaves showed the highest activity decrease at the extract concentration of 160 ppm, with a relative inhibition value of 21.35%, while allopurinol as a positive control has a relative value inhibition of 61.21%. Relative value inhibition indicated the potential of *M. oleifera* leaves as a source of medicinal plants for gout sufferers. Additionally, a computational analysis was performed to observe the molecular interaction between the primary compounds of *M. oleifera* leaves, i.e., 5-O-acetyl-thio-octyl-beta-l-rhamno furanoside, quinic acid, and 2-Dimethyl (trimethyl silylmethyl) silyloxy methyl tetrahydrofuran, and XO using the molecular docking method. The finding implied that these compounds are bound to the catalytic sites of XO by hydrogen bonds and hydrophobic interactions, indicating the primary compounds of *M. oleifera* leaves could become XO inhibitors to treat gout disease.

Keywords

Moringa oleifera leaves**,** inhibition, molecular docking, xanthine oxidase, bovine milk.

Introduction

Moringa oleifera is a medicinal plant that is widely cultivated in many tropical and subtropical countries (Boopathi and Raveendran. 2021). *M. oleifera* is a benefical plant because certain parts of its structure, such as its leaves, flowers, roots, seeds, and fruit, are widely used in a variety of applications. *M. oleifera* leaves, flowers and fruit are used as vegetables and traditional medicine. *M. oleifera* seed is a natural coagulant that can be used to purify water and as an oil extraction agent in the production of biofuel (Sagona et al. 2020). While, *M. oleifera* root has antibacterial, antioxidant, and anti-diabetic properties, according to several studies. Currently, *M. oleifera* study focused on the leaves' medical benefits. The diversity of metabolites in the leaves is indicated to have several pharmacological action. A number of studies show that the flavonoid and phenolic compounds found in *M. oleifera* leaves have antiinflammatory, antidiabetic, antibacterial, and antimicrobial activities. Several studies suggest that the flavonoid and phenolic compounds in *M. oleifera* leaves have anti-inflammatory, anti-diabetic, antibacterial, and antifungal activities (Fejér.2019); antiulcer activity is provided by sterols, terpenoids, flavonoids, tannins, and glycosides in *M. oleifera* leaves (Jincy and Sunil. 2020), whereas antioxidant activity is provided by polyphenols (Padayachee and Baijnath. 2020; Rocchetti. 2020). On the other hand, *M. oleifera* leaves other pharmacological properties include its capacity to block enzymes that act as receptors for particular diseases (Singh et al. 2020).

XO is an enzyme that plays a role in catalyzing the oxidation of hypoxanthine to xanthine, which becomes uric acid. XO is derived from the enzyme class molybdenum iron-sulfur flavin hydroxylase, mainly found in the liver, kidneys, brain, gastrointestinal tract (Maiuolo et al. 2016). The enzyme is also present in the entire cardiovascular

system. Inhibition of XO can suppress the biosynthesis of uric acid, which is one of the therapeutic approaches for treating gout, neuropathy, and kidney stones, which leads to hyperuricemia (Gliozzi et al. 2016; White. 2018).

Suppressing XO activity is the primary approach in treating hyperuricemia and gout in clinical settings because XO has an essential role in the formation of uric acid. Allopurinol, a synthetic drug used clinically to treat gout, is one of the XO inhibitors. (Seth et al. 2014). However, excessive use of allopurinol can cause nephropathy, hepatitis, digestive disorders, reduced white blood cells, allergies, and liver damage (Zeng et al. 2018). In order to avoid these undesirable side effects, the use of medicinal plants can be considered as an alternative therapeutic option (Mohos et al. 2019).

One of the plants that indicated to have the ability to inhibit XO activity is *M. oleifera* . Based on our previous study, *M. oleifera* leaves contain secondary metabolites such as flavonoids, alkaloids, tannins, and saponins. Methanolic extract of *M. oleifera* leaves showed antioxidant activity and inhibition of the enzyme α-glucosidase. (Natsir et al. 2019; Natsir et al. 2018). This study aimed to analyze the inhibitory activity of methanol extract of *M. oleifera* leaves against XO enzyme isolated from cow's milk. However, the XO enzyme was isolated and characterized first in order to determine the optimal conditions of the enzyme. Moreover, the molecular docking method was employed to investigate insight into the molecular recognition of the primary compounds of *M. oleifera* leaves in binding to the moiety of XO (Blaney and Dixon. 1993; Kitchen et al. 2004).

Materials and Methods

Chemicals and Instruments

The materials used in this study included: bovine milk obtained from cattle farmers in Enrekang Regency, South Sulawesi, M. oleifera leaf from Topoyo Subdistrict, West Sulawesi Province (Latitude: 2°02'17.21"S and Longitude: 114°15'30.36"E), $CH_3OH_{(pa)}$, NaCl, $(NH_4)_2SO_4$, $NaOH_{(pa)}$, HCl, xanthine substrate and allopurinol were purchase from Sigma Aldrich. The instruments used in this study were autoclave, centrifuge (Hermle Z336K), Alu-Lid rotor (Hermle 220.87 V20), rotary evaporator, vortex, stirrer magnetic, UV-Vis 1800 (Shimadzu-Japan), FTIR Spectrofotometer (Shimadzu-Japan), GCMS-QP2010 Ultra (Shimadzu-Japan).

Isolation of XO

The XO isolation process is a modified method from Bou-Salah (Bou-Salah et al. 2020), in which 500 mL fresh bovine milk was heated to a temperature of 30 ◦C, combined with 178.5 g of NaCl, then centrifuged at a speed of 3000 rpm for 30 minutes. The supernatant was fractionated with ammonium sulfate at 4 ◦C using an ice bath, then centrifuged at 8000 rpm at 4°C for 20 minutes using a Alu-Lid rotor (RFC 21.379/24.325 xg; angle rotor 24 x 1.5/2.0 ml; angle 45°, max. speed 15.000/16.000 rpm). The precipitate was dissolved in 0.05 M potassium phosphate buffer pH 7.5 to 250 mL.

Preparation of XO Solution

Xanthine substrate of about 15.21 mg was added to the measuring flask and then added with five drops of 1 M NaOH, shaken until dissolved. The solution was diluted with CO_2-free demineralized water to 100.0 mL (1 mM concentration). The xanthine substrate was prepared by diluting the stock solution to obtain a standard solution, with a concentration of 0.05; 0.1; 0.15; 0.2, and 0.25 mM (Kostić et al. 2015).

Allopurinol Solution

Allopurinol 1000 µg/mL stock solution was prepared by weighing 10 mg of allopurinol and dissolving it in 5 drops of 1 M NaOH. The solution was transferred to a volumetric flask with a volume of 10 mL and then diluted with CO_2-free demineralized water.
The standard allopurinol solution was prepared by diluting the stock solution to get a series of allopurinol standard solutions, with a concentration of 0.1; 0.2; 0.5; 1.0 and 2.0 µg/mL (Gong et al. 2020).

XO Crude Extract

The crude XO extract was weighed about 22.17 mg using a 25 mL weighing bottle, then the extract was added into a volumetric flask and diluted with phosphate buffer solution. The volume was diluted to the limit mark to obtain an XO solution of 0.1 unit/mL (Kostić et al. 2015).

XO Characterization

The crude extract of the enzyme was characterized to determine the optimum conditions of the enzyme, such as pH, substrate concentration, and temperature effect (Kostić et al. 2015; Natsir et al. 2002). The optimum conditions were determined by analyzing the optimum activity of the enzyme. It was calculated by Equation 1:

$$\mathrm{Ea} = \frac{(\mathrm{Ab} - \mathrm{Ac})\ \mathrm{V} \times \mathrm{df}}{12.2 \times 0{,}1}, \qquad (1)$$

Where Ea is enzyme activity (mU/mL); Ab is the absorbance of blank; Ac is the absorbance of control; V is total volume assay (mL); df is dilution factor; 12.2 is uric acid extrinsic coefficient at 290 nm (mM); and 0.1 is the volume of XO used U/mL of the enzyme.

Optimum pH

Phosphate buffer solutions of 0.2 M (3.9 mL) with a pH variation of 6; 6.5; 7; 7.5 and 8 were added 2 mL of 0.15 mM xanthine substrate solution, then pre-incubated for 10 minutes at 25 °C. 0.1 mL of XO was added to the mixtures and then incubated for 30 minutes at 25 C. The absorption of the sample was measured at λmax 232 nm using a UV-Vis spectrophotometer (Kostić et al. 2015; Natsir et al. 2002; Sharma et al. 2016).

Optimum Substrate Concentration

The optimum substrate concentration was determined by adding 2 mL of phosphate buffer solution at the optimum pH, with a xanthine substrate concentration of 0.05 mM; 0.10 mM; 0.15 mM; 0.20 mM, and 0.25 mM. After pre-incubation, 0.1 mL of XO was added to the solution, and the mixture was incubated at 25 °C for 30 minutes. A similar procedure was applied for control by replacing the crude enzyme extract using 0.1 mL of distilled water (Kostić et al. 2015; Natsir et al. 2002).

Optimum Temperature

Phosphate buffer solution 0.2 M (3.9 mL) of optimum pH was added to 2 mL of xanthine substrate with optimum concentrations, then pre-incubated for 10 minutes. The enzyme XO (0.1 mL) was added, incubated for 30 minutes at 20 °C; 25 °C; 30 °C; 35 °C, and 40 °C. After the incubation process, the absorption was measured at λmax 232 nm using a UV-Vis spectrophotometer (Sharma et al. 2019; Natsir et al. 2010).

Preparation and Extraction Moringa oleifera Leaves

M. oleifera leaves are harvested from the tree by manually collecting the 3rd to 5th petiole leaves. The leaves are washed and then dried for 7-10 days at room temperature. After drying, the leaves were then processed into a fine powder using a grinding machine. Dry *M. oleifera* leaves powder is mixed with methanol in a ratio of 1:20 (w/v). The extraction process was conducted at 45 °C for 20 minutes with constant stirring using a magnetic stirrer. The extract obtained was filtered and then evaporated using a rotary evaporator to obtain a thick methanol extract. The metabolomic profile of the methanol extract was analyzed using FTIR and GCMS. (Natsir et al. 2018; Natsir et al. 2019; Rocchetti et al. 2019).

FTIR Spectroscopic Analysis

The FTIR spectrum of M. oleifera leaves methanol extract was analyzed using an FTIR spectrophotometer (Shimadzu-Japan) at a frequency of 4000–250 cm^{-1}). The spectrum was recorded using approximately 1 mg of methanol extract (Meenakshi et al. 2020).

GCMS Analysis

The methanol extract of M. oleifera leaves was analyzed using GCMS-QP2010 Ultra (Shimadzu), which was connected to a

capillary column DB-1 (0.25 m film 0.25 mm I. d. 30 m length). The temperature of the injector was kept at 250 °C (constant). The column oven temperature was set at 50°C for 3 minutes, then raised to 280 °C for 3 minutes, and finally held at 300 °C for 10 minutes. The chromatogram results were identified by comparing the obtained spectral configurations on mass spectral databases that were readily available (NIST libraries) (Ezhilan and Neelamegam. 2012).

Inhibition Activity of Methanol Extract of Moringa Oleifera Leaves Against XO

The methanol extract of *M. oleifera* leaves were diluted to a 20 µg/mL concentration with 0.05 mM phosphate buffer solution pH 7.5. An aliquot of 3 mL extract solution was added to a reaction tube, followed by 2 mL of 0.15 mM xanthine and 0.2 mL of XO, and then incubated at room temperature for 45 minutes. After incubation, 1 mL HCl (0.58 M) was added to the mixtures to stop the enzymatic reaction. Water was used as the control solution for the negative control, and allopurinol as a positive control. The absorbance of the solution was measured using a UV-Vis spectrophotometer at λ_{max} 232 nm. Calculation of inhibition ability was obtained from the linear equation of the time versus concentration of the XO curve (Fachriyah et al. 2018).

Molecular Docking

Molecular docking was performed using the AutoDock Vina package developed by Trott and co-workers to determine the ligand's binding site into the receptor's catalytic site (Trott and Olson. 2010). In this study, the M. oleifera leaves extracts were indicated to treat gout disease induced by hyperuricemia. A study reported that 5-O-acetyl-thio-octyl-beta-l-rhamno furanoside, quinic acid, and 2-Dimethyl (trimethyl silylmethyl) silyloxy methyl tetrahydrofuran were identified as the primary compounds of *M. oleifera* and therefore used as the ligand molecules for the docking. The chemical structures of those ligands were retrieved from the PubChem database, as shown in Figure 1. All ligands were downloaded and saved as sdf extensions. Open Babel 2.4.1 program packages were applied to convert sdf files to pdbqt extension (O'Boyle et al. 2011). As for the target molecule, XO was assigned as the receptor since this enzyme is related to gout disease. The tertiary structure of the receptor was taken from a protein data bank (PDB ID: 1v97) with a resolution of 1.94 Å, as shown in Figure 2 (Okamoto et al. 2004). The polar hydrogen and Kollman's united atom charges were added to the receptor using AutoDock Tools 1.5.6 created by Morris and co-workers (Morris et al. 2009). Afterwards, the XO was saved in pdbqt format.

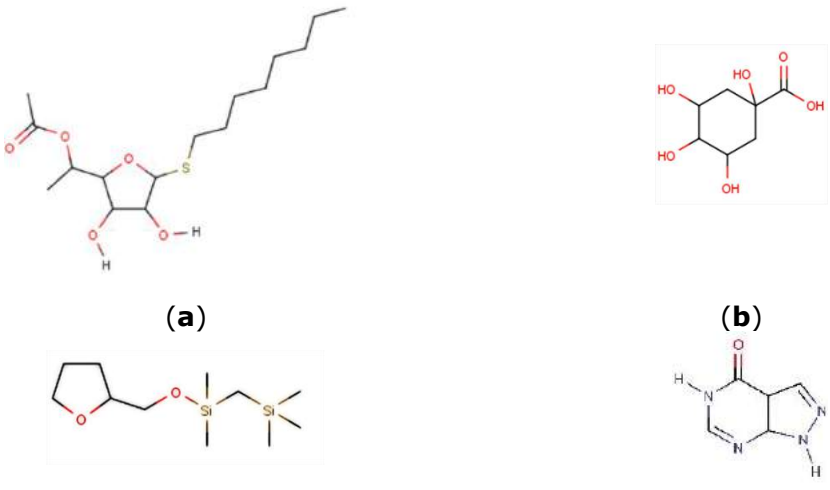


Figure 1. The chemical structure of the compounds of (**a**) 5-O-acetyl-thio-octyl-.beta.-l-rhamnofuranoside (PubChem ID: 537841), (**b**) Quinic acid (PubChem ID: 6508), (**c**) 2-Dimethyl (trimethylsilylmethyl) silyloxymethyl tetrahydrofuran (PubChem ID: 559105), and (**d**) Alluporinol (PubChem ID: 135401907) as the positive control.

In performing molecular docking, a grid box parameter is required to decide the positional and rotational of the ligand into the moiety of the receptor (Arwansyah et al. 2021). The grid box was placed on 24 × 24 × 26 points with a grid spacing of 1.00 Å. Meanwhile, the grid box centre was set at the coordinates x=148.649, y=43.411, and z= 26.399. The exhaustiveness was computed at 100. Other parameters were assigned as the default of AutoDock Vina. The Broyden-Fletcher-Goldfarb-Shanno (BFGS) algorithm was used as a search parameter to obtain the binding pose and conformation of the ligand into the receptor site. The docking protocols were set according to a similar procedure with our previous study provided in Ref. (Arwansyah. 2016).

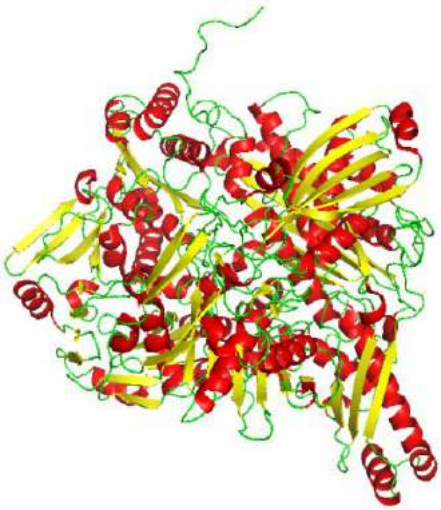


Figure 2. The tertiary structure of the created model of XO (PDB: 1v97) (Okamoto et al. 2004). The structures of α-helix, β-sheet, and turn are presented by cartoon models' red, yellow, and green colours.

Result and Discussion

Isolation results from bovine milk produced 340 mL of crude extract of XO enzyme. The enzymes used for the characterization and inhibition tests were stored at 4 °C to maintain stability and avoid denaturation.

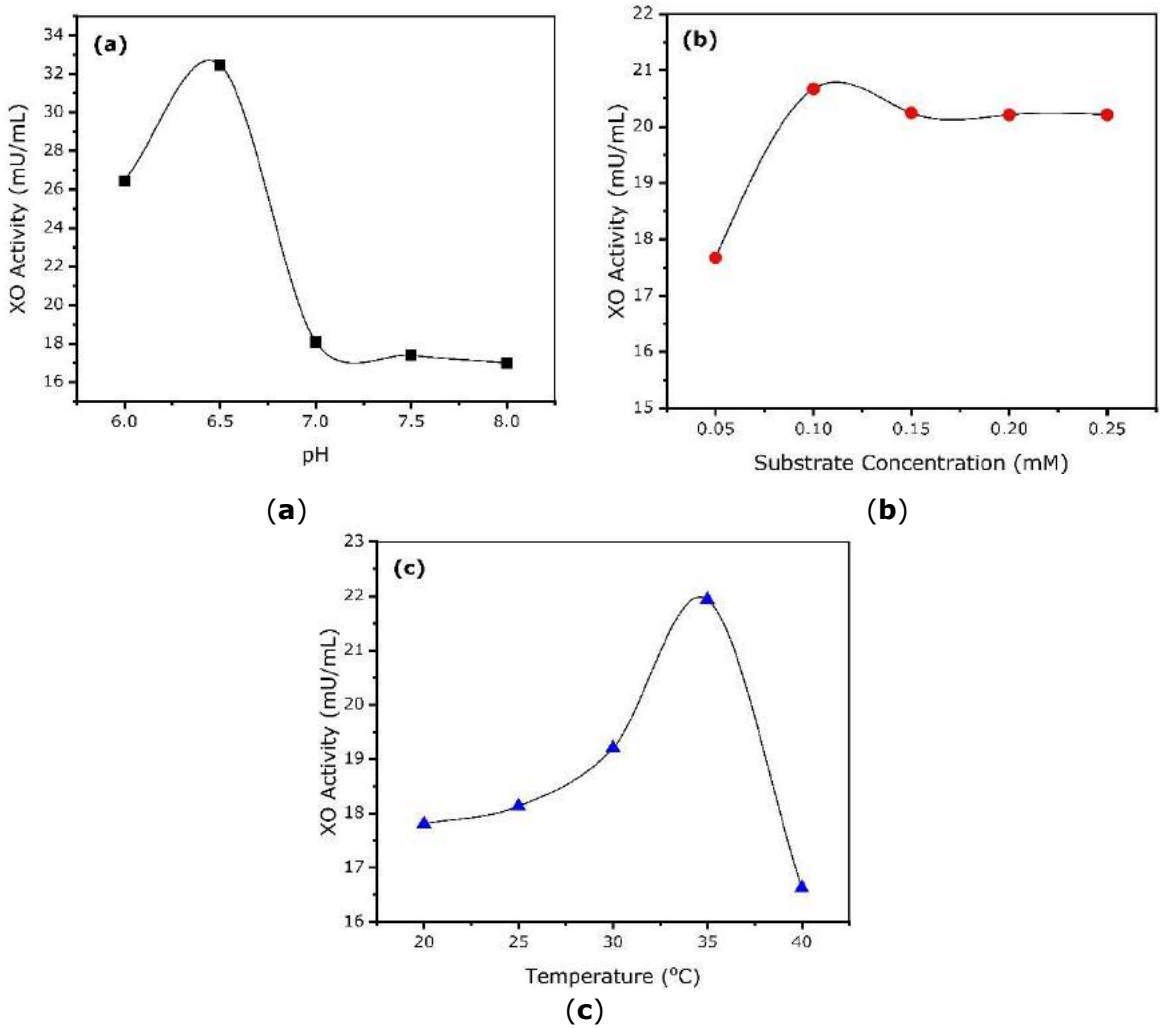
Characterization of XO

Determination of the optimum pH of the enzyme was carried out by conditioning the enzyme at a certain pH in the reaction between the enzyme and the substrate, as shown in Figure 3(**a**). Each type of enzyme has an optimum pH range, where the enzyme offers maximum activity in high stability. Generally, enzymes are amphiphilic, which means they can be acidic and base due to their active ability to provide functional groups of specific amino acid residues that are donor and acceptor proton (Singh. 2017). The XO activity showed that the optimum pH was at 6.5 with the activity of 32.47 mU/mL, and after pH increased at pH 7.0, XO activity decreased to 18.10 mU/mL. It was unveiled that the increase of enzyme activity at the optimum pH can be related to changes in ionization of the enzyme ionic group on the active site. Thus the conformation of the active site becomes more effective in binding and changing the substrate during the catalysis process (Huang et al. 2017).

The effect of substrate concentration was assessed to determine the optimum substrate concentration suitable for the enzyme. The substrate concentration used was 0.05; 0.1; 0.15; 0.2; 0.25 mM. The results obtained are shown in Figure 3. The results demonstrated that higher enzyme activity was achieved at elevated substrate concentration. However, when after it reached the optimum substrate concentration, the activity tended to decrease. We reported that the highest enzyme activity was at 0.1 mM substrate concentration, with an activity of 21.55 U/mL. The increase in substrate concentration is directly related to the reaction rate until it reaches a maximum value of V_{max}. If the substrate concentration is increased, there will be no increase in the reaction rate because the substrate has saturated the enzyme's active site (Sharma et al. 2016).

Temperature is critical in enzymatic reactions because enzymes are proteins that are easily denatured against changing environmental conditions. The change in environmental temperature will affect enzyme activity (Claaßen. 2019). The enzyme will show optimal catalytic activity at a specific temperature and denatured when exposed to extreme temperatures (Roche and Royer. 2018). When the

temperature increases to optimal, the reaction rate would be accelerated because kinetic energy increases (Marañón et al. 2018). Increased kinetic energy will accelerate the motion of vibration, translation, and rotation of both enzymes and substrates. It will increase the frequency of collisions between enzymes and substrates (Zhang et al. 2016).. In this study, the determination of the optimum temperature of the XO enzyme used a variation of incubation temperature in the range of 20; 25; 30; 35 and 40 °C. The results obtained are shown in Figure 3. From the graph, it is clearly seen that the optimum temperature was reached at 35 °C with an activity of 21.94 mU/mL.



(**a**) (**b**) (**c**)

Figure 3. Characterization of XO (**a**) pH; (**b**) Substrate concentration; (**c**) Temperature.

FTIR Spectroscopic Analysis

FT-IR spectroscopic analysis of *M. oleifera* leaves methanol extract was used to analyze the phytoconstituents in the sample based on spectral data (Figure 4).

The results of the FTIR analysis of the methanol extract of M. oleifera leaves (Table 1) showed the presence of flavonoids and phenolics from the O-H and C=O groups at 3435 cm-1 and 1732, 1714 cm-1 which indicated the presence of C-H streching. Alkaloids in the C-N band at 1460, 1411 cm-1 and N-H in the fingerprint region of 1635 cm-1 (Maobe. 2013).

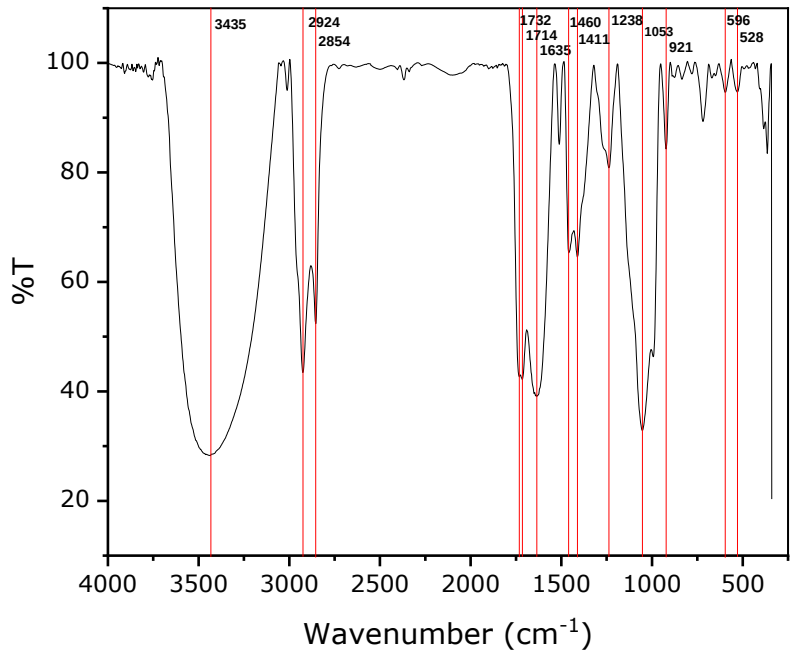


Figure 4. FTIR spectrum of methanol extract of M. oleifera leaves.

Table 1. FTIR spectra analysis of methanol extraxt of M. oleifera leaves.

Functional groups	**Wavenumber** (cm^{-1})	**Vibrations**
O-H	3435	strech
C-H	2924, 2854	strech
C=O	1732, 1714	strech
N-H	1635	bend
C-N	1460, 1411	strech
C-O	1238	strech
Si-O-C	1053	strech
C-OH	921	deformation
C-S	596	strech
C-C=O	528	bend

Tannins were discovered in the form of free phenol by stretching O-H at 3435 cm^{-1} and C-O at 1238 cm^{-1}. The C=O band is represented by the peak found at 1732, 1714 cm, and the C-O band is represented by the peak found at 1238 cm^{-1}.

GCMS profiling data showed three main compounds in the methanol extract of M. oleifera leaves, namely 5-O-acetyl-thio-octyl-.beta.-l-rhamnofuranoside, Quinic acid, 2-Dimethyl (trimethylsilylmethyl) silyloxymethyl tetrahydrofuran. The presence of 5-O-acetyl-thio-octyl-.beta.-l-rhamnofuranoside was identified from the O-H group at 3435 cm^{-1}, C-H at 2924, 2854 cm^{-1}, C=O at 1732, 1714 cm^{-1} and C-O at 1238 cm^{-1} which are strech in cyclic ethers. The presence of the C-S group at 596 cm^{-1} is a spesific band of the 5-O-acetyl-thio-octyl-.beta.-l-rhamnofuranoside compound. Quinic acid was identified from the typical strain O-H at 3435 cm^{-1}, C-O at 1238 cm^{-1}, C-C=O at 528 cm^{-1} and C-OH at 921 cm^{-1} which is a typical band of carboxylic acid groups. The compound 2-Dimethyl (trimethylsilylmethyl) silyloxymethyl tetrahydrofuran was identified from the presence of C-H band at 2924, 2854 cm^{-1}, C-O at 1238 cm^{-1}, and Si-O-C at 1053 cm^{-1} which are typical bands of this compound.

GCMS Analysis

For metabolite profiling, GCMS was used to identify bioactive compounds in a methanol

extract of M. oleifera leaves. The GCMS chromatogram (Figure. 5) showed 78 peaks which indicating the presence of 78 metabolite compounds.

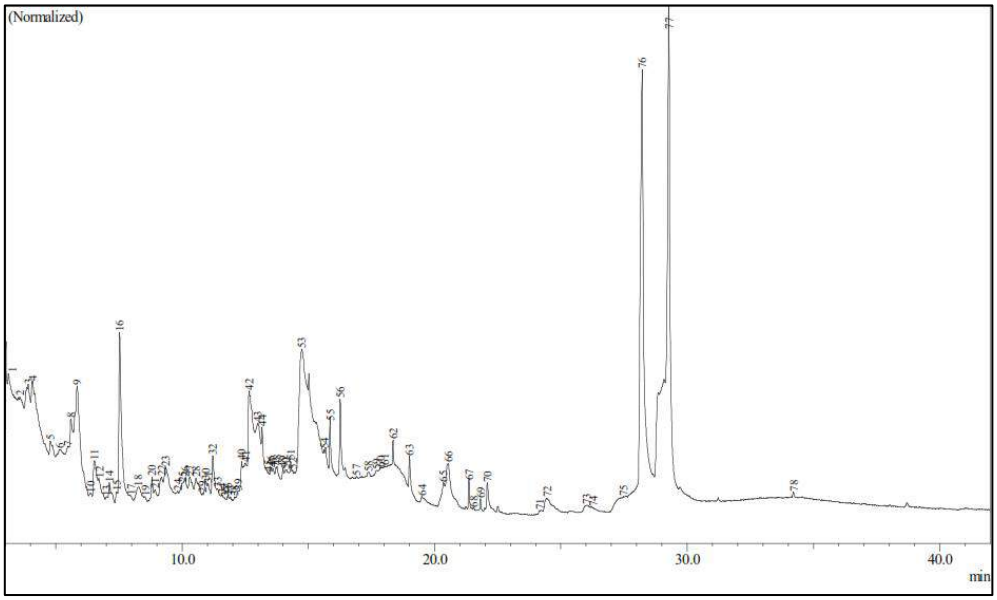


Figure 5. GCMS chromatogram of *M. oleifera* leaves methanol extracts.

The seventy eight compounds are characterized and identified through comparison of constituent mass spectra with the NIST library (Table 2).

Table 2. Phytocomponents identified in the methanol extract of *M. oleifera* leaves by GCMS.

Peak Number	Ret.Time	Name of the compounds	Peak Area (%)
1	3.123	2-Furanmethanol	0.13
2	3.568	2-[3'-(1"-Hydroxy-1"-Methylethyl)-2',2'-Dimethylcyclobutyl] Ethanal	0.06
3	3.903	1,2,4,5-TETRAZINE, 1,2,3,6-TETRAHYDRO-3,6-DIMETHYL-	0.71
4	4.079	1-Butanamine, 2-Methyl-N-(2-Methylbutylidene)-	1.34
5	4.779	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	0.25
6	5.161	6-(t-butyloxycarbonylaminopropionamido)hexanamide, N-methyl-N-[4-(1-pyrrolidinyl)-	0.49
7	5.467	N-Methyl-3-piperidinecarboxamide	0.41
8	5.594	1-Butanamine, 2-Methyl-N-(2-Methylbutylidene)-	0.97
9	5.84	1,2,3,4-Butanetetrol, [S-(R*,R*)]-	2.9
10	6.374	2-Octenoic acid, 4,5,7-trhydroxy	0.03
11	6.522	1,3,5-Triazine-2,4,6-triamine	0.77
12	6.708	Cyclopentanol	0.32
13	6.992	Benzeneethanol	0.04
14	7.116	2,4,8,10-Tetraoxaspiro[5.5]undecane	0.29
15	7.4	2-Propanamine, N-Methyl-N-Nitroso-	0.12
16	7.524	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	3.31
17	7.975	alpha-[5-Ethyl-2,3,4,5-tetrahydro-2-furyl]glycine	0.03
18	8.262	5-Methoxypyrrolidin-2-one	0.42
19	8.533	Butylamine, N-(1-Propylbutylidene)-	0.05
20	8.804	Boron, Trihydro(Morpholine-N4)-, (T-4)-	0.32
21	8.923	2,3-Dihydro-Benzofuran	0.22
22	9.183	2-Furancarboxaldehyde, 5-(hydroxymethyl)-	0.6
23	9.349	2-Propanone, 1-Phenyl-	1.31
24	9.819	Prednisolone	0.26
25	10	2-Chloroethyl vinyl sulfide	0.39
26	10.118	Cyclohexanone, 2-(2-Butynyl)-	0.51
27	10.312	2,5-Pyrrolidione, N-[2-(thienyl)acetyloxy]-	0.67
28	10.545	Propanoic acid, 2-[(tetrahydro-2H-pyran-2-yl)oxy]-	0.76
29	10.808	2-Propanone, 1-(3,5,5-trimethyl-2-cyclohexen-1-ylidene)-, (Z)-	0.09
30	10.922	2-Methyl-l-methylmannopyranoside	0.4
31	11.033	2-Piperidineacetic Acid, .Alpha.-Phenyl-, Methyl Ester	0.28

32	11.213	2-Furanmethanol, 5-ethenyltetrahydro-.alpha.,.alpha.,5-trimethyl-, cis-	0.83
33	11.4	Naphthalene, 1,2-Dihydro-1,5,8-Trimethyl-	0.25
34	11.592	Ethanone, 1-(2,3-Dihydro-1,1-Dimethyl-1h-Inden-4-Yl)-	0.12
35	11.683	4-(2,4,4-Trimethyl-cyclohexa-1,5-dienyl)-but-3-en-2-one	0.05
36	11.809	1,6,6-Trimethyl-7-(3-oxobut-1-enyl)-3,8-dioxatricyclo[5.1.0.0(2,4)]octan-5-one	0.12
37	11.917	9,10-Dimethylene-Tricyclo[4.2.1.1 2,5]Decane	0.08
38	12.075	4-(7,8-Dihydro-Tetrazolo[1,5-B][1,2,4]Triazin-7-Yl)-2,6-Dimethyl-Phenol	0.12
39	12.225	Bicyclo[4.2.1]nona-2,4,7-triene, 9-acetyl-, syn-	0.17
40	12.352	4-(2,4,4-Trimethyl-1,5-Cyclohexadien-1-Yl)-3-Buten-2-One	0.55
41	12.505	Undecane, 3-Methyl-	0.97
42	12.653	Benzeneacetonitrile, 4-hydroxy-	3.61
43	12.994	Beta.-D-Glucopyranose, 1,6-Anhydro-	2.44
44	13.166	2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-, (R)-	1.66
45	13.417	Dodecanoic Acid	0.32
46	13.512	1,3-Cyclohexanediol, 2,5-dimethyl-2-nitro-, monoacetate (ester), [1s-(1.alpha.,2.beta.,3.alpha.,5.alpha.)]-	0.42
47	13.594	Ethanediamide, N-Dodecyl-N'-(2-Thiazolyl)-	0.53
48	13.759	1,2-BENZENEDICARBOXYLIC ACID, DIETHYL ESTER	1.03
49	13.994	1,3,3-TRIMETHYL-2-(2-METHYLCYCLOPROPYL)-1-CYCLOHEXENE #	0.53
50	14.095	3-Buten-2-one, 1-(2,3,6-trimethylphenyl)-	0.65
51	14.3	Megastigmatrienone	0.79
52	14.375	3-Methyl-6-Oxo-2-Hexenyl Acetate	0.3
53	14.742	1,3,4,5-Tetrahydroxy-Cyclohexanecarboxylic Acid (Quinic Acid)	14.66
54	15.626	10,11-Dihydroxy-3,7,11-Trimethyl-2,6-Dodecadienyl Acetate	1.52
55	15.867	Tetradecanoic acid	1.75
56	16.262	2(4h)-Benzofuranone, 5,6,7,7a-Tetrahydro-6-Hydroxy-4,4,7a-Trimethyl-, (6s-Cis)-	3.1
57	16.906	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	1.17
58	17.367	1-Butyl 2-(8-Methylnonyl) Phthalate #	2.13
59	17.7	Ethanone, 1,1'-(5-Hydroxy-2,2-Dimethylbicyclo[4.1.0]Heptane-1,7-Diyl)Bis-, (1.Al	1.03
60	17.867	Octyl-.beta.-D-glucopyranoside	0.92
61	18.017	5-(Diethylamino)-3,4-Dimethyl-2(5h)-Furanone #	1.6
62	18.353	Hexadecanoic Acid, Methyl Ester	3.39
63	19.008	n-Hexadecanoic acid	1.04
64	19.5	Hexadecanoic acid, ethyl ester	0.17
65	20.35	Nonanoic Acid	0.53
66	20.539	9-Octadecenoic Acid (Z)-	1.47
67	21.364	9-Octadecenoic acid (Z)-, methyl ester	0.22
68	21.55	2-Hexadecen-1-Ol, 3,7,11,15-Tetramethyl-, [R-[R*,R*-(E)]]-	0.02
69	21.817	Octadecanoic acid, methyl ester	0.09
70	22.091	11,14,17-Eicosatrienoic acid, methyl ester	0.44
71	24.167	Geranyl isovalerate	0.08
72	24.443	Benzyl .beta.-d-glucoside	0.73
73	26.017	2-Methyl-3-(2-Methylphenyl)Propanal	0.25
74	26.267	Sclareolide	0.12
75	27.483	2-Methyl-1-[3-(1-Trimethylsilanyloxy-Pentyl)-Oxiranyl]-Propan-1-Ol	0.78
76	28.22	2-Dimethyl(trimethylsilylmethyl)silyloxymethyltetrahydrofuran	12.21
77	29.279	5-O-Acetyl-Thio-Octyl-.Beta.-L-Rhamnofuranoside	16.53
78	34.219	Hexatriacontane	0.06

The diversity of phytochemical compounds in plant extracts is closely linked to their bioactivity. However, the main compound in the highest concentration appears to play a key role on the medicinal activity of the plant (Huie. 2002). Based on the metabolite profile data in Table 2, it shows that the main compounds in the methanol extract of *M. oleifera* leaves are 5-O-acetyl-thio-octyl-.beta.-l-rhamnofuranoside (16.53%), Quinic acid (14.66) and 2-Dimethyl(trimethylsilylmethyl)silyloxymethyl tetrahydrofuran (12.21%). This is based on the highest concentration value of each compound, which is more than 10%. The mass spectra of the three main compounds identified in the methanol extract of *M. oleifera* leaves are presented in Figure 6.

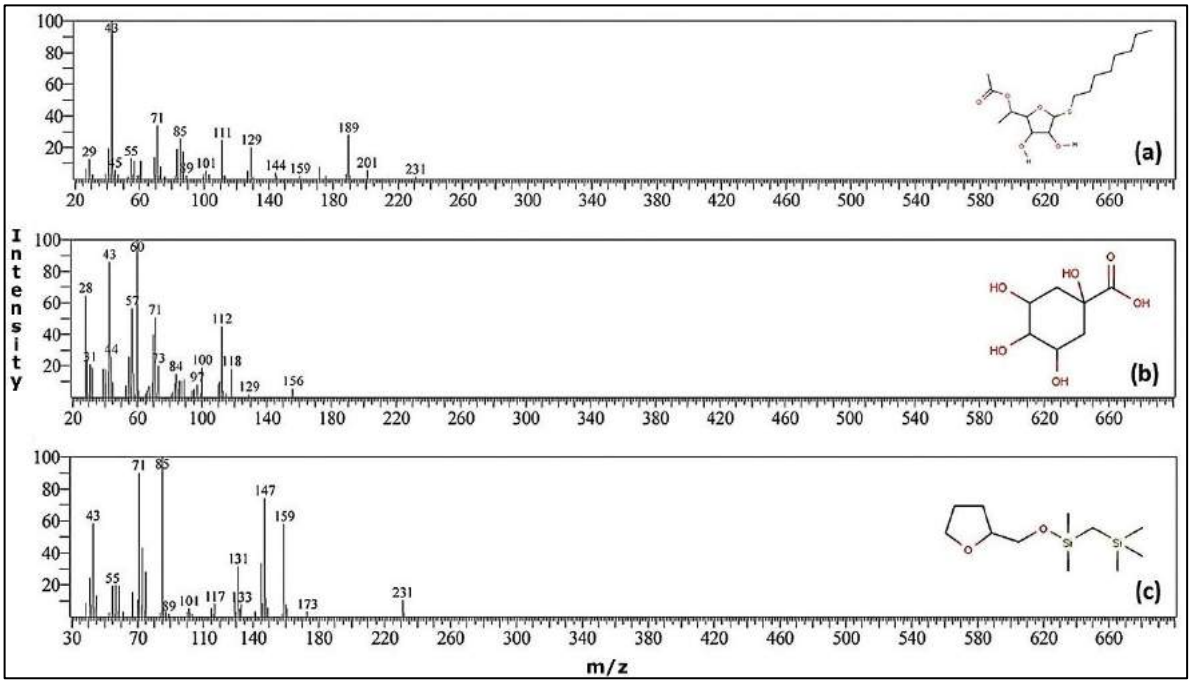


Figure 6. Mass spectrum and structure of main compounds identified by GCMS in the methanol extract of *M. oleifera*: **(a)** 5-O-acetyl-thio-octyl-.beta.-l-rhamnofuranoside; **(b)** Quinic acid; **(c)** 2-Dimethyl(trimethylsilylmethyl)silyloxymethyl tetrahydrofuran.

In general, two compounds were reported to have antioxidant activity among the three main compounds, dan no activity reported for 2-Dimethyl (trimethylsilylmethyl) silyloxymethyl tetrahydrofuran from the samples (Pero. 2009; Verma et al. 2019). Besides being an antioxidant, quinic acid has anti-inflammatory (Nam et al. 2019), anti-hepatitis B virus (Wang et al. 2009), and hepatoprotective activities (Kim et al. 2007). The biological activity of each compound is a function of their lipophilic properties, functional group properties, and its solubility in methanol (Ezhilan and Neelamegam. 2012).

Inhibition of Methanol Extract of Moringa Leaves Against XO Activity

Various concentrations of enzyme were used in the inhibition assay to determine the relationship between increasing enzyme concentration and inhibition activities. We reported that the methanol extracts of M. oleifera leaves demonstrated in vitro XO inhibition activity, and the results are presented in Table 3.

Table 3. The inhibition value of methanol extract of *M. oleifera* leaves against XO.

Sample concentrations (mg/mL)	**Methanol extract inhibition** (%)
10	5.73
20	7.04
40	8.83
80	10.02
160	21.35
Negative control	0
Allopurinol (positive control)	62.11

The analysis inhibition showed that the effectiveness of inhibition was directly proportional to the increase in extract concentration. Methanol extract at a concentration of 10 mg/mL showed inhibition values of 5.73%, while at a 160 mg/mL concentration, the inhibition value was 21.35%. The increased inhibitory

activity of the methanol extract of M. oleifera leaves was significantly linked with the metabolite content of the leaves. The presence of three main compounds in the methanol extract of *M. oleifera* leaves namely is 5-O-acetyl-thio-octyl-beta-l-rhamno furanoside, quinic acid, and 2-Dimethyl (trimethyl silylmethyl) silyloxy methyl tetrahydrofuran, become a constituent that works to prevent substrates entering the enzyme's active site.

The mechanism for binding the substituent to the active site of the XO enzyme occurs through the interaction of the O-H, C=O and C-H aliphatic functional groups of the three main compounds through hydrogen bonds and hydrophobic interactions. The interaction mechanism can be seen in Figure 5. Another study investigating the potential of *M. oleifera* leaves constituents as XO inhibitors was conducted by Tian (2021) who studied the enzymatic hydrolysis of phenolic and peptide fractions of *M. oleifera* leaves. It was discovered that the hydrolysis process significantly increased the inhibitory activity of XO as well as the antioxidant activity. These findings in the present study showed methanol extract of M. oleifera leaves activity indicates promising potential for its development as an XO inhibitor.

Molecular Docking Analysis

To analyze the molecular recognition in relation to the inhibitory activity, an advanced experimental investigation by X-ray analysis is required to obtain insight into the molecular interaction, including binding energy between the extraction of *M. oleifera* leaves and the tested enzyme (XO). However, computational analysis using the molecular docking method can currently investigate the structural and conformational changes of ligand-receptor complex (Qashqoosh et al. 2019). Therefore, this method is employed to find the viewpoints of physical and chemical properties regarding the binding action of *M. oleifera* into the moiety of XO.

Table 4. The binding energy of ligands in a complex with a receptor (XO) is obtained by molecular docking.

No.	Compound	Binding Energy (Kcal/mol)
1	5-o-acetyl-thio-octyl-.beta.-l-rhamnofuranoside	-8.2
2	Quinic acid	-6.7
3	2-Dimethyl (trimethylsilylmethyl) silyloxymethyl tetrahydrofuran	-3.6
4	Allopurinol	-6.6

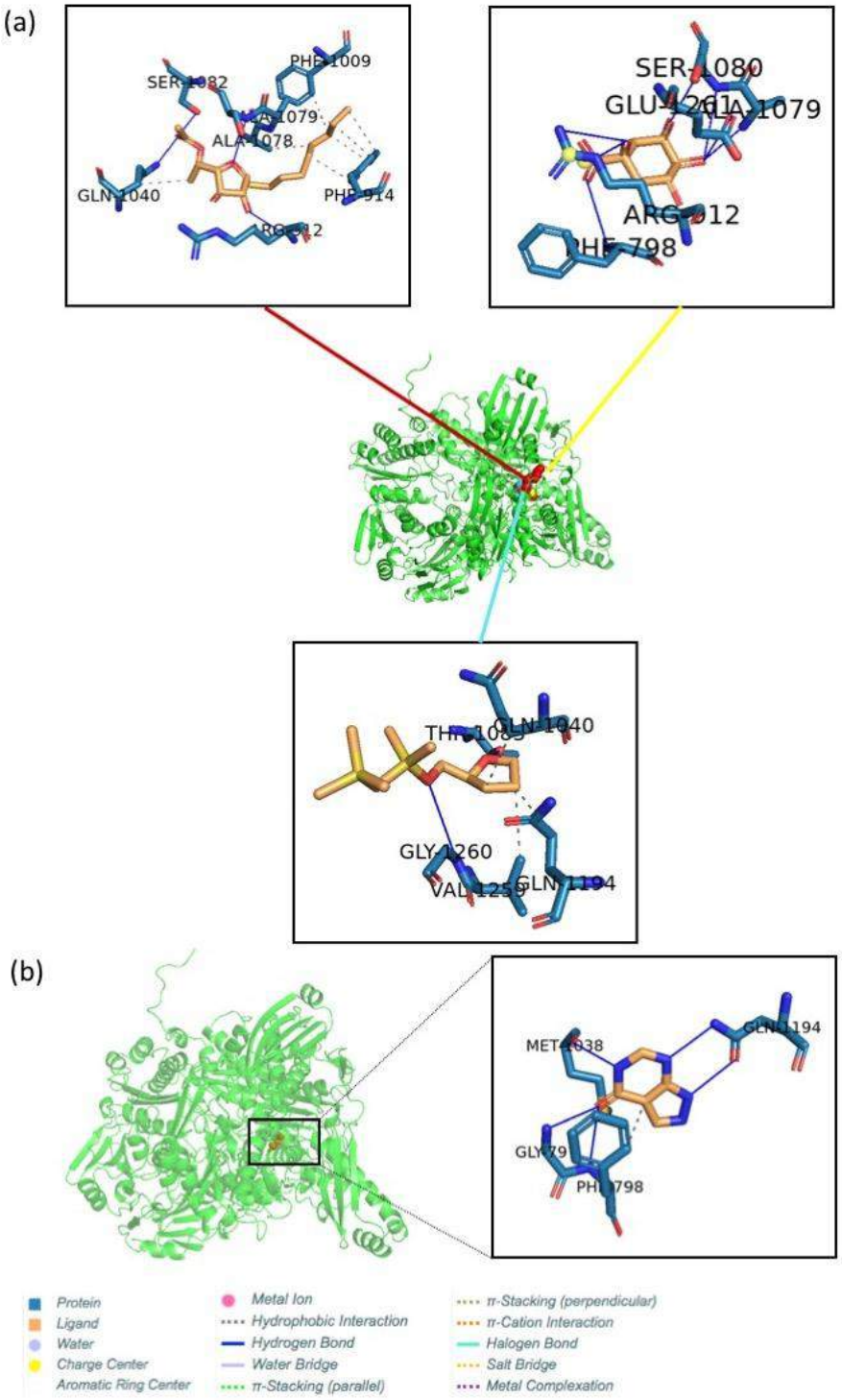


Figure 7. Binding pose of ligand in complex with a receptor (XO). Complex 1 consisted of mixed compounds where the red, yellow, and cyan lines refer to 5-O-acetyl-thio-octyl-beta-l-rhamno furanoside, quinic acid, and 2-Dimethyl (trimethyl silylmethyl) silyloxy methyl tetrahydrofuran compounds, respectively, (b) complex 2 denoted to allopurinol (control). The conformation pose of each complex is visualized by the PLIP program (Salentin et al. 2015) combined with Pymol v 2.3 program packages (DeLano. 2002).

In order to perform molecular docking, protein target (receptor) and promising drugs (ligand) are required to be prepared. As for receptor molecules, the crystal structure of XO was retrieved from the protein database (PDB: 1v97) (Okamoto et al. 2004). Meanwhile, the extracted *M. oleifera* was employed as the ligand molecules. From our docking simulations, the binding energies and the binding pose

between ligands and receptors were obtained and presented in Table 4 and Figure 7, respectively. Further, the details of molecular interaction consisting of hydrogen bonds and hydrophobic interactions between the ligands/receptor complexes were provided in Table 5 and Figure 8, respectively.

Table 5. The hydrogen bonds of ligan in complex with receptor.

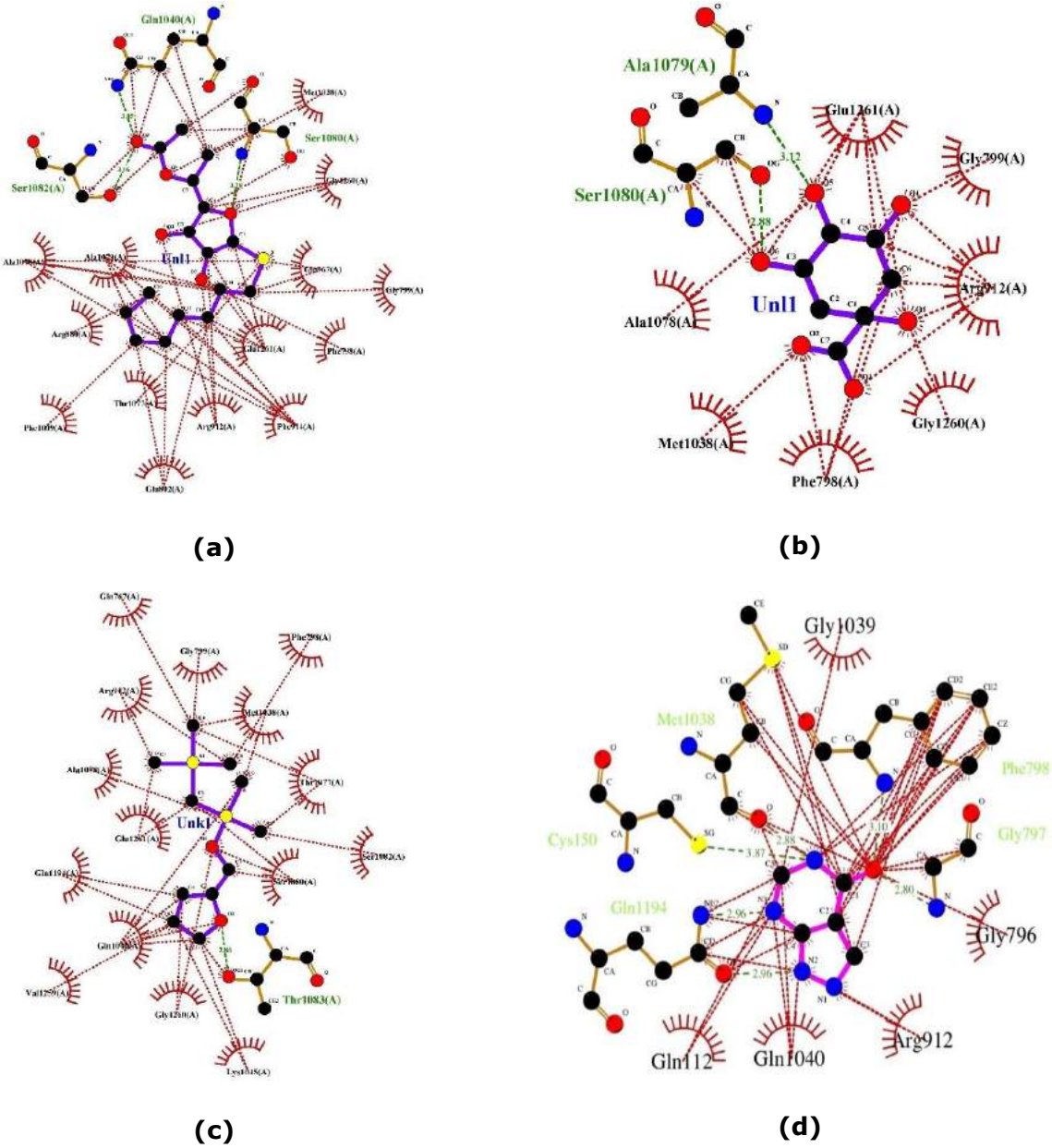
Complex	Residue	AA	Distance H-A (Å)	Distance D-A (Å)	Donor Angle	Donor Atom	Acceptor Atom
Complex 1	912A	ARG	3.47	3.8	101.02	8316 [Nam]	12332 [O3]
	1040A	GLN	2.05	3.05	164.93	9569 [Nam]	12341 [O2]
	1080A	SER	2.54	3.28	129.26	9925 [Nam]	12331 [O3]
	1082A	SER	2.68	3.06	104.92	9947 [O3]	12341 [O2]
Complex 2	912A	ARG	2.73	3.14	103.89	8324 [Ng+]	12333 [O3]
	912A	ARG	3.7	4.01	100.18	8330 [Ng+]	12333 [O3]
	1079A	ALA	2.37	3.12	129.44	9919 [Nam]	12341 [O3]
	1080A	SER	3.15	3.96	136.91	9925 [Nam]	12341 [O3]
	1080A	SER	2.52	2.88	102.01	12343 [O3]	9931 [O3]
	1261A	GLU	3.16	3.61	109.99	12341 [O3]	11647 [O2]
Complex 3	1083A	THR	2.23	2.86	123.24	9955 [O3]	12338 [O3]
	1260A	GLY	3.57	3.97	105.81	11634 [Nam]	12335 [O3]
Control	797	GLY	1.87	2.8	150.6	7210 [N]	12327 [O2]
	798	PHE	2.22	3.1	143.5	7215 [N]	12327 [O2]
	1038	MET	2.29	2.88	115.99	12337 [N]	9550 [O2]
	1194	GLN	1.95	2.96	172.11	11022 [N]	12335 [N2]
	1194	GLN	2.04	2.96	149.52	12332 [Npl]	11021 [O2]

In this research, only the primary compounds of *M. oleifera* , i.e., 5-O-acetyl-thio-octyl-beta-l-rhamno furanoside, quinic acid, and 2-Dimethyl (trimethyl silylmethyl) silyloxy methyl tetrahydrofuran, were selected to become the ligand molecules. It was not easy to simulate three ligands simultaneously in docking protocols because ligands may overlap on each atom that causes structural changes from the initial configuration. Molecular docking was performed separately for each ligand, and overall results were combined to create one configuration because of the similar receptor (XO) to overcome this issue.

Three ligands, i.e., 5-o-acetyl-thio-octyl-.beta.-l-rhamnofuranoside (PubChem ID: 537841), Quinic acid (PubChem ID: 6508), 2-Dimethyl (trimethylsilylmethyl) silyloxymethyl tetrahydrofuran (PubChem ID: 559105) against XO are extracted from the PubChem database. The possibility of ligand binding to the receptor site is achieved when the binding energy of the ligand-receptor complex is a negative value. From our finding, three complexes were found with various binding energies, as listed in Table 4.

All ligands showed negative values binding energy which are -9.3 kcal/mol, -8.2 kcal/mol, and -10.6 kcal/mol for 5-O-acetyl-thio-octyl-beta-l-rhamno furanoside, quinic acid, and 2-Dimethyl (trimethyl silylmethyl) silyloxy methyl tetrahydrofuran consecutively, indicated that the ligands could bind to the receptor, forming a ligan-receptor complex. In addition to that, the ligands of all complexes have higher binding energies than Alluporinol (a positive control), with the binding energy of -6.6 kcal/mol. This finding revealed that those 3 ligands found in the extract of *M. oleifera* leaves probably hold the potential as inhibitors for XO. Even though the inhibition activity of allopurinol is higher compared to the extract, the results still demonstrate that

the *M. oleifera* leaves extracts containing those primary compounds could inhibit the XO enzyme. Thus, to identify the molecular interactions, including hydrogen bond and hydrophobic interaction between ligand and receptor, the snapshot structure of those ligands was analyzed using PLIP server (Salentin et al. 2015) and LigPlot v.4.5.3 program packages (Wallace et al. 1995).



(a) **(b)**

(c) **(d)**

Figure 8. Hydrophobic interaction of ligand in complex with the receptor. (**a**) 5-o-acetyl-thio-octyl-.beta.-l-rhamnofuranoside, (**b**) Quinic acid, (**c**) 2-Dimethyl (trimethylsilylmethyl) silyloxymethyl tetrahydrofuran, (**d**) Alluporinol (control). The ligand refers to the stick model in magenta color. Redline is represented hydrophobic interaction between ligand and residues of the receptor.

Figure 8 illustrates the binding site and geometrical pocket of the ligands in complex with the receptor. The result of complex 1 was visualized from multiple dockings of the several ligands, then combined into one configuration. The lines in red, yellow, and colours correspond to the compounds of 5-O-acetyl-thio-octyl-beta-l-rhamno furanoside, quinic acid, and 2-Dimethyl (trimethyl silylmethyl) silyloxy methyl tetrahydrofuran, respectively. Meanwhile, complex 2 was denoted from the positive control into the site of XO. Of this figure, hydrogen bond and hydrophobic interactions

contributed to the binding of ligand into the receptor. The detailed hydrogen bonds for those complexes are listed in Table 5. It was found that 5-O-acetyl-thio-octyl-beta-l-rhamno furanoside of complex 1 participated in hydrogen bonds with residues ARG912, GLN1040,SER1080, SER1082 of the receptor. In quinic acid, the hydrogen bonds were made in the residues of ARG912, ARG912, ALA1079, SER1080, SER1080, GLU1261. For 2-Dimethyl (trimethyl silylmethyl) silyloxy methyl tetrahydrofuran, the hydrogen bonds were formed with the residues of THR1083, GLY1260. Meanwhile, Alluporinol shown in complex 2 participated in hydrogen bonds with residues GLY797, PHE798, MET1038, and GLN1194. On the other hand, the hydrophobic interactions between ligand and receptor are presented in Figure 8. 5-O-acetyl-thio-octyl-beta-l-rhamno furanoside of complex 1 formed the hydrophobic interaction with residues of the receptor, including MET1038, ARG912, GLY799, GLU1261, PHE911, PHE798, ALA1079, SER1080, GLY1260, ALA1078, THR1083, LYS1045, VAL1259, SER1082, LEU1042, GLY1039GLN1040, GLN1194, GLN112, AND CYS150. For quinic acid, the hydrophobic interaction was presented by the ligand's interaction with residues of the receptor such as MET1038, CYS150, GLN112, ARG912, PHE798, GLY799, PHE911, ALA1078, ALA1079, GLU1261, SER1080, VAL1259, LYS104, THR1083, SER1082, GLN1010, GLN1194, and GLY1039. 2-Dimethyl (trimethyl silylmethyl) silyloxy methyl tetrahydrofuran participated in hydrophobic interaction with residues of GLY799, ARG912, GLY1260, GLN1040, GLU1261, GLN1194, SER1080, ALA1079, ALA1078, PHE914, PHE1009, ARG880, GLU802, PHE798, GLN767, AND MET1038. Meanwhile, for allopurinol, the hydrophobic interaction was observed between ligand and residues, including GLY1039, PHE798, GLY797, GLY796, ARG912, GLN1040, GLN112, GLN1194, CYS150, MET1038. From these results, all primary ligands in the extracted *M. oleifera* leaves may become stable structures since the hydrogen bonds are coordinated between ligand and receptor. Also, the compounds of 5-O-acetyl-thio-octyl-beta-l-rhamno furanoside, quinic acid, and 2-Dimethyl (trimethyl silylmethyl) silyloxy methyl tetrahydrofuran bound to the similar residues of Alluporinol, indicated that those compounds have similar activity as an inhibitor for XO. Furthermore, in the paper presented by Okamoto and co-workers (Okamoto et al. 2004), the catalytic site of XO provided and visualized in the UniProt database (https://www.ebi.ac.uk/pdbe/entry/pdb/1v97/bound/MTE), which includes residues GLY797, MET1038, PHE798, ARG912, GLU1261, ALA1079, ALA1078, SER1080, GLN1040, VAL1081, SER1082, GLY1039, GLN1194, CYS150, and GLN112, is a crucial target for inhibiting the XO. Thus, the ligand that can bind to one of these residues is assumed to have a potential drug for treating goat disease. From our simulations, each ligand is bound to those residues by hydrogen bond and hydrophobic interaction, indicating those compounds can become inhibitor XO.

Conclusions

The results of enzyme characterization showed that the optimum activity of the enzyme isolated from bovine milk is at pH 6.5, substrate concentration of 0.1 mM, and reaction temperature of 35 °C. For XO enzyme inhibition, the increase in extract concentration linearly augmented the percentage of inhibition. Methanol extract of 160 mg/mL showed the highest inhibition value of 21.35%. These results indicate that the methanol extract of M. oleifera leaves has the potential as an XO inhibitor. Furthermore, computational analysis was performed to gain insight into the molecular interaction between the primary compounds of *M. oleifera* leaves, including 5-O-acetyl-thio-octyl-beta-l-rhamno furanoside, quinic acid, and 2-Dimethyl (trimethyl silylmethyl) silyloxy methyl tetrahydrofuran with XO using the molecular docking method. Our finding demonstrated that these compounds were bound to the catalytic sites of XO by hydrogen bonds and hydrophobic interaction, suggesting these primary compounds of *M. oleifera* leaves have

pharmacology activities for inhibiting the XO.

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Conflicts of Interest

The authors have no conflict of interest.

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