

**BUKTI KORESPONDENSI
JURNAL INTERNASIONAL BEREPUTASI**

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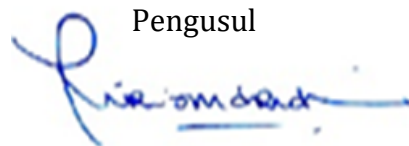
Penerbit : Institut Pertanian Bogor (IPB)

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Pengusul



Dr. apt. Ririn Suharsanti, M.Sc

1. Submission of Manuscript

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Thank you for submitting the manuscript, "Exploring the α -Amylase Inhibitory Potential of *Peronema canescens* Jack: An In Vitro and In Silico Study" to HAYATI Journal of Biosciences. With the online journal management system that we are using, you will be able to track its progress through the editorial process by logging in to the journal web site:

Manuscript URL: <https://journal.ipb.ac.id/index.php/hayati/authorDashboard/submission/59682>
Username: ryanradix

If you have any questions, please contact me. Thank you for considering this journal as a venue for your work.

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Your paper has been subjected to a blind review process by reviewers who are experts in the related fields. The full paper will be considered to be published in the HAYATI Journal of Biosciences if you revise the full paper according to the reviewer's comments. Enclosed, please find the reports from the reviewer. And we have reached a decision regarding your submission to HAYATI Journal of Biosciences, "Exploring the α -Amylase Inhibitory Potential of *Peronema canescens* Jack: An In Vitro and In Silico Study".

Our decision is : Revisions Required

The full paper will be considered to be published in the HAYATI Journal of Biosciences if you revise the full paper according to the reviewer's comments. Enclosed, please login to <https://journal.ipb.ac.id/index.php/hayati/login> in order to find the comments from the reviewers.

It is compulsory for you to revise the full paper according to the reviewer's comment and HAYATI Journal of Biosciences guideline (<https://journal.ipb.ac.id/index.php/hayati/AuthorsGuideline>). Please use the YELLOW HIGHLIGHT feature of MS-Word to make your revisions, so we can identify where your corrections have been made.

We will wait for your revision at the latest of two weeks from this email. We look forward to receiving the revised version of your full paper.

Best Regards
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Reviewer C:

1. The title clearly reflect what the article about, meanwhile there are some sentences are needed to revision.
2. The data have not been published in other article.
3. The author has adequate background for the research, but too many sentences to explain. You need to cut off some sentences, make it shorter than before.
4. The method mentioned appropriate and clearly, it showed that it mention some journal from same author.
5. The article adequately and appropriately referenced.
6. Some sentences could be removed or condensed. Please make the revision.
7. The information provided in figures, figure legends, boxes and tables clear and accurate.

Recommendation: Revisions Required

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3. Submit revised manuscript

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Date: Fri, Jan 3, 2025 at 10:24 PM
Subject: Re: [HJB] Editor Decision: Revisions Required
To: Iman Rusmana <jurnal@apps.ipb.ac.id>

Dear Editor-in-Chief,

Thank you for your email and the feedback provided by the reviewers. We have carefully revised our manuscript titled "Exploring the α -Amylase Inhibitory Potential of *Peronema canescens* Jack: An In Vitro and In Silico Study" according to the reviewer comments and the guidelines of the HAYATI Journal of Biosciences.

The revisions have been highlighted in yellow in the manuscript to clearly indicate the changes made. We have now submitted the revised version through the journal's online submission system at <https://journal.ipb.ac.id/index.php/hayati/login>.

Thank you for your consideration, and we look forward to your further feedback.

Best regards,

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Buttons: Balas, Teruskan, and a smiley face icon.

4. Acceptance Notification and Payment Request

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We hereby inform you that an article with the title "Exploring the α -Amylase Inhibitory Potential of *Peronema canescens* Jack: An In Vitro and In Silico Study" has been accepted to be published in HAYATI Journal of Biosciences. And could you please check the enclosed uncorrected proof.

For publishing your article in HAYATI Journal of Biosciences, we also ask you to pay the article contribution fee of US\$, 250 (for oversea authors) or IDR 3.000.000,- (for Indonesian authors). These fees can be sent via bank transfer to Bank BNI, Branch Bogor (Swift Code: BNINIDJABGR), account number 0154596721, Name: JURNAL HAYATI.

The proof of payment and the correction of the uncorrected proof file can be emailed to hayati.jbiosci@apps.ipb.ac.id within 14 days for further publishing process of the manuscript.

We look forward to receiving your correction of the uncorrected proof file and the proof of payment.

Best Regards

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The attachment is a PDF document titled "Exploring the α -Amylase Inhibitory Potential of *Peronema canescens* Jack: An In Vitro and In Silico Study".

6. Proof Payment

The screenshot shows a Gmail interface with a search bar containing 'hjb'. The email content is as follows:

Date: Mon, 3 Feb 2025, 10:16
Subject: The proof of payment and the correction (M. Ryan Radix R)
To: <hayati.jbiosci@apps.ipb.ac.id>

Dear Editorial Team of HAYATI Journal of Biosciences,

I hope this email finds you well.

I am writing to submit the corrected version of the *uncorrected proof* for our manuscript titled:

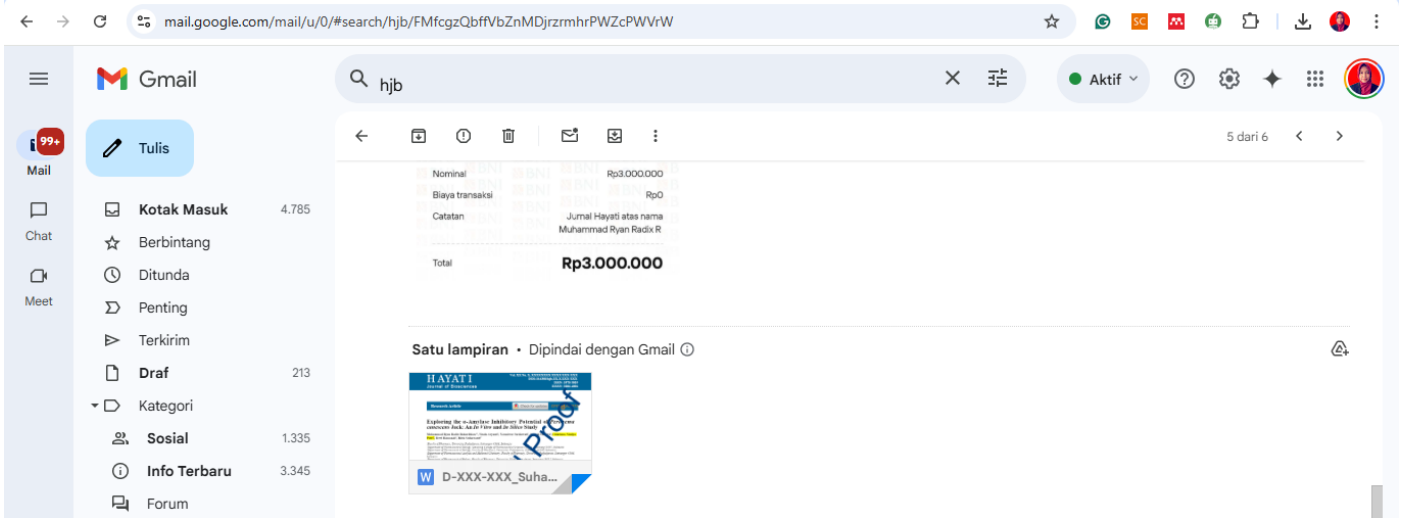
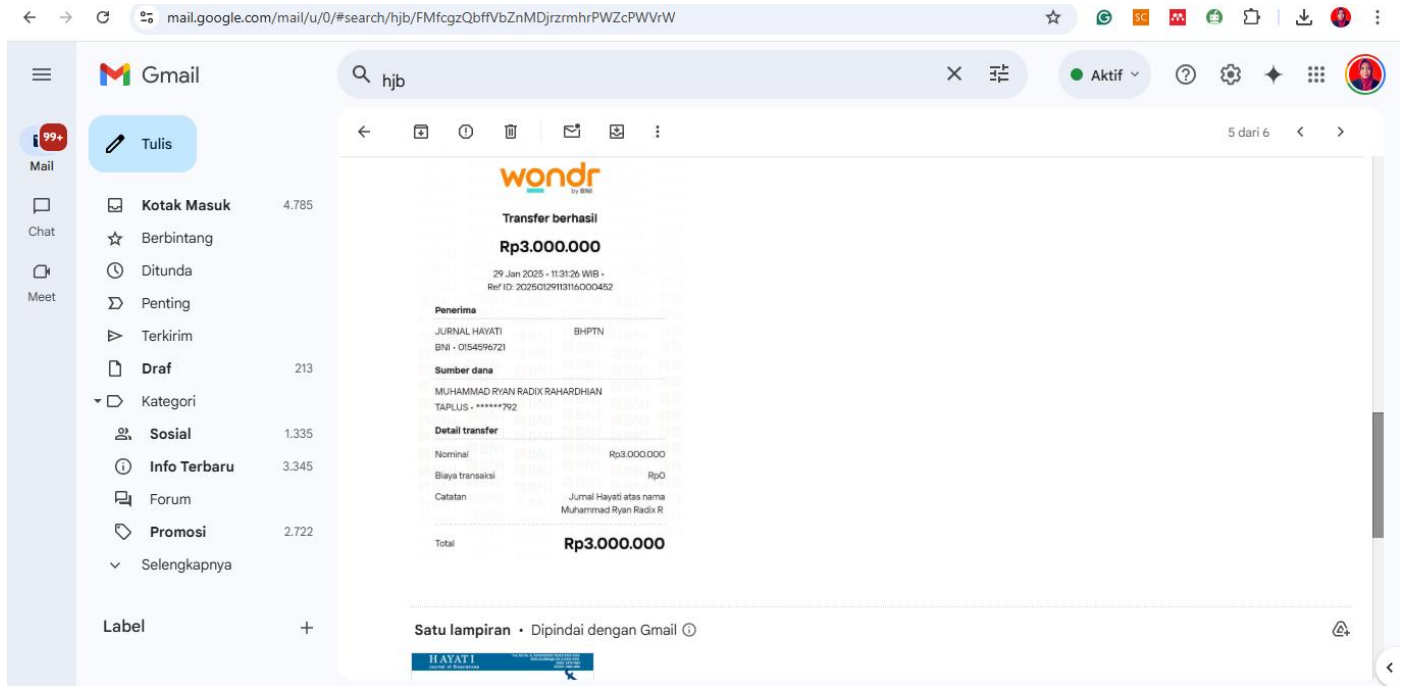
"Exploring the α -Amylase Inhibitory Potential of *Peronema canescens* Jack: An In Vitro and In Silico Study"

The corrections have been made and highlighted in yellow for easy reference. Additionally, I have attached the proof of payment for the article contribution fee as requested.

Please let me know if any further modifications are required. I look forward to the final publication of our article.

Thank you for your time and consideration.

Best regards,



7. Editor decision-send to production

The screenshot shows a Gmail interface with a search bar containing 'hjb'. The email is from 'HAYATI Journal of Biosciences' (journal@apps.ipb.ac.id) to 'Muhammad, saya'. The subject is '[HJB] Editor Decision'. The email content states: 'The editing of your submission, "Exploring the α -Amylase Inhibitory Potential of Peronema canescens Jack: An In Vitro and In Silico Study," is complete. We are now sending it to production.' It includes a submission URL: <https://journal.ipb.ac.id/index.php/hayati/authorDashboard/submission/59682>. Contact information for HAYATI Journal of Biosciences is provided: Department of Biology, Bogor Agricultural University Darmaga Campus, Bogor 16680, Indonesia. Phone/Fax: +62-251-8421258. Email: hayati_j_biosci@cbn.net.id. The Windows taskbar at the bottom shows the time as 19:42 on 05/06/2025.

8. Article Published Vol 32 issue 3 on Website

The screenshot shows the website for HAYATI Journal of Biosciences. The header features the journal title in large white letters on a blue background, with p-ISSN: 1978-3019 and e-ISSN: 2086-4094. Navigation links include 'Login' and 'New Submission'. A menu bar contains 'ABOUT', 'CURRENT ISSUE', 'ARCHIVES', 'PUBLICATION ETHICS', 'EDITORS', 'SUBMISSIONS', and 'REGISTER'. The breadcrumb trail is 'Home / Archives / Vol. 32 No. 3 (2025): May 2025 / Articles'. The main article title is 'Exploring the α -Amylase Inhibitory Potential of Peronema canescens Jack: An In Vitro and In Silico Study'. The author is 'Muhammad Ryan Radix Rahardhian', Faculty of Pharmacy, Universitas Padjadjaran, Jatinangor 45363, Indonesia, and Department of Pharmaceutical Biology, Semarang College of Pharmaceutical Sciences (Stifar), Semarang 50192, Indonesia. A 'HAYATI Journal of Biosciences' logo is also visible. On the right, there are buttons for 'OPEN ACCESS', 'MAKE NEW SUBMISSION', and a 'HAYATI Journal of Biosciences Q3' badge with 'Agricultural and Biological Sciences (miscellaneous) best quartile' and 'SJR 2024 0.24'.

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Abstract

Hyperglycemia in individuals with type 2 diabetes mellitus is primarily driven by the rapid hydrolysis of starch by the enzyme α -amylase in the pancreas and the breakdown of oligosaccharides by α -glucosidase in the intestine. *Peronema canescens* Jack. (PC) has shown promise as a potential antidiabetic agent. This study aimed to evaluate the total flavonoid, phenolic, and α -amylase inhibitory activity of extracts and fractions derived from PC leaves using both in vitro and in silico approaches. The ethanol extract of PC leaves was fractionated through liquid-liquid extraction using *n*-hexane, ethyl acetate, and water as solvents. Preliminary phytochemical screening of the extracts and fractions identified the presence of alkaloids, flavonoids, saponins, tannins, and steroids/triterpenoids. The *n*-hexane fraction exhibited the highest total flavonoid content, averaging 203.37 ± 4.38 mg QE/gram, while the ethyl acetate fraction demonstrated the highest total phenolic content, averaging 147.04 ± 0.79 mg GAE/gram.



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ABSTRACTING & INDEXING

Exploring the α -Amylase Inhibitory Potential of *Peronema canescens* Jack: An In Vitro and In Silico Study

ABSTRACT

Hyperglycemia in individuals with type 2 diabetes mellitus is primarily driven by the rapid hydrolysis of starch by the enzyme α -amylase in the pancreas and the breakdown of oligosaccharides by α -glucosidase in the intestine. *Peronema canescens* Jack. (PC) has shown promise as a potential antidiabetic agent. This study aimed to evaluate the total flavonoid, phenolic, and α -amylase inhibitory activity of extracts and fractions derived from PC leaves using both in vitro and in silico approaches. The ethanol extract of PC leaves was fractionated through liquid-liquid extraction using n-hexane, ethyl acetate, and water as solvents. Preliminary phytochemical screening of the extracts and fractions identified the presence of alkaloids, flavonoids, saponins, tannins, and steroids/triterpenoids. The n-hexane fraction exhibited the highest total flavonoid content, averaging 203.37 ± 4.38 mg QE/gram, while the ethyl acetate fraction demonstrated the highest total phenolic content, averaging 147.04 ± 0.79 mg GAE/gram. Furthermore, the ethyl acetate fraction showed the strongest α -amylase inhibitory activity, with an average inhibition rate of $70.38 \pm 1.26\%$. In silico analysis, combined with GC-MS identification, suggested that three compounds—bis(2-ethylhexyl) phthalate, myristyl oleate, and 14 beta H-pregna—may contribute to the observed α -amylase inhibitory activity. These findings highlight the potential of PC as a source of natural antidiabetic agents.

Keywords: α -amylase inhibitory activity, in vitro and in silico analysis, natural antidiabetic agents, *Peronema canescens* Jack., total flavonoid and phenolic content

1. Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both (WHO, 2023). The prevalence of diabetes has reached alarming levels globally, with the International Diabetes Federation (IDF) estimating that over 537 million adults will be living with the condition in 2021 (Webber, 2013). Experts project that this number will rise to 643 million by 2030. In Indonesia, the situation mirrors this global trend, with an increasing number of the population being diagnosed with diabetes. The current standard of care for managing diabetes includes synthetic drugs such as sulfonylureas, metformin, and thiazolidinediones, designed to enhance insulin sensitivity, stimulate insulin secretion, or reduce glucose production. However, these synthetic drugs are not without their limitations. Long-term use of these medications can lead to adverse effects such as hypoglycemia (Chaudhury et al., 2017), gastrointestinal issues, and even multidrug resistance (MDR). These limitations underscore the need for alternative treatment strategies that are both effective and safe.

41 In light of the challenges associated with synthetic drugs, there has been growing
42 interest in herbal medicine as a potential alternative or complementary approach to diabetes
43 management. Plants rich in bioactive compounds provide herbal medicines with therapeutic
44 benefits and fewer side effects (Tran et al., 2020). One such plant is *Peronema canescens* Jack.
45 (PC), a species native to Indonesia, traditionally used for its medicinal properties (Rahardhian
46 et al., 2022b) . The secondary metabolites found in PC, such as flavonoids, tannins, and
47 alkaloids (Primal and Ahriyasna, 2022), have been shown to possess various pharmacological
48 activities, including antidiabetic effects. These natural compounds may help to mitigate
49 hyperglycemia by enhancing insulin sensitivity, inhibiting glucose absorption, and modulating
50 carbohydrate metabolism, making PC a promising candidate for diabetes treatment.

51 Computer-aided drug design (CADD) advancements have provided researchers with
52 powerful tools to identify and optimize potential antidiabetic agents (Meng et al., 2011) .
53 Molecular docking simulation, a key component of CADD, allows for predicting the
54 interaction between small molecules and target proteins, thereby facilitating the identification
55 of compounds with high binding affinities (Pagadala et al., 2017) . This method has been
56 beneficial in the search for natural inhibitors of enzymes such as α -amylase, which plays a
57 crucial role in carbohydrate digestion and glucose absorption (de Sales et al., 2012) .
58 Researchers can rapidly screen and identify the most promising antidiabetic compounds
59 within complex plant extracts, such as those found in PC, by molecular docking simulations
60 (Khan et al., 2024).

61 This study focuses on the antidiabetic potential of PC, examining both its in vitro and
62 in silico activities. Gas Chromatography-Mass Spectrometry (GC-MS) analysis identified
63 PC's chemical composition and revealed various bioactive compounds (Hotmian et al., 2021a).
64 The inhibitory effect of these compounds on α -amylase was then evaluated through in vitro
65 assays, while molecular docking simulations were employed to understand the interaction
66 between these compounds and the enzyme at the molecular level (Santos et al., 2019) . The
67 physicochemical properties of PC It were also analyzed and compared with other herbal
68 medicines to highlight its unique advantages. This comprehensive approach sheds light on the
69 potential of PC as an antidiabetic agent and underscores its benefits over other herbal
70 remedies (Posadzki et al., 2013).

71 The novelty of this study lies in its integrative approach, combining in vitro and in
72 silico methods to investigate the α -amylase inhibitory potential of PC. While previous

73 research has explored the antidiabetic effects of various herbal medicines, studies focusing
74 specifically on PC remain limited. By providing a detailed analysis of its bioactive
75 compounds and their mechanisms of action, this study serves as a preliminary investigation
76 that could pave the way for future research (Altemimi et al., 2017). The findings presented
77 here are relevant for developing PC as a potential antidiabetic treatment but also offer
78 valuable insights for researchers working on similar objectives, making this study a
79 significant consideration for ongoing and future developments in the field.

80 This study aims to evaluate the α -amylase inhibitory activity of PC using both in vitro
81 and in silico methods. This research seeks to identify the specific compounds responsible for
82 this activity and to understand their mechanisms of action at the molecular level. This
83 research will contribute to the growing knowledge of natural antidiabetic agents and support
84 PC development as a potential treatment for diabetes mellitus.

85

86 **2. Materials and Methods**

87 **2.1. Materials**

88 The equipment used in this study includes a Pyrex separation funnel, a Heidolph-G3
89 rotary evaporator, Silica Gel F254 plates, UV 254 and 366 lamps (Evaco GL 220V 50Hz T8
90 15W), micropipettes (Socorex and Dragon Lab), vortex mixers, a Shimadzu UV-1780 UV-
91 Vis spectrophotometer (Serial No. A119161), a Synergy-HTX multi-mode ELISA reader
92 with 96-well plates, and a QP 2010 gas chromatography-mass spectrometer (GC-MS). The
93 materials utilized in this study include *Peronema canescens* Jack., ethanol, n-hexane, ethyl
94 acetate, FeCl₃, MgSO₄, hydrochloric-ethanolic acid mixture (1:1), hydrochloric acid,
95 Lieberman-Burchard reagent, DPPH (Sigma), quercetin, methanol, phosphate buffer, alpha-
96 amylase enzyme (Sigma Aldrich), starch, and DNS

97

98 **2.1.1. Hardware and Software**

99 The molecular docking study was conducted using a laptop equipped with an Asus ROG
100 503 VD. The software utilized in this study includes ChemDraw Professional 15.0, Chem3D
101 15.0, Biovia Discovery Studio 2021, Command Prompt, and AutoDock Tools-1.5.6.
102 Visualization of docking results and the creation of ligands and receptors were performed
103 using Biovia Discovery Studio 2021. Receptor structures were obtained from the RCSB
104 Protein Data Bank (PDB) website (<https://www.rcsb.org/>), while ligand structures were

105 downloaded from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>). Lipinski's rule
106 of five was applied to assess drug-likeness using the SCFBio website ([http://www.scfbio-
107 iitd.res.in/software/drugdesign/lipinski.jsp](http://www.scfbio-
107 iitd.res.in/software/drugdesign/lipinski.jsp)). The pk-CSM online tool evaluated
108 Pharmacokinetic and toxicological properties (<https://biosig.lab.uq.edu.au/pkcsm/>).

109

110 **2.2. Methods**

111 **2.2.1. Sample preparation, extraction, and fractionation**

112 The extraction of *Peronema canescens*. Jack (PC) used the maceration method with
113 96% ethanol as the solvent. Three hundred grams of PC was placed in a maceration jar, and
114 96% ethanol was added as the solvent. The maceration process was performed for three days,
115 with periodic solvent changes and occasional stirring to enhance extraction efficiency. The
116 resulting macerate was filtered and then concentrated using a rotary vacuum evaporator. The
117 concentrated extract was further thickened using a water bath at approximately 50°C
118 (Rahardhian et al., 2019). Ten grams of the ethanol extract of PC leaves were dissolved in 100
119 mL of water and transferred into a separatory funnel. The mixture was then partitioned by
120 adding 100 mL of n-hexane, followed by vigorous shaking. The mixture was allowed to settle
121 until two distinct phases formed: the aqueous and n-hexane. The aqueous phase, separated
122 from the n-hexane phase, was reintroduced into the separatory funnel. Subsequently, 100 mL
123 of ethyl acetate was added to the aqueous phase. The mixture was shaken several times to
124 ensure thorough mixing and then allowed to separate into its respective phases. The resulting
125 fractions were concentrated using a rotary vacuum evaporator and thickened using a water
126 bath at approximately 50°C to obtain a viscous fraction. (Rahardhian et al., 2019).

127 **2.2.2. Phytochemical screening and TLC affirmation**

128 Phytochemical screening follows the method (Putri et al., 2022). Each sample,
129 including the ethanol extract, n-hexane fraction, ethyl acetate fraction, and water fraction of
130 PC, was prepared by dissolving 30 mg of the sample in 3 mL of ethanol until completely
131 dissolved. This solution was used for the subsequent phytochemical screening tests.
132 Flavonoids: To the sample solution, magnesium (Mg) powder, 1 mL of hydrochloric acid
133 (HCl), and amyl alcohol were added. The formation of red, yellow, or orange colours
134 indicated a positive presence of flavonoids. Polyphenols: A 10% ferric chloride (FeCl₃)
135 solution was added to the sample. The appearance of a blue or blackish-green color indicated
136 the presence of polyphenols. Tannins: The sample was mixed with gelatin salts. The

137 formation of a yellowish-white precipitate indicated a positive result for tannins. Alkaloids:
138 The sample was treated with HCl and water, then heated and divided into two portions
139 (Filtrate 1 and Filtrate 2). Filtrate 1: Reacted with Mayer's reagent, forming a yellowish-
140 white precipitate, indicating the presence of alkaloids. Filtrate 2: Reacted with Dragendorff's
141 reagent, forming a brick-red precipitate, confirming the presence of alkaloids. Saponins: The
142 sample was placed in a test tube, and 10 mL of distilled water was added. The mixture was
143 shaken vigorously for 10 seconds. A positive reaction was indicated by the formation of foam
144 that persisted for 10 minutes at a height of 1-3 cm. Steroids/triterpenoids: The sample was
145 treated with chloroform and filtered. The filtrate was mixed with anhydrous acetic acid
146 (CH₃COOH) and heated, then cooled and treated with sulfuric acid (H₂SO₄). A green color
147 in the solution indicated the presence of steroids, while an orange or red color indicated the
148 presence of triterpenoids.

149 Thin-layer chromatography (TLC) was performed following the method described by
150 (Putri et al., 2022) to identify the compound content of the samples. A small amount of each
151 sample was dripped directly onto the TLC plate. Flavonoids: The mobile phase consisted of
152 n-butanol, acetic acid, and water in a ratio of 4:1:5. The TLC plate was then exposed to
153 ammonia vapor. The presence of flavonoids was indicated by the formation of yellow stains
154 after ammonia treatment. Tannins: The mobile phase was prepared using ethyl acetate,
155 methanol, and water in a ratio of 100:13.5:10. The presence of tannins was indicated by
156 visible spots formed by FeCl₃, which resulted in blackish-green patches. Alkaloids: The
157 mobile phase comprised ethyl acetate, methanol, and water in a ratio of 6:4:2. The presence
158 of alkaloids was confirmed by the appearance of brown patches upon applying Dragendorff's
159 reagent. Saponins, A solvent system of chloroform, methanol, and water in a ratio of
160 64:50:10, was utilized. Anisaldehyde-sulfuric acid was used as a visualization reagent, and
161 the TLC plate was heated on a hot plate for 5-10 minutes at 100°C. The presence of saponins
162 was indicated by the formation of colored patches, including yellow, green, red, dark blue,
163 purple, and brownish-yellow. Steroids/triterpenoids: The mobile phase consisted of n-hexane
164 and ethyl acetate in a ratio of 17:3. Anisaldehyde-sulfuric acid was again used for
165 visualization, and the TLC plate was heated on a hot plate for 5-10 minutes at 100°C. A
166 purple color indicated the presence of steroids, while a blue color indicated triterpenoids.

167

168 **2.2.3. Determination of total phenolic and total flavonoid content**

169 The total flavonoid content (TFC) and total phenolic content (TPC) of the extracts
170 were determined using established spectrophotometric methods (Rahardhian et al., 2019).
171 The total phenolic content was assessed using the Folin-Ciocalteu reagent with gallic acid as
172 the standard. A 1.0 mL aliquot of the standard solution or sample was placed in a test tube.
173 Add 4.5 mL of 10% Folin-Ciocalteu reagent to the test tube and 4.5 mL of 7.5% sodium
174 carbonate (Na_2CO_3) solution. The mixture was incubated for 90 minutes at room temperature.
175 The absorbance was measured at a wavelength of 765 nm using a Shimadzu® UV-Vis
176 Spectrophotometer (Model 1240). The total flavonoid content was determined using
177 aluminum chloride (AlCl_3) with quercetin as the standard. A 1.0 mL aliquot of the standard
178 solution or sample was placed in a test tube. Add 3.0 mL of ethanol, 0.2 mL of 10%
179 aluminum chloride (AlCl_3) solution, 0.2 mL of 20% anhydrous sodium acetate (CH_3COONa)
180 solution, and 5.6 mL of distilled water. The mixture was homogenized thoroughly. The
181 mixture was incubated for 30 minutes at room temperature. The absorbance was measured at
182 a wavelength of 420 nm using a Shimadzu® UV-Vis Spectrophotometer (Model 1240). The
183 total phenolic and flavonoid contents were calculated based on the absorbance values
184 obtained from the gallic acid and quercetin standard curves, respectively.

185 **2.2.4. α -amylase Inhibitory Activity**

186 The α -amylase inhibitory activity of the samples was evaluated using a colorimetric
187 method on a 96-well plate. The samples were dissolved in a 1% dimethyl sulfoxide (DMSO)
188 solution. Each sample, along with the negative control (1% DMSO solution) and positive
189 control (acarbose at concentrations of 50 mg and 100 mg), was pipetted into the wells of a
190 96-well plate at a volume of 20 μL per well. 50 μL of 100 mM phosphate buffer (pH 6.8) was
191 added to each well, followed by ten μL of α -amylase solution (2 U/mL). The plate was pre-
192 incubated at 37°C for 20 minutes to allow the enzyme and inhibitor to interact. After pre-
193 incubation, 20 μL of a 1% starch solution in 100 mM phosphate buffer (pH 6.8) was added to
194 each well as the substrate. The reaction mixture was further incubated at 37°C for 30 minutes.
195 After incubation, 100 μL of dinitrosalicylic acid (DNS) reagent was added to each well. The
196 plate was then incubated at 100°C for 10 minutes to develop the color. The absorbance of the
197 resulting mixture was measured at a wavelength of 540 nm using a Multiplate Reader.
198 Acarbose at various concentrations served as the standard for comparison, and the percentage
199 of α -amylase inhibition was calculated based on the absorbance values obtained from the
200 samples relative to the control wells.

$$\text{Inhibition of } \alpha\text{-amylase (\%)} = \frac{((Ac - Ac') - (As - As'))}{(Ac' - As')} \times 100$$

201 Where, Ac: Absorbance of the blank (control without any sample or inhibitor), Ac':
 202 Absorbance of the blank control (in the presence of the solvent, e.g., 1% DMSO), As:
 203 Absorbance of the sample (in the presence of the sample extract), As' : Absorbance of the
 204 sample control (sample with solvent but no substrate or enzyme)

205 **2.2.5. Identification of compounds in the active fraction of PC using GC-MS.**

206 GC-MS analysis was performed at the Integrated Laboratory of the Islamic University
 207 of Indonesia, Yogyakarta, to identify the compounds present in the samples. The columns
 208 used in the analysis were Rtx-5MS columns, thickness 0.25 μm , length 30.0 m and diameter
 209 0.25 mm, column temperature 80.0 $^{\circ}\text{C}$, injection temperature 300.00 $^{\circ}\text{C}$, with split injection
 210 mode, pressure 42.3 kPa, total flow 117.5 mL/min and column flow 0.74 mL/min. (Hotmian
 211 et al., 2021b).

212 **2.2.6. Molecular Docking**

213 Molecular docking was conducted using various software tools to analyze the
 214 interactions between the proteins and ligands. The receptors were downloaded from the
 215 Protein Data Bank (PDB) <https://www.rcsb.org/> (Pagadala et al., 2017). Water molecules
 216 surrounding the protein were removed, and the protein chain was separated from its native
 217 ligand and saved in *pdb format as a protein file. The native ligand structure was extracted
 218 by removing the corresponding portion of the protein chain and saved in *pdb format as a
 219 ligand file. The ligand's structure was initially prepared in 2D using ChemDraw Pro 12.0.
 220 (PerkinElmer, 2015), the 3D structure was constructed, and molecular mechanics (MM)
 221 geometry optimization was performed using Chem3D Pro 12.0. The optimized ligand
 222 structure was saved in PDB file format. The protein was prepared using AutoDockTools
 223 1.5.6 (Morris et al., 2009) by adding hydrogen atoms to the polar side of the structure and
 224 applying Kollman charges. The ligand was prepared by correcting its structure and adding
 225 Gasteiger charges. The prepared structures were saved in *pdbqt format. The redocking
 226 process utilized a grid box of dimensions 40 x 40 x 40 with the following coordinates: x =
 227 10.265, y = 45.877, z = 19.734. Docking Parameters Genetic Algorithm (GA), the output
 228 algorithm for docking results, was set to Lamarckian GA 4.2, and other docking parameters
 229 were set to default values. The critical parameter to evaluate the docking results was the
 230 RMSD. An acceptable RMSD value was $\leq 3.0 \text{ \AA}$, indicating reliable docking conformations.

231

232 **2.2.7. Evaluation of Drug Likelihood and ADMET**

233 Drug likeness was evaluated based on Lipinski's Rule of Five, a widely used
234 guideline in drug discovery to predict the oral bioavailability of compounds. The following
235 criteria were considered molecular weight: the compound should not exceed 500 Da, the
236 number of hydrogen bond acceptors (-H bond acceptors) should be no more than 10, the
237 number of hydrogen bond donors (-H bond donors) should not exceed 5, the log P value,
238 which indicates the compound's lipophilicity, should be less than 5 (or MlogP < 4.15)
239 (Rahardhian et al., 2022a). ADMET (Absorption, Distribution, Metabolism, Excretion, and
240 Toxicity) properties were predicted using computational methods to further assess the
241 compounds' suitability for drug development. CaCO₂ permeability was assessed to evaluate
242 intestinal absorption. Blood-brain barrier (BBB) permeability was predicted to assess the
243 ability of the compound to cross the BBB. The compound was evaluated as a substrate for
244 CYP2D6, a key enzyme in drug metabolism. Total clearance was calculated to estimate how
245 quickly the compound is eliminated from the body. AMES toxicity tests assessed potential
246 genotoxic effects (D. E. V Pires et al., 2015).

247

248 **2.3. Data Analysis**

249 Statistical analysis was performed using GraphPad Prism 9.0 (GraphPad Software Inc., CA,
250 USA). The results are presented as mean values \pm standard deviation (SD). NOVA was
251 applied to evaluate the differences among the various extracts. This method helps determine
252 whether there are statistically significant differences between the means of three or more
253 independent groups. A post hoc test was conducted following ANOVA to identify specific
254 group differences. A significance level of $p > 0.05$ was used to determine statistical
255 significance.

256










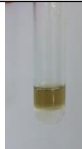













257 **3. Results**




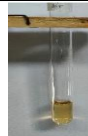




258 **3.1. Phytochemical Screening**

259 The fractionation of the ethanol extract from PC leaves resulted in the following yields:
260 32.81% for the n-hexane fraction, 16.86% for the ethyl acetate fraction, and 34.17% for the
261 water fraction. Notably, the ethyl acetate fraction yield was lower than that of the n-hexane
262 and water fractions. This variation in yield may be attributed to the differing solubility

263 profiles of the compounds present in the PC leaf extract, with nonpolar and polar compounds
 264 being more prevalent than semipolar compounds. Phytochemical screening was conducted to
 265 identify various bioactive compounds, including alkaloids, flavonoids, saponins, tannins, and
 266 triterpenoids/steroids. The results indicated that the ethanol extract of PC leaves tested
 267 positive for alkaloids, flavonoids, tannins, saponins, and triterpenoids. In contrast, the water
 268 fraction demonstrated a positive presence only for alkaloids and tannins. The results of the
 269 phytochemical screening indicate that the ethanol extract and fractions of *Peronema*
 270 *canescens* leaves contain various bioactive compounds Table 1.

271 Table 1. Phytochemical Screening Results of Ethanol Extract and Fractions of PC Leaves

Compound Group	Reagent	Ethanol Extract	n-Hexane Fraction	Ethyl Acetate Fraction	Water Fraction
Phenolic	Add 5 to 6 drops of a 1% ferric chloride (FeCl ₃)				
		(+)	(+)	(+)	(+)
Tannins	2 mL of a 10% NaCl solution mixed with 1% gelatin				
		(+)	(-)	(+)	(+)
Flavonoids	Magnesium powder combined with concentrated HCl and amyl alcohol				
		(+)	(+)	(+)	(-)
Alkaloids	Add three drops of Bouchardat's reagent.				
		(+)	(+)	(+)	(+)
		Mix 1 mL of 2N HCl with two drops of Mayer's reagent			
(+)	(+)		(+)	(-)	
Mix 1 mL of 2N HCl with two drops of Dragendorff's reagent					
	(+)	(+)	(+)	(+)	

Saponins	Add 10 mL of hot water to 2N HCl				
		(+)	(+)	(+)	(-)
Steroids	Mix 5 mL of chloroform with anhydrous acetic acid and H ₂ SO ₄ .				
		(+)	(+)	(+)	(-)

272 **Description**

273 (+) = Contains the compound

274 (-) = Does not contain the compound

275

276 **3.2. Thin Layer Chromatography (TLC)**

277 The compounds present in the ethanol extract, n-hexane fraction, ethyl acetate fraction, and
278 water fraction of PC leaves were further analyzed using Thin Layer Chromatography (TLC).

279 This technique confirmed the presence of various bioactive compounds by evaluating the R_f
280 values and the corresponding color stains formed on the TLC plates. The R_f values were
281 calculated for each compound, allowing for the identification of the specific compounds
282 present in each fraction. The results of the TLC analysis are summarized in Table 2, which
283 includes the R_f values and corresponding colors observed for each compound group in the
284 different fractions.

285 From the results of phytochemical screening and TLC tests, PC leaf extracts and fractions
286 contain several secondary metabolite compounds, including alkaloids, flavonoids, saponins,
287 tannins, terpenoids, steroids, and phenolics Table 2.

288 Table 2. TLC Results of Ethanol Extract and Fractions of PC Leaves

Compound Group	Mobile Phase	Sample	Visual	UV 254	UV 366	Spot Appearance	R _f Value
Alkaloids	ethyl acetate, methanol, and water (6:4:2)	EE	Brown	Brown	Orange	Brown	0,81
		NHF	Brown	Brown	Orange	Brown	0,79
		EAF	Orange	Brown	Orange	Brown	0,75
		WF	Brown	Brown	Blue	Brown	0,73
Flavonoid	n-butanol, acetic acid, and water (4:1:5)	EE	Green	Green	Orange	Green	0,91
		NHF	Green	Green	Orange	Green	0,91
		EAF	Green	Green	Orange	Green	0,91
		WF	Brown	Green	Blue	Yellow	0,56
Saponin	chloroform, methanol, and water (64:50:10)	EE	Green	Green	Orange	Purple	0,94
		NHF	Green	Green	Orange	Purple	0,94
		EAF	Green	Green	Orange	Green	0,90
		FAE	-	-	-	-	-
Tannins	ethyl acetate,	EE	Green	Black	-	Green	0,88

	methanol, and water (100:13.5:10)	NHF	Brown	Black	-	Blackish	
		FEA	Brown	Black	-	Ash	0,94
		WF	Brown	Black	-	Blackish	
		FEA	Brown	Black	-	Black	0,94
		WF	Brown	Black	-	Black	0,91
Steroids	n-hexane and ethyl acetate (17:3)	FAE	Green	Brown	Blue	Brown	0,91
		FNH	Green	Brown	Blue	Brown	0,91
		FEA	Green	Brown	Orange	Brown	0,22
		FA	-	-	-	Brown	-

289 **Notes:**

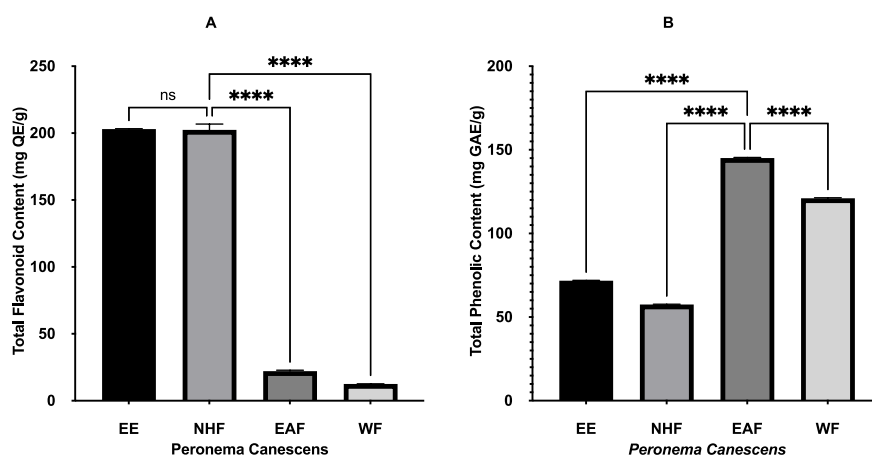
290 (+) = Contains tested compounds

291 (-) = Does not contain the tested compounds

292

293

294 3.3. Determination of total phenolic and total flavonoid content



295

296 Figure 1. Total Flavonoid Content (A) and Total Phenolic Content (B). EE: Ethanolic Extract,
 297 NHF: n-Hexane Fraction, EAF: Ethyl Acetate Fraction, WF: Water Fraction. ns:
 298 Non-significant; ****: Significant difference ($P > 0.05$)

299 Figure 1 shows that the n-hexane fraction of PC leaves exhibits the highest total flavonoid
 300 content, with an average concentration of 203.3742 ± 4.3777 mg QE/gram. In contrast, the
 301 ethyl acetate fraction of PC leaves contains the highest total phenolic content, with an
 302 average concentration of 147.0397 ± 0.7864 mg GAE/gram.

303

304 3.4. Active Fraction of PC Leaves Using GC-MS

305 The active fraction was identified using Gas Chromatography-Mass Spectrometry (GC-MS).
 306 Based on the analysis of PC leaves' active fraction (ethyl acetate fraction), the compounds
 307 identified are listed in Table 3.

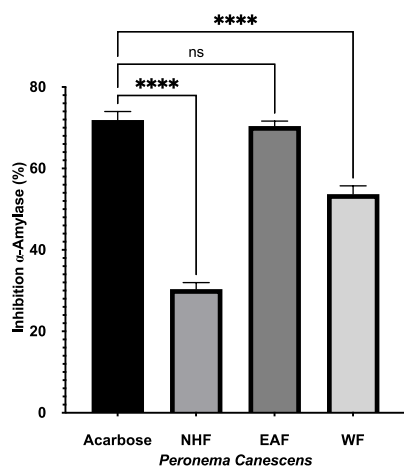
308 Table 3. Results of GC-MS Identification of Active Fraction Compounds in PC Leaves

Peak	Retention Time	Percent Area(%)	Molecular Weight	Base Peak	Similarity Index	Molecular Formula	Compound
1	20,208	1,70	390	149	97	C ₂₄ H ₃₈ O ₄	Bis(2-ethylhexyl) phthalate
2	21,656	0,58	478	57	63	C ₃₂ H ₆₂ O ₂	Myristyl oleate
3	21,942	0,57	593	57	59	C ₃₉ H ₇₆ O ₃	Oleic acid
4	22,825	2,12	288	57	64	C ₂₁ H ₃₆	14-Beta-H-Pregna
5	22,902	8,77	723	57	77	C ₄₅ H ₈₆ O ₆	Tetradecanoic acid
6	23,021	16,46	639	57	75	C ₃₉ H ₇₄ O ₆	Dodecanoic acid
7	23,142	9,25	723	57	68	C ₄₅ H ₈₆ O ₆	Tetradecanoic acid
8	23,255	27,05	751	57	77	C ₄₇ H ₉₀ O ₆	Hexadecanoic acid
9	23,301	19,04	639	57	83	C ₃₉ H ₇₄ O ₆	Dodecanoic acid
10	23,367	14,46	639	183	71	C ₃₉ H ₇₄ O ₆	Dodecanoic acid

309

310 **3.5. In Vitro Inhibition of α -Amylase Activity**

311 The α -amylase inhibitory activity of the extract and fractions of PC leaves was evaluated by
312 measuring the total reducing sugar using the dinitrosalicylic acid (DNS) method, with starch
313 as the substrate. DNS is an aromatic compound that reacts with reducing sugars to form 3-
314 amino-5-nitrosalicylic acid, which absorbs electromagnetic radiation. The α -amylase
315 inhibitory activity was quantified at a wavelength of 540 nm using a 96-well microplate
316 reader.



317

318 Figure 3. Percentage Inhibition of α -Amylase Enzyme Activity. NHF: n-hexane fraction, EAF:
 319 Ethyl acetate fraction, WF: Water fraction, ns: non-significant, ****: Significant
 320 difference ($P > 0.05$).

321 Based on Figure 3, the active fraction with the highest percentage of α -amylase enzyme
 322 inhibition was observed in the ethyl acetate fraction of PC leaves, with an average inhibition
 323 of $70.38 \pm 1.26\%$.

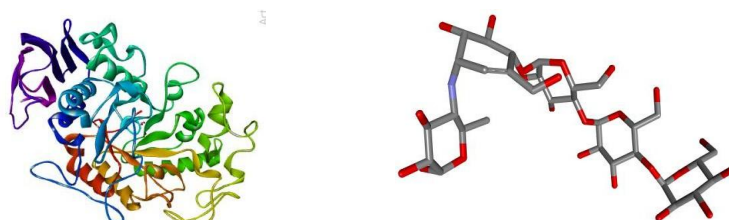
324

325 3.6. In Silico Inhibition of α -Amylase Activity

326 Molecular docking is a commonly used method in drug development to identify compounds
 327 or molecules with therapeutic activity by predicting ligand-target interactions and evaluating
 328 structural activity using computational techniques (Rahardhian et al., 2022). The validation
 329 results showed an RMSD value of 1.74 \AA , indicating that the redocking method met the
 330 required criteria. Furthermore, the binding energy affinity (ΔG) was found to be -4.17
 331 kcal/mol, with an inhibition constant (K_i) of the same magnitude obtained during the fourth
 332 docking run.

333

334



A

B

335 Figure 4. The three-dimensional structure of the α -amylase enzyme (A) and its natural ligands
 336 (B)

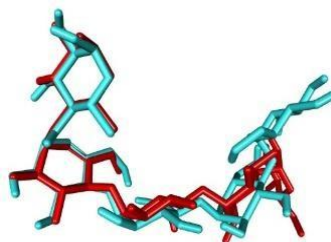


Figure 5. The overlay of the acarbose confirmation before (blue) and after (red) redocking

337 Table 4. Results of Molecular Docking Analysis of Ethyl Acetate Fraction Compounds of PC
 338 Leaves

No	Compound	ΔG (kcal/mol)	Ki (mM)	Amino acids Residue
1	Bis(2-ethylhexyl) phthalate	-4.59	432.20	Tyr 62, Leu 162, His 201, Lys 200, Ile 235, His 299
2	Myristyl oleate	-3.32	3.66	Ile 51, Leu 165, Pro 54, His 101, Trp 59, His 305, Tyr 62, Asp 300
3	14 Beta H pregna	-7.81	1.90	Tent 162, Tent 165, Trp 58, Trp 62, His 299
4	Natural ligand (Acarbose)	-4,17	-	The 163, His 305, Asp 300, Glu 233, His 101, Arg 195, Asp 197, His 299

339 Based on Table 4, three bioactive compounds exhibit favorable binding energies and amino
 340 acid interactions with the same target protein as the natural ligands. The binding energy
 341 values reflect the spontaneity of the interaction between the protein and the ligand; the more
 342 negative the binding energy, the more rapid the binding process occurs. This suggests that
 343 these compounds may serve as effective α -amylase inhibitors, warranting further
 344 investigation into their potential therapeutic applications.

345 Table 5. Results of ADME Analysis of Active Fraction Compounds of PC Leaves

No	Compound	Absorption (Caco2 permeability) (log Papp in y) 10 ⁻⁶ cm/s)	Distribution (BBB permeability) (log BB)	Metabolism (CYP2D6 substrate) (Yes/No)	Excretion (Total clearance) ml/min/kg)	Toxicity (AMES) (log (Yes/No))
----	----------	---	---	---	---	--------------------------------------

1	Bis(2-ethylhexyl) phthalate	1,408	-0,175	No	1,898	No
2	Myristyl oleate	1,306	1,014	No	2,149	No
3	14 Beta H pregna	1,405	0,885	No	0,67	No

346 Table 5 presents the results of the ADME (Absorption, Distribution, Metabolism, and
 347 Excretion) analysis for the bioactive compounds identified in the active fraction of PC leaves.
 348 This analysis is crucial for evaluating the pharmacokinetic properties of these compounds,
 349 which can influence their effectiveness as potential therapeutic agents. The results provide
 350 insights into the compounds' absorption rates, distribution patterns in the body, metabolic
 351 stability, and excretion profiles, helping to assess their suitability for drug development. The
 352 ADME (Absorption, Distribution, Metabolism, Excretion) and toxicity profiles of the
 353 bioactive compounds identified in the active fraction of PC leaves. The analysis indicates that
 354 the bioactive compounds Bis(2-ethylhexyl) phthalate, Myristyl oleate, and 14 Beta H pregna
 355 meet the criteria for a favorable ADME profile, suggesting efficient absorption and
 356 distribution within the body.

357 Table 6. Results of the Lipinski analysis for the bioactive compounds found in the active

No	Compound	Molecular weight	Log P	Donor hydrogen bonding	Hydrogen acceptor bonding	Surface area
1	Bis(2-ethylhexyl) phthalate	390,564	6,433	0	4	170,550
2	Myristyl oleate	478,846	112,682	0	2	214,638
6	14 Beta H pregna	274,492	60,553	0	0	125,650

358 fraction of PC leaves

359
 360 Table 6 presents the results of the Lipinski analysis for the bioactive compounds found in the
 361 active fraction of PC leaves. Lipinski's rule of five evaluates the drug-likeness of compounds
 362 based on their molecular properties, which predict good absorption and permeation. The
 363 analysis assesses molecular weight, hydrogen bond donors and acceptors, and logP values.
 364 Compounds that comply with these criteria will likely exhibit favorable pharmacokinetic
 365 properties, making them suitable candidates for further drug development. The bioactive
 366 compounds in the active fraction of PC leaves meet Lipinski's rule of five requirements.

367 Specifically, these compounds exhibit a molecular weight of less than 500 mg/mol, a logP
368 value between 0.4 and 5, a hydrogen bond donor count of ≤ 10 , a hydrogen bond acceptor
369 count of ≤ 5 , and a molar refractivity below 130. Consequently, it can be concluded that these
370 bioactive compounds are considered safe for oral use.

371

372 **4. Discussion**

373 The fractionation of the ethanol extract from PC leaves yielded 32.81% for the n-hexane
374 fraction, 16.86% for the ethyl acetate fraction, and 34.17% for the water fraction. The lower
375 yield of the ethyl acetate fraction indicates a higher abundance of nonpolar and polar
376 compounds relative to semipolar compounds in the extract (Duistermaat and Kolk, 2000) .
377 This pattern reflects the principles of solvent polarity, where nonpolar solvents like n-hexane
378 extract nonpolar compounds such as lipids, while polar solvents like water preferentially
379 extract polar substances like sugars and certain phenolic compounds(Altemimi et al., 2017).

380 Phytochemical screening revealed alkaloids, flavonoids, tannins, saponins, and
381 triterpenoids/steroids in the ethanol extract. However, the water fraction was only positive for
382 alkaloids and tannins. This selective presence underscores the solubility characteristics of the
383 compounds, with flavonoids and triterpenoids being more soluble in ethyl acetate, explaining
384 their absence in the water fraction. The limited presence of flavonoids and triterpenoids in the
385 water fraction of PC leaves aligns with other studies on medicinal plants, suggesting that these
386 compounds are more concentrated in less polar solvents. The phytochemical screening of the
387 ethanol extract from PC leaves, along with its fractions, revealed a diverse array of bioactive
388 compounds. The tests utilized various reagents to identify specific compound groups,
389 including phenolics, tannins, flavonoids, alkaloids, saponins, and steroids. This suggests that
390 phenolics, The research results are consistent with (Rahardhian et al., 2022c), indicating that
391 PC contains phenolic. The presence of phenolic compounds was confirmed in all fractions,
392 including the ethanol extract, n-hexane, ethyl acetate, and water fractions, as indicated by the
393 positive reaction with ferric chloride. Tannins were present in the ethanol extract and water
394 fraction but absent in the n-hexane fraction. This distribution indicates that tannins, which are
395 polyphenolic compounds with astringent properties, are more soluble in polar solvents like
396 water and ethanol. The screening indicated flavonoids were present in the ethanol, n-hexane,
397 and ethyl acetate fractions but absent in the water fraction. This result highlights the
398 preferential solubility of flavonoids in less polar solvents, supporting findings in other studies

399 that suggest flavonoids are typically more concentrated in ethyl acetate and hexane fractions.
400 All fractions tested positive for alkaloids, with multiple reagents confirming their presence.
401 Saponins were present in the ethanol, n-hexane, and ethyl acetate fractions but absent in the
402 water fraction. This suggests that while saponins can dissolve in polar solvents, they may
403 require a particular concentration or temperature to effectively extract, as indicated by their
404 absence in the water fraction after the hot water test. Steroids were found in the ethanol, n-
405 hexane, and ethyl acetate fractions but not in the water fraction. This aligns with their
406 chemical nature, as these compounds are generally more soluble in nonpolar or moderately
407 polar solvents. The phytochemical screening demonstrates that PC leaves are rich in bioactive
408 compounds across different fractions. The solubility profiles of these compounds indicate
409 their potential utility in therapeutic applications, as well as the need for further research to
410 explore their pharmacological properties and mechanisms of action (Suryanti et al., 2022).

411 The thin-layer chromatography (TLC) analysis of PC leaves provides a comprehensive
412 overview of its bioactive compound profile, including alkaloids, flavonoids, saponins, tannins,
413 and steroids (Ladeska et al., 2024). The visualizations and R_f values obtained during the
414 analysis allow for a comparative understanding of the composition and potential therapeutic
415 applications of these compounds (Yodha et al., 2024). The presence of alkaloids was
416 confirmed across all samples, with R_f values ranging from 0.73 to 0.81. The consistent brown
417 coloration under UV light and the appearance of orange spots following Dragendorff's
418 reagent application suggest a robust alkaloid profile in PC leaves. These findings align with
419 research (Li et al., 2020), which noted that alkaloids often exhibit similar R_f values due to
420 their polar characteristics, influencing their solubility and mobility in chromatographic
421 conditions. Alkaloids are recognized for their pharmacological activities, including analgesic
422 and antimicrobial properties, reinforcing the potential medicinal value of PC. Flavonoids
423 displayed R_f values of 0.56 to 0.91, with the ethanol, n-hexane, and ethyl acetate fractions
424 exhibiting a distinct green coloration. The yellow stains after ammonia treatment are
425 consistent with flavonoid presence, as supported by studies like those of (Panche et al., 2016)
426 which indicate that the coloration is indicative of flavonoid derivatives. The consistent R_f
427 values across the fractions suggest that PC leaves contain a diverse range of flavonoids, which
428 may enhance their therapeutic profile. The analysis showed a strong presence of saponins,
429 with R_f values around 0.90 to 0.94 and vibrant colored patches indicating their presence. This
430 finding corroborates previous studies, such as those by (Cheok et al., 2014). Tannins were

431 identified with Rf values of 0.88 to 0.94, displaying green-blackish spots after FeCl₃ treatment.
432 This aligns with findings from (Das et al., 2020) , which suggest that tannins' astringent
433 properties and their ability to form complexes with proteins are responsible for their
434 bioactivity. The presence of tannins in PC leaves may contribute to their potential health
435 benefits, particularly in antioxidant and antimicrobial applications. Steroids were detected,
436 with Rf values of 0.22 to 0.91. The purple color indicating steroids and the blue for
437 triterpenoids under UV light corresponds with findings from (Tarigan et al., 2023), which
438 report that these compounds have significant roles in various biological activities, including
439 anti-inflammatory effects. The varying Rf values suggest differing solubility characteristics
440 among the compounds, indicating the complexity of the steroidal profile in PC leaves.

441 The n-hexane fraction demonstrated the highest total flavonoid content, averaging
442 203.3742 ± 4.3777 mg QE/gram. The predominance of flavonoids in the n-hexane fraction
443 suggests that these compounds have a higher solubility in nonpolar solvents(Putri et al., 2023).
444 This aligns with findings from several studies indicating that nonpolar extraction methods are
445 effective for isolating lipophilic flavonoid compounds. For instance, research by (Putri et al.,
446 2022) supports the idea that nonpolar solvents can enhance the extraction of certain flavonoid
447 derivatives, potentially improving their bioavailability and pharmacological efficacy. In
448 contrast, the ethyl acetate fraction exhibited the highest total phenolic content, averaging
449 147.0397 ± 0.7864 mg GAE/gram. Phenolic compounds are well-known for their potent
450 antioxidant properties, contributing to the plant's protective mechanisms against oxidative
451 stress. The higher concentration of phenolics in the ethyl acetate fraction indicates their
452 preferential solubility in moderately polar solvents, which has been corroborated by studies
453 such as those by (Rahardhian et al., 2019). These findings highlight the effectiveness of ethyl
454 acetate in extracting bioactive phenolic compounds, which may have implications for their
455 therapeutic applications in treating oxidative stress-related diseases. The observed differences
456 in flavonoid and phenolic concentrations between the two solvent fractions emphasize the
457 importance of solvent polarity in the extraction process. This selective extraction allows for a
458 deeper understanding of the phytochemical profile of PC leaves, which can guide further
459 research into their potential health benefits. Notably, the high total flavonoid content in the n-
460 hexane fraction suggests that PC may be a valuable source of flavonoid-rich extracts that
461 could be utilized in nutraceutical formulations targeting antihyperglycemic.

462

463 The gas chromatography-mass spectrometry (GC-MS) analysis of PC leaves provided
464 valuable insights into the chemical composition of its extracts, revealing a range of fatty acids
465 and esters. Each compound's retention time, molecular weight, and similarity index contribute
466 to understanding the plant's phytochemical profile and its potential applications in health and
467 nutrition (Hotmian et al., 2021a) . Among the identified compounds, notable fatty acids
468 include tetradecanoic acid ($C_{14}H_{28}O_2$) and hexadecanoic acid ($C_{16}H_{32}O_2$), which accounted for
469 significant peak areas of 8.77% and 27.05%, respectively. The predominance of hexadecanoic
470 acid is particularly interesting, as it has been associated with various health benefits, including
471 anti-inflammatory and antimicrobial properties. This aligns with research indicating that fatty
472 acids play crucial roles in cellular functions and may cause diabetes mellitus diseases (Berry,
473 1997) . The presence of bis(2-ethylhexyl) phthalate ($C_{24}H_{38}O_4$), with a peak area of 1.70%,
474 indicates the plant's potential to contain plasticizer compounds, which can have implications
475 for environmental health. Additionally, oleic acid ($C_{18}H_{34}O_2$), which appeared in the analysis,
476 is a well-known monounsaturated fatty acid praised for its heart health benefits, including its
477 ability to lower harmful cholesterol levels (Eleazu et al., 2018). The molecular weight of oleic
478 acid (593) and its retention time further suggests its bioactive potential. The diversity of
479 compounds, including myristyl oleate ($C_{32}H_{62}O_2$) and 14-Beta-H-Pregna ($C_{21}H_{36}$), with
480 respective peak areas of 0.58% and 2.12%, points to the complex nature of the phytochemical
481 constituents of PC. The variety of fatty acids and esters indicates that the plant could serve as
482 a source of bioactive compounds with diverse pharmacological activities, ranging from anti
483 diabetic mellitus to antioxidant effects. The similarity index values, ranging from 57 to 97,
484 highlight the reliability of the GC-MS identification process, reflecting the compounds'
485 structural integrity and known bioactivities. The higher similarity indices suggest that the
486 identified compounds are well-documented in the literature, supporting their potential
487 utilization in therapeutic applications. In summary, the GC-MS analysis of PC leaves reveals
488 a rich array of fatty acids and esters, emphasizing the plant's potential as a source of bioactive
489 compounds

490 The findings from this study reveal significant insights into the α -amylase inhibitory
491 potential of PC, particularly highlighting the efficacy of the ethyl acetate fraction. The
492 observed average inhibition percentage of 70.38% for the ethyl acetate fraction is notably
493 higher than the 30.37% and 53.18% inhibition observed in the n-hexane and water fractions,
494 respectively. This variation in inhibitory activity suggests a differential distribution of

495 bioactive compounds in the various fractions, likely attributable to their solubility properties
496 and polarity. The ethyl acetate fraction's superior inhibition effect is particularly noteworthy,
497 as it closely approaches the inhibition level of acarbose, a well-known α -amylase inhibitor,
498 which demonstrated an average inhibition percentage of 74.29%. The lack of significant
499 difference between the ethyl acetate fraction and acarbose indicates that the compounds
500 present in this fraction possess potent α -amylase inhibitory activity. This finding is consistent
501 with existing literature, which suggests that ethyl acetate often extracts a variety of bioactive
502 compounds, such as flavonoids and phenolic compounds, that are known for their enzyme-
503 inhibitory effects (Nyambe-Silavwe et al., 2015). The lower inhibition percentages observed
504 in the n-hexane fraction may be attributed to the nonpolar nature of the compounds extracted,
505 which are less likely to interact effectively with the active site of the α -amylase enzyme.
506 Conversely, the water fraction exhibited moderate inhibition, possibly due to the presence of
507 polar compounds, but it was less effective than the ethyl acetate fraction, highlighting the
508 significance of solvent polarity in influencing the extraction of bioactive constituents. This
509 study demonstrates that the ethyl acetate fraction of PC exhibits significant α -amylase
510 inhibitory potential, comparable to acarbose, suggesting its potential as a natural alternative
511 for diabetes management and warranting further research into its pharmacological
512 applications (Lestari et al., 2018).

513 The molecular docking results presented in this study provide critical insights into the
514 binding affinities and inhibitory potentials of various compounds against α -amylase (Lolok et
515 al., 2022). The free energy of binding (ΔG) and inhibition constants (K_i) serve as valuable
516 indicators of the interactions between these compounds and the enzyme (Yuningtyas et al.,
517 2024), shedding light on their potential as α -amylase inhibitors. Bis(2-ethylhexyl) phthalate
518 exhibited a binding free energy of -4.59 kcal/mol with an inhibition constant (K_i) of 432.20
519 mM. The interaction of this compound with the enzyme involves critical amino acid residues
520 such as Tyr 62, Leu 162, and His 201. While the negative ΔG value indicates a favorable
521 binding, the relatively high K_i value suggests that this compound may not be a potent
522 inhibitor of α -amylase. The interactions with critical residues indicate a potential for activity,
523 but further modifications may be needed to enhance its inhibitory potency. Myristyl oleate
524 showed a lower binding affinity, with ΔG of -3.32 kcal/mol and a K_i value of 3.66 mM. The
525 presence of interactions with multiple residues, including Ile 51, Leu 165, and His 101,
526 suggests that this compound forms multiple favorable contacts with the enzyme. The

527 significantly lower K_i indicates that myristyl oleate could act as a more potent inhibitor
528 compared to bis(2-ethylhexyl) phthalate, possibly due to its ability to engage critical active
529 site residues effectively. 14 Beta H-pregna demonstrated the strongest binding affinity with a
530 ΔG of -7.81 kcal/mol and a low K_i of 1.90 mM. The strong interactions with amino acids such
531 as Trp 58 and Trp 62 indicate that this compound may have a significant impact on the active
532 site configuration of α -amylase. The natural ligand (acarbose), known for its clinical efficacy
533 in inhibiting α -amylase, had a ΔG of -4.17 kcal/mol but did not present a K_i value as this
534 compound acts through a different mechanism. The amino acid residues involved in binding,
535 such as The 163 and His 305, reflect the established interactions that allow acarbose to inhibit
536 the enzyme competitively. This comparison highlights the relative effectiveness of the newly
537 identified compounds against a standard inhibitor, demonstrating that certain derivatives may
538 offer similar or improved efficacy.

539 The physicochemical properties of the compounds derived from PC were evaluated
540 based on their molecular weight, lipophilicity (Log P), hydrogen bonding capacity, and
541 surface area. Understanding these characteristics is critical for assessing their bioavailability,
542 interactions, and overall pharmacological potential (Pires et al., 2015). Absorption of Bis(2-
543 ethylhexyl) phthalate exhibited an absorption value of 1.408 (log Papp in 10^{-6} cm/s),
544 indicating a relatively favorable permeability across the intestinal barrier. This suggests that it
545 may be effectively absorbed after oral administration. Myristyl oleate, with a slightly lower
546 absorption value of 1.306, also shows good permeability, although it is less than that of bis(2-
547 ethylhexyl) phthalate. 14 Beta H-pregna displayed an absorption value similar to bis(2-
548 ethylhexyl) phthalate (1.405), indicating its potential for adequate oral bioavailability. Overall,
549 the absorption profiles of these compounds suggest they may achieve adequate systemic
550 exposure following administration. Distribution of Myristyl oleate had the highest distribution
551 coefficient (log BB = 1.014), indicating a favorable ability to cross the blood-brain barrier
552 (BBB). This characteristic may allow for central nervous system (CNS) activity, potentially
553 making it useful for conditions where CNS targeting is desired. In contrast, bis(2-ethylhexyl)
554 phthalate showed a negative log BB value (-0.175), indicating limited distribution to the brain.
555 14 Beta H-pregna also demonstrated a positive log BB value (0.885), suggesting moderate
556 ability to penetrate the BBB. These distribution characteristics are vital when considering the
557 therapeutic application of these compounds, particularly in relation to CNS effects.
558 Metabolism All three compounds were determined not to be substrates for CYP2D6,

559 suggesting they are less likely to be metabolized through this common liver enzyme. This
560 could imply a potentially favorable metabolic profile, as compounds that are not extensively
561 metabolized may maintain higher bioavailability. Excretion clearance rates provide insights
562 into the elimination of these compounds from the body. Myristyl oleate had the highest total
563 clearance (2.149 log ml/min/kg), suggesting it may be rapidly eliminated, which could
564 influence dosing regimens. Conversely, 14 Beta H-pregna exhibited a significantly lower
565 clearance rate (0.67 logs ml/min/kg), indicating a slower elimination and potentially more
566 prolonged duration of action. Notably, none of the compounds tested positive for
567 mutagenicity in the Ames test, indicating a favorable toxicity profile. This is an encouraging
568 finding, as it suggests these compounds may have a lower risk of causing genetic damage.

569 Bis(2-ethylhexyl) phthalate (MW = 390.564 g/mol) and Myristyl oleate (MW = 478.846
570 g/mol) are relatively large molecules, which may influence their absorption and distribution in
571 biological systems. Larger molecules tend to exhibit lower permeability through biological
572 membranes, which could affect their therapeutic efficacy. In contrast, 14 Beta H-pregna has a
573 lower molecular weight (MW = 274.492 g/mol), potentially facilitating better absorption and
574 distribution, as smaller compounds typically diffuse more easily through cell membranes. The
575 Log P values provide insights into the hydrophobicity of the compounds. Myristyl oleate
576 exhibits an extraordinarily high Log P value (112.682), indicating its lipophilic solid nature,
577 which could enhance its ability to cross lipid membranes. However, such high lipophilicity
578 may also lead to challenges in solubility in aqueous environments, potentially limiting its
579 bioavailability when administered orally. Bis(2-ethylhexyl) phthalate has a significant Log P
580 value of 6.433, also reflecting its lipophilicity and suggesting a potential for membrane
581 permeation. Conversely, 14 Beta H-pregna has a Log P of 60.553, which is comparatively
582 lower than the other two compounds but still suggests a reasonable level of lipophilicity. The
583 varied lipophilicity of these compounds indicates that their absorption and distribution will
584 differ significantly, influencing their pharmacological activities. None of the compounds
585 displayed any donor hydrogen bonding, indicating that they may not participate in hydrogen
586 bond donation, which could limit their interactions with target proteins or enzymes. This
587 property could influence the compounds' binding affinities and their overall efficacy in
588 biological systems. The hydrogen acceptor bonding also varied among the compounds. Bis(2-
589 ethylhexyl) phthalate exhibited four acceptor sites, while Myristyl oleate had two, and 14 Beta
590 H-pregna had none. The presence of hydrogen bond acceptors in bis(2-ethylhexyl) phthalate

591 could facilitate its interaction with biomolecules, enhancing its potential activity. On the other
592 hand, the lack of hydrogen bonding capacity in 14 Beta H-pregna may limit its interactions
593 with proteins, potentially reducing its efficacy. Surface area is an important parameter that can
594 influence drug absorption and permeability. Myristyl oleate has the largest surface area
595 (214.638 \AA^2), which might affect its interactions with biological membranes and potential
596 targets. The larger surface area could facilitate a more significant interaction with the lipid
597 bilayer, impacting absorption rates. Bis(2-ethylhexyl) phthalate has a moderate surface area
598 (170.550 \AA^2), while 14 Beta H-pregna has the smallest surface area (125.650 \AA^2). The smaller
599 surface area of 14 Beta H-pregna could correlate with its lower molecular weight, potentially
600 leading to better permeability.

601

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726

Exploring the α -Amylase Inhibitory Potential of *Peronema canescens* Jack: An In Vitro and In Silico Study

ABSTRACT

Hyperglycemia in individuals with type 2 diabetes mellitus is primarily driven by the rapid hydrolysis of starch by the enzyme α -amylase in the pancreas and the breakdown of oligosaccharides by α -glucosidase in the intestine. *Peronema canescens* Jack. (PC) has shown promise as a potential antidiabetic agent. This study aimed to evaluate the total flavonoid, phenolic, and α -amylase inhibitory activity of extracts and fractions derived from PC leaves using both in vitro and in silico approaches. The ethanol extract of PC leaves was fractionated through liquid-liquid extraction using n-hexane, ethyl acetate, and water as solvents. Preliminary phytochemical screening of the extracts and fractions identified the presence of alkaloids, flavonoids, saponins, tannins, and steroids/triterpenoids. The n-hexane fraction exhibited the highest total flavonoid content, averaging 203.37 ± 4.38 mg QE/gram, while the ethyl acetate fraction demonstrated the highest total phenolic content, averaging 147.04 ± 0.79 mg GAE/gram. Furthermore, the ethyl acetate fraction showed the strongest α -amylase inhibitory activity, with an average inhibition rate of $70.38 \pm 1.26\%$. In silico analysis, combined with GC-MS identification, suggested that three compounds—bis(2-ethylhexyl) phthalate, myristyl oleate, and 14 beta H-pregna—may contribute to the observed α -amylase inhibitory activity. These findings highlight the potential of PC as a source of natural antidiabetic agents.

Keywords: α -amylase inhibitory activity, in vitro and in silico analysis, natural antidiabetic agents, *Peronema canescens* Jack., total flavonoid and phenolic content

1. Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both (WHO, 2023). The prevalence of diabetes has reached alarming levels globally, with the International Diabetes Federation (IDF) estimating that over 537 million adults will be living with the condition in 2021 (Webber, 2013). Experts project that this number will rise to 643 million by 2030. In Indonesia, the situation mirrors this global trend, with an increasing number of the population being diagnosed with diabetes. The current standard of care for managing diabetes includes synthetic drugs such as sulfonylureas, metformin, and thiazolidinediones, designed to enhance insulin sensitivity, stimulate insulin secretion, or reduce glucose production. However, these synthetic drugs are not without their limitations. Long-term use of these medications can lead to adverse effects such as hypoglycemia (Chaudhury et al., 2017), gastrointestinal issues, and even multidrug resistance (MDR). These limitations underscore the need for alternative treatment strategies that are both effective and safe.

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41 In light of the challenges associated with synthetic drugs, there has been growing
42 interest in herbal medicine as a potential alternative or complementary approach to diabetes
43 management. Plants rich in bioactive compounds provide herbal medicines with therapeutic
44 benefits and fewer side effects (Tran et al., 2020). One such plant is *Peronema canescens* Jack.
45 (PC), a species native to Indonesia, traditionally used for its medicinal properties (Rahardhian
46 et al., 2022b) . The secondary metabolites found in PC, such as flavonoids, tannins, and
47 alkaloids (Primal and Ahriyasna, 2022), have been shown to possess various pharmacological
48 activities, including antidiabetic effects. These natural compounds may help to mitigate
49 hyperglycemia by enhancing insulin sensitivity, inhibiting glucose absorption, and modulating
50 carbohydrate metabolism, making PC a promising candidate for diabetes treatment.

51 Computer-aided drug design (CADD) advancements have provided researchers with
52 powerful tools to identify and optimize potential antidiabetic agents (Meng et al., 2011) .
53 Molecular docking simulation, a key component of CADD, allows for predicting the
54 interaction between small molecules and target proteins, thereby facilitating the identification
55 of compounds with high binding affinities (Pagadala et al., 2017) . This method has been
56 beneficial in the search for natural inhibitors of enzymes such as α -amylase, which plays a
57 crucial role in carbohydrate digestion and glucose absorption (de Sales et al., 2012) .
58 Researchers can rapidly screen and identify the most promising antidiabetic compounds
59 within complex plant extracts, such as those found in PC, by molecular docking simulations
60 (Khan et al., 2024).

61 This study focuses on the antidiabetic potential of PC, examining both its in vitro and
62 in silico activities. Gas Chromatography-Mass Spectrometry (GC-MS) analysis identified
63 PC's chemical composition and revealed various bioactive compounds (Hotmian et al., 2021a).
64 The inhibitory effect of these compounds on α -amylase was then evaluated through in vitro
65 assays, while molecular docking simulations were employed to understand the interaction
66 between these compounds and the enzyme at the molecular level (Santos et al., 2019). The
67 physicochemical properties of PC It were also analyzed and compared with other herbal
68 medicines to highlight its unique advantages. This comprehensive approach sheds light on the
69 potential of PC as an antidiabetic agent and underscores its benefits over other herbal
70 remedies (Posadzki et al., 2013).

71 The novelty of this study lies in its integrative approach, combining in vitro and in
72 silico methods to investigate the α -amylase inhibitory potential of PC. While previous

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73 research has explored the antidiabetic effects of various herbal medicines, studies focusing
74 specifically on PC remain limited. By providing a detailed analysis of its bioactive
75 compounds and their mechanisms of action, this study serves as a preliminary investigation
76 that could pave the way for future research (Altemimi et al., 2017). The findings presented
77 here are relevant for developing PC as a potential antidiabetic treatment but also offer
78 valuable insights for researchers working on similar objectives, making this study a
79 significant consideration for ongoing and future developments in the field.

80 This study aims to evaluate the α -amylase inhibitory activity of PC using both in vitro
81 and in silico methods. This research seeks to identify the specific compounds responsible for
82 this activity and to understand their mechanisms of action at the molecular level. This
83 research will contribute to the growing knowledge of natural antidiabetic agents and support
84 PC development as a potential treatment for diabetes mellitus.

86 2. Materials and Methods

87 2.1. Materials

88 The equipment used in this study includes a Pyrex separation funnel, a Heidolph-G3
89 rotary evaporator, Silica Gel F254 plates, UV 254 and 366 lamps (Evaco GL 220V 50Hz T8
90 15W), micropipettes (Socorex and Dragon Lab), vortex mixers, a Shimadzu UV-1780 UV-
91 Vis spectrophotometer (Serial No. A119161), a Synergy-HTX multi-mode ELISA reader
92 with 96-well plates, and a QP 2010 gas chromatography-mass spectrometer (GC-MS). The
93 materials utilized in this study include *Peronema canescens* Jack., ethanol, n-hexane, ethyl
94 acetate, FeCl₃, MgSO₄, hydrochloric-ethanolic acid mixture (1:1), hydrochloric acid,
95 Lieberman-Burchard reagent, DPPH (Sigma), quercetin, methanol, phosphate buffer, alpha-
96 amylase enzyme (Sigma Aldrich), starch, and DNS

Comment[DS1]: Do not use abbreviation.

Comment[DS2]: Do not use abbreviation.

98 2.1.1. Hardware and Software

99 The molecular docking study was conducted using a laptop equipped with an Asus ROG
100 503 VD. The software utilized in this study includes ChemDraw Professional 15.0, Chem3D
101 15.0, Biovia Discovery Studio 2021, Command Prompt, and AutoDock Tools-1.5.6.
102 Visualization of docking results and the creation of ligands and receptors were performed
103 using Biovia Discovery Studio 2021. Receptor structures were obtained from the RCSB
104 Protein Data Bank (PDB) website (<https://www.rcsb.org/>), while ligand structures were

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105 downloaded from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>). Lipinski's rule
106 of five was applied to assess drug-likeness using the SCFBio website ([http://www.scfbio-](http://www.scfbio-iitd.res.in/software/drugdesign/lipinski.jsp)
107 [iitd.res.in/software/drugdesign/lipinski.jsp](http://www.scfbio-iitd.res.in/software/drugdesign/lipinski.jsp)). The pk-CSM online tool evaluated
108 Pharmacokinetic and toxicological properties (<https://biosig.lab.uq.edu.au/pkcsml/>).
109

110 2.2. Methods

111 2.2.1. Sample preparation, extraction, and fractionation

112 The extraction of *Peronema canescens*. Jack (PC) used the maceration method with
113 96% ethanol as the solvent. Three hundred grams of PC was placed in a maceration jar, and
114 96% ethanol was added as the solvent. The maceration process was performed for three days,
115 with periodic solvent changes and occasional stirring to enhance extraction efficiency. The
116 resulting macerate was filtered and then concentrated using a rotary vacuum evaporator. The
117 concentrated extract was further thickened using a water bath at approximately 50°C
118 (Rahardhian et al., 2019). Ten grams of the ethanol extract of PC leaves were dissolved in 100
119 mL of water and transferred into a separatory funnel. The mixture was then partitioned by
120 adding 100 mL of n-hexane, followed by vigorous shaking. The mixture was allowed to settle
121 until two distinct phases formed: the aqueous and n-hexane. The aqueous phase, separated
122 from the n-hexane phase, was reintroduced into the separatory funnel. Subsequently, 100 mL
123 of ethyl acetate was added to the aqueous phase. The mixture was shaken several times to
124 ensure thorough mixing and then allowed to separate into its respective phases. The resulting
125 fractions were concentrated using a rotary vacuum evaporator and thickened using a water
126 bath at approximately 50°C to obtain a viscous fraction. (Rahardhian et al., 2019).

127 2.2.2. Phytochemical screening and TLC affirmation

128 Phytochemical screening follows the method (Putri et al., 2022). Each sample,
129 including the ethanol extract, n-hexane fraction, ethyl acetate fraction, and water fraction of
130 PC, was prepared by dissolving 30 mg of the sample in 3 mL of ethanol until completely
131 dissolved. This solution was used for the subsequent phytochemical screening tests.
132 Flavonoids: To the sample solution, magnesium (Mg) powder, 1 mL of hydrochloric acid
133 (HCl), and amyl alcohol were added. The formation of red, yellow, or orange colours
134 indicated a positive presence of flavonoids. Polyphenols: A 10% ferric chloride (FeCl₃)
135 solution was added to the sample. The appearance of a blue or blackish-green color indicated
136 the presence of polyphenols. Tannins: The sample was mixed with gelatin salts. The

Comment[DS3]: ?

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137 formation of a yellowish-white precipitate indicated a positive result for tannins. Alkaloids:
138 The sample was treated with HCl and water, then heated and divided into two portions
139 (Filtrate 1 and Filtrate 2). Filtrate 1: Reacted with Mayer's reagent, forming a yellowish-
140 white precipitate, indicating the presence of alkaloids. Filtrate 2: Reacted with Dragendorff's
141 reagent, forming a brick-red precipitate, confirming the presence of alkaloids. Saponins: The
142 sample was placed in a test tube, and 10 mL of distilled water was added. The mixture was
143 shaken vigorously for 10 seconds. A positive reaction was indicated by the formation of foam
144 that persisted for 10 minutes at a height of 1-3 cm. Steroids/triterpenoids: The sample was
145 treated with chloroform and filtered. The filtrate was mixed with anhydrous acetic acid
146 (CH₃COOH) and heated, then cooled and treated with sulfuric acid (H₂SO₄). A green color
147 in the solution indicated the presence of steroids, while an orange or red color indicated the
148 presence of triterpenoids.

Comment[DS4]: Subscript for 3

Comment[DS5]: Idem.

149 Thin-layer chromatography (TLC) was performed following the method described by
150 (Putri et al., 2022) to identify the compound content of the samples. A small amount of each
151 sample was dripped directly onto the TLC plate. Flavonoids: The mobile phase consisted of
152 n-butanol, acetic acid, and water in a ratio of 4:1:5. The TLC plate was then exposed to
153 ammonia vapor. The presence of flavonoids was indicated by the formation of yellow stains
154 after ammonia treatment. Tannins: The mobile phase was prepared using ethyl acetate,
155 methanol, and water in a ratio of 100:13.5:10. The presence of tannins was indicated by
156 visible spots formed by FeCl₃, which resulted in blackish-green patches. Alkaloids: The
157 mobile phase comprised ethyl acetate, methanol, and water in a ratio of 6:4:2. The presence
158 of alkaloids was confirmed by the appearance of brown patches upon applying Dragendorff's
159 reagent. Saponins, A solvent system of chloroform, methanol, and water in a ratio of
160 64:50:10, was utilized. Anisaldehyde-sulfuric acid was used as a visualization reagent, and
161 the TLC plate was heated on a hot plate for 5-10 minutes at 100°C. The presence of saponins
162 was indicated by the formation of colored patches, including yellow, green, red, dark blue,
163 purple, and brownish-yellow. Steroids/triterpenoids: The mobile phase consisted of n-hexane
164 and ethyl acetate in a ratio of 17:3. Anisaldehyde-sulfuric acid was again used for
165 visualization, and the TLC plate was heated on a hot plate for 5-10 minutes at 100°C. A
166 purple color indicated the presence of steroids, while a blue color indicated triterpenoids.

167

168 2.2.3. Determination of total phenolic and total flavonoid content

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169 The total flavonoid content (TFC) and total phenolic content (TPC) of the extracts
170 were determined using established spectrophotometric methods (Rahardhian et al., 2019).

171 The total phenolic content was assessed using the Folin-Ciocalteu reagent with gallic acid as
172 the standard. A 1.0 mL aliquot of the standard solution or sample was placed in a test tube.

173 Add 4.5 mL of 10% Folin-Ciocalteu reagent to the test tube and 4.5 mL of 7.5% sodium
174 carbonate (Na_2CO_3) solution. The mixture was incubated for 90 minutes at room temperature.

175 The absorbance was measured at a wavelength of 765 nm using a Shimadzu® UV-Vis
176 Spectrophotometer (Model 1240). The total flavonoid content was determined using
177 aluminum chloride (AlCl_3) with quercetin as the standard. A 1.0 mL aliquot of the standard
178 solution or sample was placed in a test tube. Add 3.0 mL of ethanol, 0.2 mL of 10%
179 aluminum chloride (AlCl_3) solution, 0.2 mL of 20% anhydrous sodium acetate (CH_3COONa)
180 solution, and 5.6 mL of distilled water. The mixture was homogenized thoroughly. The
181 mixture was incubated for 30 minutes at room temperature. The absorbance was measured at
182 a wavelength of 420 nm using a Shimadzu® UV-Vis Spectrophotometer (Model 1240). The
183 total phenolic and flavonoid contents were calculated based on the absorbance values
184 obtained from the gallic acid and quercetin standard curves, respectively.

185 2.2.4. α -amylase Inhibitory Activity

186 The α -amylase inhibitory activity of the samples was evaluated using a colorimetric
187 method on a 96-well plate. The samples were dissolved in a 1% dimethyl sulfoxide (DMSO)
188 solution. Each sample, along with the negative control (1% DMSO solution) and positive
189 control (acarbose at concentrations of 50 mg and 100 mg), was pipetted into the wells of a
190 96-well plate at a volume of 20 μL per well. 50 μL of 100 mM phosphate buffer (pH 6.8) was
191 added to each well, followed by ten μL of α -amylase solution (2 U/mL). The plate was pre-
192 incubated at 37°C for 20 minutes to allow the enzyme and inhibitor to interact. After pre-
193 incubation, 20 μL of a 1% starch solution in 100 mM phosphate buffer (pH 6.8) was added to
194 each well as the substrate. The reaction mixture was further incubated at 37°C for 30 minutes.
195 After incubation, 100 μL of dinitrosalicylic acid (DNS) reagent was added to each well. The
196 plate was then incubated at 100°C for 10 minutes to develop the color. The absorbance of the
197 resulting mixture was measured at a wavelength of 540 nm using a Multiplate Reader.
198 Acarbose at various concentrations served as the standard for comparison, and the percentage
199 of α -amylase inhibition was calculated based on the absorbance values obtained from the
200 samples relative to the control wells.

Comment[DS6]: Too long sentences. Make it simple, if the method was mentioned exactly the same as someone else's research before (without modification), just mentioned it.

$$\text{Inhibition of } \alpha\text{-amylase (\%)} = \frac{((Ac - Ac') - (As - As'))}{(Ac' - As')} \times 100$$

201 Where, Ac: Absorbance of the blank (control without any sample or inhibitor), Ac':
 202 Absorbance of the blank control (in the presence of the solvent, e.g., 1% DMSO), As:
 203 Absorbance of the sample (in the presence of the sample extract), As' : Absorbance of the
 204 sample control (sample with solvent but no substrate or enzyme)

205 **2.2.5. Identification of compounds in the active fraction of PC using GC-MS.**

206 GC-MS analysis was performed at the Integrated Laboratory of the Islamic University
 207 of Indonesia, Yogyakarta, to identify the compounds present in the samples. The columns
 208 used in the analysis were Rtx-5MS columns, thickness 0.25 μm , length 30.0 m and diameter
 209 0.25 mm, column temperature 80.0 $^{\circ}\text{C}$, injection temperature 300.00 $^{\circ}\text{C}$, with split injection
 210 mode, pressure 42.3 kPa, total flow 117.5 mL/min and column flow 0.74 mL/min. (Hotmian
 211 et al., 2021b).

212 **2.2.6. Molecular Docking**

213 Molecular docking was conducted using various software tools to analyze the
 214 interactions between the proteins and ligands. The receptors were downloaded from the
 215 Protein Data Bank (PDB) <https://www.rcsb.org/> (Pagadala et al., 2017). Water molecules
 216 surrounding the protein were removed, and the protein chain was separated from its native
 217 ligand and saved in *pdb format as a protein file. The native ligand structure was extracted
 218 by removing the corresponding portion of the protein chain and saved in *pdb format as a
 219 ligand file. The ligand's structure was initially prepared in 2D using ChemDraw Pro 12.0.
 220 (PerkinElmer, 2015), the 3D structure was constructed, and molecular mechanics (MM)
 221 geometry optimization was performed using Chem3D Pro 12.0. The optimized ligand
 222 structure was saved in PDB file format. The protein was prepared using AutoDockTools
 223 1.5.6 (Morris et al., 2009) by adding hydrogen atoms to the polar side of the structure and
 224 applying Kollman charges. The ligand was prepared by correcting its structure and adding
 225 Gasteiger charges. The prepared structures were saved in *pdbqt format. The redocking
 226 process utilized a grid box of dimensions 40 x 40 x 40 with the following coordinates: x =
 227 10.265, y = 45.877, z = 19.734. Docking Parameters Genetic Algorithm (GA), the output
 228 algorithm for docking results, was set to Lamarckian GA 4.2, and other docking parameters
 229 were set to default values. The critical parameter to evaluate the docking results was the
 230 RMSD. An acceptable RMSD value was $\leq 3.0 \text{ \AA}$, indicating reliable docking conformations.

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231

232 2.2.7. Evaluation of Drug Likelihood and ADMET

233 Drug likeness was evaluated based on Lipinski's Rule of Five, a widely used
234 guideline in drug discovery to predict the oral bioavailability of compounds. The following
235 criteria were considered molecular weight: the compound should not exceed 500 Da, the
236 number of hydrogen bond acceptors (-H bond acceptors) should be no more than 10, the
237 number of hydrogen bond donors (-H bond donors) should not exceed 5, the log P value,
238 which indicates the compound's lipophilicity, should be less than 5 (or MlogP < 4.15)
239 (Rahardhian et al., 2022a). ADMET (Absorption, Distribution, Metabolism, Excretion, and
240 Toxicity) properties were predicted using computational methods to further assess the
241 compounds' suitability for drug development. CaCO₂ permeability was assessed to evaluate
242 intestinal absorption. Blood-brain barrier (BBB) permeability was predicted to assess the
243 ability of the compound to cross the BBB. The compound was evaluated as a substrate for
244 CYP2D6, a key enzyme in drug metabolism. Total clearance was calculated to estimate how
245 quickly the compound is eliminated from the body. AMES toxicity tests assessed potential
246 genotoxic effects (D. E. V Pires et al., 2015).

Comment[DS7]: Abbreviation do not put in the first sentence.

247

248 2.3. Data Analysis

249 Statistical analysis was performed using GraphPad Prism 9.0 (GraphPad Software Inc., CA,
250 USA). The results are presented as mean values ± standard deviation (SD). NOVA was
251 applied to evaluate the differences among the various extracts. This method helps determine
252 whether there are statistically significant differences between the means of three or more
253 independent groups. A post hoc test was conducted following ANOVA to identify specific
254 group differences. A significance level of $p > 0.05$ was used to determine statistical
255 significance.

Comment[DS9]: ANOVA?

256

257 3. Results






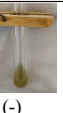






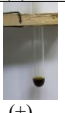



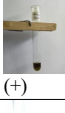

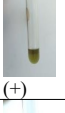
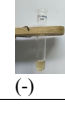
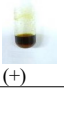
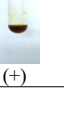
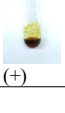
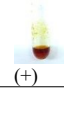
258 3.1. Phytochemical Screening

259 The fractionation of the ethanol extract from PC leaves resulted in the following yields:
260 32.81% for the n-hexane fraction, 16.86% for the ethyl acetate fraction, and 34.17% for the
261 water fraction. Notably, the ethyl acetate fraction yield was lower than that of the n-hexane
262 and water fractions. This variation in yield may be attributed to the differing solubility

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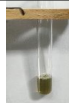







263 profiles of the compounds present in the PC leaf extract, with nonpolar and polar compounds
 264 being more prevalent than semipolar compounds. Phytochemical screening was conducted to
 265 identify various bioactive compounds, including alkaloids, flavonoids, saponins, tannins, and
 266 triterpenoids/steroids. The results indicated that the ethanol extract of PC leaves tested
 267 positive for alkaloids, flavonoids, tannins, saponins, and triterpenoids. In contrast, the water
 268 fraction demonstrated a positive presence only for alkaloids and tannins. The results of the
 269 phytochemical screening indicate that the ethanol extract and fractions of *Peronema*
 270 *canescens* leaves contain various bioactive compounds Table 1.

271 Table 1. Phytochemical Screening Results of Ethanol Extract and Fractions of PC Leaves

Compound Group	Reagent	Ethanol Extract	n-Hexane Fraction	Ethyl Acetate Fraction	Water Fraction
Phenolic	Add 5 to 6 drops of a 1% ferric chloride (FeCl ₃)	 (+)	 (+)	 (+)	 (+)
Tannins	2 mL of a 10% NaCl solution mixed with 1% gelatin	 (+)	 (-)	 (+)	 (+)
Flavonoids	Magnesium powder combined with concentrated HCl and amyl alcohol	 (+)	 (+)	 (+)	 (-)
Alkaloids	Add three drops of Bouchardat's reagent.	 (+)	 (+)	 (+)	 (+)
	Mix 1 mL of 2N HCl with two drops of Mayer's reagent	 (+)	 (+)	 (+)	 (-)
	Mix 1 mL of 2N HCl with two drops of Dragendorff's reagent	 (+)	 (+)	 (+)	 (+)

Comment[DS10]: Give the description of the color or other finish reaction which provide positive reaction. Is there any color changes or precipitation?

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Saponins	Add 10 mL of hot water to 2N HCl				
		(+)	(+)	(+)	(-)
Steroids	Mix 5 mL of chloroform with anhydrous acetic acid and H ₂ SO ₄ .				
		(+)	(+)	(+)	(-)

Description

(+) = Contains the compound

(-) = Does not contain the compound

275

3.2. Thin Layer Chromatography (TLC)

276 The compounds present in the ethanol extract, n-hexane fraction, ethyl acetate fraction, and
 277 water fraction of PC leaves were further analyzed using Thin Layer Chromatography (TLC).
 278 This technique confirmed the presence of various bioactive compounds by evaluating the R_f
 279 values and the corresponding color stains formed on the TLC plates. The R_f values were
 280 calculated for each compound, allowing for the identification of the specific compounds
 281 present in each fraction. The results of the TLC analysis are summarized in Table 2, which
 282 includes the R_f values and corresponding colors observed for each compound group in the
 283 different fractions.

284 From the results of phytochemical screening and TLC tests, PC leaf extracts and fractions
 285 contain several secondary metabolite compounds, including alkaloids, flavonoids, saponins,
 286 tannins, terpenoids, steroids, and phenolics Table 2.

288 Table 2. TLC Results of Ethanol Extract and Fractions of PC Leaves

Compound Group	Mobile Phase	Sample	Visual	UV 254	UV 366	Spot Appearance	R _f Value
Alkaloids	ethyl acetate, methanol, and water (6:4:2)	EE	Brown	Brown	Orange	Brown	0,81
		NHF	Brown	Brown	Orange	Brown	0,79
		EAF	Orange	Brown	Orange	Brown	0,75
		WF	Brown	Brown	Blue	Brown	0,73
Flavonoid	n-butanol, acetic acid, and water (4:1:5)	EE	Green	Green	Orange	Green	0,91
		NHF	Green	Green	Orange	Green	0,91
		EAF	Green	Green	Orange	Green	0,91
		WF	Brown	Green	Blue	Yellow	0,56
Saponin	chloroform, methanol, and water (64:50:10)	EE	Green	Green	Orange	Purple	0,94
		NHF	Green	Green	Orange	Purple	0,94
		EAF	Green	Green	Orange	Green	0,90
		FAE	-	-	-	-	-
Tannins	ethyl acetate,	EE	Green	Black	-	Green	0,88

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	methanol, and water (100:13.5:10)	NHF	Brown	Black	-	Ash	0,94
		FEA	Brown	Black	-	Blackish	0,94
		WF	Brown	Black	-	Black	0,91
Steroids	n-hexane and ethyl acetate (17:3)	FAE	Green	Brown	Blue	Brown	0,91
		FNH	Green	Brown	Blue	Brown	0,91
		FEA	Green	Brown	Orange	Brown	0,22
		FA	-	-	-	Brown	-

289 **Notes:**

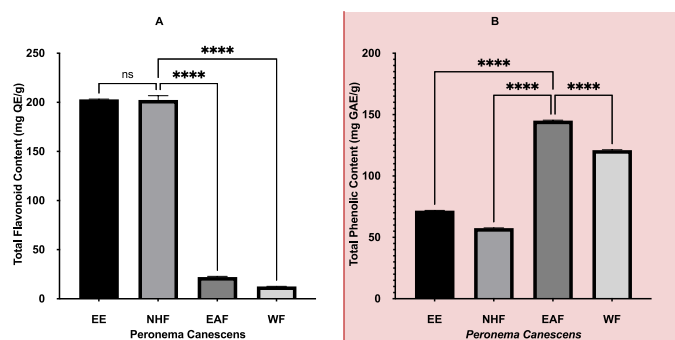
290 (+) = Contains tested compounds

291 (-) = Does not contain the tested compounds

292

293

294 **3.3. Determination of total phenolic and total flavonoid content**



295

296 Figure 1. Total Flavonoid Content (A) and Total Phenolic Content (B). EE: Ethanolic Extract,
297 NHF: n-Hexane Fraction, EAF: Ethyl Acetate Fraction, WF: Water Fraction. ns:
298 Non-significant; ****: Significant difference ($P > 0.05$)

299 Figure 1 shows that the n-hexane fraction of PC leaves exhibits the highest total flavonoid
300 content, with an average concentration of 203.3742 ± 4.3777 mg QE/gram. In contrast, the
301 ethyl acetate fraction of PC leaves contains the highest total phenolic content, with an
302 average concentration of 147.0397 ± 0.7864 mg GAE/gram.

303

304 **3.4. Active Fraction of PC Leaves Using GC-MS**

305 The active fraction was identified using Gas Chromatography-Mass Spectrometry (GC-MS).

306 Based on the analysis of PC leaves' active fraction (ethyl acetate fraction), the compounds
307 identified are listed in Table 3.

Comment[DS11]: *Peronema canescens*. Revision of the binomial nomenclature

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308 Table 3. Results of GC-MS Identification of Active Fraction Compounds in PC Leaves

Peak	Retention Time	Percent Area(%)	Molecular Weight	Base Peak	Similarity Index	Molecular Formula	Compound
1	20,208	1,70	390	149	97	C ₂₄ H ₃₈ O ₄	Bis(2-ethylhexyl) phthalate
2	21,656	0,58	478	57	63	C ₃₂ H ₆₂ O ₂	Myristyl oleate
3	21,942	0,57	593	57	59	C ₃₉ H ₇₆ O ₃	Oleic acid
4	22,825	2,12	288	57	64	C ₂₁ H ₃₆	14-Beta-H-Pregna
5	22,902	8,77	723	57	77	C ₄₅ H ₈₆ O ₆	Tetradecanoic acid
6	23,021	16,46	639	57	75	C ₃₉ H ₇₄ O ₆	Dodecanoic acid
7	23,142	9,25	723	57	68	C ₄₅ H ₈₆ O ₆	Tetradecanoic acid
8	23,255	27,05	751	57	77	C ₄₇ H ₉₀ O ₆	Hexadecanoic acid
9	23,301	19,04	639	57	83	C ₃₉ H ₇₄ O ₆	Dodecanoic acid
10	23,367	14,46	639	183	71	C ₃₉ H ₇₄ O ₆	Dodecanoic acid

Comment[DS12]: Subscript for the number. And also below.

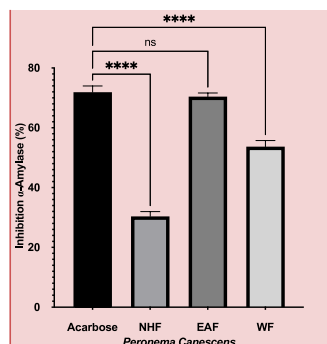
309

310 3.5. In Vitro Inhibition of α -Amylase Activity

311 The α -amylase inhibitory activity of the extract and fractions of PC leaves was evaluated by
 312 measuring the total reducing sugar using the dinitrosalicylic acid (DNS) method, with starch
 313 as the substrate. DNS is an aromatic compound that reacts with reducing sugars to form 3-
 314 amino-5-nitrosalicylic acid, which absorbs electromagnetic radiation. The α -amylase
 315 inhibitory activity was quantified at a wavelength of 540 nm using a 96-well microplate
 316 reader.

Comment[DS13]: Do not put the abbreviation at the first sentence.

Comment[DS14]: Idem.



317

318 Figure 3. Percentage Inhibition of α -Amylase Enzyme Activity. NHF: n-hexane fraction, EAF:
 319 Ethyl acetate fraction, WF: Water fraction, ns: non-significant, ****: Significant
 320 difference ($P > 0.05$).

321 Based on Figure 3, the active fraction with the highest percentage of α -amylase enzyme
 322 inhibition was observed in the ethyl acetate fraction of PC leaves, with an average inhibition
 323 of $70.38 \pm 1.26\%$.

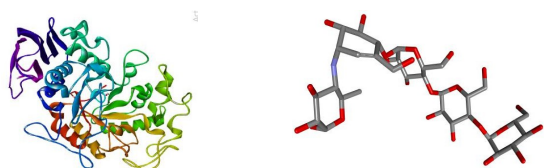
324

325 3.6. In Silico Inhibition of α -Amylase Activity

326 Molecular docking is a commonly used method in drug development to identify compounds
 327 or molecules with therapeutic activity by predicting ligand-target interactions and evaluating
 328 structural activity using computational techniques (Rahardhian et al., 2022). The validation
 329 results showed an RMSD value of 1.74 \AA , indicating that the redocking method met the
 330 required criteria. Furthermore, the binding energy affinity (ΔG) was found to be -4.17
 331 kcal/mol, with an inhibition constant (K_i) of the same magnitude obtained during the fourth
 332 docking run.

333

334



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A

B

335 Figure 4. The three-dimensional structure of the α -amylase enzyme (A) and its natural ligands
336 (B)

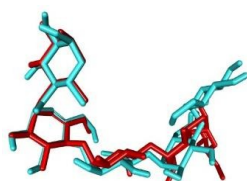


Figure 5. The overlay of the acarbose confirmation before (blue) and after (red) redocking

337 Table 4. Results of Molecular Docking Analysis of Ethyl Acetate Fraction Compounds of PC
338 Leaves

No	Compound	ΔG (kcal/mol)	Ki (mM)	Amino acids Residue
1	Bis(2-ethylhexyl) phthalate	-4.59	432.20	Tyr 62, Leu 162, His 201, Lys 200, Ile 235, His 299
2	Myristyl oleate	-3.32	3.66	Ile 51, Leu 165, Pro 54, His 101, Trp 59, His 305, Tyr 62, Asp 300
3	14 Beta H pregna	-7.81	1.90	Tent 162, Tent 165, Trp 58, Trp 62, His 299
4	Natural ligand (Acarbose)	-4,17	-	The 163, His 305, Asp 300, Glu 233, His 101, Arg 195, Asp 197, His 299

339 Based on Table 4, three bioactive compounds exhibit favorable binding energies and amino
340 acid interactions with the same target protein as the natural ligands. The binding energy
341 values reflect the spontaneity of the interaction between the protein and the ligand; the more
342 negative the binding energy, the more rapid the binding process occurs. This suggests that
343 these compounds may serve as effective α -amylase inhibitors, warranting further
344 investigation into their potential therapeutic applications.

345 Table 5. Results of ADME Analysis of Active Fraction Compounds of PC Leaves

No	Compound	Absorption (Caco2 permeability) (log Papp in y) 10 ⁶ cm/s)	Distribution (BBB permeability) (log BB)	Metabolism (CYP2D6 substrate) (Yes/No)	Excretion (Total clearance) ml/min/kg)	Toxicity (AMES) (log (Yes/No))
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1	Bis(2-ethylhexyl) phthalate	1,408	-0,175	No	1,898	No
2	Myristyl oleate	1,306	1,014	No	2,149	No
3	14 Beta H pregna	1,405	0,885	No	0,67	No

346 Table 5 presents the results of the ADME (Absorption, Distribution, Metabolism, and
 347 Excretion) analysis for the bioactive compounds identified in the active fraction of PC leaves.
 348 This analysis is crucial for evaluating the pharmacokinetic properties of these compounds,
 349 which can influence their effectiveness as potential therapeutic agents. The results provide
 350 insights into the compounds' absorption rates, distribution patterns in the body, metabolic
 351 stability, and excretion profiles, helping to assess their suitability for drug development. The
 352 ADME (Absorption, Distribution, Metabolism, Excretion) and toxicity profiles of the
 353 bioactive compounds identified in the active fraction of PC leaves. The analysis indicates that
 354 the bioactive compounds Bis(2-ethylhexyl) phthalate, Myristyl oleate, and 14 Beta H pregna
 355 meet the criteria for a favorable ADME profile, suggesting efficient absorption and
 356 distribution within the body.

357 Table 6. Results of the Lipinski analysis for the bioactive compounds found in the active

No	Compound	Molecular weight	Log P	Donor hydrogen bonding	Hydrogen acceptor bonding	Surface area
1	Bis(2-ethylhexyl) phthalate	390,564	6,433	0	4	170,550
2	Myristyl oleate	478,846	112,682	0	2	214,638
6	14 Beta H pregna	274,492	60,553	0	0	125,650

358 fraction of PC leaves

359

360 Table 6 presents the results of the Lipinski analysis for the bioactive compounds found in the
 361 active fraction of PC leaves. Lipinski's rule of five evaluates the drug-likeness of compounds
 362 based on their molecular properties, which predict good absorption and permeation. The
 363 analysis assesses molecular weight, hydrogen bond donors and acceptors, and logP values.
 364 Compounds that comply with these criteria will likely exhibit favorable pharmacokinetic
 365 properties, making them suitable candidates for further drug development. The bioactive
 366 compounds in the active fraction of PC leaves meet Lipinski's rule of five requirements.

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367 Specifically, these compounds exhibit a molecular weight of less than 500 mg/mol, a logP
368 value between 0.4 and 5, a hydrogen bond donor count of ≤ 10 , a hydrogen bond acceptor
369 count of ≤ 5 , and a molar refractivity below 130. Consequently, it can be concluded that these
370 bioactive compounds are considered safe for oral use.

371

372 **4. Discussion**

373 The fractionation of the ethanol extract from PC leaves yielded 32.81% for the n-hexane
374 fraction, 16.86% for the ethyl acetate fraction, and 34.17% for the water fraction. The lower
375 yield of the ethyl acetate fraction indicates a higher abundance of nonpolar and polar
376 compounds relative to semipolar compounds in the extract (Duistermaat and Kolk, 2000) .
377 This pattern reflects the principles of solvent polarity, where nonpolar solvents like n-hexane
378 extract nonpolar compounds such as lipids, while polar solvents like water preferentially
379 extract polar substances like sugars and certain phenolic compounds(Altemimi et al., 2017).

380 Phytochemical screening revealed alkaloids, flavonoids, tannins, saponins, and
381 triterpenoids/steroids in the ethanol extract. However, the water fraction was only positive for
382 alkaloids and tannins. This selective presence underscores the solubility characteristics of the
383 compounds, with flavonoids and triterpenoids being more soluble in ethyl acetate, explaining
384 their absence in the water fraction. The limited presence of flavonoids and triterpenoids in the
385 water fraction of PC leaves aligns with other studies on medicinal plants, suggesting that these
386 compounds are more concentrated in less polar solvents. The phytochemical screening of the
387 ethanol extract from PC leaves, along with its fractions, revealed a diverse array of bioactive
388 compounds. The tests utilized various reagents to identify specific compound groups,
389 including phenolics, tannins, flavonoids, alkaloids, saponins, and steroids. This suggests that
390 phenolics, The research results are consistent with (Rahardhian et al., 2022c), indicating that
391 PC contains phenolic. The presence of phenolic compounds was confirmed in all fractions,
392 including the ethanol extract, n-hexane, ethyl acetate, and water fractions, as indicated by the
393 positive reaction with ferric chloride. Tannins were present in the ethanol extract and water
394 fraction but absent in the n-hexane fraction. This distribution indicates that tannins, which are
395 polyphenolic compounds with astringent properties, are more soluble in polar solvents like
396 water and ethanol. The screening indicated flavonoids were present in the ethanol, n-hexane,
397 and ethyl acetate fractions but absent in the water fraction. This result highlights the
398 preferential solubility of flavonoids in less polar solvents, supporting findings in other studies

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399 that suggest flavonoids are typically more concentrated in ethyl acetate and hexane fractions.
400 All fractions tested positive for alkaloids, with multiple reagents confirming their presence.
401 Saponins were present in the ethanol, n-hexane, and ethyl acetate fractions but absent in the
402 water fraction. This suggests that while saponins can dissolve in polar solvents, they may
403 require a particular concentration or temperature to effectively extract, as indicated by their
404 absence in the water fraction after the hot water test. Steroids were found in the ethanol, n-
405 hexane, and ethyl acetate fractions but not in the water fraction. This aligns with their
406 chemical nature, as these compounds are generally more soluble in nonpolar or moderately
407 polar solvents. The phytochemical screening demonstrates that PC leaves are rich in bioactive
408 compounds across different fractions. The solubility profiles of these compounds indicate
409 their potential utility in therapeutic applications, as well as the need for further research to
410 explore their pharmacological properties and mechanisms of action (Suryanti et al., 2022).

411 The thin-layer chromatography (TLC) analysis of PC leaves provides a comprehensive
412 overview of its bioactive compound profile, including alkaloids, flavonoids, saponins, tannins,
413 and steroids (Ladeska et al., 2024). The visualizations and Rf values obtained during the
414 analysis allow for a comparative understanding of the composition and potential therapeutic
415 applications of these compounds (Yodha et al., 2024). The presence of alkaloids was
416 confirmed across all samples, with Rf values ranging from 0.73 to 0.81. The consistent brown
417 coloration under UV light and the appearance of orange spots following Dragendorff's
418 reagent application suggest a robust alkaloid profile in PC leaves. These findings align with
419 research (Li et al., 2020), which noted that alkaloids often exhibit similar Rf values due to
420 their polar characteristics, influencing their solubility and mobility in chromatographic
421 conditions. Alkaloids are recognized for their pharmacological activities, including analgesic
422 and antimicrobial properties, reinforcing the potential medicinal value of PC. Flavonoids
423 displayed Rf values of 0.56 to 0.91, with the ethanol, n-hexane, and ethyl acetate fractions
424 exhibiting a distinct green coloration. The yellow stains after ammonia treatment are
425 consistent with flavonoid presence, as supported by studies like those of (Panche et al., 2016)
426 which indicate that the coloration is indicative of flavonoid derivatives. The consistent Rf
427 values across the fractions suggest that PC leaves contain a diverse range of flavonoids, which
428 may enhance their therapeutic profile. The analysis showed a strong presence of saponins,
429 with Rf values around 0.90 to 0.94 and vibrant colored patches indicating their presence. This
430 finding corroborates previous studies, such as those by (Cheok et al., 2014). Tannins were

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431 identified with Rf values of 0.88 to 0.94, displaying green-blackish spots after FeCl₃ treatment.
432 This aligns with findings from (Das et al., 2020) , which suggest that tannins' astringent
433 properties and their ability to form complexes with proteins are responsible for their
434 bioactivity. The presence of tannins in PC leaves may contribute to their potential health
435 benefits, particularly in antioxidant and antimicrobial applications. Steroids were detected,
436 with Rf values of 0.22 to 0.91. The purple color indicating steroids and the blue for
437 triterpenoids under UV light corresponds with findings from (Tarigan et al., 2023), which
438 report that these compounds have significant roles in various biological activities, including
439 anti-inflammatory effects. The varying Rf values suggest differing solubility characteristics
440 among the compounds, indicating the complexity of the steroidal profile in PC leaves.

441 The n-hexane fraction demonstrated the highest total flavonoid content, averaging
442 203.3742 ± 4.3777 mg QE/gram. The predominance of flavonoids in the n-hexane fraction
443 suggests that these compounds have a higher solubility in nonpolar solvents(Putri et al., 2023).
444 This aligns with findings from several studies indicating that nonpolar extraction methods are
445 effective for isolating lipophilic flavonoid compounds. For instance, research by (Putri et al.,
446 2022) supports the idea that nonpolar solvents can enhance the extraction of certain flavonoid
447 derivatives, potentially improving their bioavailability and pharmacological efficacy. In
448 contrast, the ethyl acetate fraction exhibited the highest total phenolic content, averaging
449 147.0397 ± 0.7864 mg GAE/gram. Phenolic compounds are well-known for their potent
450 antioxidant properties, contributing to the plant's protective mechanisms against oxidative
451 stress. The higher concentration of phenolics in the ethyl acetate fraction indicates their
452 preferential solubility in moderately polar solvents, which has been corroborated by studies
453 such as those by (Rahardhian et al., 2019). These findings highlight the effectiveness of ethyl
454 acetate in extracting bioactive phenolic compounds, which may have implications for their
455 therapeutic applications in treating oxidative stress-related diseases. The observed differences
456 in flavonoid and phenolic concentrations between the two solvent fractions emphasize the
457 importance of solvent polarity in the extraction process. This selective extraction allows for a
458 deeper understanding of the phytochemical profile of PC leaves, which can guide further
459 research into their potential health benefits. Notably, the high total flavonoid content in the n-
460 hexane fraction suggests that PC may be a valuable source of flavonoid-rich extracts that
461 could be utilized in nutraceutical formulations targeting antihyperglycemic.

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463 The gas chromatography-mass spectrometry (GC-MS) analysis of PC leaves provided
464 valuable insights into the chemical composition of its extracts, revealing a range of fatty acids
465 and esters. Each compound's retention time, molecular weight, and similarity index contribute
466 to understanding the plant's phytochemical profile and its potential applications in health and
467 nutrition (Hotmian et al., 2021a). Among the identified compounds, notable fatty acids
468 include tetradecanoic acid ($C_{14}H_{28}O_2$) and hexadecanoic acid ($C_{16}H_{32}O_2$), which accounted for
469 significant peak areas of 8.77% and 27.05%, respectively. The predominance of hexadecanoic
470 acid is particularly interesting, as it has been associated with various health benefits, including
471 anti-inflammatory and antimicrobial properties. This aligns with research indicating that fatty
472 acids play crucial roles in cellular functions and may cause diabetes mellitus diseases (Berry,
473 1997). The presence of bis(2-ethylhexyl) phthalate ($C_{24}H_{38}O_4$), with a peak area of 1.70%,
474 indicates the plant's potential to contain plasticizer compounds, which can have implications
475 for environmental health. Additionally, oleic acid ($C_{18}H_{34}O_2$), which appeared in the analysis,
476 is a well-known monounsaturated fatty acid praised for its heart health benefits, including its
477 ability to lower harmful cholesterol levels (Eleazu et al., 2018). The molecular weight of oleic
478 acid (593) and its retention time further suggests its bioactive potential. The diversity of
479 compounds, including myristyl oleate ($C_{32}H_{62}O_2$) and 14-Beta-H-Pregna ($C_{21}H_{36}$), with
480 respective peak areas of 0.58% and 2.12%, points to the complex nature of the phytochemical
481 constituents of PC. The variety of fatty acids and esters indicates that the plant could serve as
482 a source of bioactive compounds with diverse pharmacological activities, ranging from anti
483 diabetic mellitus to antioxidant effects. The similarity index values, ranging from 57 to 97,
484 highlight the reliability of the GC-MS identification process, reflecting the compounds'
485 structural integrity and known bioactivities. The higher similarity indices suggest that the
486 identified compounds are well-documented in the literature, supporting their potential
487 utilization in therapeutic applications. In summary, the GC-MS analysis of PC leaves reveals
488 a rich array of fatty acids and esters, emphasizing the plant's potential as a source of bioactive
489 compounds

490 The findings from this study reveal significant insights into the α -amylase inhibitory
491 potential of PC, particularly highlighting the efficacy of the ethyl acetate fraction. The
492 observed average inhibition percentage of 70.38% for the ethyl acetate fraction is notably
493 higher than the 30.37% and 53.18% inhibition observed in the n-hexane and water fractions,
494 respectively. This variation in inhibitory activity suggests a differential distribution of

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495 bioactive compounds in the various fractions, likely attributable to their solubility properties
496 and polarity. The ethyl acetate fraction's superior inhibition effect is particularly noteworthy,
497 as it closely approaches the inhibition level of acarbose, a well-known α -amylase inhibitor,
498 which demonstrated an average inhibition percentage of 74.29%. The lack of significant
499 difference between the ethyl acetate fraction and acarbose indicates that the compounds
500 present in this fraction possess potent α -amylase inhibitory activity. This finding is consistent
501 with existing literature, which suggests that ethyl acetate often extracts a variety of bioactive
502 compounds, such as flavonoids and phenolic compounds, that are known for their enzyme-
503 inhibitory effects (Nyambe-Silavwe et al., 2015). The lower inhibition percentages observed
504 in the n-hexane fraction may be attributed to the nonpolar nature of the compounds extracted,
505 which are less likely to interact effectively with the active site of the α -amylase enzyme.
506 Conversely, the water fraction exhibited moderate inhibition, possibly due to the presence of
507 polar compounds, but it was less effective than the ethyl acetate fraction, highlighting the
508 significance of solvent polarity in influencing the extraction of bioactive constituents. This
509 study demonstrates that the ethyl acetate fraction of PC exhibits significant α -amylase
510 inhibitory potential, comparable to acarbose, suggesting its potential as a natural alternative
511 for diabetes management and warranting further research into its pharmacological
512 applications (Lestari et al., 2018).

513 The molecular docking results presented in this study provide critical insights into the
514 binding affinities and inhibitory potentials of various compounds against α -amylase (Lolok et
515 al., 2022). The free energy of binding (ΔG) and inhibition constants (K_i) serve as valuable
516 indicators of the interactions between these compounds and the enzyme (Yuningtyas et al.,
517 2024), shedding light on their potential as α -amylase inhibitors. Bis(2-ethylhexyl) phthalate
518 exhibited a binding free energy of -4.59 kcal/mol with an inhibition constant (K_i) of 432.20
519 mM. The interaction of this compound with the enzyme involves critical amino acid residues
520 such as Tyr 62, Leu 162, and His 201. While the negative ΔG value indicates a favorable
521 binding, the relatively high K_i value suggests that this compound may not be a potent
522 inhibitor of α -amylase. The interactions with critical residues indicate a potential for activity,
523 but further modifications may be needed to enhance its inhibitory potency. Myristyl oleate
524 showed a lower binding affinity, with ΔG of -3.32 kcal/mol and a K_i value of 3.66 mM. The
525 presence of interactions with multiple residues, including Ile 51, Leu 165, and His 101,
526 suggests that this compound forms multiple favorable contacts with the enzyme. The

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527 significantly lower K_i indicates that myristyl oleate could act as a more potent inhibitor
528 compared to bis(2-ethylhexyl) phthalate, possibly due to its ability to engage critical active
529 site residues effectively. 14 Beta H-pregna demonstrated the strongest binding affinity with a
530 ΔG of -7.81 kcal/mol and a low K_i of 1.90 mM. The strong interactions with amino acids such
531 as Trp 58 and Trp 62 indicate that this compound may have a significant impact on the active
532 site configuration of α -amylase. The natural ligand (acarbose), known for its clinical efficacy
533 in inhibiting α -amylase, had a ΔG of -4.17 kcal/mol but did not present a K_i value as this
534 compound acts through a different mechanism. The amino acid residues involved in binding,
535 such as The 163 and His 305, reflect the established interactions that allow acarbose to inhibit
536 the enzyme competitively. This comparison highlights the relative effectiveness of the newly
537 identified compounds against a standard inhibitor, demonstrating that certain derivatives may
538 offer similar or improved efficacy.

539 The physicochemical properties of the compounds derived from PC were evaluated
540 based on their molecular weight, lipophilicity (Log P), hydrogen bonding capacity, and
541 surface area. Understanding these characteristics is critical for assessing their bioavailability,
542 interactions, and overall pharmacological potential (Pires et al., 2015). Absorption of Bis(2-
543 ethylhexyl) phthalate exhibited an absorption value of 1.408 (log Papp in 10⁻⁶ cm/s),
544 indicating a relatively favorable permeability across the intestinal barrier. This suggests that it
545 may be effectively absorbed after oral administration. Myristyl oleate, with a slightly lower
546 absorption value of 1.306, also shows good permeability, although it is less than that of bis(2-
547 ethylhexyl) phthalate. 14 Beta H-pregna displayed an absorption value similar to bis(2-
548 ethylhexyl) phthalate (1.405), indicating its potential for adequate oral bioavailability. Overall,
549 the absorption profiles of these compounds suggest they may achieve adequate systemic
550 exposure following administration. Distribution of Myristyl oleate had the highest distribution
551 coefficient (log BB = 1.014), indicating a favorable ability to cross the blood-brain barrier
552 (BBB). This characteristic may allow for central nervous system (CNS) activity, potentially
553 making it useful for conditions where CNS targeting is desired. In contrast, bis(2-ethylhexyl)
554 phthalate showed a negative log BB value (-0.175), indicating limited distribution to the brain.
555 14 Beta H-pregna also demonstrated a positive log BB value (0.885), suggesting moderate
556 ability to penetrate the BBB. These distribution characteristics are vital when considering the
557 therapeutic application of these compounds, particularly in relation to CNS effects.
558 Metabolism All three compounds were determined not to be substrates for CYP2D6,

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559 suggesting they are less likely to be metabolized through this common liver enzyme. This
560 could imply a potentially favorable metabolic profile, as compounds that are not extensively
561 metabolized may maintain higher bioavailability. Excretion clearance rates provide insights
562 into the elimination of these compounds from the body. Myristyl oleate had the highest total
563 clearance (2.149 log ml/min/kg), suggesting it may be rapidly eliminated, which could
564 influence dosing regimens. Conversely, 14 Beta H-pregna exhibited a significantly lower
565 clearance rate (0.67 logs ml/min/kg), indicating a slower elimination and potentially more
566 prolonged duration of action. Notably, none of the compounds tested positive for
567 mutagenicity in the Ames test, indicating a favorable toxicity profile. This is an encouraging
568 finding, as it suggests these compounds may have a lower risk of causing genetic damage.

569 Bis(2-ethylhexyl) phthalate (MW = 390.564 g/mol) and Myristyl oleate (MW = 478.846
570 g/mol) are relatively large molecules, which may influence their absorption and distribution in
571 biological systems. Larger molecules tend to exhibit lower permeability through biological
572 membranes, which could affect their therapeutic efficacy. In contrast, 14 Beta H-pregna has a
573 lower molecular weight (MW = 274.492 g/mol), potentially facilitating better absorption and
574 distribution, as smaller compounds typically diffuse more easily through cell membranes. The
575 Log P values provide insights into the hydrophobicity of the compounds. Myristyl oleate
576 exhibits an extraordinarily high Log P value (112.682), indicating its lipophilic solid nature,
577 which could enhance its ability to cross lipid membranes. However, such high lipophilicity
578 may also lead to challenges in solubility in aqueous environments, potentially limiting its
579 bioavailability when administered orally. Bis(2-ethylhexyl) phthalate has a significant Log P
580 value of 6.433, also reflecting its lipophilicity and suggesting a potential for membrane
581 permeation. Conversely, 14 Beta H-pregna has a Log P of 60.553, which is comparatively
582 lower than the other two compounds but still suggests a reasonable level of lipophilicity. The
583 varied lipophilicity of these compounds indicates that their absorption and distribution will
584 differ significantly, influencing their pharmacological activities. None of the compounds
585 displayed any donor hydrogen bonding, indicating that they may not participate in hydrogen
586 bond donation, which could limit their interactions with target proteins or enzymes. This
587 property could influence the compounds' binding affinities and their overall efficacy in
588 biological systems. The hydrogen acceptor bonding also varied among the compounds. Bis(2-
589 ethylhexyl) phthalate exhibited four acceptor sites, while Myristyl oleate had two, and 14 Beta
590 H-pregna had none. The presence of hydrogen bond acceptors in bis(2-ethylhexyl) phthalate

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591 could facilitate its interaction with biomolecules, enhancing its potential activity. On the other
592 hand, the lack of hydrogen bonding capacity in 14 Beta H-pregna may limit its interactions
593 with proteins, potentially reducing its efficacy. Surface area is an important parameter that can
594 influence drug absorption and permeability. Myristyl oleate has the largest surface area
595 (214.638 Å²), which might affect its interactions with biological membranes and potential
596 targets. The larger surface area could facilitate a more significant interaction with the lipid
597 bilayer, impacting absorption rates. Bis(2-ethylhexyl) phthalate has a moderate surface area
598 (170.550 Å²), while 14 Beta H-pregna has the smallest surface area (125.650 Å²). The smaller
599 surface area of 14 Beta H-pregna could correlate with its lower molecular weight, potentially
600 leading to better permeability.

601

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Exploring the α -Amylase Inhibitory Potential of *Peronema canescens* Jack: An *In Vitro* and *In Silico* Study

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ABSTRACT

Hyperglycemia in individuals with type 2 diabetes mellitus is primarily driven by the rapid hydrolysis of starch by the enzyme α -amylase in the pancreas and the breakdown of oligosaccharides by α -glucosidase in the intestine. *Peronema canescens* Jack. (PC) has shown promise as a potential antidiabetic agent. This study aimed to evaluate the total flavonoid, phenolic, and α -amylase inhibitory activity of extracts and fractions derived from PC leaves using both *in vitro* and *in silico* approaches. The ethanol extract of PC leaves was fractionated through liquid-liquid extraction using *n*-hexane, ethyl acetate, and water as solvents. Preliminary phytochemical screening of the extracts and fractions identified the presence of alkaloids, flavonoids, saponins, tannins, and steroids/triterpenoids. The *n*-hexane fraction exhibited the highest total flavonoid content, averaging 203.37 \pm 4.38 mg QE/gram, while the ethyl acetate fraction demonstrated the highest total phenolic content, averaging 147.04 \pm 0.79 mg GAE/gram. Furthermore, the ethyl acetate fraction showed the strongest α -amylase inhibitory activity, with an average inhibition rate of 70.38 \pm 1.26%. *In silico* analysis, combined with GC-MS identification, suggested that three compounds, bis(2-ethylhexyl) phthalate, myristyl oleate, and 14 beta H-pregna may contribute to the observed α -amylase inhibitory activity. These findings highlight the potential of PC as a source of natural antidiabetic agents.



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1. Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both (WHO 2023). The prevalence of diabetes has reached alarming levels globally, with the International Diabetes Federation (IDF) estimating that over 537 million adults will be living with the condition in 2021 (Webber 2013). Experts project that this number will rise to 643 million by 2030. In Indonesia, the situation mirrors this global

trend, with an increasing number of the population being diagnosed with diabetes. The current standard of care for managing diabetes includes synthetic drugs such as sulfonylureas, metformin, and thiazolidinediones, designed to enhance insulin sensitivity, stimulate insulin secretion, or reduce glucose production. However, these synthetic drugs are not without their limitations. Long-term use of these medications can lead to adverse effects such as hypoglycemia (Chaudhury *et al.* 2017), gastrointestinal issues, and even multidrug resistance (MDR). These limitations underscore the need for alternative treatment strategies that are both effective and safe.

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In light of the challenges associated with synthetic drugs, there has been growing interest in herbal medicine as a potential alternative or complementary approach to diabetes management. Plants rich in bioactive compounds provide herbal medicines with therapeutic benefits and fewer side effects (Tran *et al.* 2020). One such plant is *Peronema canescens* Jack. (PC), a species native to Indonesia, traditionally used for its medicinal properties (Rahardhian *et al.* 2022b). The secondary metabolites found in PC, such as flavonoids, tannins, and alkaloids (Primal and Ahriyasna 2022), have been shown to possess various pharmacological activities, including antidiabetic effects. These natural compounds may help to mitigate hyperglycemia by enhancing insulin sensitivity, inhibiting glucose absorption, and modulating carbohydrate metabolism, making PC a promising candidate for diabetes treatment.

Computer-aided drug design (CADD) advancements have provided researchers with powerful tools to identify and optimize potential antidiabetic agents (Meng *et al.* 2011). Molecular docking simulation, a key component of CADD, allows for predicting the interaction between small molecules and target proteins, thereby facilitating the identification of compounds with high binding affinities (Pagadala *et al.* 2017). This method has been beneficial in the search for natural inhibitors of enzymes such as α -amylase, which plays a crucial role in carbohydrate digestion and glucose absorption (de Sales *et al.* 2012). Researchers can rapidly screen and identify the most promising antidiabetic compounds within complex plant extracts, such as those found in PC, by molecular docking simulations (Khan *et al.* 2024).

This study focuses on the antidiabetic potential of PC, examining both its *in vitro* and *in silico* activities. Gas Chromatography-Mass Spectrometry (GC-MS) analysis identified PC's chemical composition and revealed various bioactive compounds (Hotmian *et al.* 2021a). The inhibitory effect of these compounds on α -amylase was then evaluated through *in vitro* assays, while molecular docking simulations were employed to understand the interaction between these compounds and the enzyme at the molecular level (Santos *et al.* 2019). The physicochemical properties of PC were also analyzed and compared with other herbal medicines to highlight its unique advantages. This comprehensive approach sheds light on the potential of PC as an antidiabetic agent and underscores its benefits over other herbal remedies (Posadzki *et al.* 2013).

The novelty of this study lies in its integrative approach, combining *in vitro* and *in silico* methods to

investigate the α -amylase inhibitory potential of PC. While previous research has explored the antidiabetic effects of various herbal medicines, studies focusing specifically on PC remain limited. By providing a detailed analysis of its bioactive compounds and their mechanisms of action, this study serves as a preliminary investigation that could pave the way for future research (Altemimi *et al.* 2017). The findings presented here are relevant for developing PC as a potential antidiabetic treatment but also offer valuable insights for researchers working on similar objectives, making this study a significant consideration for ongoing and future developments in the field.

This study aims to evaluate the α -amylase inhibitory activity of PC using both *in vitro* and *in silico* methods. This research seeks to identify the specific compounds responsible for this activity and to understand their mechanisms of action at the molecular level. This research will contribute to the growing knowledge of natural antidiabetic agents and support PC development as a potential treatment for diabetes mellitus.

2. Materials and Methods

2.1. Materials

The equipment used in this study includes a Pyrex separation funnel, a Heidolph-G3 rotary evaporator, Silica Gel F254 plates, UV 254 and 366 lamps (Evaco GL 220V 50Hz T8 15W), micropipettes (Socorex and Dragon Lab), vortex mixers, a Shimadzu UV-1780 UV-Vis spectrophotometer (Serial No. A119161), a Synergy-HTX multi-mode ELISA reader with 96-well plates, and a QP 2010 gas chromatography-mass spectrometer (GC-MS). The materials utilized in this study include *Peronema canescens* Jack., ethanol, *n*-hexane, ethyl acetate, FeCl₃, MgSO₄, hydrochloric-ethanolic acid mixture (1:1), hydrochloric acid, Lieberman-Burchard reagent, 2,2-diphenyl-1-picrylhydrazyl (Sigma), quercetin, methanol, phosphate buffer, alpha-amylase enzyme (Sigma Aldrich), starch, and 3,5-dinitrosalicylic acid.

2.1.1. Hardware and Software

The molecular docking study was conducted using a laptop equipped with an Asus ROG 503 VD. The software utilized in this study includes ChemDraw Professional 15.0, Chem3D 15.0, Biovia Discovery Studio 2021, Command Prompt, and AutoDock Tools-1.5.6. Visualization of docking results and the creation of ligands and receptors were performed using

Biovia Discovery Studio 2021. Receptor structures were obtained from the RCSB Protein Data Bank (PDB) website (<https://www.rcsb.org/>), while ligand structures were downloaded from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>). Lipinski's rule of five was applied to assess drug-likeness using the SCFBio website (<http://www.scfbio-iitd.res.in/software/drugdesign/lipinski.jsp>). The pk-CSM online tool evaluated Pharmacokinetic and toxicological properties (<https://biosig.lab.uq.edu.au/pkcsfm/>).

2.2. Methods

2.2.1. Sample Preparation, Extraction, and Fractionation

The extraction of *Peronema canescens*. Jack (PC) used the maceration method with 96% ethanol as the solvent. Three hundred grams of PC was placed in a maceration jar, and 96% ethanol was added as the solvent. The maceration process was performed for three days, with periodic solvent changes and occasional stirring to enhance extraction efficiency. The resulting macerate was filtered and then concentrated using a rotary vacuum evaporator. The concentrated extract was further thickened using a water bath at approximately 50°C (Rahardhian *et al.* 2019). Ten grams of the ethanol extract of PC leaves were dissolved in 100 ml of water and transferred into a separatory funnel. The mixture was then partitioned by adding 100 ml of *n*-hexane, followed by vigorous shaking. The mixture was allowed to settle until two distinct phases formed: the aqueous and *n*-hexane. The aqueous phase, separated from the *n*-hexane phase, was reintroduced into the separatory funnel. Subsequently, 100 ml of ethyl acetate was added to the aqueous phase. The mixture was shaken several times to ensure thorough mixing and then allowed to separate into its respective phases. The resulting fractions were concentrated using a rotary vacuum evaporator and thickened using a water bath at approximately 50°C to obtain a viscous fraction. (Rahardhian *et al.* 2019).

2.2.2. Phytochemical screening and TLC

Phytochemical screening follows the method (Putri *et al.* 2022). Each sample, including the ethanol extract, *n*-hexane fraction, ethyl acetate fraction, and water fraction of PC, was prepared by dissolving 30 mg of the sample in 3 ml of ethanol until completely dissolved. This solution was used for the subsequent phytochemical screening tests. Flavonoids: To the sample solution, magnesium (Mg) powder, 1 ml of hydrochloric acid (HCl), and amyl alcohol were added. The formation

of red, yellow, or orange colors indicated a positive presence of flavonoids. Polyphenols: A 10% ferric chloride (FeCl₃) solution was added to the sample. The appearance of a blue or blackish-green color indicated the presence of polyphenols. Tannins: The sample was mixed with gelatin salts. The formation of a yellowish-white precipitate indicated a positive result for tannins. Alkaloids: The sample was treated with HCl and water, then heated and divided into two portions (Filtrate 1 and Filtrate 2). Filtrate 1: Reacted with Mayer's reagent, forming a yellowish-white precipitate, indicating the presence of alkaloids. Filtrate 2: Reacted with Dragendorff's reagent, forming a brick-red precipitate, confirming the presence of alkaloids. Saponins: The sample was placed in a test tube, and 10 ml of distilled water was added. The mixture was shaken vigorously for 10 seconds. A positive reaction was indicated by the formation of foam that persisted for 10 minutes at a height of 1-3 cm. Steroids/triterpenoids: The sample was treated with chloroform and filtered. The filtrate was mixed with anhydrous acetic acid (CH₃COOH) and heated, then cooled and treated with sulfuric acid (H₂SO₄). A green color in the solution indicated the presence of steroids, while an orange or red color indicated the presence of triterpenoids.

Thin-layer chromatography (TLC) was performed following the method described by (Putri *et al.* 2022) to identify the compound content of the samples. A small amount of each sample was dripped directly onto the TLC plate. Flavonoids: The mobile phase consisted of *n*-butanol, acetic acid, and water in a ratio of 4:1:5. The TLC plate was then exposed to ammonia vapor. The presence of flavonoids was indicated by the formation of yellow stains after ammonia treatment. Tannins: The mobile phase was prepared using ethyl acetate, methanol, and water in a ratio of 100:13.5:10. The presence of tannins was indicated by visible spots formed by FeCl₃, which resulted in blackish-green patches. Alkaloids: The mobile phase comprised ethyl acetate, methanol, and water in a ratio of 6:4:2. The presence of alkaloids was confirmed by the appearance of brown patches upon applying Dragendorff's reagent. Saponins, A solvent system of chloroform, methanol, and water in a ratio of 64:50:10, was utilized. Anisaldehyde-sulfuric acid was used as a visualization reagent, and the TLC plate was heated on a hot plate for 5-10 minutes at 100°C. The presence of saponins was indicated by the formation of colored patches, including yellow, green, red, dark blue, purple, and brownish-yellow. Steroids/triterpenoids: The mobile phase consisted of *n*-hexane and ethyl acetate

in a ratio of 17:3. Anisaldehyde-sulfuric acid was again used for visualization, and the TLC plate was heated on a hot plate for 5-10 minutes at 100°C. A purple color indicated the presence of steroids, while a blue color indicated triterpenoids.

2.2.3. Determination of Total Phenolic and Total Flavonoid Content

The total flavonoid content (TFC) and total phenolic content (TPC) of the extracts were determined using established spectrophotometric methods (Rahardhian *et al.* 2019). TPC was assessed using the Folin-Ciocalteu reagent with gallic acid as the standard, and absorbance was measured at 765 nm. TFC was determined using aluminum chloride (AlCl₃) with quercetin as the standard, and absorbance was measured at 420 nm. Both analyses were conducted using a Shimadzu® UV-Vis Spectrophotometer (Model 1240). The total phenolic and flavonoid contents were calculated based on the absorbance values obtained from the gallic acid and quercetin standard curves, respectively.

2.2.4. α -amylase Inhibitory Activity

The α -amylase inhibitory activity of the samples was evaluated using a colorimetric method on a 96-well plate. The samples were dissolved in a 1% dimethyl sulfoxide (DMSO) solution. Each sample, along with the negative control (1% DMSO solution) and positive control (acarbose at concentrations of 50 mg and 100 mg), was pipetted into the wells of a 96-well plate at a volume of 20 μ L per well. 50 μ L of 100 mM phosphate buffer (pH 6.8) was added to each well, followed by ten μ L of α -amylase solution (2 U/ml). The plate was pre-incubated at 37°C for 20 minutes to allow the enzyme and inhibitor to interact. After pre-incubation, 20 μ L of a 1% starch solution in 100 mM phosphate buffer (pH 6.8) was added to each well as the substrate. The reaction mixture was further incubated at 37°C for 30 minutes. After incubation, 100 μ L of 3,5-dinitrosalicylic acid (DNS) reagent was added to each well. The plate was then incubated at 100°C for 10 minutes to develop the color. The absorbance of the resulting mixture was measured at a wavelength of 540 nm using a Multiplate Reader. Acarbose at various concentrations served as the standard for comparison, and the percentage of α -amylase inhibition was calculated based on the absorbance values obtained from the samples relative to the control wells.

$$\text{Inhibition of } \alpha\text{-amylase (\%)} = \frac{((Ac - Ac') - (As - As'))}{(Ac' - As')} \times 100$$

Where:

- Ac : Absorbance of the blank (control without any sample or inhibitor)
- Ac' : Absorbance of the blank control (in the presence of the solvent, e.g., 1% DMSO)
- As : Absorbance of the sample (in the presence of the sample extract)
- As' : Absorbance of the sample control (sample with solvent but no substrate or enzyme)

2.2.5. Identification of Compounds in the Active Fraction of PC using GC-MS

GC-MS analysis was performed at the Integrated Laboratory of the Islamic University of Indonesia, Yogyakarta, to identify the compounds present in the samples. The columns used in the analysis were Rtx-5MS columns, thickness 0.25 μ m, length 30.0 m and diameter 0.25 mm, column temperature 80.0°C, injection temperature 300.00°C, with split injection mode, pressure 42.3 kPa, total flow 117.5 ml/min and column flow 0.74 m/min. (Hotmian *et al.* 2021b).

2.2.6. Molecular Docking

Molecular docking was conducted using various software tools to analyze the interactions between the proteins and ligands. The receptors were downloaded from the Protein Data Bank (PDB) <https://www.rcsb.org/> (Pagadala *et al.* 2017). Water molecules surrounding the protein were removed, and the protein chain was separated from its native ligand and saved in *.pdb format as a protein file. The native ligand structure was extracted by removing the corresponding portion of the protein chain and saved in *.pdb format as a ligand file. The ligand's structure was initially prepared in 2D using ChemDraw Pro 12.0. (PerkinElmer 2015), the 3D structure was constructed, and molecular mechanics (MM) geometry optimization was performed using Chem3D Pro 12.0. The optimized ligand structure was saved in PDB file format. The protein was prepared using AutoDockTools 1.5.6 (Morris *et al.* 2009) by adding hydrogen atoms to the polar side of the structure and applying Kollman charges. The ligand was prepared by correcting its structure and adding Gasteiger charges. The prepared structures were saved in *.pdbqt format. The redocking process utilized a grid box of dimensions 40 \times 40 \times 40 with the following coordinates: x = 10.265, y = 45.877, z = 19.734. Docking Parameters Genetic Algorithm (GA), the output algorithm for docking results, was set to Lamarckian GA 4.2, and other docking parameters were set to default values. The

critical parameter to evaluate the docking results was the RMSD. An acceptable RMSD value was ≤ 3.0 Å, indicating reliable docking conformations.

2.2.7. Evaluation of Drug Likelihood and ADMET

Drug likeness was evaluated based on Lipinski's Rule of Five, a widely used guideline in drug discovery to predict the oral bioavailability of compounds. The following criteria were considered molecular weight: the compound should not exceed 500 Da, the number of hydrogen bond acceptors (-H bond acceptors) should be no more than 10, the number of hydrogen bond donors (-H bond donors) should not exceed 5, the log P value, which indicates the compound's lipophilicity, should be less than 5 (or MlogP <4.15) (Rahardhian *et al.* 2022a). Absorption, Distribution, Metabolism, Excretion, and Toxicity (ADMET) properties were predicted using computational methods further to assess the compounds' suitability for drug development. CaCO₂ permeability was assessed to evaluate intestinal absorption. Blood-brain barrier (BBB) permeability was predicted to assess the ability of the compound to cross the BBB. The compound was evaluated as a substrate for CYP2D6, a key enzyme in drug metabolism. Total clearance was calculated to estimate how quickly the compound is eliminated from the body. The AMES (Ames Mutagenicity Test) toxicity tests assessed potential genotoxic effects (Pires *et al.* 2015).

2.3. Data Analysis

Statistical analysis was performed using GraphPad Prism 9.0 (GraphPad Software Inc., CA, USA). The results are presented as mean values \pm standard deviation (SD). ANOVA was applied to evaluate the differences among the various extracts. This method helps determine whether there are statistically significant differences between the means of three or more independent groups. A post hoc test was conducted following ANOVA to identify specific group differences. A significance level of $p > 0.05$ was used to determine statistical significance.

3. Results

3.1. Phytochemical Screening

The fractionation of the ethanol extract from PC leaves resulted in the following yields: 32.81% for the n-hexane fraction, 16.86% for the ethyl acetate fraction, and 34.17% for the water fraction. Notably, the ethyl acetate fraction yield was lower than that of

the n-hexane and water fractions. This variation in yield may be attributed to the differing solubility profiles of the compounds present in the PC leaf extract, with nonpolar and polar compounds being more prevalent than semipolar compounds. Phytochemical screening was conducted to identify various bioactive compounds, including alkaloids, flavonoids, saponins, tannins, and triterpenoids/steroids. The results indicated that the ethanol extract of PC leaves tested positive for alkaloids, flavonoids, tannins, saponins, and triterpenoids. In contrast, the water fraction demonstrated a positive presence only for alkaloids and tannins. The results of the phytochemical screening indicate that the ethanol extract and fractions of *Peronema canescens* leaves contain various bioactive compounds Table 1.

3.2. Thin Layer Chromatography (TLC)

The compounds present in the ethanol extract, n-hexane fraction, ethyl acetate fraction, and water fraction of PC leaves were further analyzed using Thin Layer Chromatography (TLC). This technique confirmed the presence of various bioactive compounds by evaluating the R_f values and the corresponding color stains formed on the TLC plates. The R_f values were calculated for each compound, allowing for the identification of the specific compounds present in each fraction. The results of the TLC analysis are summarized in Table 2, which includes the R_f values and corresponding colors observed for each compound group in the different fractions.

From the results of phytochemical screening and TLC tests, PC leaf extracts and fractions contain several secondary metabolite compounds, including alkaloids, flavonoids, saponins, tannins, terpenoids, steroids, and phenolics Table 2.

3.3. Determination of Total Phenolic and Total Flavonoid Content

Figure 1 shows that the n-hexane fraction of PC leaves exhibit the highest total flavonoid content, with an average concentration of 203.3742 ± 4.3777 mg QE/gram. In contrast, the ethyl acetate fraction of PC leaves contains the highest total phenolic content, with an average concentration of 147.0397 ± 0.7864 mg GAE/gram.






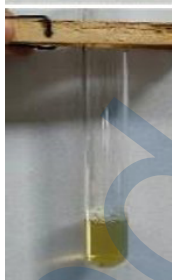

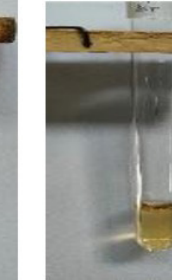
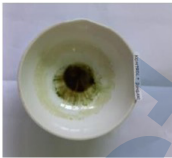



3.4. Active Fraction of PC Leaves Using GC-MS

The active fraction was identified using Gas Chromatography-Mass Spectrometry (GC-MS). Based

Table 1. Phytochemical screening results of ethanol extract and fractions of PC leaves

Compound group	Reagent	Ethanol extract	<i>n</i> -hexane fraction	Ethyl acetate fraction	Water fraction
Phenolic	Add 5 to 6 drops of a 1% ferric chloride (FeCl_3)				
Tannins	2 ml of a 10% NaCl solution mixed with 1% gelatin				
Flavonoids	Magnesium powder combined with concentrated HCl and amyl alcohol				
Alkaloids	Add three drops of Bouchardat's reagent				
	Mix 1 ml of 2N HCl with two drops of Mayer's reagent				

Table 1. Continued

Compound group	Reagent	Ethanol extract	<i>n</i> -hexane fraction	Ethyl acetate fraction	Water fraction
Saponins	Mix 1 ml of 2N HCl with two drops of Dragendorff's reagent				
	Add 10 ml of hot water to 2N HCl				
Steroids	Mix 5 ml of chloroform with anhydrous acetic acid and H ₂ SO ₄				

(+): contains the compound, (-): does not contain the compound

Table 2. TLC results of ethanol extract and fractions of PC leaves

Compound group	Mobile phase	Sample	Visual	UV 254	UV 366	Spot appearance	Rf value
Alkaloids	ethyl acetate, methanol, and water (6:4:2)	EE	Brown	Brown	Orange	Brown	0.81
		NHF	Brown	Brown	Orange	Brown	0.79
		EAF	Orange	Brown	Orange	Brown	0.75
		WF	Brown	Brown	Blue	Brown	0.73
Flavonoid	<i>n</i> -butanol, acetic acid, and water (4:1:5)	EE	Green	Green	Orange	Green	0.91
		NHF	Green	Green	Orange	Green	0.91
		EAF	Green	Green	Orange	Green	0.91
		WF	Brown	Green	Blue	Yellow	0.56
Saponin	chloroform, methanol, and water (64:50:10)	EE	Green	Green	Orange	Purple	0,94
		NHF	Green	Green	Orange	Purple	0,94
		EAF	Green	Green	Orange	Green	0,90
		FAE	-	-	-	-	-
Tannins	ethyl acetate, methanol, and water (100:13.5:10)	EE	Green	Black	-	Green Blackish	0.88
		NHF	Brown	Black	-	Ash Blackish	0.94
		FEA	Brown	Black	-	Black	0.94
		WF	Brown	Black	-	Black	0.91
Steroids	<i>n</i> -hexane and ethyl acetate (17:3)	FAE	Green	Brown	Blue	Brown	0.91
		FNH	Green	Brown	Blue	Brown	0.91
		FEA	Green	Brown	Orange	Brown	0.22
		FA	-	-	-	Brown	-

(+): contains the compound, (-): does not contain the compound

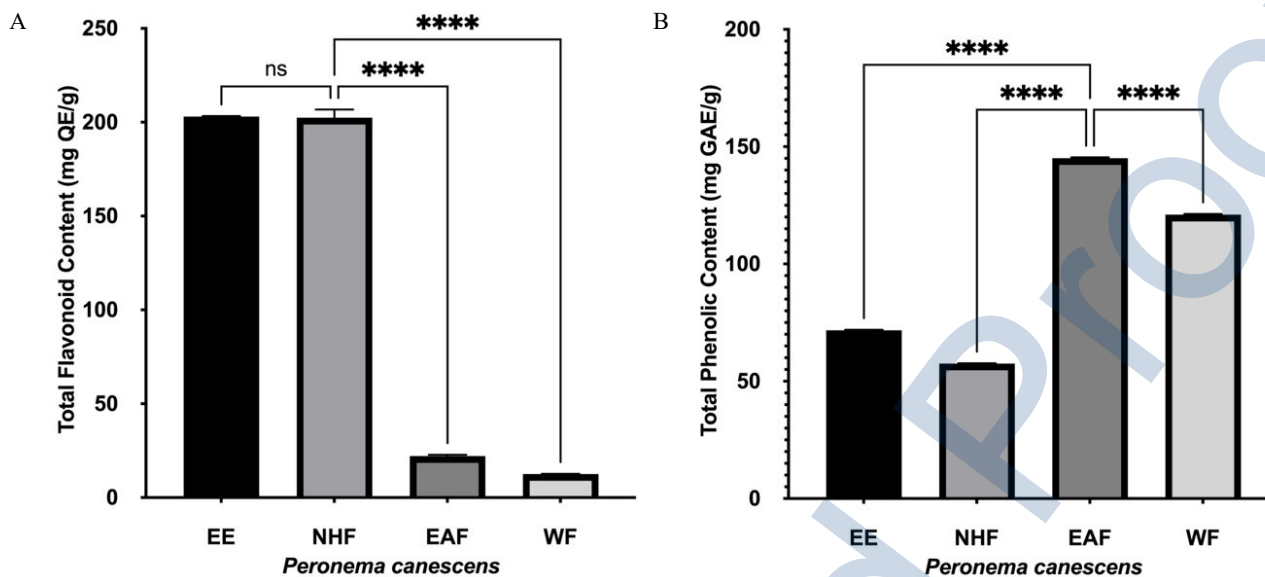


Figure 1. (A) Total flavonoid content and (B) total phenolic content. EE: ethanolic extract, NHF: *n*-hexane fraction, EAF: ethyl acetate fraction, WF: water fraction. ns: non-significant; ****: significant difference ($P < 0.05$)

on the analysis of PC leaves' active fraction (ethyl acetate fraction), the compounds identified are listed in Table 3.

3.5. *In Vitro* Inhibition of α -Amylase Activity

The α -amylase inhibitory activity of the extract and fractions of PC leaves was evaluated by measuring the total reducing sugar using the 3,5-dinitrosalicylic acid (DNS) method, with starch as the substrate. 3,5-dinitrosalicylic acid is an aromatic compound that reacts with reducing sugars to form 3-amino-5-nitrosalicylic acid, which absorbs electromagnetic radiation. The α -amylase inhibitory activity was quantified at a wavelength of 540 nm using a 96-well microplate reader.

Based on Figure 2, the active fraction with the highest percentage of α -amylase enzyme inhibition was observed in the ethyl acetate fraction of PC leaves, with an average inhibition of $70.38 \pm 1.26\%$.

3.6. *In Silico* Inhibition of α -Amylase Activity

Molecular docking is a commonly used method in drug development to identify compounds or molecules with therapeutic activity by predicting ligand-target interactions and evaluating structural activity using computational techniques (Rahardhian *et al.* 2022). The validation results showed an RMSD value of 1.74 Å, indicating that the redocking method met the required criteria. Furthermore, the binding energy affinity (ΔG) was found to be -4.17 kcal/mol, with an inhibition

constant (K_i) of the same magnitude obtained during the fourth docking run.

Based on Table 4, three bioactive compounds exhibit favorable binding energies and amino acid interactions with the same target protein as the natural ligands. The binding energy values reflect the spontaneity of the interaction between the protein and the ligand; the more negative the binding energy, the more rapid the binding process occurs. This suggests that these compounds may serve as effective α -amylase inhibitors, warranting further investigation into their potential therapeutic applications.

Table 5 presents the results of the ADME (Absorption, Distribution, Metabolism, and Excretion) analysis for the bioactive compounds identified in the active fraction of PC leaves. This analysis is crucial for evaluating the pharmacokinetic properties of these compounds, which can influence their effectiveness as potential therapeutic agents. The results provide insights into the compounds' absorption rates, distribution patterns in the body, metabolic stability, and excretion profiles, helping to assess their suitability for drug development. The ADME (Absorption, Distribution, Metabolism, Excretion) and toxicity profiles of the bioactive compounds identified in the active fraction of PC leaves. The analysis indicates that the bioactive compounds Bis(2-ethylhexyl) phthalate, Myristyl oleate, and 14 Beta H pregna meet the criteria for a favorable ADME profile, suggesting efficient absorption and distribution within the body.

Table 3. Results of GC-MS identification of active fraction compounds in PC leaves

Peak	Retention time	Percent area(%)	Molecular weight	Base peak	Similarity index	Molecular formula	Compound
1	20,208	1.70	390	149	97	C ₂₄ H ₃₈ O ₄	Bis(2-ethylhexyl) phthalate
2	21,656	0.58	478	57	63	C ₃₂ H ₆₂ O ₂	Myristyl oleate
3	21,942	0.57	593	57	59	C ₃₉ H ₇₆ O ₃	Oleic acid
4	22,825	2.12	288	57	64	C ₂₁ H ₃₆	14-Beta-H-Pregna
5	22,902	8.77	723	57	77	C ₄₅ H ₈₆ O ₆	Tetradecanoic acid
6	23,021	16.46	639	57	75	C ₃₉ H ₇₄ O ₆	Dodecanoic acid
7	23,142	9.25	723	57	68	C ₄₅ H ₈₆ O ₆	Tetradecanoic acid
8	23,255	27.05	751	57	77	C ₄₇ H ₉₀ O ₆	Hexadecanoic acid
9	23,301	19.04	639	57	83	C ₃₉ H ₇₄ O ₆	Dodecanoic acid
10	23,367	14.46	639	183	71	C ₃₉ H ₇₄ O ₆	Dodecanoic acid

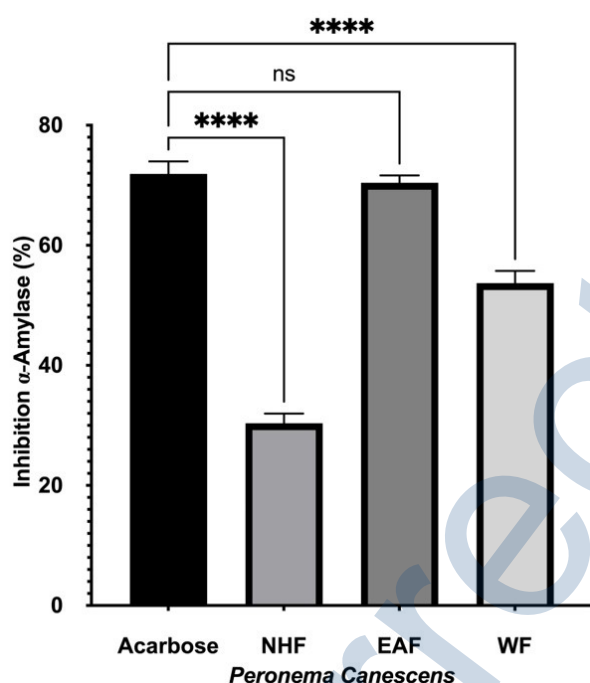


Figure 2. Percentage inhibition of α -amylase enzyme activity. NHF: *n*-hexane fraction, EAF: ethyl acetate fraction, WF: water fraction, ns: non-significant, ****: Significant difference ($P > 0.05$)

Table 6 presents the results of the Lipinski analysis for the bioactive compounds found in the active fraction of PC leaves. Lipinski's rule of five evaluates the drug-likeness of compounds based on their molecular properties, which predict good absorption and permeation. The analysis assesses molecular weight, hydrogen bond donors and acceptors, and logP values. Compounds that comply with these criteria will likely exhibit favorable pharmacokinetic properties, making them suitable candidates for further drug development. The bioactive compounds in the active fraction of PC leaves meet Lipinski's rule of five

Table 4. Results of molecular docking analysis of ethyl acetate fraction compounds of PC leaves

Compound	ΔG (kcal/mol)	Ki (mM)	Amino acids residue
Bis(2-ethylhexyl) phthalate	-4.59	432.20	Tyr 62, Leu 162, His 201, Lys 200, Ile 235, His 299
Myristyl oleate	-3.32	3.66	Ile 51, Leu 165, Pro 54, His 101, Trp 59, His 305, Tyr 62, Asp 300
14 Beta H pregna	-7.81	1.90	Tent 162, Tent 165, Trp 58, Trp 62, His 299
Natural ligand (Acarbose)	-4.17	-	The 163, His 305, Asp 300, Glu 233, His 101, Arg 195, Asp 197, His 299

requirements. Specifically, these compounds exhibit a molecular weight of less than 500 mg/mol, a logP value between 0.4 and 5, a hydrogen bond donor count of ≤ 10 , a hydrogen bond acceptor count of ≤ 5 , and a molar refractivity below 130. Consequently, it can be concluded that these bioactive compounds are considered safe for oral use.

4. Discussion

The fractionation of the ethanol extract from PC leaves yielded 32.81% for the *n*-hexane fraction, 16.86% for the ethyl acetate fraction, and 34.17% for the water fraction. The lower yield of the ethyl acetate fraction indicates a higher abundance of nonpolar and polar compounds relative to semipolar compounds in the extract (Duistermaat and Kolk 2000). This pattern reflects the principles of solvent polarity, where nonpolar solvents like *n*-hexane extract nonpolar compounds such as lipids, while polar solvents like water preferentially extract polar substances like sugars and certain phenolic compounds (Altemimi *et al.* 2017).

Table 5. Results of ADME analysis of active fraction compounds of PC leaves

Compound	Absorption (Caco2 permeability) (log Papp in 10 ⁻⁶ cm/s)	Distribution (BBB permeability y) (log BB)	Excretion (Total clearance) (log ml/min/kg)	Toxicity (AMES) (Yes/No)
Bis(2-ethylhexyl) phthalate	1,408	-0.175	-0.175	No
Myristyl oleate	1,306	1.014	1.014	No
14 Beta H pregna	1,405	0.885	0.885	No

Table 6. Results of the lipinski analysis for the bioactive compounds found in the active fraction of PC leaves

Compound	Molecular weight	Log P	Donor hydrogen bonding	Hydrogen acceptor bonding	Surface area
Bis(2-ethylhexyl) phthalate	390,564	6,433	0	4	170,550
Myristyl oleate	478,846	112,682	0	2	214,638
14 Beta H pregna	274,492	60,553	0	0	125,650

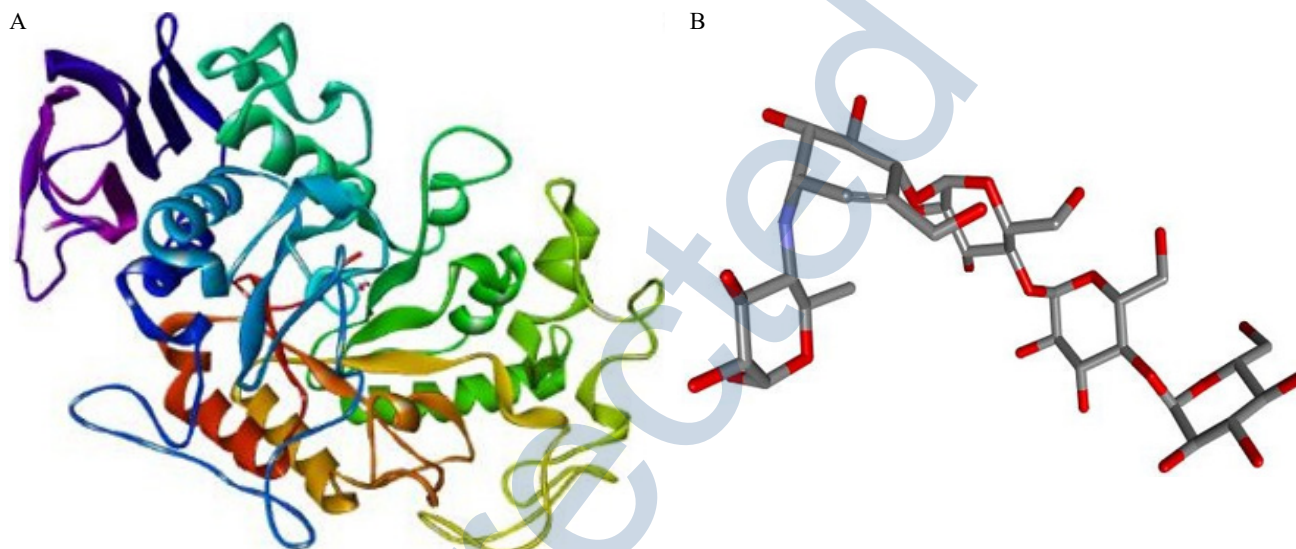
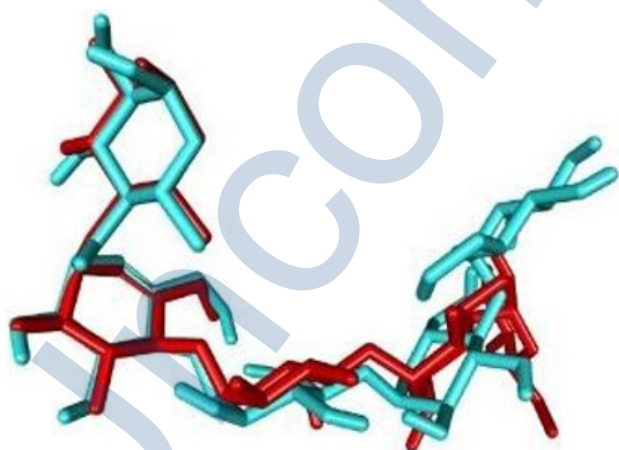
Figure 3. (A) The three-dimensional structure of the α -amylase enzyme and (B) its natural ligands

Figure 4. The overlay of the acarbose confirmation before (blue) and after (red) redocking

Phytochemical screening revealed alkaloids, flavonoids, tannins, saponins, and triterpenoids/steroids in the ethanol extract. However, the water fraction was only positive for alkaloids and tannins. This selective presence underscores the solubility characteristics of the compounds, with flavonoids and triterpenoids being more soluble in ethyl acetate, explaining their absence in the water fraction. The limited presence of flavonoids and triterpenoids in the water fraction of PC leaves aligns with other studies on medicinal plants, suggesting that these compounds are more concentrated in less polar solvents. The phytochemical screening of the ethanol extract from PC leaves, along with its fractions, revealed a diverse array of bioactive compounds. The tests utilized various reagents to identify specific compound groups, including

phenolics, tannins, flavonoids, alkaloids, saponins, and steroids. This suggests that phenolics, The research results are consistent with (Rahardhian *et al.* 2022c), indicating that PC contains phenolic.

The presence of phenolic compounds was confirmed in all fractions, including the ethanol extract, *n*-hexane, ethyl acetate, and water fractions, as indicated by the positive reaction with ferric chloride. Tannins were present in the ethanol extract and water fraction but absent in the *n*-hexane fraction. This distribution indicates that tannins, which are polyphenolic compounds with astringent properties, are more soluble in polar solvents like water and ethanol. The screening indicated flavonoids were present in the ethanol, *n*-hexane, and ethyl acetate fractions but absent in the water fraction. This result highlights the preferential solubility of flavonoids in less polar solvents, supporting findings in other studies that suggest flavonoids are typically more concentrated in ethyl acetate and hexane fractions. All fractions tested positive for alkaloids, with multiple reagents confirming their presence. Saponins were present in the ethanol, *n*-hexane, and ethyl acetate fractions but absent in the water fraction. This suggests that while saponins can dissolve in polar solvents, they may require a particular concentration or temperature to effectively extract, as indicated by their absence in the water fraction after the hot water test. Steroids were found in the ethanol, *n*-hexane, and ethyl acetate fractions but not in the water fraction. This aligns with their chemical nature, as these compounds are generally more soluble in nonpolar or moderately polar solvents. The phytochemical screening demonstrates that PC leaves are rich in bioactive compounds across different fractions. The solubility profiles of these compounds indicate their potential utility in therapeutic applications, as well as the need for further research to explore their pharmacological properties and mechanisms of action (Suryanti *et al.* 2022).

The thin-layer chromatography (TLC) analysis of PC leaves provides a comprehensive overview of its bioactive compound profile, including alkaloids, flavonoids, saponins, tannins, and steroids (Ladeska *et al.* 2024). The visualizations and Rf values obtained during the analysis allow for a comparative understanding of the composition and potential therapeutic applications of these compounds (Yodha *et al.* 2024). The presence of alkaloids was confirmed across all samples, with Rf values ranging from 0.73 to 0.81. The consistent brown coloration under UV light and the appearance of orange spots following Dragendorff's reagent application suggest a robust alkaloid profile in PC leaves. These findings align with research (Li *et al.* 2020), which noted that

alkaloids often exhibit similar Rf values due to their polar characteristics, influencing their solubility and mobility in chromatographic conditions. Alkaloids are recognized for their pharmacological activities, including analgesic and antimicrobial properties, reinforcing the potential medicinal value of PC. Flavonoids displayed Rf values of 0.56 to 0.91, with the ethanol, *n*-hexane, and ethyl acetate fractions exhibiting a distinct green coloration. The yellow stains after ammonia treatment are consistent with flavonoid presence, as supported by studies like those of (Panche *et al.* 2016), which indicate that the coloration is indicative of flavonoid derivatives. The consistent Rf values across the fractions suggest that PC leaves contain a diverse range of flavonoids, which may enhance their therapeutic profile. The analysis showed a strong presence of saponins, with Rf values around 0.90 to 0.94 and vibrant colored patches indicating their presence. This finding corroborates previous studies, such as those by (Cheok *et al.* 2014). Tannins were identified with Rf values of 0.88 to 0.94, displaying green-blackish spots after FeCl₃ treatment. This aligns with findings from (Das *et al.* 2020), which suggest that tannins' astringent properties and their ability to form complexes with proteins are responsible for their bioactivity. The presence of tannins in PC leaves may contribute to their potential health benefits, particularly in antioxidant and antimicrobial applications. Steroids were detected, with Rf values of 0.22 to 0.91. The purple color indicating steroids and the blue for triterpenoids under UV light corresponds with findings from (Tarigan *et al.* 2023), which report that these compounds have significant roles in various biological activities, including anti-inflammatory effects. The varying Rf values suggest differing solubility characteristics among the compounds, indicating the complexity of the steroidal profile in PC leaves.

The *n*-hexane fraction demonstrated the highest total flavonoid content, averaging 203.3742±4.3777 mg QE/gram. The predominance of flavonoids in the *n*-hexane fraction suggests that these compounds have a higher solubility in nonpolar solvents (Putri *et al.* 2023). This aligns with findings from several studies indicating that nonpolar extraction methods are effective for isolating lipophilic flavonoid compounds. For instance, research by (Putri *et al.* 2022) supports the idea that nonpolar solvents can enhance the extraction of certain flavonoid derivatives, potentially improving their bioavailability and pharmacological efficacy. In contrast, the ethyl acetate fraction exhibited the highest total phenolic content, averaging 147.0397±0.7864 mg GAE/gram. Phenolic compounds are well-known for their potent antioxidant properties, contributing to the plant's

protective mechanisms against oxidative stress. The higher concentration of phenolics in the ethyl acetate fraction indicates their preferential solubility in moderately polar solvents, which has been corroborated by studies such as those by (Rahardhian *et al.* 2019). These findings highlight the effectiveness of ethyl acetate in extracting bioactive phenolic compounds, which may have implications for their therapeutic applications in treating oxidative stress-related diseases. The observed differences in flavonoid and phenolic concentrations between the two solvent fractions emphasize the importance of solvent polarity in the extraction process. This selective extraction allows for a deeper understanding of the phytochemical profile of PC leaves, which can guide further research into their potential health benefits. Notably, the high total flavonoid content in the *n*-hexane fraction suggests that PC may be a valuable source of flavonoid-rich extracts that could be utilized in nutraceutical formulations targeting antihyperglycemic.

The gas chromatography-mass spectrometry (GC-MS) analysis of PC leaves provided valuable insights into the chemical composition of its extracts, revealing a range of fatty acids and esters. Each compound's retention time, molecular weight, and similarity index contribute to understanding the plant's phytochemical profile and its potential applications in health and nutrition (Hotmian *et al.* 2021a). Among the identified compounds, notable fatty acids include tetradecanoic acid ($C_{14}H_{28}O_2$) and hexadecanoic acid ($C_{16}H_{32}O_2$), which accounted for significant peak areas of 8.77% and 27.05%, respectively. The predominance of hexadecanoic acid is particularly interesting, as it has been associated with various health benefits, including anti-inflammatory and antimicrobial properties. This aligns with research indicating that fatty acids play crucial roles in cellular functions and may cause diabetes mellitus diseases (Berry 1997). The presence of bis(2-ethylhexyl) phthalate ($C_{24}H_{38}O_4$), with a peak area of 1.70%, indicates the plant's potential to contain plasticizer compounds, which can have implications for environmental health.

Additionally, oleic acid ($C_{18}H_{34}O_2$), which appeared in the analysis, is a well-known monounsaturated fatty acid praised for its heart health benefits, including its ability to lower harmful cholesterol levels (Eleazu *et al.* 2018). The molecular weight of oleic acid (282) and its retention time further suggests its bioactive potential. The diversity of compounds points to the complex nature of the phytochemical constituents of PC, including myristyl oleate ($C_{32}H_{62}O_2$) and 14-Beta-H-Pregna ($C_{21}H_{36}$), with respective peak areas of 0.58% and 2.12%. The variety of fatty acids and esters indicates that the plant could serve as a source of bioactive compounds with diverse

pharmacological activities, ranging from antidiabetic mellitus to antioxidant effects. The similarity index values, ranging from 57 to 97, highlight the reliability of the GC-MS identification process, reflecting the compounds' structural integrity and known bioactivities. The higher similarity indices suggest that the identified compounds are well-documented in the literature, supporting their potential utilization in therapeutic applications. In summary, the GC-MS analysis of PC leaves reveals a rich array of fatty acids and esters, emphasizing the plant's potential as a source of bioactive compounds.

The findings from this study reveal significant insights into the α -amylase inhibitory potential of PC, particularly highlighting the efficacy of the ethyl acetate fraction. The observed average inhibition percentage of 70.38% for the ethyl acetate fraction is notably higher than the 30.37% and 53.18% inhibition observed in the *n*-hexane and water fractions, respectively. This variation in inhibitory activity suggests a differential distribution of bioactive compounds in the various fractions, likely attributable to their solubility properties and polarity. The ethyl acetate fraction's superior inhibition effect is particularly noteworthy, as it closely approaches the inhibition level of acarbose, a well-known α -amylase inhibitor, which demonstrated an average inhibition percentage of 74.29%. The lack of significant difference between the ethyl acetate fraction and acarbose indicates that the compounds present in this fraction possess potent α -amylase inhibitory activity. This finding is consistent with existing literature, which suggests that ethyl acetate often extracts a variety of bioactive compounds, such as flavonoids and phenolic compounds, that are known for their enzyme-inhibitory effects (Nyambe-Silavwe *et al.* 2015). The lower inhibition percentages observed in the *n*-hexane fraction may be attributed to the nonpolar nature of the compounds extracted, which are less likely to interact effectively with the active site of the α -amylase enzyme. Conversely, the water fraction exhibited moderate inhibition, possibly due to the presence of polar compounds, but it was less effective than the ethyl acetate fraction, highlighting the significance of solvent polarity in influencing the extraction of bioactive constituents. This study demonstrates that the ethyl acetate fraction of PC exhibits significant α -amylase inhibitory potential, comparable to acarbose, suggesting its potential as a natural alternative for diabetes management and warranting further research into its pharmacological applications (Lestari *et al.* 2018).

The molecular docking results presented in this study provide critical insights into the binding affinities and inhibitory potentials of various compounds against α -amylase (Lolok *et al.* 2022). The free energy of binding

(ΔG) and inhibition constants (K_i) serve as valuable indicators of the interactions between these compounds and the enzyme (Yuningtyas *et al.* 2024), shedding light on their potential as α -amylase inhibitors. Bis(2-ethylhexyl) phthalate exhibited a binding free energy of -4.59 kcal/mol with an inhibition constant (K_i) of 432.20 mM. The interaction of this compound with the enzyme involves critical amino acid residues such as Tyr 62, Leu 162, and His 201. While the negative ΔG value indicates a favorable binding, the relatively high K_i value suggests that this compound may not be a potent inhibitor of α -amylase. The interactions with critical residues indicate a potential for activity, but further modifications may be needed to enhance its inhibitory potency. Myristyl oleate showed a lower binding affinity, with ΔG of -3.32 kcal/mol and a K_i value of 3.66 mM. The presence of interactions with multiple residues, including Ile 51, Leu 165, and His 101, suggests that this compound forms multiple favorable contacts with the enzyme. The significantly lower K_i indicates that myristyl oleate could act as a more potent inhibitor compared to bis(2-ethylhexyl) phthalate, possibly due to its ability to engage critical active site residues effectively. 14 Beta H-pregna demonstrated the strongest binding affinity with a ΔG of -7.81 kcal/mol and a low K_i of 1.90 mM. The strong interactions with amino acids such as Trp 58 and Trp 62 indicate that this compound may have a significant impact on the active site configuration of α -amylase. The natural ligand (acarbose), known for its clinical efficacy in inhibiting α -amylase, had a ΔG of -4.17 kcal/mol but did not present a K_i value as this compound acts through a different mechanism. The amino acid residues involved in binding, such as The 163 and His 305, reflect the established interactions that allow acarbose to inhibit the enzyme competitively. This comparison highlights the relative effectiveness of the newly identified compounds against a standard inhibitor, demonstrating that certain derivatives may offer similar or improved efficacy.

The physicochemical properties of the compounds derived from PC were evaluated based on their molecular weight, lipophilicity (Log P), hydrogen bonding capacity, and surface area. Understanding these characteristics is critical for assessing their bioavailability, interactions, and overall pharmacological potential (Pires *et al.* 2015). Absorption of Bis(2-ethylhexyl) phthalate exhibited an absorption value of 1.408 (log Papp in 10⁻⁶ cm/s), indicating a relatively favorable permeability across the intestinal barrier. This suggests that it may be effectively absorbed after oral administration. Myristyl oleate, with a slightly lower absorption value of 1.306, also shows good permeability, although it is less than that of bis(2-

ethylhexyl) phthalate. 14 Beta H-pregna displayed an absorption value similar to bis(2-ethylhexyl) phthalate (1.405), indicating its potential for adequate oral bioavailability. Overall, the absorption profiles of these compounds suggest they may achieve adequate systemic exposure following administration.

Distribution of Myristyl oleate had the highest distribution coefficient (log BB = 1.014), indicating a favorable ability to cross the blood-brain barrier (BBB). This characteristic may allow for central nervous system (CNS) activity, potentially making it useful for conditions where CNS targeting is desired. In contrast, bis(2-ethylhexyl) phthalate showed a negative log BB value (-0.175), indicating limited distribution to the brain. 14 Beta H-pregna also demonstrated a positive log BB value (0.885), suggesting moderate ability to penetrate the BBB. These distribution characteristics are vital when considering the therapeutic application of these compounds, particularly in relation to CNS effects. Metabolism All three compounds were determined not to be substrates for CYP2D6, suggesting they are less likely to be metabolized through this common liver enzyme. This could imply a potentially favorable metabolic profile, as compounds that are not extensively metabolized may maintain higher bioavailability. Excretion clearance rates provide insights into the elimination of these compounds from the body. Myristyl oleate had the highest total clearance (2.149 log ml/min/kg), suggesting it may be rapidly eliminated, which could influence dosing regimens. Conversely, 14 Beta H-pregna exhibited a significantly lower clearance rate (0.67 logs ml/min/kg), indicating a slower elimination and potentially more prolonged duration of action. Notably, none of the compounds tested positive for mutagenicity in the Ames test, indicating a favorable toxicity profile. This is an encouraging finding, as it suggests these compounds may have a lower risk of causing genetic damage.

Bis(2-ethylhexyl) phthalate (MW = 390.564 g/mol) and Myristyl oleate (MW = 478.846 g/mol) are relatively large molecules, which may influence their absorption and distribution in biological systems. Larger molecules tend to exhibit lower permeability through biological membranes, which could affect their therapeutic efficacy. In contrast, 14 Beta H-pregna has a lower molecular weight (MW = 274.492 g/mol), potentially facilitating better absorption and distribution, as smaller compounds typically diffuse more easily through cell membranes. The Log P values provide insights into the hydrophobicity of the compounds. Myristyl oleate exhibits an extraordinarily high Log P value (112.682), indicating its lipophilic solid nature, which could enhance its ability to cross

lipid membranes. However, such high lipophilicity may also lead to challenges in solubility in aqueous environments, potentially limiting its bioavailability when administered orally. Bis(2-ethylhexyl) phthalate has a significant Log P value of 6.433, also reflecting its lipophilicity and suggesting a potential for membrane permeation. Conversely, 14 Beta H-pregna has a Log P of 60.553, which is comparatively lower than the other two compounds but still suggests a reasonable level of lipophilicity.

The varied lipophilicity of these compounds indicates that their absorption and distribution will differ significantly, influencing their pharmacological activities. None of the compounds displayed any donor hydrogen bonding, indicating that they may not participate in hydrogen bond donation, which could limit their interactions with target proteins or enzymes. This property could influence the compounds' binding affinities and their overall efficacy in biological systems. The hydrogen acceptor bonding also varied among the compounds. Bis(2-ethylhexyl) phthalate exhibited four acceptor sites, while Myristyl oleate had two, and 14 Beta H-pregna had none. The presence of hydrogen bond acceptors in bis(2-ethylhexyl) phthalate could facilitate its interaction with biomolecules, enhancing its potential activity.

On the other hand, the lack of hydrogen bonding capacity in 14 Beta H-pregna may limit its interactions with proteins, potentially reducing its efficacy. Surface area is an important parameter that can influence drug absorption and permeability. Myristyl oleate has the largest surface area (214.638 Å²), which might affect its interactions with biological membranes and potential targets. The larger surface area could facilitate a more significant interaction with the lipid bilayer, impacting absorption rates. Bis(2-ethylhexyl) phthalate has a moderate surface area (170.550 Å²), while 14 Beta H-pregna has the smallest surface area (125.650 Å²). The smaller surface area of 14 Beta H-pregna could correlate with its lower molecular weight, potentially leading to better permeability.

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Exploring the α -Amylase Inhibitory Potential of *Peronema canescens* Jack: An In Vitro and In Silico Study

ABSTRACT

Hyperglycemia in individuals with type 2 diabetes mellitus is primarily driven by the rapid hydrolysis of starch by the enzyme α -amylase in the pancreas and the breakdown of oligosaccharides by α -glucosidase in the intestine. *Peronema canescens* Jack. (PC) has shown promise as a potential antidiabetic agent. This study aimed to evaluate the total flavonoid, phenolic, and α -amylase inhibitory activity of extracts and fractions derived from PC leaves using both in vitro and in silico approaches. The ethanol extract of PC leaves was fractionated through liquid-liquid extraction using n-hexane, ethyl acetate, and water as solvents. Preliminary phytochemical screening of the extracts and fractions identified the presence of alkaloids, flavonoids, saponins, tannins, and steroids/triterpenoids. The n-hexane fraction exhibited the highest total flavonoid content, averaging 203.37 ± 4.38 mg QE/gram, while the ethyl acetate fraction demonstrated the highest total phenolic content, averaging 147.04 ± 0.79 mg GAE/gram. Furthermore, the ethyl acetate fraction showed the strongest α -amylase inhibitory activity, with an average inhibition rate of $70.38 \pm 1.26\%$. In silico analysis, combined with GC-MS identification, suggested that three compounds bis(2-ethylhexyl) phthalate, myristyl oleate, and 14 beta H-pregna may contribute to the observed α -amylase inhibitory activity. These findings highlight the potential of PC as a source of natural antidiabetic agents.

Keywords: α -amylase inhibitory activity, in vitro and in silico analysis, natural antidiabetic agents, *Peronema canescens* Jack., total flavonoid and phenolic content

1. Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by hyperglycaemia resulting from defects in insulin secretion, insulin action, or both (WHO, 2023). The prevalence of diabetes has reached alarming levels globally, with the International Diabetes Federation (IDF) estimating that over 537 million adults will be living with the condition in 2021 (Webber, 2013). Experts project that this number will rise to 643 million by 2030. In Indonesia, the situation mirrors this global trend, with an increasing number of the population being diagnosed with diabetes. The current standard of care for managing diabetes includes synthetic drugs such as sulfonylureas, metformin, and thiazolidinediones, designed to enhance insulin sensitivity, stimulate insulin secretion, or reduce glucose production. However, these synthetic drugs are not without their limitations. Long-term use of these medications can lead to adverse effects such as hypoglycaemia (Chaudhury et al., 2017), gastrointestinal issues, and even multidrug resistance (MDR). These limitations underscore the need for alternative treatment strategies that are both effective and safe.

41 In light of the challenges associated with synthetic drugs, there has been growing
42 interest in herbal medicine as a potential alternative or complementary approach to diabetes
43 management. Plants rich in bioactive compounds provide herbal medicines with therapeutic
44 benefits and fewer side effects (Tran et al., 2020). One such plant is *Peronema canescens* Jack.
45 (PC), a species native to Indonesia, traditionally used for its medicinal properties (Rahardhian
46 et al., 2022b) . The secondary metabolites found in PC, such as flavonoids, tannins, and
47 alkaloids (Primal and Ahriyasna, 2022), have been shown to possess various pharmacological
48 activities, including antidiabetic effects. These natural compounds may help to mitigate
49 hyperglycemia by enhancing insulin sensitivity, inhibiting glucose absorption, and modulating
50 carbohydrate metabolism, making PC a promising candidate for diabetes treatment.

51 Computer-aided drug design (CADD) advancements have provided researchers with
52 powerful tools to identify and optimize potential antidiabetic agents (Meng et al., 2011) .
53 Molecular docking simulation, a key component of CADD, allows for predicting the
54 interaction between small molecules and target proteins, thereby facilitating the identification
55 of compounds with high binding affinities (Pagadala et al., 2017) . This method has been
56 beneficial in the search for natural inhibitors of enzymes such as α -amylase, which plays a
57 crucial role in carbohydrate digestion and glucose absorption (de Sales et al., 2012) .
58 Researchers can rapidly screen and identify the most promising antidiabetic compounds
59 within complex plant extracts, such as those found in PC, by molecular docking simulations
60 (Khan et al., 2024).

61 This study focuses on the antidiabetic potential of PC, examining both its in vitro and
62 in silico activities. Gas Chromatography-Mass Spectrometry (GC-MS) analysis identified
63 PC's chemical composition and revealed various bioactive compounds (Hotmian et al., 2021a).
64 The inhibitory effect of these compounds on α -amylase was then evaluated through in vitro
65 assays, while molecular docking simulations were employed to understand the interaction
66 between these compounds and the enzyme at the molecular level (Santos et al., 2019) . The
67 physicochemical properties of PC It were also analyzed and compared with other herbal
68 medicines to highlight its unique advantages. This comprehensive approach sheds light on the
69 potential of PC as an antidiabetic agent and underscores its benefits over other herbal
70 remedies (Posadzki et al., 2013).

71 The novelty of this study lies in its integrative approach, combining in vitro and in
72 silico methods to investigate the α -amylase inhibitory potential of PC. While previous

73 research has explored the antidiabetic effects of various herbal medicines, studies focusing
74 specifically on PC remain limited. By providing a detailed analysis of its bioactive
75 compounds and their mechanisms of action, this study serves as a preliminary investigation
76 that could pave the way for future research (Altemimi et al., 2017). The findings presented
77 here are relevant for developing PC as a potential antidiabetic treatment but also offer
78 valuable insights for researchers working on similar objectives, making this study a
79 significant consideration for ongoing and future developments in the field.

80 This study aims to evaluate the α -amylase inhibitory activity of PC using both in vitro
81 and in silico methods. This research seeks to identify the specific compounds responsible for
82 this activity and to understand their mechanisms of action at the molecular level. This
83 research will contribute to the growing knowledge of natural antidiabetic agents and support
84 PC development as a potential treatment for diabetes mellitus.

85

86 2. Materials and Methods

87 2.1. Materials

88 The equipment used in this study includes a Pyrex separation funnel, a Heidolph-G3
89 rotary evaporator, Silica Gel F254 plates, UV 254 and 366 lamps (Evaco GL 220V 50Hz T8
90 15W), micropipettes (Socorex and Dragon Lab), vortex mixers, a Shimadzu UV-1780 UV-
91 Vis spectrophotometer (Serial No. A119161), a Synergy-HTX multi-mode ELISA reader
92 with 96-well plates, and a QP 2010 gas chromatography-mass spectrometer (GC-MS). The
93 materials utilized in this study include *Peronema canescens* Jack., ethanol, n-hexane, ethyl
94 acetate, FeCl₃, MgSO₄, hydrochloric-ethanolic acid mixture (1:1), hydrochloric acid,
95 Lieberman-Burchard reagent, 2,2-diphenyl-1-picrylhydrazyl (Sigma), quercetin, methanol,
96 phosphate buffer, alpha-amylase enzyme (Sigma Aldrich), starch, and 3,5-dinitrosalicylic
97 acid.

98

99 2.1.1. Hardware and Software

100 The molecular docking study was conducted using a laptop equipped with an Asus ROG
101 503 VD. The software utilized in this study includes ChemDraw Professional 15.0, Chem3D
102 15.0, Biovia Discovery Studio 2021, Command Prompt, and AutoDock Tools-1.5.6.
103 Visualization of docking results and the creation of ligands and receptors were performed
104 using Biovia Discovery Studio 2021. Receptor structures were obtained from the RCSB

105 Protein Data Bank (PDB) website (<https://www.rcsb.org/>), while ligand structures were
106 downloaded from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>). Lipinski's rule
107 of five was applied to assess drug-likeness using the SCFBio website ([http://www.scfbio-
108 iitd.res.in/software/drugdesign/lipinski.jsp](http://www.scfbio-
108 iitd.res.in/software/drugdesign/lipinski.jsp)). The pk-CSM online tool evaluated
109 Pharmacokinetic and toxicological properties (<https://biosig.lab.uq.edu.au/pkcsm/>).

110

111 **2.2. Methods**

112 **2.2.1. Sample preparation, extraction, and fractionation**

113 The extraction of *Peronema canescens*. Jack (PC) used the maceration method with
114 96% ethanol as the solvent. Three hundred grams of PC was placed in a maceration jar, and
115 96% ethanol was added as the solvent. The maceration process was performed for three days,
116 with periodic solvent changes and occasional stirring to enhance extraction efficiency. The
117 resulting macerate was filtered and then concentrated using a rotary vacuum evaporator. The
118 concentrated extract was further thickened using a water bath at approximately 50°C
119 (Rahardhian et al., 2019). Ten grams of the ethanol extract of PC leaves were dissolved in 100
120 mL of water and transferred into a separatory funnel. The mixture was then partitioned by
121 adding 100 mL of n-hexane, followed by vigorous shaking. The mixture was allowed to settle
122 until two distinct phases formed: the aqueous and n-hexane. The aqueous phase, separated
123 from the n-hexane phase, was reintroduced into the separatory funnel. Subsequently, 100 mL
124 of ethyl acetate was added to the aqueous phase. The mixture was shaken several times to
125 ensure thorough mixing and then allowed to separate into its respective phases. The resulting
126 fractions were concentrated using a rotary vacuum evaporator and thickened using a water
127 bath at approximately 50°C to obtain a viscous fraction. (Rahardhian et al., 2019).

128 **2.2.2. Phytochemical screening and TLC**

129 Phytochemical screening follows the method (Putri et al., 2022). Each sample,
130 including the ethanol extract, n-hexane fraction, ethyl acetate fraction, and water fraction of
131 PC, was prepared by dissolving 30 mg of the sample in 3 mL of ethanol until completely
132 dissolved. This solution was used for the subsequent phytochemical screening tests.
133 Flavonoids: To the sample solution, magnesium (Mg) powder, 1 mL of hydrochloric acid
134 (HCl), and amyl alcohol were added. The formation of red, yellow, or orange colours
135 indicated a positive presence of flavonoids. Polyphenols: A 10% ferric chloride (FeCl₃)
136 solution was added to the sample. The appearance of a blue or blackish-green color indicated

137 the presence of polyphenols. Tannins: The sample was mixed with gelatin salts. The
138 formation of a yellowish-white precipitate indicated a positive result for tannins. Alkaloids:
139 The sample was treated with HCl and water, then heated and divided into two portions
140 (Filtrate 1 and Filtrate 2). Filtrate 1: Reacted with Mayer's reagent, forming a yellowish-
141 white precipitate, indicating the presence of alkaloids. Filtrate 2: Reacted with Dragendorff's
142 reagent, forming a brick-red precipitate, confirming the presence of alkaloids. Saponins: The
143 sample was placed in a test tube, and 10 mL of distilled water was added. The mixture was
144 shaken vigorously for 10 seconds. A positive reaction was indicated by the formation of foam
145 that persisted for 10 minutes at a height of 1-3 cm. Steroids/triterpenoids: The sample was
146 treated with chloroform and filtered. The filtrate was mixed with anhydrous acetic acid
147 (CH_3COOH) and heated, then cooled and treated with sulfuric acid (H_2SO_4). A green color in
148 the solution indicated the presence of steroids, while an orange or red color indicated the
149 presence of triterpenoids.

150 Thin-layer chromatography (TLC) was performed following the method described by
151 (Putri et al., 2022) to identify the compound content of the samples. A small amount of each
152 sample was dripped directly onto the TLC plate. Flavonoids: The mobile phase consisted of
153 n-butanol, acetic acid, and water in a ratio of 4:1:5. The TLC plate was then exposed to
154 ammonia vapor. The presence of flavonoids was indicated by the formation of yellow stains
155 after ammonia treatment. Tannins: The mobile phase was prepared using ethyl acetate,
156 methanol, and water in a ratio of 100:13.5:10. The presence of tannins was indicated by
157 visible spots formed by FeCl_3 , which resulted in blackish-green patches. Alkaloids: The
158 mobile phase comprised ethyl acetate, methanol, and water in a ratio of 6:4:2. The presence
159 of alkaloids was confirmed by the appearance of brown patches upon applying Dragendorff's
160 reagent. Saponins, A solvent system of chloroform, methanol, and water in a ratio of
161 64:50:10, was utilized. Anisaldehyde-sulfuric acid was used as a visualization reagent, and
162 the TLC plate was heated on a hot plate for 5-10 minutes at 100°C . The presence of saponins
163 was indicated by the formation of colored patches, including yellow, green, red, dark blue,
164 purple, and brownish-yellow. Steroids/triterpenoids: The mobile phase consisted of n-hexane
165 and ethyl acetate in a ratio of 17:3. Anisaldehyde-sulfuric acid was again used for
166 visualization, and the TLC plate was heated on a hot plate for 5-10 minutes at 100°C . A
167 purple color indicated the presence of steroids, while a blue color indicated triterpenoids.

168

2.2.3. Determination of total phenolic and total flavonoid content

The total flavonoid content (TFC) and total phenolic content (TPC) of the extracts were determined using established spectrophotometric methods (Rahardhian et al., 2019). TPC was assessed using the Folin-Ciocalteu reagent with gallic acid as the standard, and absorbance was measured at 765 nm. TFC was determined using aluminum chloride (AlCl₃) with quercetin as the standard, and absorbance was measured at 420 nm. Both analyses were conducted using a Shimadzu® UV-Vis Spectrophotometer (Model 1240). The total phenolic and flavonoid contents were calculated based on the absorbance values obtained from the gallic acid and quercetin standard curves, respectively.

2.2.4. α -amylase Inhibitory Activity

The α -amylase inhibitory activity of the samples was evaluated using a colorimetric method on a 96-well plate. The samples were dissolved in a 1% dimethyl sulfoxide (DMSO) solution. Each sample, along with the negative control (1% DMSO solution) and positive control (acarbose at concentrations of 50 mg and 100 mg), was pipetted into the wells of a 96-well plate at a volume of 20 μ L per well. 50 μ L of 100 mM phosphate buffer (pH 6.8) was added to each well, followed by ten μ L of α -amylase solution (2 U/mL). The plate was pre-incubated at 37°C for 20 minutes to allow the enzyme and inhibitor to interact. After pre-incubation, 20 μ L of a 1% starch solution in 100 mM phosphate buffer (pH 6.8) was added to each well as the substrate. The reaction mixture was further incubated at 37°C for 30 minutes. After incubation, 100 μ L of 3,5-dinitrosalicylic acid (DNS) reagent was added to each well. The plate was then incubated at 100°C for 10 minutes to develop the color. The absorbance of the resulting mixture was measured at a wavelength of 540 nm using a Multiplate Reader. Acarbose at various concentrations served as the standard for comparison, and the percentage of α -amylase inhibition was calculated based on the absorbance values obtained from the samples relative to the control wells.

$$\text{Inhibition of } \alpha - \text{amylase (\%)} = \frac{((Ac - Ac') - (As - As'))}{(Ac' - As')} \times 100$$

Where, Ac: Absorbance of the blank (control without any sample or inhibitor), Ac': Absorbance of the blank control (in the presence of the solvent, e.g., 1% DMSO), As: Absorbance of the sample (in the presence of the sample extract), As' : Absorbance of the sample control (sample with solvent but no substrate or enzyme)

2.2.5. Identification of compounds in the active fraction of PC using GC-MS.

199 GC-MS analysis was performed at the Integrated Laboratory of the Islamic University
200 of Indonesia, Yogyakarta, to identify the compounds present in the samples. The columns
201 used in the analysis were Rtx-5MS columns, thickness 0.25 μm , length 30.0 m and diameter
202 0.25 mm, column temperature 80.0 $^{\circ}\text{C}$, injection temperature 300.00 $^{\circ}\text{C}$, with split injection
203 mode, pressure 42.3 kPa, total flow 117.5 mL/min and column flow 0.74 mL/min. (Hotmian
204 et al., 2021b).

205 **2.2.6. Molecular Docking**

206 Molecular docking was conducted using various software tools to analyze the
207 interactions between the proteins and ligands. The receptors were downloaded from the
208 Protein Data Bank (PDB) <https://www.rcsb.org/> (Pagadala et al., 2017). Water molecules
209 surrounding the protein were removed, and the protein chain was separated from its native
210 ligand and saved in *pdb format as a protein file. The native ligand structure was extracted
211 by removing the corresponding portion of the protein chain and saved in *pdb format as a
212 ligand file. The ligand's structure was initially prepared in 2D using ChemDraw Pro 12.0.
213 (PerkinElmer, 2015), the 3D structure was constructed, and molecular mechanics (MM)
214 geometry optimization was performed using Chem3D Pro 12.0. The optimized ligand
215 structure was saved in PDB file format. The protein was prepared using AutoDockTools
216 1.5.6 (Morris et al., 2009) by adding hydrogen atoms to the polar side of the structure and
217 applying Kollman charges. The ligand was prepared by correcting its structure and adding
218 Gasteiger charges. The prepared structures were saved in *pdbqt format. The redocking
219 process utilized a grid box of dimensions 40 x 40 x 40 with the following coordinates: x =
220 10.265, y = 45.877, z = 19.734. Docking Parameters Genetic Algorithm (GA), the output
221 algorithm for docking results, was set to Lamarckian GA 4.2, and other docking parameters
222 were set to default values. The critical parameter to evaluate the docking results was the
223 RMSD. An acceptable RMSD value was $\leq 3.0 \text{ \AA}$, indicating reliable docking conformations.

225 **2.2.7. Evaluation of Drug Likelihood and ADMET**

226 Drug likeness was evaluated based on Lipinski's Rule of Five, a widely used
227 guideline in drug discovery to predict the oral bioavailability of compounds. The following
228 criteria were considered molecular weight: the compound should not exceed 500 Da, the
229 number of hydrogen bond acceptors (-H bond acceptors) should be no more than 10, the
230 number of hydrogen bond donors (-H bond donors) should not exceed 5, the log P value,

231 which indicates the compound's lipophilicity, should be less than 5 (or MlogP < 4.15)
232 (Rahardhian et al., 2022a). **Absorption, Distribution, Metabolism, Excretion, and Toxicity**
233 **(ADMET)** properties were predicted using computational methods to further assess the
234 compounds' suitability for drug development. CaCO₂ permeability was assessed to evaluate
235 intestinal absorption. Blood-brain barrier (BBB) permeability was predicted to assess the
236 ability of the compound to cross the BBB. The compound was evaluated as a substrate for
237 CYP2D6, a key enzyme in drug metabolism. Total clearance was calculated to estimate how
238 quickly the compound is eliminated from the body. The **AMES (Ames Mutagenicity Test)**
239 toxicity tests assessed potential genotoxic effects (Pires et al., 2015).

240

241 **2.3. Data Analysis**

242 Statistical analysis was performed using GraphPad Prism 9.0 (GraphPad Software Inc., CA,
243 USA). The results are presented as mean values \pm standard deviation (SD). **ANOVA** was
244 applied to evaluate the differences among the various extracts. This method helps determine
245 whether there are statistically significant differences between the means of three or more
246 independent groups. A post hoc test was conducted following ANOVA to identify specific
247 group differences. A significance level of $p > 0.05$ was used to determine statistical
248 significance.

249










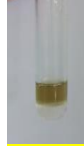


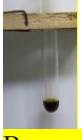











250 **3. Results**









251 **3.1. Phytochemical Screening**

252 The fractionation of the ethanol extract from PC leaves resulted in the following yields:
253 32.81% for the n-hexane fraction, 16.86% for the ethyl acetate fraction, and 34.17% for the
254 water fraction. Notably, the ethyl acetate fraction yield was lower than that of the n-hexane
255 and water fractions. This variation in yield may be attributed to the differing solubility
256 profiles of the compounds present in the PC leaf extract, with nonpolar and polar compounds
257 being more prevalent than semipolar compounds. Phytochemical screening was conducted to
258 identify various bioactive compounds, including alkaloids, flavonoids, saponins, tannins, and
259 triterpenoids/steroids. The results indicated that the ethanol extract of PC leaves tested
260 positive for alkaloids, flavonoids, tannins, saponins, and triterpenoids. In contrast, the water
261 fraction demonstrated a positive presence only for alkaloids and tannins. The results of the

262 phytochemical screening indicate that the ethanol extract and fractions of *Peronema*
 263 *canescens* leaves contain various bioactive compounds Table 1.

264 Table 1. Phytochemical Screening Results of Ethanol Extract and Fractions of PC Leaves

Compound Group	Reagent	Ethanol Extract	n-Hexane Fraction	Ethyl Acetate Fraction	Water Fraction
Phenolic	Add 5 to 6 drops of a 1% ferric chloride (FeCl ₃)	 Blue-black color (+)	 Blue-black color (+)	 Blue-black color (+)	 Blue-black color (+)
Tannins	2 mL of a 10% NaCl solution mixed with 1% gelatin	 White precipitate (+)	 No precipitate (-)	 White precipitate (+)	 White precipitate (+)
Flavonoids	Magnesium powder combined with concentrated HCl and amyl alcohol	 Orange color on the amyl alcohol layer (+)	 Orange color on the amyl alcohol layer (+)	 Orange color on the amyl alcohol layer (+)	 No Orange color on the amyl alcohol (-)
Alkaloids	Add three drops of Bouchardat's reagent.	 Brown precipitate (+)	 Brown precipitate (+)	 Brown precipitate (+)	 Brown precipitate (+)
	Mix 1 mL of 2N HCl with two drops of Mayer's reagent	 White precipitate (+)	 White precipitate (+)	 White precipitate (+)	 No precipitate (-)
	Mix 1 mL of 2N HCl with two drops of Dragendorff's reagent	 Brown precipitate (+)	 Brown precipitate (+)	 Brown precipitate (+)	 Brown precipitate (+)

Saponins	Add 10 mL of hot water to 2N HCl				
		Stable foam (+)	Stable foam (+)	Stable foam (+)	No foam (-)
Steroids	Mix 5 mL of chloroform with anhydrous acetic acid and H ₂ SO ₄ .				
		Green color (+)	Green color (+)	Green color (+)	No Green color (+)

265 **Description**

266 (+) = Contains the compound

267 (-) = Does not contain the compound

268

269 **3.2. Thin Layer Chromatography (TLC)**

270 The compounds present in the ethanol extract, n-hexane fraction, ethyl acetate fraction, and
271 water fraction of PC leaves were further analyzed using Thin Layer Chromatography (TLC).

272 This technique confirmed the presence of various bioactive compounds by evaluating the R_f
273 values and the corresponding color stains formed on the TLC plates. The R_f values were
274 calculated for each compound, allowing for the identification of the specific compounds
275 present in each fraction. The results of the TLC analysis are summarized in Table 2, which
276 includes the R_f values and corresponding colors observed for each compound group in the
277 different fractions.

278 From the results of phytochemical screening and TLC tests, PC leaf extracts and fractions
279 contain several secondary metabolite compounds, including alkaloids, flavonoids, saponins,
280 tannins, terpenoids, steroids, and phenolics Table 2.

281 Table 2. TLC Results of Ethanol Extract and Fractions of PC Leaves

Compound Group	Mobile Phase	Sample	Visual	UV 254	UV 366	Spot Appearance	R _f Value
Alkaloids	ethyl acetate, methanol, and water (6:4:2)	EE	Brown	Brown	Orange	Brown	0,81
		NHF	Brown	Brown	Orange	Brown	0,79
		EAF	Orange	Brown	Orange	Brown	0,75
		WF	Brown	Brown	Blue	Brown	0,73
Flavonoid	n-butanol, acetic acid, and water (4:1:5)	EE	Green	Green	Orange	Green	0,91
		NHF	Green	Green	Orange	Green	0,91
		EAF	Green	Green	Orange	Green	0,91
		WF	Brown	Green	Blue	Yellow	0,56
Saponin	chloroform,	EE	Green	Green	Orange	Purple	0,94

	methanol, and water (64:50:10)	NHF	Green	Green	Orange	Purple	0,94
		EAF	Green	Green	Orange	Green	0,90
		FAE	-	-	-	-	-
Tannins	ethyl acetate, methanol, and water (100:13.5:10)	EE	Green	Black	-	Green	0,88
		NHF	Brown	Black	-	Ash Blackish	0,94
		FEA	Brown	Black	-	Black	0,94
		WF	Brown	Black	-	Black	0,91
Steroids	n-hexane and ethyl acetate (17:3)	FAE	Green	Brown	Blue	Brown	0,91
		FNH	Green	Brown	Blue	Brown	0,91
		FEA	Green	Brown	Orange	Brown	0,22
		FA	-	-	-	Brown	-

282 **Notes:**

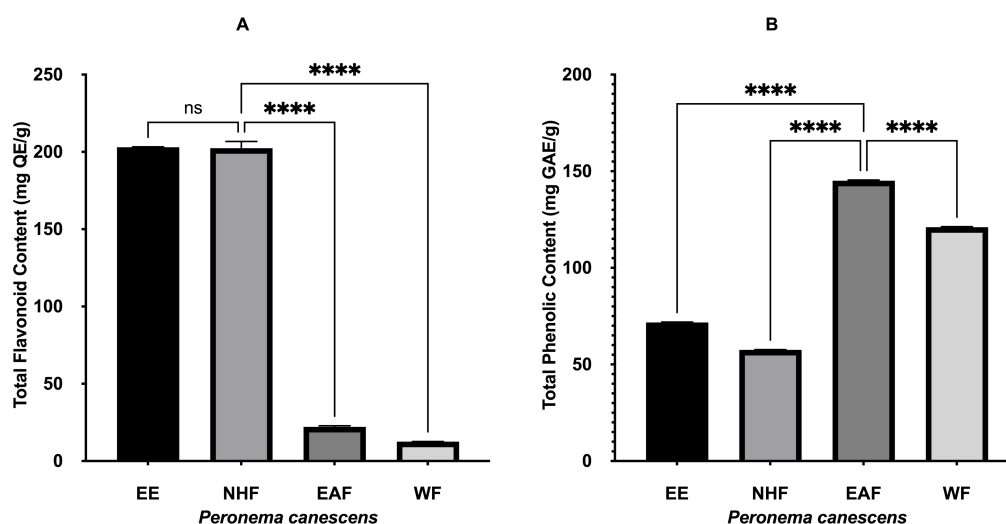
283 (+) = Contains tested compounds

284 (-) = Does not contain the tested compounds

285

286

287 **3.3. Determination of total phenolic and total flavonoid content**



288

289 Figure 1. Total Flavonoid Content (A) and Total Phenolic Content (B). EE: Ethanolic Extract,
 290 NHF: n-Hexane Fraction, EAF: Ethyl Acetate Fraction, WF: Water Fraction. ns:
 291 Non-significant; ****: Significant difference (P > 0.05)

292 Figure 1 shows that the n-hexane fraction of PC leaves exhibits the highest total flavonoid
 293 content, with an average concentration of 203.3742 ± 4.3777 mg QE/gram. In contrast, the
 294 ethyl acetate fraction of PC leaves contains the highest total phenolic content, with an
 295 average concentration of 147.0397 ± 0.7864 mg GAE/gram.

296

297 **3.4. Active Fraction of PC Leaves Using GC-MS**

298 The active fraction was identified using Gas Chromatography-Mass Spectrometry (GC-MS).
 299 Based on the analysis of PC leaves' active fraction (ethyl acetate fraction), the compounds
 300 identified are listed in Table 3.

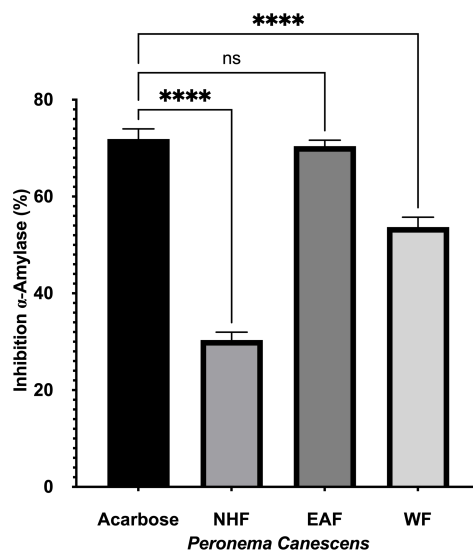
301 Table 3. Results of GC-MS Identification of Active Fraction Compounds in PC Leaves

Peak	Retention Time	Percent Area(%)	Molecular Weight	Base Peak	Similarity Index	Molecular Formula	Compound
1	20,208	1,70	390	149	97	C ₂₄ H ₃₈ O ₄	Bis(2-ethylhexyl) phthalate
2	21,656	0,58	478	57	63	C ₃₂ H ₆₂ O ₂	Myristyl oleate
3	21,942	0,57	593	57	59	C ₃₉ H ₇₆ O ₃	Oleic acid
4	22,825	2,12	288	57	64	C ₂₁ H ₃₆	14-Beta-H-Pregna
5	22,902	8,77	723	57	77	C ₄₅ H ₈₆ O ₆	Tetradecanoic acid
6	23,021	16,46	639	57	75	C ₃₉ H ₇₄ O ₆	Dodecanoic acid
7	23,142	9,25	723	57	68	C ₄₅ H ₈₆ O ₆	Tetradecanoic acid
8	23,255	27,05	751	57	77	C ₄₇ H ₉₀ O ₆	Hexadecanoic acid
9	23,301	19,04	639	57	83	C ₃₉ H ₇₄ O ₆	Dodecanoic acid
10	23,367	14,46	639	183	71	C ₃₉ H ₇₄ O ₆	Dodecanoic acid

302

303 3.5. In Vitro Inhibition of α -Amylase Activity

304 The α -amylase inhibitory activity of the extract and fractions of PC leaves was evaluated by
 305 measuring the total reducing sugar using the 3,5-dinitrosalicylic acid (DNS) method, with
 306 starch as the substrate. 3,5-dinitrosalicylic acid is an aromatic compound that reacts with
 307 reducing sugars to form 3-amino-5-nitrosalicylic acid, which absorbs electromagnetic
 308 radiation. The α -amylase inhibitory activity was quantified at a wavelength of 540 nm using a
 309 96-well microplate reader.



310

311 Figure 3. Percentage Inhibition of α -Amylase Enzyme Activity. NHF: n-hexane fraction, EAF:
 312 Ethyl acetate fraction, WF: Water fraction, ns: non-significant, *****: Significant
 313 difference ($P > 0.05$).

314 Based on Figure 3, the active fraction with the highest percentage of α -amylase enzyme
 315 inhibition was observed in the ethyl acetate fraction of PC leaves, with an average inhibition
 316 of $70.38 \pm 1.26\%$.

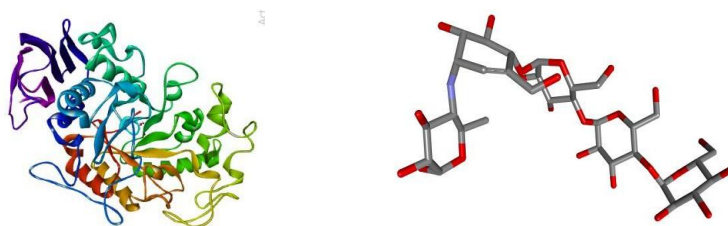
317

318 3.6. In Silico Inhibition of α -Amylase Activity

319 Molecular docking is a commonly used method in drug development to identify compounds
 320 or molecules with therapeutic activity by predicting ligand-target interactions and evaluating
 321 structural activity using computational techniques (Rahardhian et al., 2022). The validation
 322 results showed an RMSD value of 1.74 \AA , indicating that the redocking method met the
 323 required criteria. Furthermore, the binding energy affinity (ΔG) was found to be -4.17
 324 kcal/mol, with an inhibition constant (K_i) of the same magnitude obtained during the fourth
 325 docking run.

326

327



A

B

328 Figure 4. The three-dimensional structure of the α -amylase enzyme (A) and its natural ligands
 329 (B)

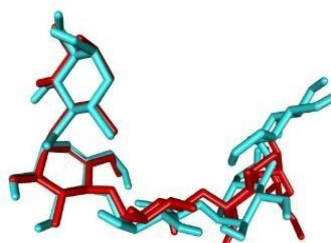


Figure 5. The overlay of the acarbose confirmation before (blue) and after (red) redocking

330 Table 4. Results of Molecular Docking Analysis of Ethyl Acetate Fraction Compounds of PC
 331 Leaves

No	Compound	ΔG (kcal/mol)	Ki (mM)	Amino acids Residue
1	Bis(2-ethylhexyl) phthalate	-4.59	432.20	Tyr 62, Leu 162, His 201, Lys 200, Ile 235, His 299
2	Myristyl oleate	-3.32	3.66	Ile 51, Leu 165, Pro 54, His 101, Trp 59, His 305, Tyr 62, Asp 300
3	14 Beta H pregna	-7.81	1.90	Tent 162, Tent 165, Trp 58, Trp 62, His 299
4	Natural ligand (Acarbose)	-4,17	-	The 163, His 305, Asp 300, Glu 233, His 101, Arg 195, Asp 197, His 299

332 Based on Table 4, three bioactive compounds exhibit favorable binding energies and amino
 333 acid interactions with the same target protein as the natural ligands. The binding energy
 334 values reflect the spontaneity of the interaction between the protein and the ligand; the more
 335 negative the binding energy, the more rapid the binding process occurs. This suggests that
 336 these compounds may serve as effective α -amylase inhibitors, warranting further
 337 investigation into their potential therapeutic applications.

338 Table 5. Results of ADME Analysis of Active Fraction Compounds of PC Leaves

No	Compound	Absorption (Caco2 permeability) (log Papp 10 ⁻⁶ cm/s)	Distribution (BBB permeability) (log BB)	Metabolism (CYP2D6 substrate) (Yes/No)	Excretion (Total clearance) (ml/min/kg)	Toxicity (AMES) (log (Yes/No))
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1	Bis(2-ethylhexyl) phthalate	1,408	-0,175	No	1,898	No
2	Myristyl oleate	1,306	1,014	No	2,149	No
3	14 Beta H pregna	1,405	0,885	No	0,67	No

339 Table 5 presents the results of the ADME (Absorption, Distribution, Metabolism, and
340 Excretion) analysis for the bioactive compounds identified in the active fraction of PC leaves.
341 This analysis is crucial for evaluating the pharmacokinetic properties of these compounds,
342 which can influence their effectiveness as potential therapeutic agents. The results provide
343 insights into the compounds' absorption rates, distribution patterns in the body, metabolic
344 stability, and excretion profiles, helping to assess their suitability for drug development. The
345 ADME (Absorption, Distribution, Metabolism, Excretion) and toxicity profiles of the
346 bioactive compounds identified in the active fraction of PC leaves. The analysis indicates that
347 the bioactive compounds Bis(2-ethylhexyl) phthalate, Myristyl oleate, and 14 Beta H pregna
348 meet the criteria for a favorable ADME profile, suggesting efficient absorption and
349 distribution within the body.

350 Table 6. Results of the Lipinski analysis for the bioactive compounds found in the active

No	Compound	Molecular weight	Log P	Donor hydrogen bonding	Hydrogen acceptor bonding	Surface area
1	Bis(2-ethylhexyl) phthalate	390,564	6,433	0	4	170,550
2	Myristyl oleate	478,846	112,682	0	2	214,638
6	14 Beta H pregna	274,492	60,553	0	0	125,650

351 fraction of PC leaves

352
353 Table 6 presents the results of the Lipinski analysis for the bioactive compounds found in the
354 active fraction of PC leaves. Lipinski's rule of five evaluates the drug-likeness of compounds
355 based on their molecular properties, which predict good absorption and permeation. The
356 analysis assesses molecular weight, hydrogen bond donors and acceptors, and logP values.
357 Compounds that comply with these criteria will likely exhibit favorable pharmacokinetic
358 properties, making them suitable candidates for further drug development. The bioactive
359 compounds in the active fraction of PC leaves meet Lipinski's rule of five requirements.

360 Specifically, these compounds exhibit a molecular weight of less than 500 mg/mol, a logP
361 value between 0.4 and 5, a hydrogen bond donor count of ≤ 10 , a hydrogen bond acceptor
362 count of ≤ 5 , and a molar refractivity below 130. Consequently, it can be concluded that these
363 bioactive compounds are considered safe for oral use.

364

365 **4. Discussion**

366 The fractionation of the ethanol extract from PC leaves yielded 32.81% for the n-hexane
367 fraction, 16.86% for the ethyl acetate fraction, and 34.17% for the water fraction. The lower
368 yield of the ethyl acetate fraction indicates a higher abundance of nonpolar and polar
369 compounds relative to semipolar compounds in the extract (Duistermaat and Kolk, 2000) .
370 This pattern reflects the principles of solvent polarity, where nonpolar solvents like n-hexane
371 extract nonpolar compounds such as lipids, while polar solvents like water preferentially
372 extract polar substances like sugars and certain phenolic compounds(Altemimi et al., 2017).

373 Phytochemical screening revealed alkaloids, flavonoids, tannins, saponins, and
374 triterpenoids/steroids in the ethanol extract. However, the water fraction was only positive for
375 alkaloids and tannins. This selective presence underscores the solubility characteristics of the
376 compounds, with flavonoids and triterpenoids being more soluble in ethyl acetate, explaining
377 their absence in the water fraction. The limited presence of flavonoids and triterpenoids in the
378 water fraction of PC leaves aligns with other studies on medicinal plants, suggesting that these
379 compounds are more concentrated in less polar solvents. The phytochemical screening of the
380 ethanol extract from PC leaves, along with its fractions, revealed a diverse array of bioactive
381 compounds. The tests utilized various reagents to identify specific compound groups,
382 including phenolics, tannins, flavonoids, alkaloids, saponins, and steroids. This suggests that
383 phenolics, The research results are consistent with (Rahardhian et al., 2022c), indicating that
384 PC contains phenolic. The presence of phenolic compounds was confirmed in all fractions,
385 including the ethanol extract, n-hexane, ethyl acetate, and water fractions, as indicated by the
386 positive reaction with ferric chloride. Tannins were present in the ethanol extract and water
387 fraction but absent in the n-hexane fraction. This distribution indicates that tannins, which are
388 polyphenolic compounds with astringent properties, are more soluble in polar solvents like
389 water and ethanol. The screening indicated flavonoids were present in the ethanol, n-hexane,
390 and ethyl acetate fractions but absent in the water fraction. This result highlights the
391 preferential solubility of flavonoids in less polar solvents, supporting findings in other studies

392 that suggest flavonoids are typically more concentrated in ethyl acetate and hexane fractions.
393 All fractions tested positive for alkaloids, with multiple reagents confirming their presence.
394 Saponins were present in the ethanol, n-hexane, and ethyl acetate fractions but absent in the
395 water fraction. This suggests that while saponins can dissolve in polar solvents, they may
396 require a particular concentration or temperature to effectively extract, as indicated by their
397 absence in the water fraction after the hot water test. Steroids were found in the ethanol, n-
398 hexane, and ethyl acetate fractions but not in the water fraction. This aligns with their
399 chemical nature, as these compounds are generally more soluble in nonpolar or moderately
400 polar solvents. The phytochemical screening demonstrates that PC leaves are rich in bioactive
401 compounds across different fractions. The solubility profiles of these compounds indicate
402 their potential utility in therapeutic applications, as well as the need for further research to
403 explore their pharmacological properties and mechanisms of action (Suryanti et al., 2022).

404 The thin-layer chromatography (TLC) analysis of PC leaves provides a comprehensive
405 overview of its bioactive compound profile, including alkaloids, flavonoids, saponins, tannins,
406 and steroids (Ladeska et al., 2024). The visualizations and Rf values obtained during the
407 analysis allow for a comparative understanding of the composition and potential therapeutic
408 applications of these compounds (Yodha et al., 2024). The presence of alkaloids was
409 confirmed across all samples, with Rf values ranging from 0.73 to 0.81. The consistent brown
410 coloration under UV light and the appearance of orange spots following Dragendorff's
411 reagent application suggest a robust alkaloid profile in PC leaves. These findings align with
412 research (Li et al., 2020), which noted that alkaloids often exhibit similar Rf values due to
413 their polar characteristics, influencing their solubility and mobility in chromatographic
414 conditions. Alkaloids are recognized for their pharmacological activities, including analgesic
415 and antimicrobial properties, reinforcing the potential medicinal value of PC. Flavonoids
416 displayed Rf values of 0.56 to 0.91, with the ethanol, n-hexane, and ethyl acetate fractions
417 exhibiting a distinct green coloration. The yellow stains after ammonia treatment are
418 consistent with flavonoid presence, as supported by studies like those of (Panche et al., 2016)
419 which indicate that the coloration is indicative of flavonoid derivatives. The consistent Rf
420 values across the fractions suggest that PC leaves contain a diverse range of flavonoids, which
421 may enhance their therapeutic profile. The analysis showed a strong presence of saponins,
422 with Rf values around 0.90 to 0.94 and vibrant colored patches indicating their presence. This
423 finding corroborates previous studies, such as those by (Cheok et al., 2014). Tannins were

424 identified with Rf values of 0.88 to 0.94, displaying green-blackish spots after FeCl₃ treatment.
425 This aligns with findings from (Das et al., 2020) , which suggest that tannins' astringent
426 properties and their ability to form complexes with proteins are responsible for their
427 bioactivity. The presence of tannins in PC leaves may contribute to their potential health
428 benefits, particularly in antioxidant and antimicrobial applications. Steroids were detected,
429 with Rf values of 0.22 to 0.91. The purple color indicating steroids and the blue for
430 triterpenoids under UV light corresponds with findings from (Tarigan et al., 2023), which
431 report that these compounds have significant roles in various biological activities, including
432 anti-inflammatory effects. The varying Rf values suggest differing solubility characteristics
433 among the compounds, indicating the complexity of the steroidal profile in PC leaves.

434 The n-hexane fraction demonstrated the highest total flavonoid content, averaging
435 203.3742 ± 4.3777 mg QE/gram. The predominance of flavonoids in the n-hexane fraction
436 suggests that these compounds have a higher solubility in nonpolar solvents(Putri et al., 2023).
437 This aligns with findings from several studies indicating that nonpolar extraction methods are
438 effective for isolating lipophilic flavonoid compounds. For instance, research by (Putri et al.,
439 2022) supports the idea that nonpolar solvents can enhance the extraction of certain flavonoid
440 derivatives, potentially improving their bioavailability and pharmacological efficacy. In
441 contrast, the ethyl acetate fraction exhibited the highest total phenolic content, averaging
442 147.0397 ± 0.7864 mg GAE/gram. Phenolic compounds are well-known for their potent
443 antioxidant properties, contributing to the plant's protective mechanisms against oxidative
444 stress. The higher concentration of phenolics in the ethyl acetate fraction indicates their
445 preferential solubility in moderately polar solvents, which has been corroborated by studies
446 such as those by (Rahardhian et al., 2019). These findings highlight the effectiveness of ethyl
447 acetate in extracting bioactive phenolic compounds, which may have implications for their
448 therapeutic applications in treating oxidative stress-related diseases. The observed differences
449 in flavonoid and phenolic concentrations between the two solvent fractions emphasize the
450 importance of solvent polarity in the extraction process. This selective extraction allows for a
451 deeper understanding of the phytochemical profile of PC leaves, which can guide further
452 research into their potential health benefits. Notably, the high total flavonoid content in the n-
453 hexane fraction suggests that PC may be a valuable source of flavonoid-rich extracts that
454 could be utilized in nutraceutical formulations targeting antihyperglycemic.

455 The gas chromatography-mass spectrometry (GC-MS) analysis of PC leaves provided
456 valuable insights into the chemical composition of its extracts, revealing a range of fatty acids
457 and esters. Each compound's retention time, molecular weight, and similarity index contribute
458 to understanding the plant's phytochemical profile and its potential applications in health and
459 nutrition (Hotmian et al., 2021a) . Among the identified compounds, notable fatty acids
460 include tetradecanoic acid ($C_{14}H_{28}O_2$) and hexadecanoic acid ($C_{16}H_{32}O_2$), which accounted for
461 significant peak areas of 8.77% and 27.05%, respectively. The predominance of hexadecanoic
462 acid is particularly interesting, as it has been associated with various health benefits, including
463 anti-inflammatory and antimicrobial properties. This aligns with research indicating that fatty
464 acids play crucial roles in cellular functions and may cause diabetes mellitus diseases (Berry,
465 1997) . The presence of bis(2-ethylhexyl) phthalate ($C_{24}H_{38}O_4$), with a peak area of 1.70%,
466 indicates the plant's potential to contain plasticizer compounds, which can have implications
467 for environmental health. Additionally, oleic acid ($C_{18}H_{34}O_2$), which appeared in the analysis,
468 is a well-known monounsaturated fatty acid praised for its heart health benefits, including its
469 ability to lower harmful cholesterol levels (Eleazu et al., 2018). The molecular weight of oleic
470 acid (593) and its retention time further suggests its bioactive potential. The diversity of
471 compounds, including myristyl oleate ($C_{32}H_{62}O_2$) and 14-Beta-H-Pregna ($C_{21}H_{36}$), with
472 respective peak areas of 0.58% and 2.12%, points to the complex nature of the phytochemical
473 constituents of PC. The variety of fatty acids and esters indicates that the plant could serve as
474 a source of bioactive compounds with diverse pharmacological activities, ranging from anti
475 diabetic mellitus to antioxidant effects. The similarity index values, ranging from 57 to 97,
476 highlight the reliability of the GC-MS identification process, reflecting the compounds'
477 structural integrity and known bioactivities. The higher similarity indices suggest that the
478 identified compounds are well-documented in the literature, supporting their potential
479 utilization in therapeutic applications. In summary, the GC-MS analysis of PC leaves reveals
480 a rich array of fatty acids and esters, emphasizing the plant's potential as a source of bioactive
481 compounds

482 The findings from this study reveal significant insights into the α -amylase inhibitory
483 potential of PC, particularly highlighting the efficacy of the ethyl acetate fraction. The
484 observed average inhibition percentage of 70.38% for the ethyl acetate fraction is notably
485 higher than the 30.37% and 53.18% inhibition observed in the n-hexane and water fractions,
486 respectively. This variation in inhibitory activity suggests a differential distribution of

487 bioactive compounds in the various fractions, likely attributable to their solubility properties
488 and polarity. The ethyl acetate fraction's superior inhibition effect is particularly noteworthy,
489 as it closely approaches the inhibition level of acarbose, a well-known α -amylase inhibitor,
490 which demonstrated an average inhibition percentage of 74.29%. The lack of significant
491 difference between the ethyl acetate fraction and acarbose indicates that the compounds
492 present in this fraction possess potent α -amylase inhibitory activity. This finding is consistent
493 with existing literature, which suggests that ethyl acetate often extracts a variety of bioactive
494 compounds, such as flavonoids and phenolic compounds, that are known for their enzyme-
495 inhibitory effects (Nyambe-Silavwe et al., 2015). The lower inhibition percentages observed
496 in the n-hexane fraction may be attributed to the nonpolar nature of the compounds extracted,
497 which are less likely to interact effectively with the active site of the α -amylase enzyme.
498 Conversely, the water fraction exhibited moderate inhibition, possibly due to the presence of
499 polar compounds, but it was less effective than the ethyl acetate fraction, highlighting the
500 significance of solvent polarity in influencing the extraction of bioactive constituents. This
501 study demonstrates that the ethyl acetate fraction of PC exhibits significant α -amylase
502 inhibitory potential, comparable to acarbose, suggesting its potential as a natural alternative
503 for diabetes management and warranting further research into its pharmacological
504 applications (Lestari et al., 2018).

505 The molecular docking results presented in this study provide critical insights into the
506 binding affinities and inhibitory potentials of various compounds against α -amylase (Lolok et
507 al., 2022). The free energy of binding (ΔG) and inhibition constants (K_i) serve as valuable
508 indicators of the interactions between these compounds and the enzyme (Yuningtyas et al.,
509 2024), shedding light on their potential as α -amylase inhibitors. Bis(2-ethylhexyl) phthalate
510 exhibited a binding free energy of -4.59 kcal/mol with an inhibition constant (K_i) of 432.20
511 mM. The interaction of this compound with the enzyme involves critical amino acid residues
512 such as Tyr 62, Leu 162, and His 201. While the negative ΔG value indicates a favorable
513 binding, the relatively high K_i value suggests that this compound may not be a potent
514 inhibitor of α -amylase. The interactions with critical residues indicate a potential for activity,
515 but further modifications may be needed to enhance its inhibitory potency. Myristyl oleate
516 showed a lower binding affinity, with ΔG of -3.32 kcal/mol and a K_i value of 3.66 mM. The
517 presence of interactions with multiple residues, including Ile 51, Leu 165, and His 101,
518 suggests that this compound forms multiple favorable contacts with the enzyme. The

519 significantly lower K_i indicates that myristyl oleate could act as a more potent inhibitor
520 compared to bis(2-ethylhexyl) phthalate, possibly due to its ability to engage critical active
521 site residues effectively. 14 Beta H-pregna demonstrated the strongest binding affinity with a
522 ΔG of -7.81 kcal/mol and a low K_i of 1.90 mM. The strong interactions with amino acids such
523 as Trp 58 and Trp 62 indicate that this compound may have a significant impact on the active
524 site configuration of α -amylase. The natural ligand (acarbose), known for its clinical efficacy
525 in inhibiting α -amylase, had a ΔG of -4.17 kcal/mol but did not present a K_i value as this
526 compound acts through a different mechanism. The amino acid residues involved in binding,
527 such as The 163 and His 305, reflect the established interactions that allow acarbose to inhibit
528 the enzyme competitively. This comparison highlights the relative effectiveness of the newly
529 identified compounds against a standard inhibitor, demonstrating that certain derivatives may
530 offer similar or improved efficacy.

531 The physicochemical properties of the compounds derived from PC were evaluated
532 based on their molecular weight, lipophilicity (Log P), hydrogen bonding capacity, and
533 surface area. Understanding these characteristics is critical for assessing their bioavailability,
534 interactions, and overall pharmacological potential (Pires et al., 2015). Absorption of Bis(2-
535 ethylhexyl) phthalate exhibited an absorption value of 1.408 (log Papp in 10^{-6} cm/s),
536 indicating a relatively favorable permeability across the intestinal barrier. This suggests that it
537 may be effectively absorbed after oral administration. Myristyl oleate, with a slightly lower
538 absorption value of 1.306, also shows good permeability, although it is less than that of bis(2-
539 ethylhexyl) phthalate. 14 Beta H-pregna displayed an absorption value similar to bis(2-
540 ethylhexyl) phthalate (1.405), indicating its potential for adequate oral bioavailability. Overall,
541 the absorption profiles of these compounds suggest they may achieve adequate systemic
542 exposure following administration. Distribution of Myristyl oleate had the highest distribution
543 coefficient (log BB = 1.014), indicating a favorable ability to cross the blood-brain barrier
544 (BBB). This characteristic may allow for central nervous system (CNS) activity, potentially
545 making it useful for conditions where CNS targeting is desired. In contrast, bis(2-ethylhexyl)
546 phthalate showed a negative log BB value (-0.175), indicating limited distribution to the brain.
547 14 Beta H-pregna also demonstrated a positive log BB value (0.885), suggesting moderate
548 ability to penetrate the BBB. These distribution characteristics are vital when considering the
549 therapeutic application of these compounds, particularly in relation to CNS effects.
550 Metabolism All three compounds were determined not to be substrates for CYP2D6,

551 suggesting they are less likely to be metabolized through this common liver enzyme. This
552 could imply a potentially favorable metabolic profile, as compounds that are not extensively
553 metabolized may maintain higher bioavailability. Excretion clearance rates provide insights
554 into the elimination of these compounds from the body. Myristyl oleate had the highest total
555 clearance (2.149 log ml/min/kg), suggesting it may be rapidly eliminated, which could
556 influence dosing regimens. Conversely, 14 Beta H-pregna exhibited a significantly lower
557 clearance rate (0.67 logs ml/min/kg), indicating a slower elimination and potentially more
558 prolonged duration of action. Notably, none of the compounds tested positive for
559 mutagenicity in the Ames test, indicating a favorable toxicity profile. This is an encouraging
560 finding, as it suggests these compounds may have a lower risk of causing genetic damage.

561 Bis(2-ethylhexyl) phthalate (MW = 390.564 g/mol) and Myristyl oleate (MW = 478.846
562 g/mol) are relatively large molecules, which may influence their absorption and distribution in
563 biological systems. Larger molecules tend to exhibit lower permeability through biological
564 membranes, which could affect their therapeutic efficacy. In contrast, 14 Beta H-pregna has a
565 lower molecular weight (MW = 274.492 g/mol), potentially facilitating better absorption and
566 distribution, as smaller compounds typically diffuse more easily through cell membranes. The
567 Log P values provide insights into the hydrophobicity of the compounds. Myristyl oleate
568 exhibits an extraordinarily high Log P value (112.682), indicating its lipophilic solid nature,
569 which could enhance its ability to cross lipid membranes. However, such high lipophilicity
570 may also lead to challenges in solubility in aqueous environments, potentially limiting its
571 bioavailability when administered orally. Bis(2-ethylhexyl) phthalate has a significant Log P
572 value of 6.433, also reflecting its lipophilicity and suggesting a potential for membrane
573 permeation. Conversely, 14 Beta H-pregna has a Log P of 60.553, which is comparatively
574 lower than the other two compounds but still suggests a reasonable level of lipophilicity. The
575 varied lipophilicity of these compounds indicates that their absorption and distribution will
576 differ significantly, influencing their pharmacological activities. None of the compounds
577 displayed any donor hydrogen bonding, indicating that they may not participate in hydrogen
578 bond donation, which could limit their interactions with target proteins or enzymes. This
579 property could influence the compounds' binding affinities and their overall efficacy in
580 biological systems. The hydrogen acceptor bonding also varied among the compounds. Bis(2-
581 ethylhexyl) phthalate exhibited four acceptor sites, while Myristyl oleate had two, and 14 Beta
582 H-pregna had none. The presence of hydrogen bond acceptors in bis(2-ethylhexyl) phthalate

583 could facilitate its interaction with biomolecules, enhancing its potential activity. On the other
584 hand, the lack of hydrogen bonding capacity in 14 Beta H-pregna may limit its interactions
585 with proteins, potentially reducing its efficacy. Surface area is an important parameter that can
586 influence drug absorption and permeability. Myristyl oleate has the largest surface area
587 (214.638 Å²), which might affect its interactions with biological membranes and potential
588 targets. The larger surface area could facilitate a more significant interaction with the lipid
589 bilayer, impacting absorption rates. Bis(2-ethylhexyl) phthalate has a moderate surface area
590 (170.550 Å²), while 14 Beta H-pregna has the smallest surface area (125.650 Å²). The smaller
591 surface area of 14 Beta H-pregna could correlate with its lower molecular weight, potentially
592 leading to better permeability.

593

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Exploring the α -Amylase Inhibitory Potential of *Peronema canescens* Jack: An *In Vitro* and *In Silico* Study

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ABSTRACT

Hyperglycemia in individuals with type 2 diabetes mellitus is primarily driven by the rapid hydrolysis of starch by the enzyme α -amylase in the pancreas and the breakdown of oligosaccharides by α -glucosidase in the intestine. *Peronema canescens* Jack. (PC) has shown promise as a potential antidiabetic agent. This study aimed to evaluate the total flavonoid, phenolic, and α -amylase inhibitory activity of extracts and fractions derived from PC leaves using both *in vitro* and *in silico* approaches. The ethanol extract of PC leaves was fractionated through liquid-liquid extraction using *n*-hexane, ethyl acetate, and water as solvents. Preliminary phytochemical screening of the extracts and fractions identified the presence of alkaloids, flavonoids, saponins, tannins, and steroids/triterpenoids. The *n*-hexane fraction exhibited the highest total flavonoid content, averaging 203.37 \pm 4.38 mg QE/gram, while the ethyl acetate fraction demonstrated the highest total phenolic content, averaging 147.04 \pm 0.79 mg GAE/gram. Furthermore, the ethyl acetate fraction showed the strongest α -amylase inhibitory activity, with an average inhibition rate of 70.38 \pm 1.26%. *In silico* analysis, combined with GC-MS identification, suggested that three compounds, bis(2-ethylhexyl) phthalate, myristyl oleate, and 14 beta H-pregna may contribute to the observed α -amylase inhibitory activity. These findings highlight the potential of PC as a source of natural antidiabetic agents.



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1. Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both (WHO 2023). The prevalence of diabetes has reached alarming levels globally, with the International Diabetes Federation (IDF) estimating that over 537 million adults will be living with the condition in 2021 (Webber 2013). Experts project that this number will rise to 643 million by 2030. In Indonesia, the situation mirrors this global

trend, with an increasing number of the population being diagnosed with diabetes. The current standard of care for managing diabetes includes synthetic drugs such as sulfonylureas, metformin, and thiazolidinediones, designed to enhance insulin sensitivity, stimulate insulin secretion, or reduce glucose production. However, these synthetic drugs are not without their limitations. Long-term use of these medications can lead to adverse effects such as hypoglycemia (Chaudhury *et al.* 2017), gastrointestinal issues, and even multidrug resistance (MDR). These limitations underscore the need for alternative treatment strategies that are both effective and safe.

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In light of the challenges associated with synthetic drugs, there has been growing interest in herbal medicine as a potential alternative or complementary approach to diabetes management. Plants rich in bioactive compounds provide herbal medicines with therapeutic benefits and fewer side effects (Tran *et al.* 2020). One such plant is *Peronema canescens* Jack. (PC), a species native to Indonesia, traditionally used for its medicinal properties (Rahardhian *et al.* 2022b). The secondary metabolites found in PC, such as flavonoids, tannins, and alkaloids (Primal and Ahriyasna 2022), have been shown to possess various pharmacological activities, including antidiabetic effects. These natural compounds may help to mitigate hyperglycemia by enhancing insulin sensitivity, inhibiting glucose absorption, and modulating carbohydrate metabolism, making PC a promising candidate for diabetes treatment.

Computer-aided drug design (CADD) advancements have provided researchers with powerful tools to identify and optimize potential antidiabetic agents (Meng *et al.* 2011). Molecular docking simulation, a key component of CADD, allows for predicting the interaction between small molecules and target proteins, thereby facilitating the identification of compounds with high binding affinities (Pagadala *et al.* 2017). This method has been beneficial in the search for natural inhibitors of enzymes such as α -amylase, which plays a crucial role in carbohydrate digestion and glucose absorption (de Sales *et al.* 2012). Researchers can rapidly screen and identify the most promising antidiabetic compounds within complex plant extracts, such as those found in PC, by molecular docking simulations (Khan *et al.* 2024).

This study focuses on the antidiabetic potential of PC, examining both its *in vitro* and *in silico* activities. Gas Chromatography-Mass Spectrometry (GC-MS) analysis identified PC's chemical composition and revealed various bioactive compounds (Hotmian *et al.* 2021a). The inhibitory effect of these compounds on α -amylase was then evaluated through *in vitro* assays, while molecular docking simulations were employed to understand the interaction between these compounds and the enzyme at the molecular level (Santos *et al.* 2019). The physicochemical properties of PC were also analyzed and compared with other herbal medicines to highlight its unique advantages. This comprehensive approach sheds light on the potential of PC as an antidiabetic agent and underscores its benefits over other herbal remedies (Posadzki *et al.* 2013).

The novelty of this study lies in its integrative approach, combining *in vitro* and *in silico* methods to

investigate the α -amylase inhibitory potential of PC. While previous research has explored the antidiabetic effects of various herbal medicines, studies focusing specifically on PC remain limited. By providing a detailed analysis of its bioactive compounds and their mechanisms of action, this study serves as a preliminary investigation that could pave the way for future research (Altemimi *et al.* 2017). The findings presented here are relevant for developing PC as a potential antidiabetic treatment but also offer valuable insights for researchers working on similar objectives, making this study a significant consideration for ongoing and future developments in the field.

This study aims to evaluate the α -amylase inhibitory activity of PC using both *in vitro* and *in silico* methods. This research seeks to identify the specific compounds responsible for this activity and to understand their mechanisms of action at the molecular level. This research will contribute to the growing knowledge of natural antidiabetic agents and support PC development as a potential treatment for diabetes mellitus.

2. Materials and Methods

2.1. Materials

The equipment used in this study includes a Pyrex separation funnel, a Heidolph-G3 rotary evaporator, Silica Gel F254 plates, UV 254 and 366 lamps (Evaco GL 220V 50Hz T8 15W), micropipettes (Socorex and Dragon Lab), vortex mixers, a Shimadzu UV-1780 UV-Vis spectrophotometer (Serial No. A119161), a Synergy-HTX multi-mode ELISA reader with 96-well plates, and a QP 2010 gas chromatography-mass spectrometer (GC-MS). The materials utilized in this study include *Peronema canescens* Jack., ethanol, *n*-hexane, ethyl acetate, FeCl₃, MgSO₄, hydrochloric-ethanolic acid mixture (1:1), hydrochloric acid, Lieberman-Burchard reagent, 2,2-diphenyl-1-picrylhydrazyl (Sigma), quercetin, methanol, phosphate buffer, alpha-amylase enzyme (Sigma Aldrich), starch, and 3,5-dinitrosalicylic acid.

2.1.1. Hardware and Software

The molecular docking study was conducted using a laptop equipped with an Asus ROG 503 VD. The software utilized in this study includes ChemDraw Professional 15.0, Chem3D 15.0, Biovia Discovery Studio 2021, Command Prompt, and AutoDock Tools-1.5.6. Visualization of docking results and the creation of ligands and receptors were performed using

Biovia Discovery Studio 2021. Receptor structures were obtained from the RCSB Protein Data Bank (PDB) website (<https://www.rcsb.org/>), while ligand structures were downloaded from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>). Lipinski's rule of five was applied to assess drug-likeness using the SCFBio website (<http://www.scfbio-iitd.res.in/software/drugdesign/lipinski.jsp>). The pk-CSM online tool evaluated Pharmacokinetic and toxicological properties (<https://biosig.lab.uq.edu.au/pkcsml/>).

2.2. Methods

2.2.1. Sample Preparation, Extraction, and Fractionation

The extraction of *Peronema canescens*. Jack (PC) used the maceration method with 96% ethanol as the solvent. Three hundred grams of PC was placed in a maceration jar, and 96% ethanol was added as the solvent. The maceration process was performed for three days, with periodic solvent changes and occasional stirring to enhance extraction efficiency. The resulting macerate was filtered and then concentrated using a rotary vacuum evaporator. The concentrated extract was further thickened using a water bath at approximately 50°C (Rahardhian *et al.* 2019). Ten grams of the ethanol extract of PC leaves were dissolved in 100 ml of water and transferred into a separatory funnel. The mixture was then partitioned by adding 100 ml of *n*-hexane, followed by vigorous shaking. The mixture was allowed to settle until two distinct phases formed: the aqueous and *n*-hexane. The aqueous phase, separated from the *n*-hexane phase, was reintroduced into the separatory funnel. Subsequently, 100 ml of ethyl acetate was added to the aqueous phase. The mixture was shaken several times to ensure thorough mixing and then allowed to separate into its respective phases. The resulting fractions were concentrated using a rotary vacuum evaporator and thickened using a water bath at approximately 50°C to obtain a viscous fraction. (Rahardhian *et al.* 2019).

2.2.2. Phytochemical screening and TLC

Phytochemical screening follows the method (Putri *et al.* 2022). Each sample, including the ethanol extract, *n*-hexane fraction, ethyl acetate fraction, and water fraction of PC, was prepared by dissolving 30 mg of the sample in 3 ml of ethanol until completely dissolved. This solution was used for the subsequent phytochemical screening tests. Flavonoids: To the sample solution, magnesium (Mg) powder, 1 ml of hydrochloric acid (HCl), and amyl alcohol were added. The formation

of red, yellow, or orange colors indicated a positive presence of flavonoids. Polyphenols: A 10% ferric chloride (FeCl₃) solution was added to the sample. The appearance of a blue or blackish-green color indicated the presence of polyphenols. Tannins: The sample was mixed with gelatin salts. The formation of a yellowish-white precipitate indicated a positive result for tannins. Alkaloids: The sample was treated with HCl and water, then heated and divided into two portions (Filtrate 1 and Filtrate 2). Filtrate 1: Reacted with Mayer's reagent, forming a yellowish-white precipitate, indicating the presence of alkaloids. Filtrate 2: Reacted with Dragendorff's reagent, forming a brick-red precipitate, confirming the presence of alkaloids. Saponins: The sample was placed in a test tube, and 10 ml of distilled water was added. The mixture was shaken vigorously for 10 seconds. A positive reaction was indicated by the formation of foam that persisted for 10 minutes at a height of 1-3 cm. Steroids/triterpenoids: The sample was treated with chloroform and filtered. The filtrate was mixed with anhydrous acetic acid (CH₃COOH) and heated, then cooled and treated with sulfuric acid (H₂SO₄). A green color in the solution indicated the presence of steroids, while an orange or red color indicated the presence of triterpenoids.

Thin-layer chromatography (TLC) was performed following the method described by (Putri *et al.* 2022) to identify the compound content of the samples. A small amount of each sample was dripped directly onto the TLC plate. Flavonoids: The mobile phase consisted of *n*-butanol, acetic acid, and water in a ratio of 4:1:5. The TLC plate was then exposed to ammonia vapor. The presence of flavonoids was indicated by the formation of yellow stains after ammonia treatment. Tannins: The mobile phase was prepared using ethyl acetate, methanol, and water in a ratio of 100:13.5:10. The presence of tannins was indicated by visible spots formed by FeCl₃, which resulted in blackish-green patches. Alkaloids: The mobile phase comprised ethyl acetate, methanol, and water in a ratio of 6:4:2. The presence of alkaloids was confirmed by the appearance of brown patches upon applying Dragendorff's reagent. Saponins, A solvent system of chloroform, methanol, and water in a ratio of 64:50:10, was utilized. Anisaldehyde-sulfuric acid was used as a visualization reagent, and the TLC plate was heated on a hot plate for 5-10 minutes at 100°C. The presence of saponins was indicated by the formation of colored patches, including yellow, green, red, dark blue, purple, and brownish-yellow. Steroids/triterpenoids: The mobile phase consisted of *n*-hexane and ethyl acetate

in a ratio of 17:3. Anisaldehyde-sulfuric acid was again used for visualization, and the TLC plate was heated on a hot plate for 5-10 minutes at 100°C. A purple color indicated the presence of steroids, while a blue color indicated triterpenoids.

2.2.3. Determination of Total Phenolic and Total Flavonoid Content

The total flavonoid content (TFC) and total phenolic content (TPC) of the extracts were determined using established spectrophotometric methods (Rahardhian *et al.* 2019). TPC was assessed using the Folin-Ciocalteu reagent with gallic acid as the standard, and absorbance was measured at 765 nm. TFC was determined using aluminum chloride (AlCl₃) with quercetin as the standard, and absorbance was measured at 420 nm. Both analyses were conducted using a Shimadzu® UV-Vis Spectrophotometer (Model 1240). The total phenolic and flavonoid contents were calculated based on the absorbance values obtained from the gallic acid and quercetin standard curves, respectively.

2.2.4. α-amylase Inhibitory Activity

The α-amylase inhibitory activity of the samples was evaluated using a colorimetric method on a 96-well plate. The samples were dissolved in a 1% dimethyl sulfoxide (DMSO) solution. Each sample, along with the negative control (1% DMSO solution) and positive control (acarbose at concentrations of 50 mg and 100 mg), was pipetted into the wells of a 96-well plate at a volume of 20 µL per well. 50 µL of 100 mM phosphate buffer (pH 6.8) was added to each well, followed by ten µL of α-amylase solution (2 U/ml). The plate was pre-incubated at 37°C for 20 minutes to allow the enzyme and inhibitor to interact. After pre-incubation, 20 µL of a 1% starch solution in 100 mM phosphate buffer (pH 6.8) was added to each well as the substrate. The reaction mixture was further incubated at 37°C for 30 minutes. After incubation, 100 µL of 3,5-dinitrosalicylic acid (DNS) reagent was added to each well. The plate was then incubated at 100°C for 10 minutes to develop the color. The absorbance of the resulting mixture was measured at a wavelength of 540 nm using a Multiplate Reader. Acarbose at various concentrations served as the standard for comparison, and the percentage of α-amylase inhibition was calculated based on the absorbance values obtained from the samples relative to the control wells.

$$\text{Inhibition of } \alpha\text{-amylase (\%)} = \frac{((Ac - Ac') - (As - As'))}{(Ac' - As')} \times 100$$

Where:

- Ac : Absorbance of the blank (control without any sample or inhibitor)
- Ac' : Absorbance of the blank control (in the presence of the solvent, e.g., 1% DMSO)
- As : Absorbance of the sample (in the presence of the sample extract)
- As' : Absorbance of the sample control (sample with solvent but no substrate or enzyme)

2.2.5. Identification of Compounds in the Active Fraction of PC using GC-MS

GC-MS analysis was performed at the Integrated Laboratory of the Islamic University of Indonesia, Yogyakarta, to identify the compounds present in the samples. The columns used in the analysis were Rtx-5MS columns, thickness 0.25 µm, length 30.0 m and diameter 0.25 mm, column temperature 80.0°C, injection temperature 300.00°C, with split injection mode, pressure 42.3 kPa, total flow 117.5 ml/min and column flow 0.74 m/min. (Hotmian *et al.* 2021b).

2.2.6. Molecular Docking

Molecular docking was conducted using various software tools to analyze the interactions between the proteins and ligands. The receptors were downloaded from the Protein Data Bank (PDB) <https://www.rcsb.org/> (Pagadala *et al.* 2017). Water molecules surrounding the protein were removed, and the protein chain was separated from its native ligand and saved in *pdb format as a protein file. The native ligand structure was extracted by removing the corresponding portion of the protein chain and saved in *pdb format as a ligand file. The ligand's structure was initially prepared in 2D using ChemDraw Pro 12.0. (PerkinElmer 2015), the 3D structure was constructed, and molecular mechanics (MM) geometry optimization was performed using Chem3D Pro 12.0. The optimized ligand structure was saved in PDB file format. The protein was prepared using AutoDockTools 1.5.6 (Morris *et al.* 2009) by adding hydrogen atoms to the polar side of the structure and applying Kollman charges. The ligand was prepared by correcting its structure and adding Gasteiger charges. The prepared structures were saved in *pdbqt format. The redocking process utilized a grid box of dimensions 40 × 40 × 40 with the following coordinates: x = 10.265, y = 45.877, z = 19.734. Docking Parameters Genetic Algorithm (GA), the output algorithm for docking results, was set to Lamarckian GA 4.2, and other docking parameters were set to default values. The

critical parameter to evaluate the docking results was the RMSD. An acceptable RMSD value was ≤ 3.0 Å, indicating reliable docking conformations.

2.2.7. Evaluation of Drug Likelihood and ADMET

Drug likeness was evaluated based on Lipinski's Rule of Five, a widely used guideline in drug discovery to predict the oral bioavailability of compounds. The following criteria were considered molecular weight: the compound should not exceed 500 Da, the number of hydrogen bond acceptors (-H bond acceptors) should be no more than 10, the number of hydrogen bond donors (-H bond donors) should not exceed 5, the log P value, which indicates the compound's lipophilicity, should be less than 5 (or MlogP <4.15) (Rahardhian *et al.* 2022a). Absorption, Distribution, Metabolism, Excretion, and Toxicity (ADMET) properties were predicted using computational methods further to assess the compounds' suitability for drug development. CaCO₂ permeability was assessed to evaluate intestinal absorption. Blood-brain barrier (BBB) permeability was predicted to assess the ability of the compound to cross the BBB. The compound was evaluated as a substrate for CYP2D6, a key enzyme in drug metabolism. Total clearance was calculated to estimate how quickly the compound is eliminated from the body. The AMES (Ames Mutagenicity Test) toxicity tests assessed potential genotoxic effects (Pires *et al.* 2015).

2.3. Data Analysis

Statistical analysis was performed using GraphPad Prism 9.0 (GraphPad Software Inc., CA, USA). The results are presented as mean values \pm standard deviation (SD). ANOVA was applied to evaluate the differences among the various extracts. This method helps determine whether there are statistically significant differences between the means of three or more independent groups. A post hoc test was conducted following ANOVA to identify specific group differences. A significance level of $p > 0.05$ was used to determine statistical significance.

3. Results

3.1. Phytochemical Screening

The fractionation of the ethanol extract from PC leaves resulted in the following yields: 32.81% for the *n*-hexane fraction, 16.86% for the ethyl acetate fraction, and 34.17% for the water fraction. Notably, the ethyl acetate fraction yield was lower than that of

the *n*-hexane and water fractions. This variation in yield may be attributed to the differing solubility profiles of the compounds present in the PC leaf extract, with nonpolar and polar compounds being more prevalent than semipolar compounds. Phytochemical screening was conducted to identify various bioactive compounds, including alkaloids, flavonoids, saponins, tannins, and triterpenoids/steroids. The results indicated that the ethanol extract of PC leaves tested positive for alkaloids, flavonoids, tannins, saponins, and triterpenoids. In contrast, the water fraction demonstrated a positive presence only for alkaloids and tannins. The results of the phytochemical screening indicate that the ethanol extract and fractions of *Peronema canescens* leaves contain various bioactive compounds Table 1.

3.2. Thin Layer Chromatography (TLC)

The compounds present in the ethanol extract, *n*-hexane fraction, ethyl acetate fraction, and water fraction of PC leaves were further analyzed using Thin Layer Chromatography (TLC). This technique confirmed the presence of various bioactive compounds by evaluating the R_f values and the corresponding color stains formed on the TLC plates. The R_f values were calculated for each compound, allowing for the identification of the specific compounds present in each fraction. The results of the TLC analysis are summarized in Table 2, which includes the R_f values and corresponding colors observed for each compound group in the different fractions.

From the results of phytochemical screening and TLC tests, PC leaf extracts and fractions contain several secondary metabolite compounds, including alkaloids, flavonoids, saponins, tannins, terpenoids, steroids, and phenolics Table 2.

3.3. Determination of Total Phenolic and Total Flavonoid Content

Figure 1 shows that the *n*-hexane fraction of PC leaves exhibit the highest total flavonoid content, with an average concentration of 203.3742 ± 4.3777 mg QE/gram. In contrast, the ethyl acetate fraction of PC leaves contains the highest total phenolic content, with an average concentration of 147.0397 ± 0.7864 mg GAE/gram.

3.4. Active Fraction of PC Leaves Using GC-MS

The active fraction was identified using Gas Chromatography-Mass Spectrometry (GC-MS). Based

Table 1. Phytochemical screening results of ethanol extract and fractions of PC leaves












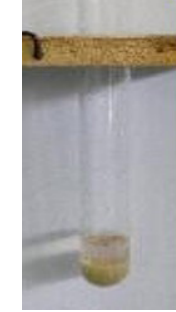















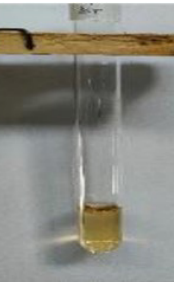
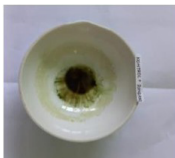



Compound group	Reagent	Ethanol extract	<i>n</i> -hexane fraction	Ethyl acetate fraction	Water fraction
Phenolic	Add 5 to 6 drops of a 1% ferric chloride (FeCl ₃)				
Tannins	2 ml of a 10% NaCl solution mixed with 1% gelatin				
Flavonoids	Magnesium powder combined with concentrated HCl and amyl alcohol				
Alkaloids	Add three drops of Bouchardat's reagent				
	Mix 1 ml of 2N HCl with two drops of Mayer's reagent				

Table 1. Continued

Compound group	Reagent	Ethanol extract	<i>n</i> -hexane fraction	Ethyl acetate fraction	Water fraction
Saponins	Mix 1 ml of 2N HCl with two drops of Dragendorff's reagent				
	Add 10 ml of hot water to 2N HCl				
Steroids	Mix 5 ml of chloroform with anhydrous acetic acid and H ₂ SO ₄				

(+): contains the compound, (-): does not contain the compound

Table 2. TLC results of ethanol extract and fractions of PC leaves

Compound group	Mobile phase	Sample	Visual	UV 254	UV 366	Spot appearance	Rf value
Alkaloids	ethyl acetate, methanol, and water (6:4:2)	EE	Brown	Brown	Orange	Brown	0.81
		NHF	Brown	Brown	Orange	Brown	0.79
		EAF	Orange	Brown	Orange	Brown	0.75
		WF	Brown	Brown	Blue	Brown	0.73
Flavonoid	<i>n</i> -butanol, acetic acid, and water (4:1:5)	EE	Green	Green	Orange	Green	0.91
		NHF	Green	Green	Orange	Green	0.91
		EAF	Green	Green	Orange	Green	0.91
		WF	Brown	Green	Blue	Yellow	0.56
Saponin	chloroform, methanol, and water (64:50:10)	EE	Green	Green	Orange	Purple	0.94
		NHF	Green	Green	Orange	Purple	0.94
		EAF	Green	Green	Orange	Green	0.90
		FAE	-	-	-	-	-
Tannins	ethyl acetate, methanol, and water (100:13.5:10)	EE	Green	Black	-	Green Blackish	0.88
		NHF	Brown	Black	-	Ash Blackish	0.94
		FEA	Brown	Black	-	Black	0.94
		WF	Brown	Black	-	Black	0.91
Steroids	<i>n</i> -hexane and ethyl acetate (17:3)	FAE	Green	Brown	Blue	Brown	0.91
		FNH	Green	Brown	Blue	Brown	0.91
		FEA	Green	Brown	Orange	Brown	0.22
		FA	-	-	-	Brown	-

(+): contains the compound, (-): does not contain the compound

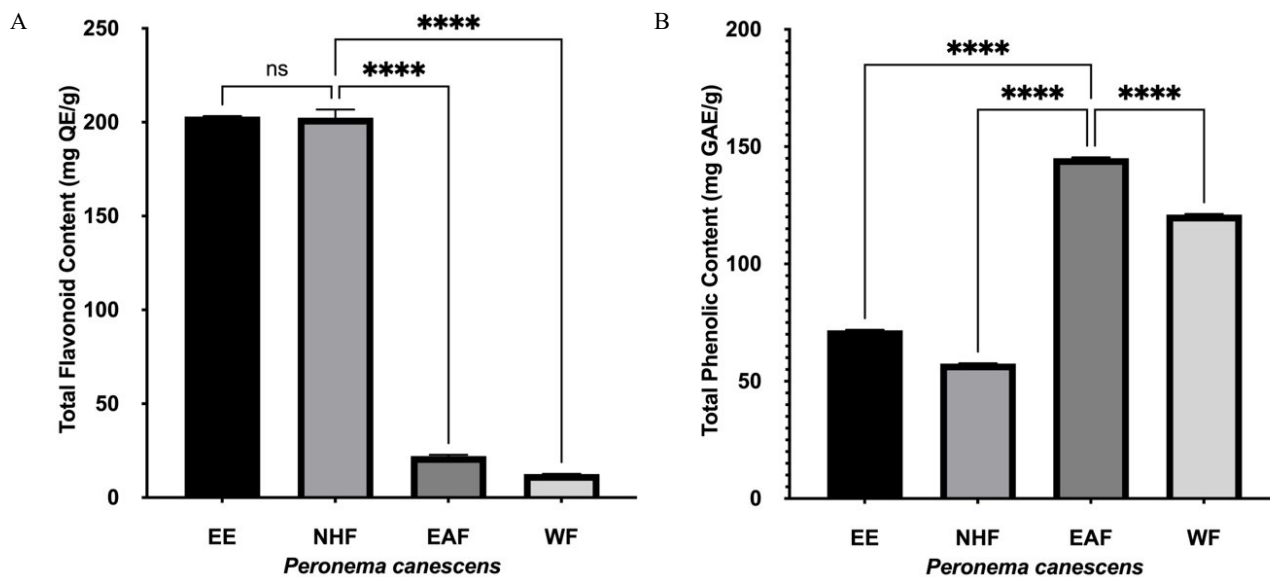


Figure 1. (A) Total flavonoid content and (B) total phenolic content. EE: ethanolic extract, NHF: *n*-hexane fraction, EAF: ethyl acetate fraction, WF: water fraction. ns: non-significant; ****: significant difference ($P > 0.05$)

on the analysis of PC leaves' active fraction (ethyl acetate fraction), the compounds identified are listed in Table 3.

3.5. *In Vitro* Inhibition of α -Amylase Activity

The α -amylase inhibitory activity of the extract and fractions of PC leaves was evaluated by measuring the total reducing sugar using the 3,5-dinitrosalicylic acid (DNS) method, with starch as the substrate. 3,5-dinitrosalicylic acid is an aromatic compound that reacts with reducing sugars to form 3-amino-5-nitrosalicylic acid, which absorbs electromagnetic radiation. The α -amylase inhibitory activity was quantified at a wavelength of 540 nm using a 96-well microplate reader.

Based on Figure 2, the active fraction with the highest percentage of α -amylase enzyme inhibition was observed in the ethyl acetate fraction of PC leaves, with an average inhibition of $70.38 \pm 1.26\%$.

3.6. *In Silico* Inhibition of α -Amylase Activity

Molecular docking is a commonly used method in drug development to identify compounds or molecules with therapeutic activity by predicting ligand-target interactions and evaluating structural activity using computational techniques. The validation results showed an RMSD value of 1.74 Å, indicating that the redocking method met the required criteria. Furthermore, the binding energy affinity (ΔG) was found to be -4.17 kcal/mol, with an inhibition constant

(K_i) of the same magnitude obtained during the fourth docking run.

Based on Table 4, three bioactive compounds exhibit favorable binding energies and amino acid interactions with the same target protein as the natural ligands. The binding energy values reflect the spontaneity of the interaction between the protein and the ligand; the more negative the binding energy, the more rapid the binding process occurs. This suggests that these compounds may serve as effective α -amylase inhibitors, warranting further investigation into their potential therapeutic applications.

Table 5 presents the results of the ADME (Absorption, Distribution, Metabolism, and Excretion) analysis for the bioactive compounds identified in the active fraction of PC leaves. This analysis is crucial for evaluating the pharmacokinetic properties of these compounds, which can influence their effectiveness as potential therapeutic agents. The results provide insights into the compounds' absorption rates, distribution patterns in the body, metabolic stability, and excretion profiles, helping to assess their suitability for drug development. The ADME (Absorption, Distribution, Metabolism, Excretion) and toxicity profiles of the bioactive compounds identified in the active fraction of PC leaves. The analysis indicates that the bioactive compounds Bis(2-ethylhexyl) phthalate, Myristyl oleate, and 14 Beta H pregna meet the criteria for a favorable ADME profile, suggesting efficient absorption and distribution within the body.

Table 3. Results of GC-MS identification of active fraction compounds in PC leaves

Peak	Retention time	Percent area(%)	Molecular weight	Base peak	Similarity index	Molecular formula	Compound
1	20,208	1.70	390	149	97	C ₂₄ H ₃₈ O ₄	Bis(2-ethylhexyl) phthalate
2	21,656	0.58	478	57	63	C ₃₂ H ₆₂ O ₂	Myristyl oleate
3	21,942	0.57	593	57	59	C ₃₉ H ₇₆ O ₃	Oleic acid
4	22,825	2.12	288	57	64	C ₂₁ H ₃₆	14-Beta-H-Pregna
5	22,902	8.77	723	57	77	C ₄₅ H ₈₆ O ₆	Tetradecanoic acid
6	23,021	16.46	639	57	75	C ₃₉ H ₇₄ O ₆	Dodecanoic acid
7	23,142	9.25	723	57	68	C ₄₅ H ₈₆ O ₆	Tetradecanoic acid
8	23,255	27.05	751	57	77	C ₄₇ H ₉₀ O ₆	Hexadecanoic acid
9	23,301	19.04	639	57	83	C ₃₉ H ₇₄ O ₆	Dodecanoic acid
10	23,367	14.46	639	183	71	C ₃₉ H ₇₄ O ₆	Dodecanoic acid

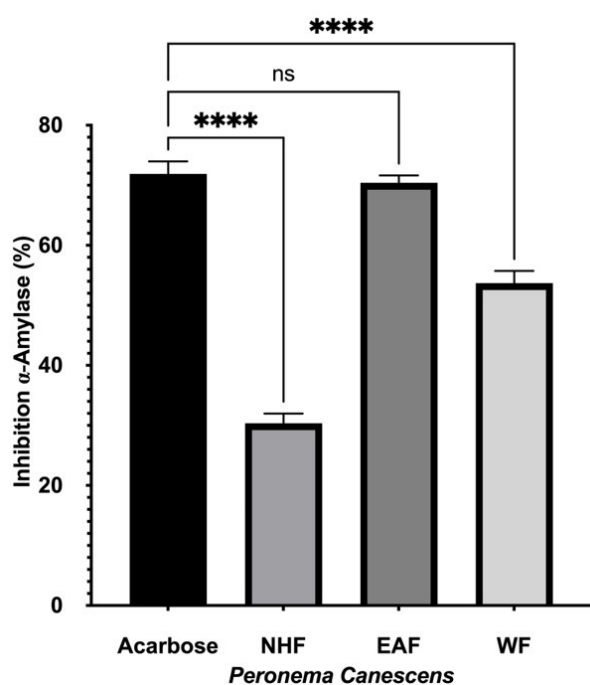


Figure 2. Percentage inhibition of α -amylase enzyme activity. NHF: *n*-hexane fraction, EAF: ethyl acetate fraction, WF: water fraction, ns: non-significant, ****: Significant difference ($P > 0.05$)

Table 6 presents the results of the Lipinski analysis for the bioactive compounds found in the active fraction of PC leaves. Lipinski's rule of five evaluates the drug-likeness of compounds based on their molecular properties, which predict good absorption and permeation. The analysis assesses molecular weight, hydrogen bond donors and acceptors, and logP values. Compounds that comply with these criteria will likely exhibit favorable pharmacokinetic properties, making them suitable candidates for further drug development. The bioactive compounds in the active fraction of PC leaves meet Lipinski's rule of five

Table 4. Results of molecular docking analysis of ethyl acetate fraction compounds of PC leaves

Compound	ΔG (kcal/mol)	Ki (mM)	Amino acids residue
Bis(2-ethylhexyl) phthalate	-4.59	432.20	Tyr 62, Leu 162, His 201, Lys 200, Ile 235, His 299
Myristyl oleate	-3.32	3.66	Ile 51, Leu 165, Pro 54, His 101, Trp 59, His 305, Tyr 62, Asp 300
14 Beta H pregna	-7.81	1.90	Tent 162, Tent 165, Trp 58, Trp 62, His 299
Natural ligand (Acarbose)	-4.17	-	The 163, His 305, Asp 300, Glu 233, His 101, Arg 195, Asp 197, His 299

requirements. Specifically, these compounds exhibit a molecular weight of less than 500 mg/mol, a logP value between 0.4 and 5, a hydrogen bond donor count of ≤ 10 , a hydrogen bond acceptor count of ≤ 5 , and a molar refractivity below 130. Consequently, it can be concluded that these bioactive compounds are considered safe for oral use.

4. Discussion

The fractionation of the ethanol extract from PC leaves yielded 32.81% for the *n*-hexane fraction, 16.86% for the ethyl acetate fraction, and 34.17% for the water fraction. The lower yield of the ethyl acetate fraction indicates a higher abundance of nonpolar and polar compounds relative to semipolar compounds in the extract (Duistermaat and Kolk 2000). This pattern reflects the principles of solvent polarity, where nonpolar solvents like *n*-hexane extract nonpolar compounds such as lipids, while polar solvents like water preferentially extract polar substances like sugars and certain phenolic compounds (Altemimi *et al.* 2017).

Table 5. Results of ADME analysis of active fraction compounds of PC leaves

Compound	Absorption (Caco2 permeability) (log Papp in 10 ⁻⁶ cm/s)	Distribution (BBB permeability y) (log BB)	Excretion (Total clearance) (log ml/min/kg)	Toxicity (AMES) (Yes/No)
Bis(2-ethylhexyl) phthalate	1,408	-0.175	-0.175	No
Myristyl oleate	1,306	1.014	1.014	No
14 Beta H pregna	1,405	0.885	0.885	No

Table 6. Results of the lipinski analysis for the bioactive compounds found in the active fraction of PC leaves

Compound	Molecular weight	Log P	Donor hydrogen bonding	Hydrogen acceptor bonding	Surface area
Bis(2-ethylhexyl) phthalate	390,564	6,433	0	4	170,550
Myristyl oleate	478,846	112,682	0	2	214,638
14 Beta H pregna	274,492	60,553	0	0	125,650

Phytochemical screening revealed alkaloids, flavonoids, tannins, saponins, and triterpenoids/steroids in the ethanol extract. However, the water fraction was only positive for alkaloids and tannins. This selective presence underscores the solubility characteristics of the compounds, with flavonoids and triterpenoids being more soluble in ethyl acetate, explaining their absence in the water fraction. The limited presence of flavonoids and triterpenoids in the water fraction of PC leaves aligns with other studies on medicinal plants, suggesting that these compounds are more concentrated in less polar solvents. The phytochemical screening of the ethanol extract from PC leaves, along with its fractions, revealed a diverse array of bioactive compounds. The tests utilized various reagents to identify specific compound groups, including phenolics, tannins, flavonoids, alkaloids, saponins, and steroids. This suggests that phenolics, The research results are consistent with (Rahardhian *et al.* 2022b), indicating that PC contains phenolic.

The presence of phenolic compounds was confirmed in all fractions, including the ethanol extract, *n*-hexane, ethyl acetate, and water fractions, as indicated by the positive reaction with ferric chloride. Tannins were present in the ethanol extract and water fraction but absent in the *n*-hexane fraction. This distribution indicates that tannins, which are polyphenolic compounds with astringent properties, are more soluble in polar solvents like water and ethanol. The screening indicated flavonoids were present in the ethanol, *n*-hexane, and ethyl acetate fractions but absent in the water fraction. This result highlights the preferential solubility of flavonoids in less polar solvents, supporting findings in other studies that suggest flavonoids are typically more concentrated in ethyl acetate and hexane fractions. All fractions tested positive for alkaloids, with multiple reagents confirming their

presence. Saponins were present in the ethanol, *n*-hexane, and ethyl acetate fractions but absent in the water fraction. This suggests that while saponins can dissolve in polar solvents, they may require a particular concentration or temperature to effectively extract, as indicated by their absence in the water fraction after the hot water test. Steroids were found in the ethanol, *n*-hexane, and ethyl acetate fractions but not in the water fraction. This aligns with their chemical nature, as these compounds are generally more soluble in nonpolar or moderately polar solvents. The phytochemical screening demonstrates that PC leaves are rich in bioactive compounds across different fractions. The solubility profiles of these compounds indicate their potential utility in therapeutic applications, as well as the need for further research to explore their pharmacological properties and mechanisms of action (Suryanti *et al.* 2022).

The thin-layer chromatography (TLC) analysis of PC leaves provides a comprehensive overview of its bioactive compound profile, including alkaloids, flavonoids, saponins, tannins, and steroids (Ladeska *et al.* 2024). The visualizations and Rf values obtained during the analysis allow for a comparative understanding of the composition and potential therapeutic applications of these compounds (Yodha *et al.* 2024). The presence of alkaloids was confirmed across all samples, with Rf values ranging from 0.73 to 0.81. The consistent brown coloration under UV light and the appearance of orange spots following Dragendorff's reagent application suggest a robust alkaloid profile in PC leaves. These findings align with research (Li *et al.* 2020), which noted that alkaloids often exhibit similar Rf values due to their polar characteristics, influencing their solubility and mobility in chromatographic conditions. Alkaloids are recognized for their pharmacological activities, including analgesic

and antimicrobial properties, reinforcing the potential medicinal value of PC. Flavonoids displayed Rf values of 0.56 to 0.91, with the ethanol, *n*-hexane, and ethyl acetate fractions exhibiting a distinct green coloration. The yellow stains after ammonia treatment are consistent with flavonoid presence, as supported by studies like those of (Panche *et al.* 2016), which indicate that the coloration is indicative of flavonoid derivatives. The consistent Rf values across the fractions suggest that PC leaves contain a diverse range of flavonoids, which may enhance their therapeutic profile. The analysis showed a strong presence of saponins, with Rf values around 0.90 to 0.94 and vibrant colored patches indicating their presence. This finding corroborates previous studies, such as those by (Cheok *et al.* 2014). Tannins were identified with Rf values of 0.88 to 0.94, displaying green-blackish spots after FeCl₃ treatment. This aligns with findings from (Das *et al.* 2020), which suggest that tannins' astringent properties and their ability to form complexes with proteins are responsible for their bioactivity. The presence of tannins in PC leaves may contribute to their potential health benefits, particularly in antioxidant and antimicrobial applications. Steroids were detected, with Rf values of 0.22 to 0.91. The purple color indicating steroids and the blue for triterpenoids under UV light corresponds with findings from (Tarigan *et al.* 2023), which report that these compounds have significant roles in various biological activities, including anti-inflammatory effects. The varying Rf values suggest differing solubility characteristics among the compounds, indicating the complexity of the steroidal profile in PC leaves.

The *n*-hexane fraction demonstrated the highest total flavonoid content, averaging 203.3742±4.3777 mg QE/gram. The predominance of flavonoids in the *n*-hexane fraction suggests that these compounds have a higher solubility in nonpolar solvents (Putri *et al.* 2023). This aligns with findings from several studies indicating that nonpolar extraction methods are effective for isolating lipophilic flavonoid compounds. For instance, research by (Putri *et al.* 2022) supports the idea that nonpolar solvents can enhance the extraction of certain flavonoid derivatives, potentially improving their bioavailability and pharmacological efficacy. In contrast, the ethyl acetate fraction exhibited the highest total phenolic content, averaging 147.0397±0.7864 mg GAE/gram. Phenolic compounds are well-known for their potent antioxidant properties, contributing to the plant's protective mechanisms against oxidative stress. The higher concentration of phenolics in the ethyl acetate fraction indicates their preferential solubility in moderately polar

solvents, which has been corroborated by studies such as those by (Rahardhian *et al.* 2019). These findings highlight the effectiveness of ethyl acetate in extracting bioactive phenolic compounds, which may have implications for their therapeutic applications in treating oxidative stress-related diseases. The observed differences in flavonoid and phenolic concentrations between the two solvent fractions emphasize the importance of solvent polarity in the extraction process. This selective extraction allows for a deeper understanding of the phytochemical profile of PC leaves, which can guide further research into their potential health benefits. Notably, the high total flavonoid content in the *n*-hexane fraction suggests that PC may be a valuable source of flavonoid-rich extracts that could be utilized in nutraceutical formulations targeting antihyperglycemic.

The gas chromatography-mass spectrometry (GC-MS) analysis of PC leaves provided valuable insights into the chemical composition of its extracts, revealing a range of fatty acids and esters. Each compound's retention time, molecular weight, and similarity index contribute to understanding the plant's phytochemical profile and its potential applications in health and nutrition (Hotmian *et al.* 2021a). Among the identified compounds, notable fatty acids include tetradecanoic acid (C₁₄H₂₈O₂) and hexadecanoic acid (C₁₆H₃₂O₂), which accounted for significant peak areas of 8.77% and 27.05%, respectively. The predominance of hexadecanoic acid is particularly interesting, as it has been associated with various health benefits, including anti-inflammatory and antimicrobial properties. This aligns with research indicating that fatty acids play crucial roles in cellular functions and may cause diabetes mellitus diseases (Berry 1997). The presence of bis(2-ethylhexyl) phthalate (C₂₄H₃₈O₄), with a peak area of 1.70%, indicates the plant's potential to contain plasticizer compounds, which can have implications for environmental health.

Additionally, oleic acid (C₁₈H₃₄O₂), which appeared in the analysis, is a well-known monounsaturated fatty acid praised for its heart health benefits, including its ability to lower harmful cholesterol levels (Eleazu *et al.* 2018). The molecular weight of oleic acid (593) and its retention time further suggests its bioactive potential. The diversity of compounds points to the complex nature of the phytochemical constituents of PC, including myristyl oleate (C₃₂H₆₂O₂) and 14-Beta-H-Pregna (C₂₁H₃₆), with respective peak areas of 0.58% and 2.12%. The variety of fatty acids and esters indicates that the plant could serve as a source of bioactive compounds with diverse pharmacological activities, ranging from antidiabetic

mellitus to antioxidant effects. The similarity index values, ranging from 57 to 97, highlight the reliability of the GC-MS identification process, reflecting the compounds' structural integrity and known bioactivities. The higher similarity indices suggest that the identified compounds are well-documented in the literature, supporting their potential utilization in therapeutic applications. In summary, the GC-MS analysis of PC leaves reveals a rich array of fatty acids and esters, emphasizing the plant's potential as a source of bioactive compounds.

The findings from this study reveal significant insights into the α -amylase inhibitory potential of PC, particularly highlighting the efficacy of the ethyl acetate fraction. The observed average inhibition percentage of 70.38% for the ethyl acetate fraction is notably higher than the 30.37% and 53.18% inhibition observed in the *n*-hexane and water fractions, respectively. This variation in inhibitory activity suggests a differential distribution of bioactive compounds in the various fractions, likely attributable to their solubility properties and polarity. The ethyl acetate fraction's superior inhibition effect is particularly noteworthy, as it closely approaches the inhibition level of acarbose, a well-known α -amylase inhibitor, which demonstrated an average inhibition percentage of 74.29%. The lack of significant difference between the ethyl acetate fraction and acarbose indicates that the compounds present in this fraction possess potent α -amylase inhibitory activity. This finding is consistent with existing literature, which suggests that ethyl acetate often extracts a variety of bioactive compounds, such as flavonoids and phenolic compounds, that are known for their enzyme-inhibitory effects (Nyambe-Silavwe *et al.* 2015). The lower inhibition percentages observed

in the *n*-hexane fraction may be attributed to the nonpolar nature of the compounds extracted, which are less likely to interact effectively with the active site of the α -amylase enzyme. Conversely, the water fraction exhibited moderate inhibition, possibly due to the presence of polar compounds, but it was less effective than the ethyl acetate fraction, highlighting the significance of solvent polarity in influencing the extraction of bioactive constituents. This study demonstrates that the ethyl acetate fraction of PC exhibits significant α -amylase inhibitory potential, comparable to acarbose, suggesting its potential as a natural alternative for diabetes management and warranting further research into its pharmacological applications (Lestari *et al.* 2018).

The molecular docking results presented in this study provide critical insights into the binding affinities and inhibitory potentials of various compounds against α -amylase (Lolok *et al.* 2022). The three-dimensional structure of the α -amylase enzyme and its natural ligands is presented in Figure 3. The free energy of binding (ΔG) and inhibition constants (K_i) serve as valuable indicators of the interactions between these compounds and the enzyme (Yuningtyas *et al.* 2024), shedding light on their potential as α -amylase inhibitors. The overlay of the acarbose ligand confirmation before and after redocking is depicted in Figure 4. Bis(2-ethylhexyl) phthalate exhibited a binding free energy of -4.59 kcal/mol with an inhibition constant (K_i) of 432.20 mM. The interaction of this compound with the enzyme involves critical amino acid residues such as Tyr 62, Leu 162, and His 201. While the negative ΔG value indicates a favorable binding, the relatively high K_i value suggests that this compound may not be a potent inhibitor of α -amylase.

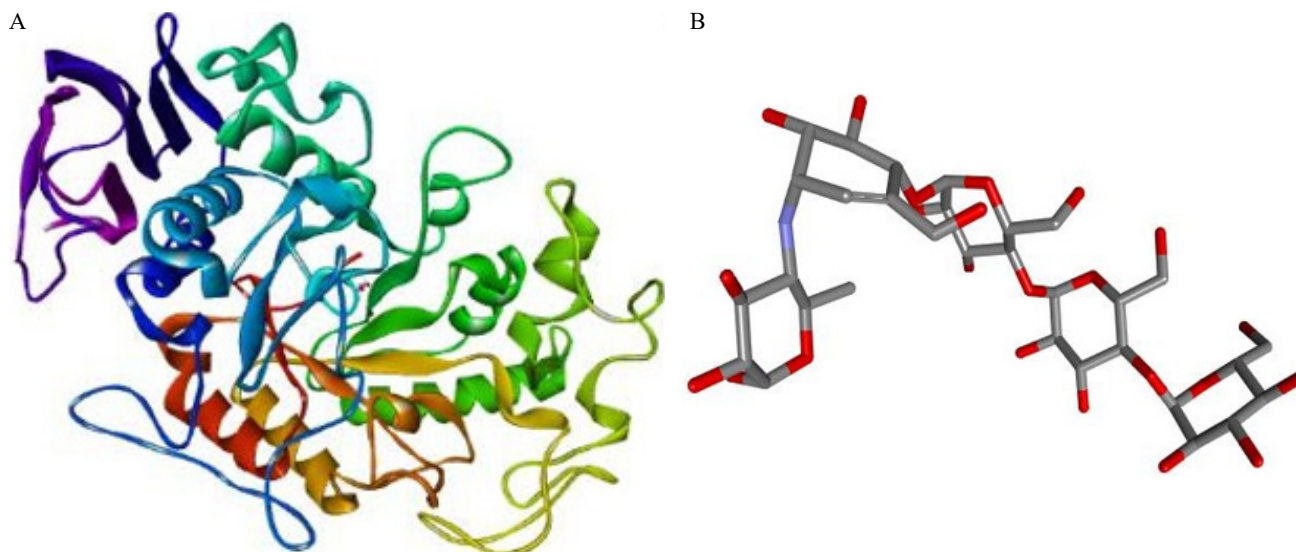


Figure 3. (A) The three-dimensional structure of the α -amylase enzyme and (B) its natural ligands

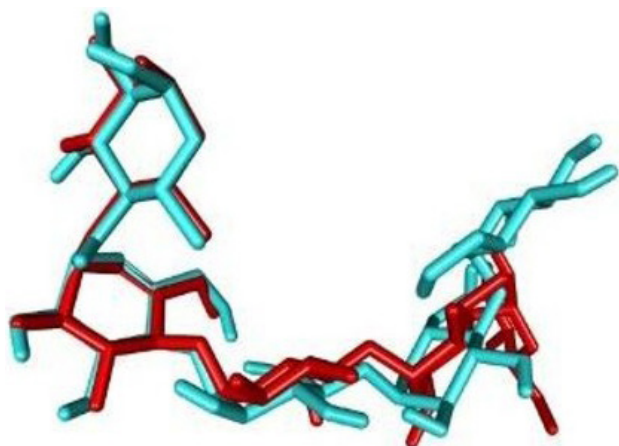


Figure 4. The overlay of the acarbose confirmation before (blue) and after (red) redocking

The interactions with critical residues indicate a potential for activity, but further modifications may be needed to enhance its inhibitory potency. Myristyl oleate showed a lower binding affinity, with ΔG of -3.32 kcal/mol and a K_i value of 3.66 mM. The presence of interactions with multiple residues, including Ile 51, Leu 165, and His 101, suggests that this compound forms multiple favorable contacts with the enzyme. The significantly lower K_i indicates that myristyl oleate could act as a more potent inhibitor compared to bis(2-ethylhexyl) phthalate, possibly due to its ability to engage critical active site residues effectively. 14 Beta H-pregna demonstrated the strongest binding affinity with a ΔG of -7.81 kcal/mol and a low K_i of 1.90 mM. The strong interactions with amino acids such as Trp 58 and Trp 62 indicate that this compound may have a significant impact on the active site configuration of α -amylase. The natural ligand (acarbose), known for its clinical efficacy in inhibiting α -amylase, had a ΔG of -4.17 kcal/mol but did not present a K_i value as this compound acts through a different mechanism. The amino acid residues involved in binding, such as The 163 and His 305, reflect the established interactions that allow acarbose to inhibit the enzyme competitively. This comparison highlights the relative effectiveness of the newly identified compounds against a standard inhibitor, demonstrating that certain derivatives may offer similar or improved efficacy.

The physicochemical properties of the compounds derived from PC were evaluated based on their molecular weight, lipophilicity (Log P), hydrogen bonding capacity, and surface area. Understanding these characteristics is critical for assessing their bioavailability, interactions, and overall pharmacological potential (Pires *et al.* 2015). Absorption of Bis(2-ethylhexyl) phthalate exhibited an absorption value of 1.408 (log Papp in 10^{-6} cm/s),

indicating a relatively favorable permeability across the intestinal barrier. This suggests that it may be effectively absorbed after oral administration. Myristyl oleate, with a slightly lower absorption value of 1.306 , also shows good permeability, although it is less than that of bis(2-ethylhexyl) phthalate. 14 Beta H-pregna displayed an absorption value similar to bis(2-ethylhexyl) phthalate (1.405), indicating its potential for adequate oral bioavailability. Overall, the absorption profiles of these compounds suggest they may achieve adequate systemic exposure following administration.

Distribution of Myristyl oleate had the highest distribution coefficient (log BB = 1.014), indicating a favorable ability to cross the blood-brain barrier (BBB). This characteristic may allow for central nervous system (CNS) activity, potentially making it useful for conditions where CNS targeting is desired. In contrast, bis(2-ethylhexyl) phthalate showed a negative log BB value (-0.175), indicating limited distribution to the brain. 14 Beta H-pregna also demonstrated a positive log BB value (0.885), suggesting moderate ability to penetrate the BBB. These distribution characteristics are vital when considering the therapeutic application of these compounds, particularly in relation to CNS effects. Metabolism All three compounds were determined not to be substrates for CYP2D6, suggesting they are less likely to be metabolized through this common liver enzyme. This could imply a potentially favorable metabolic profile, as compounds that are not extensively metabolized may maintain higher bioavailability. Excretion clearance rates provide insights into the elimination of these compounds from the body. Myristyl oleate had the highest total clearance (2.149 log ml/min/kg), suggesting it may be rapidly eliminated, which could influence dosing regimens. Conversely, 14 Beta H-pregna exhibited a significantly lower clearance rate (0.67 logs ml/min/kg), indicating a slower elimination and potentially more prolonged duration of action. Notably, none of the compounds tested positive for mutagenicity in the Ames test, indicating a favorable toxicity profile. This is an encouraging finding, as it suggests these compounds may have a lower risk of causing genetic damage.

Bis(2-ethylhexyl) phthalate (MW = 390.564 g/mol) and Myristyl oleate (MW = 478.846 g/mol) are relatively large molecules, which may influence their absorption and distribution in biological systems. Larger molecules tend to exhibit lower permeability through biological membranes, which could affect their therapeutic efficacy. In contrast, 14 Beta H-pregna has a lower molecular weight (MW = 274.492 g/mol), potentially facilitating better absorption and distribution, as smaller compounds

typically diffuse more easily through cell membranes. The Log P values provide insights into the hydrophobicity of the compounds. Myristyl oleate exhibits an extraordinarily high Log P value (112.682), indicating its lipophilic solid nature, which could enhance its ability to cross lipid membranes. However, such high lipophilicity may also lead to challenges in solubility in aqueous environments, potentially limiting its bioavailability when administered orally. Bis(2-ethylhexyl) phthalate has a significant Log P value of 6.433, also reflecting its lipophilicity and suggesting a potential for membrane permeation. Conversely, 14 Beta H-pregna has a Log P of 60.553, which is comparatively lower than the other two compounds but still suggests a reasonable level of lipophilicity.

The varied lipophilicity of these compounds indicates that their absorption and distribution will differ significantly, influencing their pharmacological activities. None of the compounds displayed any donor hydrogen bonding, indicating that they may not participate in hydrogen bond donation, which could limit their interactions with target proteins or enzymes. This property could influence the compounds' binding affinities and their overall efficacy in biological systems. The hydrogen acceptor bonding also varied among the compounds. Bis(2-ethylhexyl) phthalate exhibited four acceptor sites, while Myristyl oleate had two, and 14 Beta H-pregna had none. The presence of hydrogen bond acceptors in bis(2-ethylhexyl) phthalate could facilitate its interaction with biomolecules, enhancing its potential activity.

On the other hand, the lack of hydrogen bonding capacity in 14 Beta H-pregna may limit its interactions with proteins, potentially reducing its efficacy. Surface area is an important parameter that can influence drug absorption and permeability. Myristyl oleate has the largest surface area (214.638 Å²), which might affect its interactions with biological membranes and potential targets. The larger surface area could facilitate a more significant interaction with the lipid bilayer, impacting absorption rates. Bis(2-ethylhexyl) phthalate has a moderate surface area (170.550 Å²), while 14 Beta H-pregna has the smallest surface area (125.650 Å²). The smaller surface area of 14 Beta H-pregna could correlate with its lower molecular weight, potentially leading to better permeability.

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