BUKTI KORESPONDENSI
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Judul artikel	:	Exploring the α-Amylase Inhibitory Potential of Peronema canescens Jack: An In Vitro and In Silico Study
Jurnal	:	HAYATI Journal of Biosciences
Edisi	:	Tahun 2025, Vol 10 No 2, hal 116–120
Penerbit	:	Institut Pertanian Bogor (IPB)
Penulis	:	Muhammad Ryan Radix Rahardhian, Ninda Aryanti, Yasmiwar Susilawati, Sri Adi Sumiwi, Chintiana Nindya Putri, Dewi Ramonah, Ririn Suharsanti

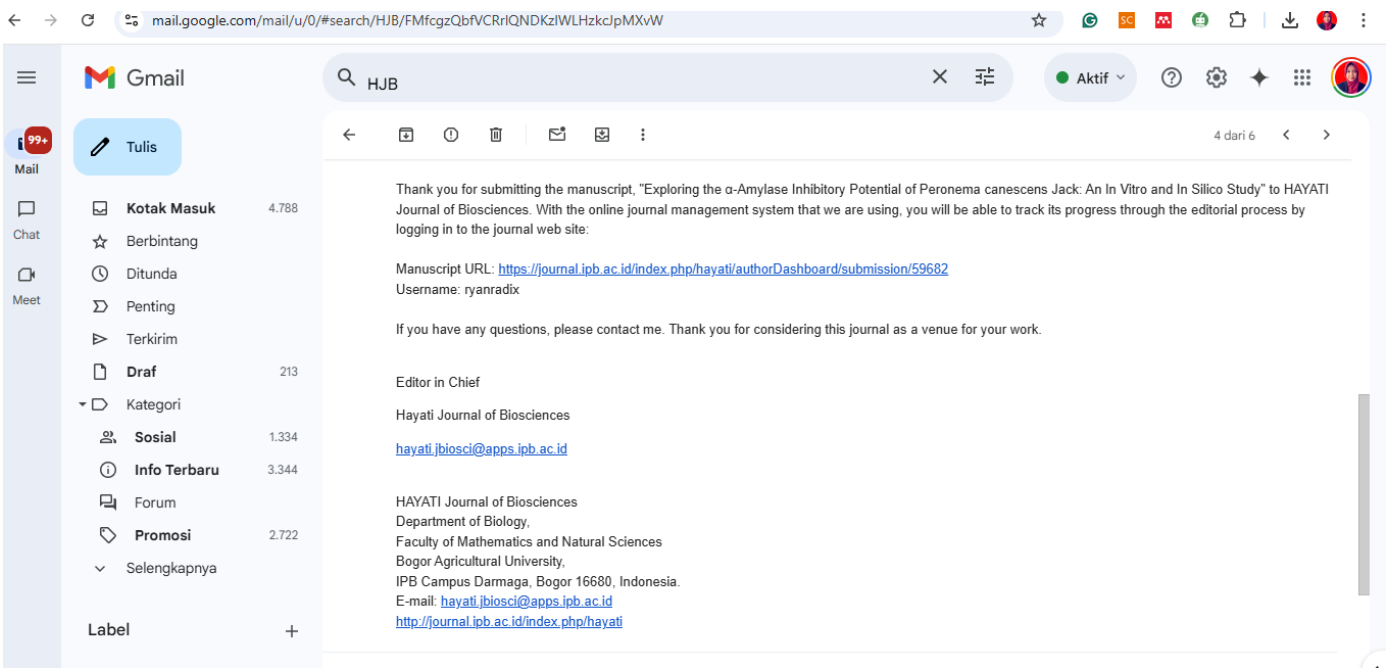
Tabel Tahapan Publikasi Artikel

No	Tahapan Publikasi	Tanggal	Halaman
1	Submission of manuscript	14 Oktober 2024	2
2	Manuscript revision request	2 Januari 2025	2
3	Submit revised manuscript	3 Januari 2025	3
4	Acceptance Notification and Payment Request	25 Januari 2025	3
5	Copyediting Revision Request	25 Januari 2025	4
6	The proof of payment and the correction gallery proof	3 Februari 2025	4
7	Editor decision : send to production	4 Februari 2025	6
8	Article published Vol 32 issue 3, 2025 https://journal.ipb.ac.id/index.php/hayati/article/view/59682/30113	4 Februari 2025	6
9	Lampiran bukti korespondensi		8-127

Pengusul

Dr. apt. Ririn Suharsanti, M.Sc

1. Submission of Manuscript



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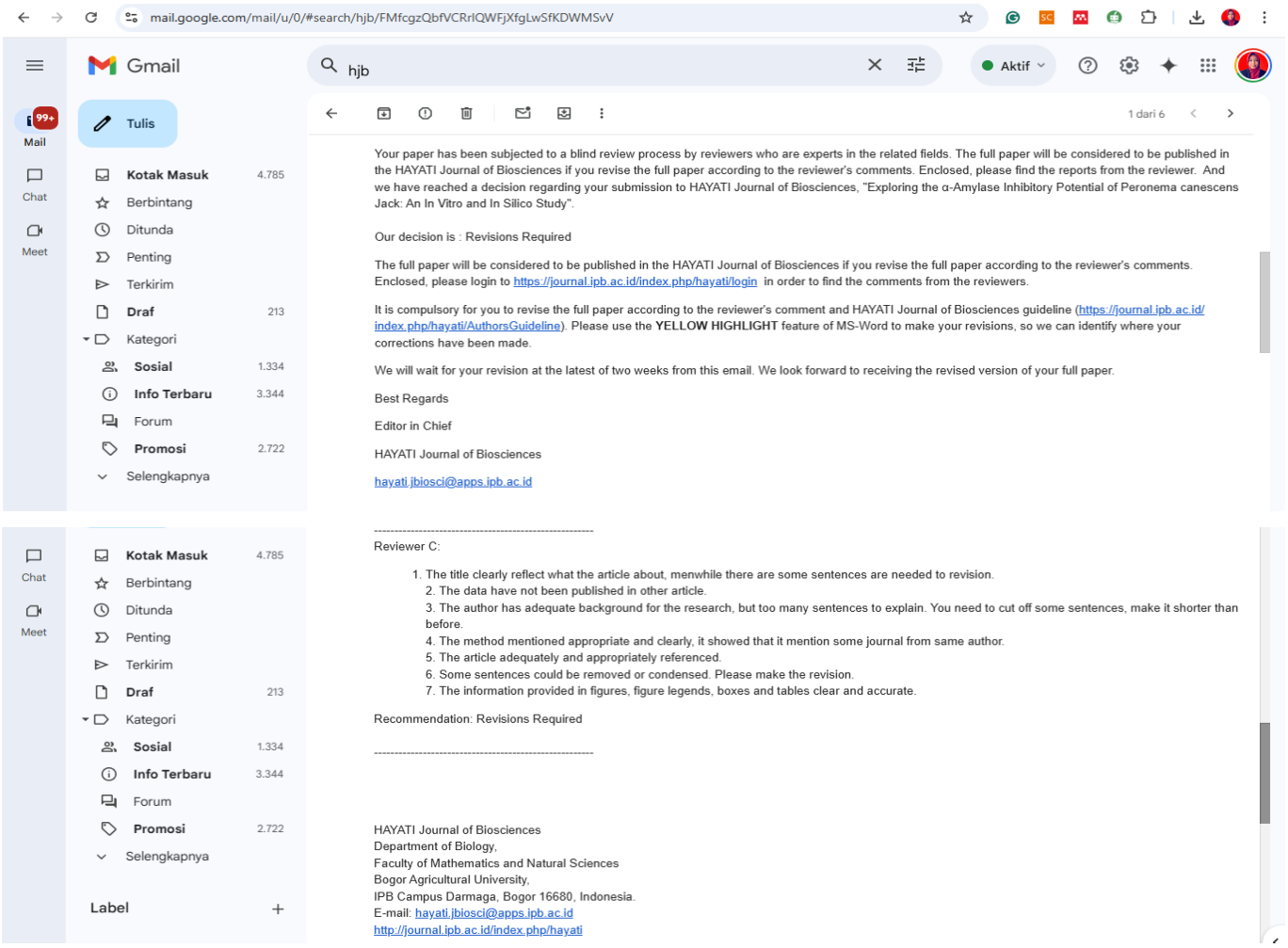

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Reviewer C:

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2. The data have not been published in other article.
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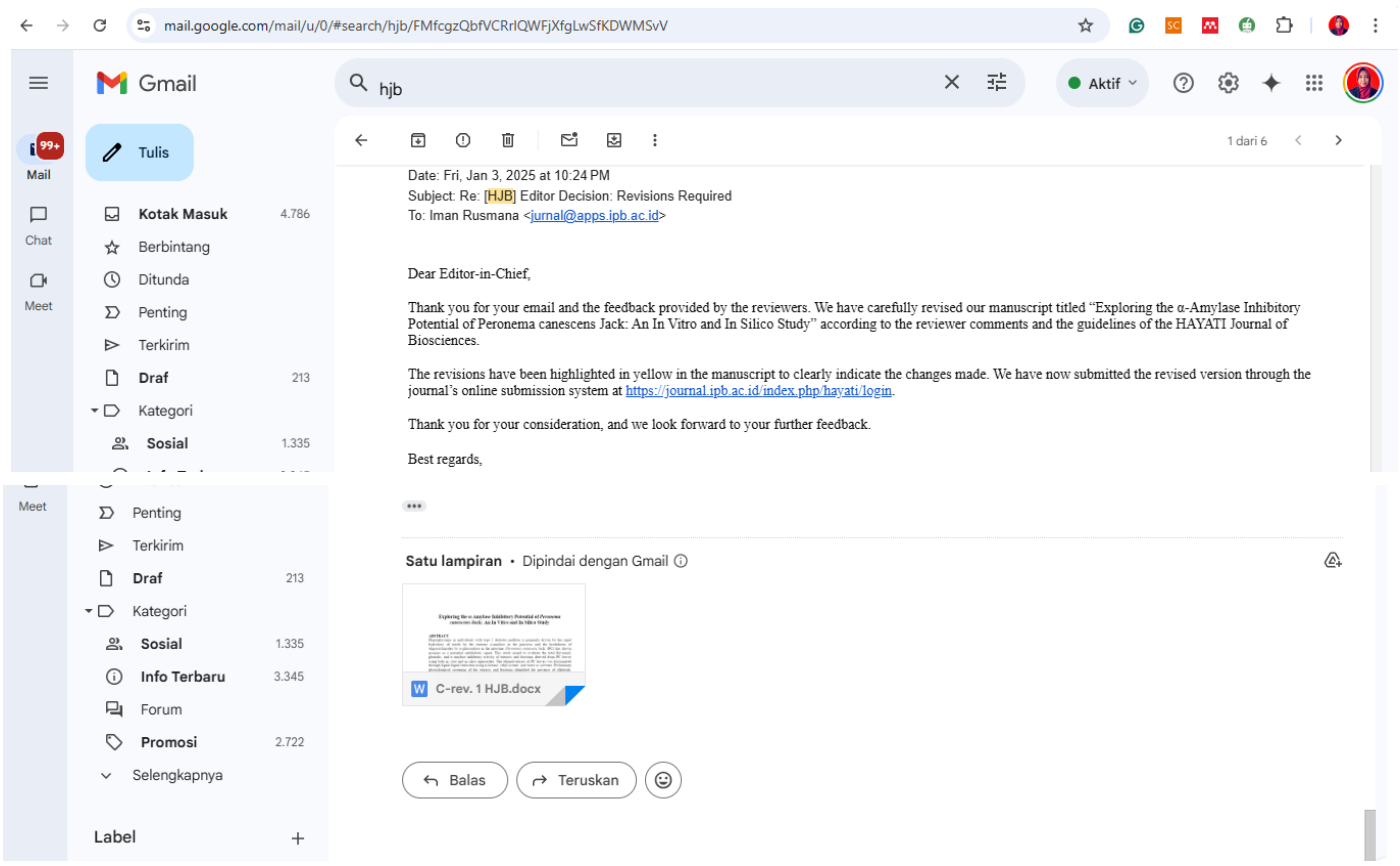

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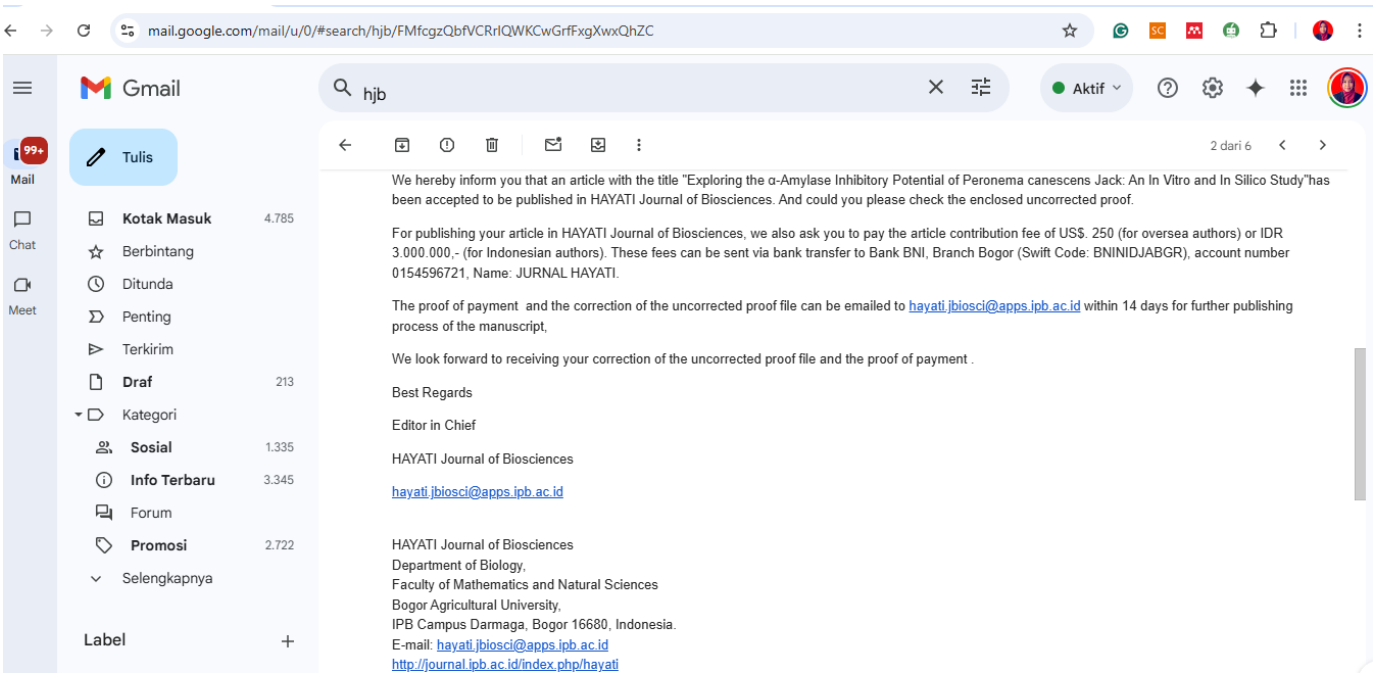

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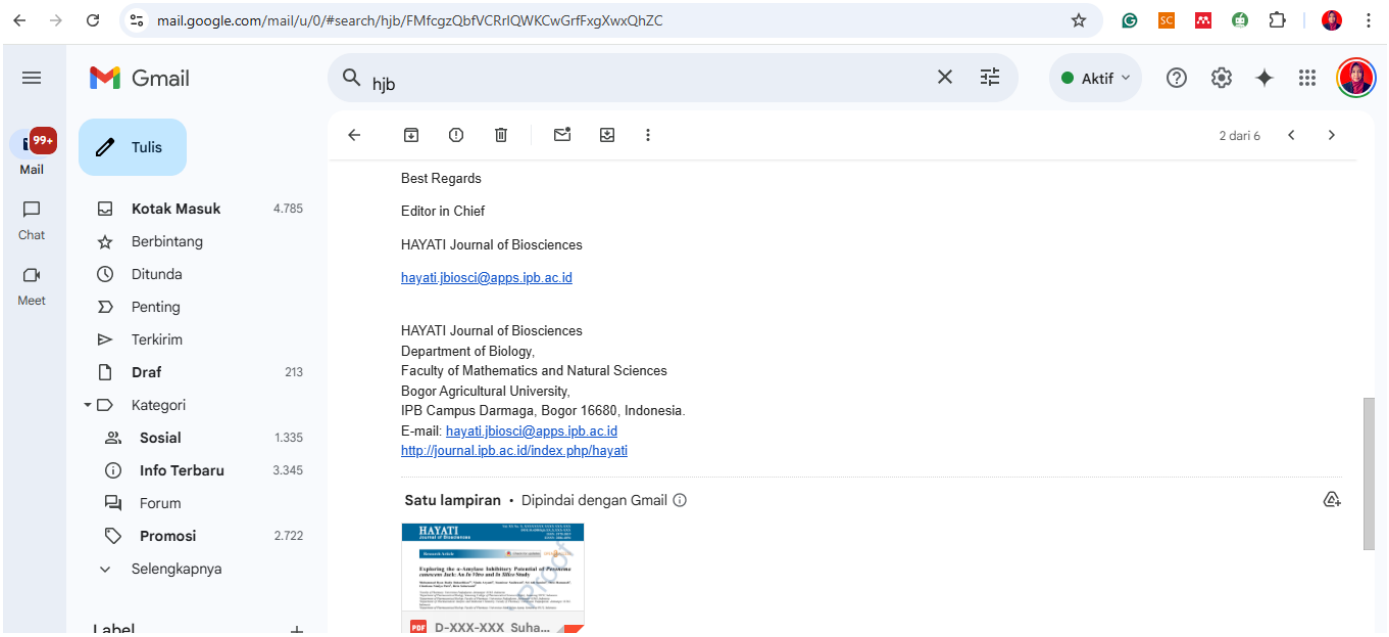

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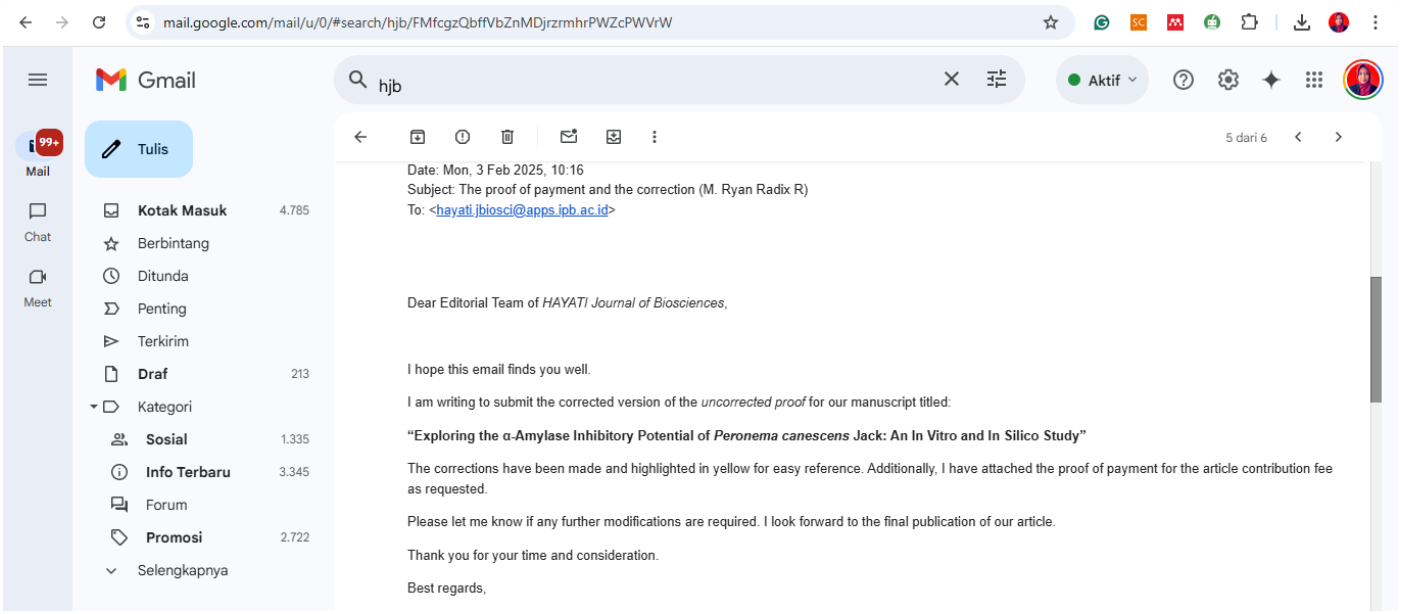

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Date: Mon, 3 Feb 2025, 10:16
Subject: The proof of payment and the correction (M. Ryan Radix R)
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Dear Editorial Team of *HAYATI Journal of Biosciences*,

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.

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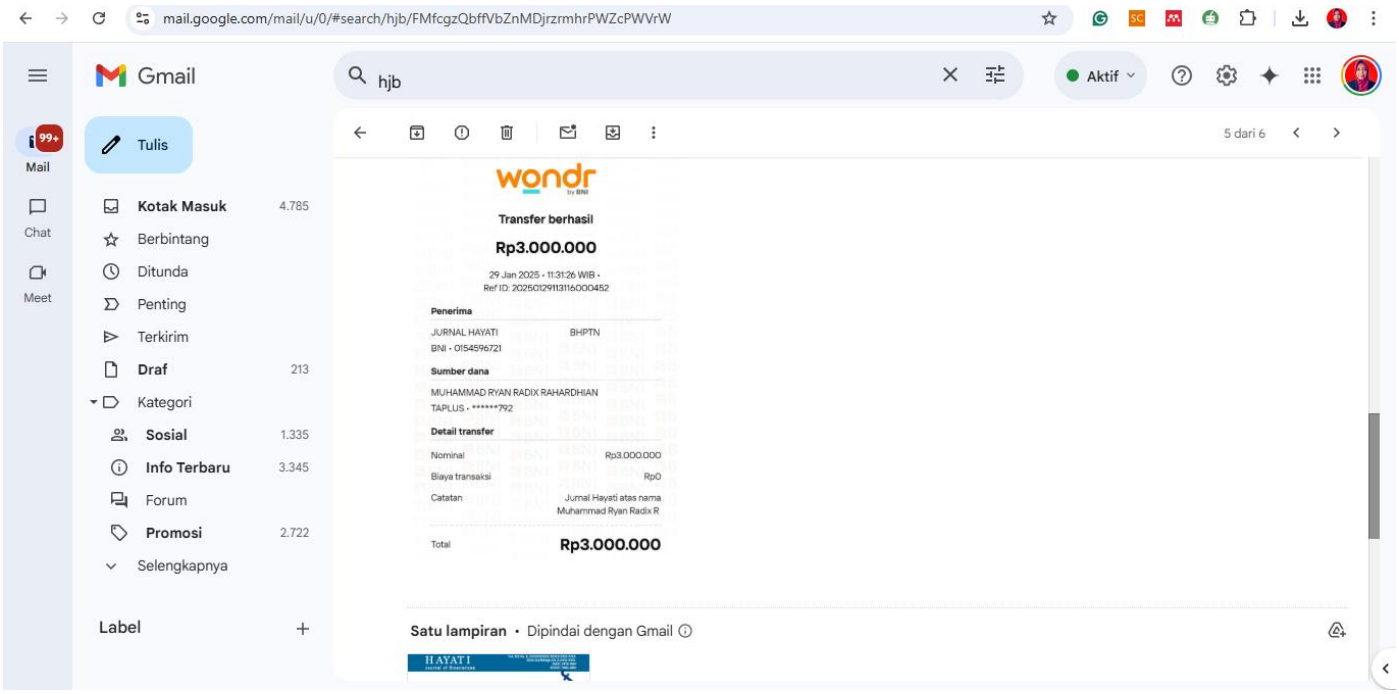
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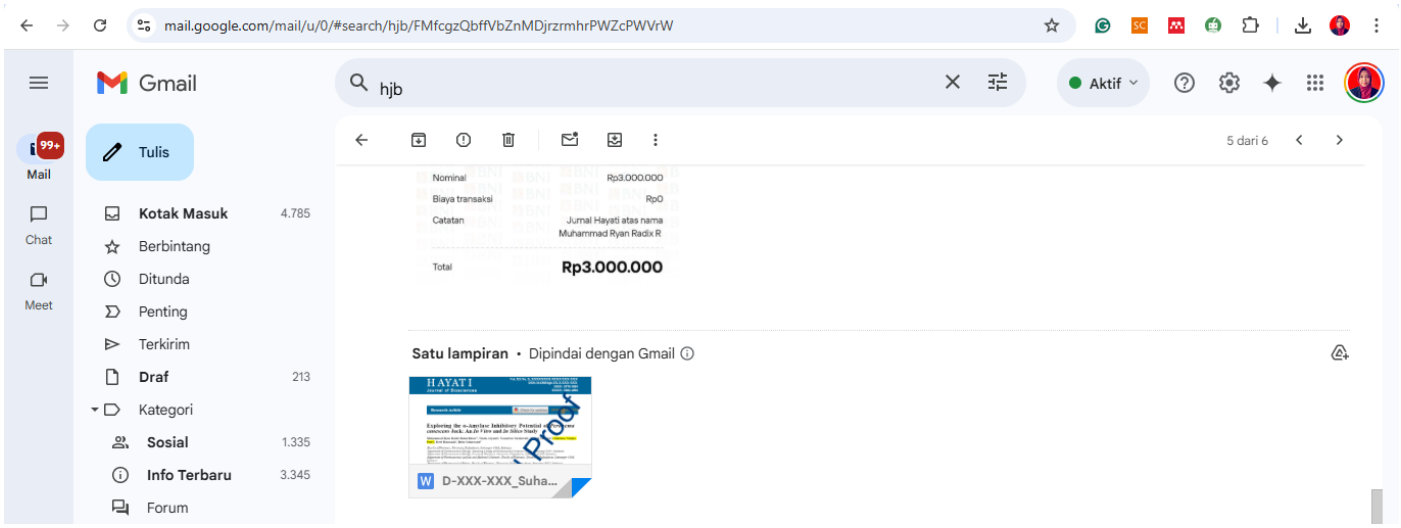
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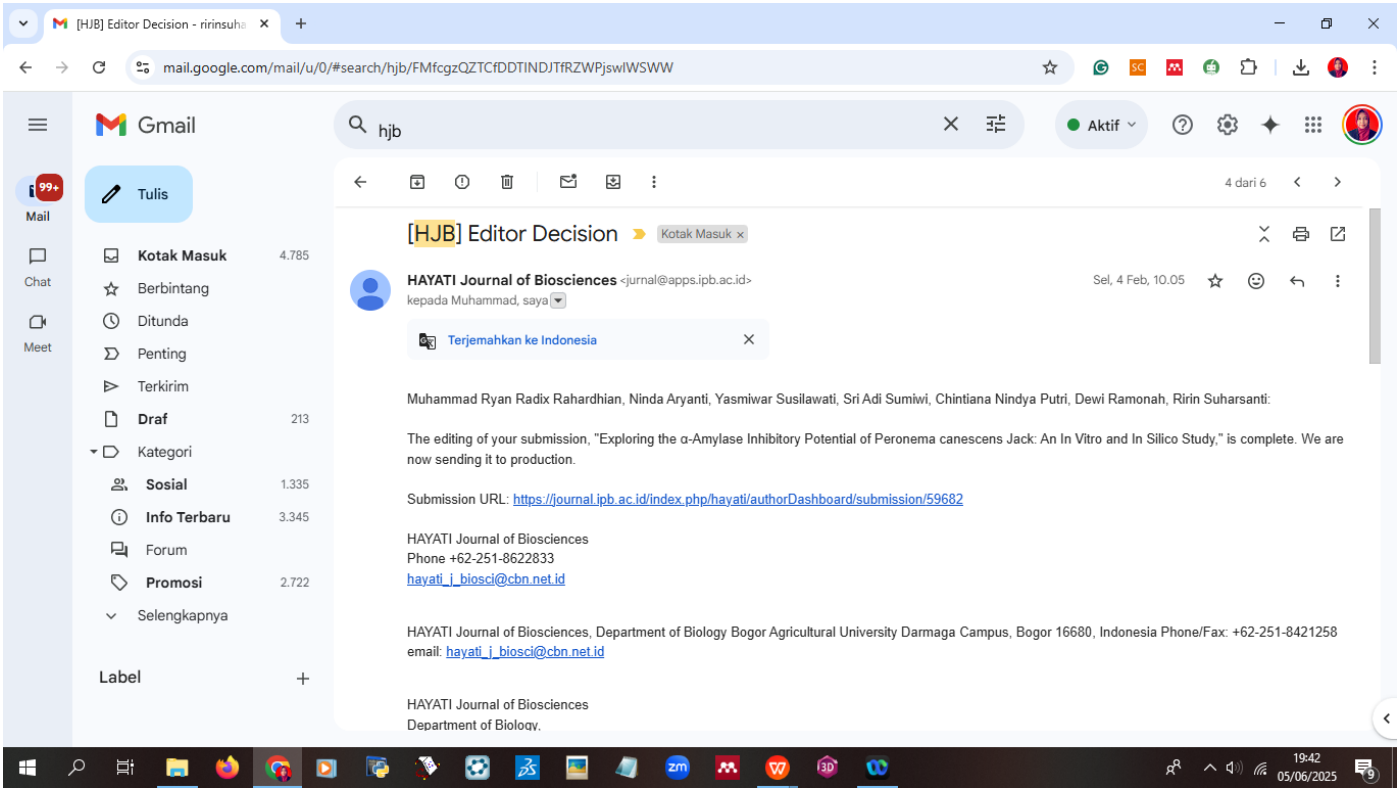

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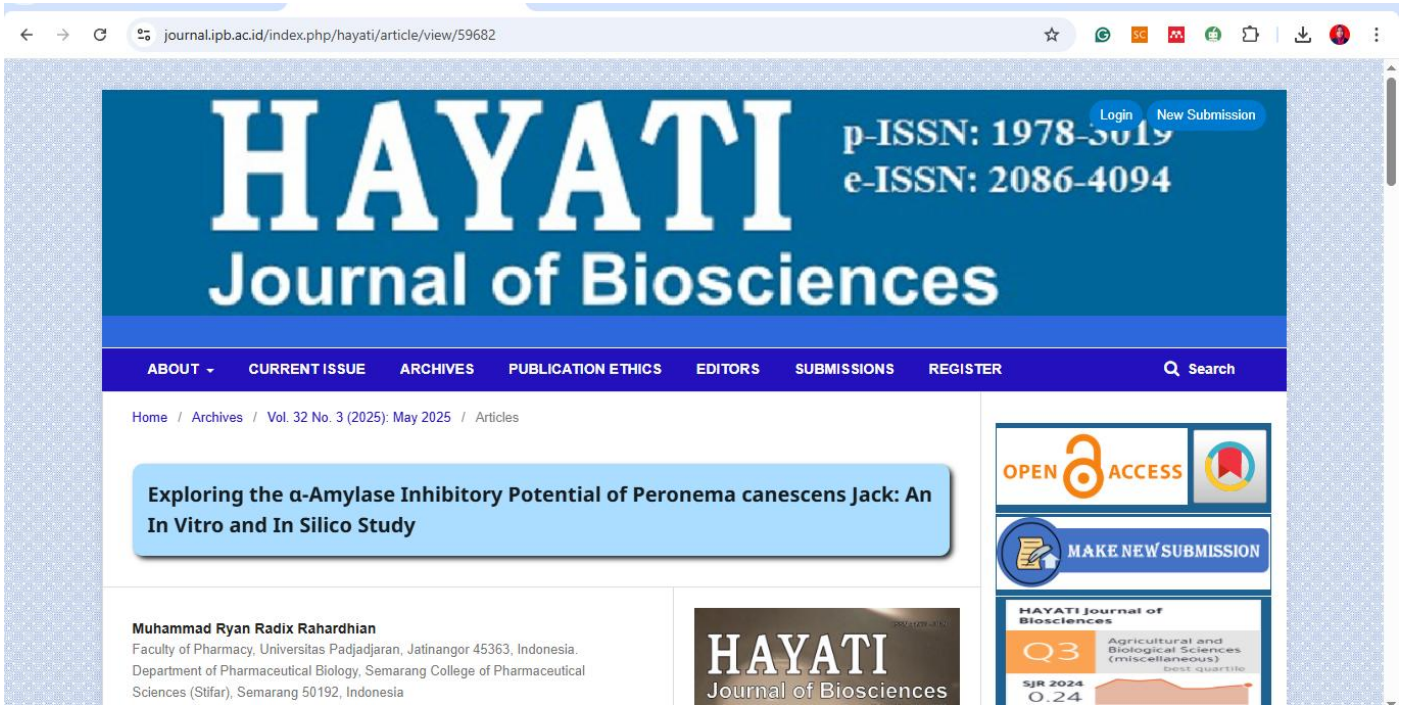

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DOI: https://doi.org/10.4308/hjb.32.3.712-726

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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2025-02-04

How to Cite
RahardhianM. R. R., AryantiN., SusilawatiY., SumiwiS. A., PutriC. N., RamonahD., & SuharsantiR. (2025). Exploring the α-Amylase Inhibitory Potential of Peronema canescens Jack: An In Vitro and In Silico Study. *HAYATI Journal of Biosciences*, *32*(3), 712-726. https://doi.org/10.4308/hjb.32.3.712-726

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Issue
Vol. 32 No. 3 (2025): May 2025

Section
Articles

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±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.,o 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.

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 It 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, DPPH (Sigma), quercetin, methanol, phosphate buffer, alpha-amylase enzyme (Sigma Aldrich), starch, and DNS

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/pkcsm/).

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 affirmation

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 colours 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) . The total phenolic content was assessed using the Folin-Ciocalteu reagent with gallic acid as the standard. A 1.0 mL aliquot of the standard solution or sample was placed in a test tube. Add 4.5 mL of 10% Folin-Ciocalteu reagent to the test tube and 4.5 mL of 7.5% sodium carbonate (Na_2CO_3) solution. The mixture was incubated for 90 minutes at room temperature. The absorbance was measured at a wavelength of 765 nm using a Shimadzu® UV-Vis Spectrophotometer (Model 1240). The total flavonoid content was determined using aluminum chloride ($AlCl_3$) with quercetin as the standard. A 1.0 mL aliquot of the standard solution or sample was placed in a test tube. Add 3.0 mL of ethanol, 0.2 mL of 10% aluminum chloride ($AlCl_3$) solution, 0.2 mL of 20% anhydrous sodium acetate (CH_3COONa) solution, and 5.6 mL of distilled water. The mixture was homogenized thoroughly. The mixture was incubated for 30 minutes at room temperature. The absorbance was measured at a wavelength of 420 nm 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 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.

$$\text{Inhibition of } \alpha - \text{amylase } (\%) = \frac{((Ac - Ac') - (As - As'))}{(Ac' - As')} x100$$

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 mL/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 x 40 x 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 Likeliness 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) . ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) properties were predicted using computational methods to further 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. AMES toxicity tests assessed potential genotoxic effects (D. E. V 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 ± standard deviation (SD). NOVA 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

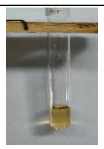
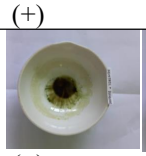
3.1. Phytochemical Screening

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

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_2SO_4.	 (+)	 (+)	 (+)	 (-)

Description

(+) = Contains the compound

(-) = Does not contain the compound

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 Rf values and the corresponding color stains formed on the TLC plates. The Rf 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 Rf 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.

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	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)				Blackish	
		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	-

Notes:

(+) = Contains tested compounds

(-) = Does not contain the tested compounds

3.3. Determination of total phenolic and total flavonoid content

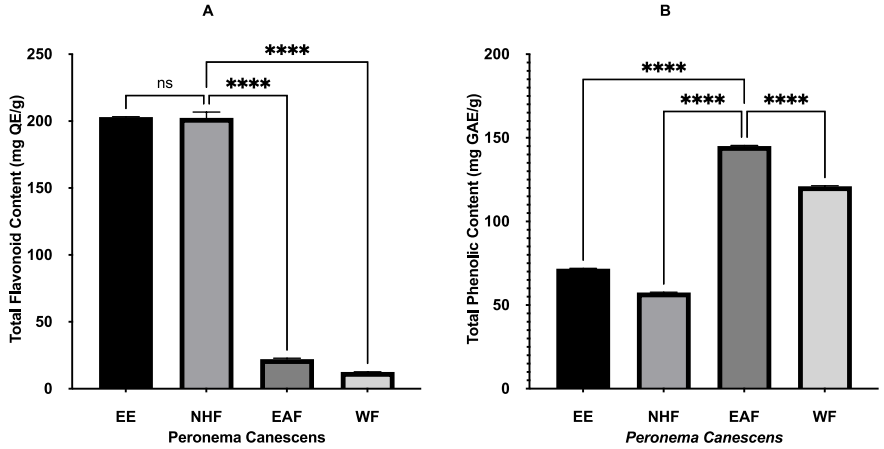


Figure 1. Total Flavonoid Content (A) and Total Phenolic Content (B). EE: Ethanolic Extract, NHF: n-Hexane Fraction, EAF: Ethyl Acetate Fraction, WF: Water Fraction. ns: Non-significant; ****: Significant difference (P > 0.05)

Figure 1 shows that the n-hexane fraction of PC leaves exhibits 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 on the analysis of PC leaves' active fraction (ethyl acetate fraction), the compounds identified are listed in Table 3.

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	C24 H38 O4	Bis(2-ethylhexyl) phthalate
2	21,656	0,58	478	57	63	C32 H62 O2	Myristyl oleate
3	21,942	0,57	593	57	59	C39 H76 O3	Oleic acid
4	22,825	2,12	288	57	64	C21 H36	14-Beta-H-Pregna
5	22,902	8,77	723	57	77	C45 H86 O6	Tetradecanoic acid
6	23,021	16,46	639	57	75	C39 H74 O6	Dodecanoic acid
7	23,142	9,25	723	57	68	C45 H86 O6	Tetradecanoic acid
8	23,255	27,05	751	57	77	C47 H90 O6	Hexadecanoic acid
9	23,301	19,04	639	57	83	C39 H74 O6	Dodecanoic acid
10	23,367	14,46	639	183	71	C39 H74 O6	Dodecanoic acid

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 dinitrosalicylic acid (DNS) method, with starch as the substrate. DNS 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.

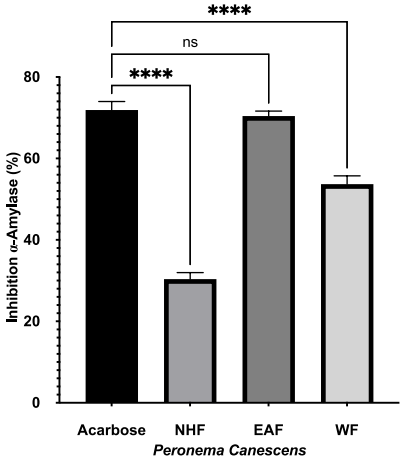
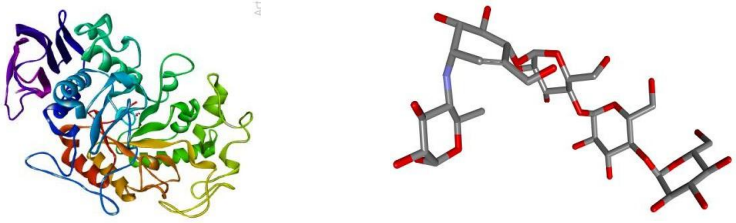


Figure 3. Percentage Inhibition of α-Amylase Enzyme Activity. NHF: n-hexane fraction, EAF: Ethyl acetate fraction, WF: Water fraction, ns: non-significant, ****: Significant difference (P > 0.05).

Based on Figure 3, 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±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 (Ki) of the same magnitude obtained during the fourth docking run.



A B

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

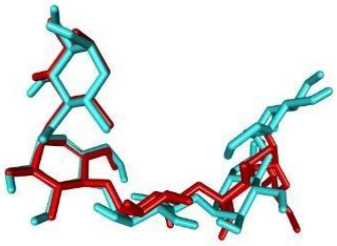


Figure 5. The overlay of the acarbose confirmation before (blue) and after (red) redocking

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

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. Results of ADME Analysis of Active Fraction Compounds of PC Leaves

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

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

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

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 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).

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

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

identified with Rf values of 0.88 to 0.94, displaying green-blackish spots after $FeCl_3$ 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 (593) and its retention time further suggests its bioactive potential. The diversity of compounds, 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%, points to the complex nature of the phytochemical constituents of PC. 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 anti diabetic 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 (Ki) 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 (Ki) 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 Ki 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 Ki 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 Ki 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 Ki 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 Ki 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,

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

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.

5. Acknowledgements

This research was supported by Hibah Penelitian dan Pengabdian Kepada Masyarakat Akademik Pendidikan Tinggi Vokasi under Contract Number: 103/SPK/D.D4/PPK.01.APTV/III/2024 and Derivative Contract Number: 018/LL6/PPM-V/AL.04/2024.

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Submission of manuscript

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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±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.,o 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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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 It 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, $FeCl_3$, $MgSO_4$, hydrochloric-ethanolic acid mixture (1:1), hydrochloric acid, Lieberman-Burchard reagent, DPPH (Sigma), quercetin, methanol, phosphate buffer, alpha-amylase enzyme (Sigma Aldrich), starch, and DNS

Comment[DS1]: Do not use abbreviation.

Comment[DS2]: Do not use abbreviation.

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/pkcsm/).

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 affirmation

Comment[DS3]: ?

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 colours 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.

Comment[DS4]: Subscript for 3

Comment[DS5]: Idem.

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) . The total phenolic content was assessed using the Folin-Ciocalteu reagent with gallic acid as the standard. A 1.0 mL aliquot of the standard solution or sample was placed in a test tube. Add 4.5 mL of 10% Folin-Ciocalteu reagent to the test tube and 4.5 mL of 7.5% sodium carbonate (Na_2CO_3) solution. The mixture was incubated for 90 minutes at room temperature. The absorbance was measured at a wavelength of 765 nm using a Shimadzu® UV-Vis Spectrophotometer (Model 1240). The total flavonoid content was determined using aluminum chloride ($AlCl_3$) with quercetin as the standard. A 1.0 mL aliquot of the standard solution or sample was placed in a test tube. Add 3.0 mL of ethanol, 0.2 mL of 10% aluminum chloride ($AlCl_3$) solution, 0.2 mL of 20% anhydrous sodium acetate (CH_3COONa) solution, and 5.6 mL of distilled water. The mixture was homogenized thoroughly. The mixture was incubated for 30 minutes at room temperature. The absorbance was measured at a wavelength of 420 nm 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.

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.

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 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.

$$\text{Inhibition of } \alpha - \text{amylase } (\%) = \frac{((Ac - Ac') - (As - As^{'}))}{(Ac^{'} - As^{'})} x100$$

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 mL/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 x 40 x 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 Likeliness 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) . ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) properties were predicted using computational methods to further 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. AMES toxicity tests assessed potential genotoxic effects (D. E. V Pires et al., 2015).

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

Comment[DS8]: Means?

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 ± standard deviation (SD). NOVA 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.

Comment[DS9]: ANOVA?

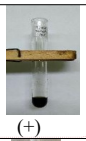
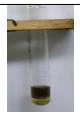
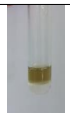
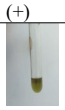
3. Results

3.1. Phytochemical Screening

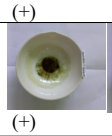
The fractionation of the ethanol extract from PC leaves resulted in the following yields: 32.81% for the n-hexane fraction, 16.86% for the ethyl acetate fraction, and 34.17% for the water fraction. Notably, the ethyl acetate fraction yield was lower than that of the n-hexane and water fractions. This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

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?

Saponins	Add 10 mL of hot water to 2N HCl	 (+)	 (+)	 (+)	 (-)
Steroids	Mix 5 mL of chloroform with anhydrous acetic acid and H_2SO_4.	 (+)	 (+)	 (+)	 (-)

Description

(+) = Contains the compound

(-) = Does not contain the compound

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 Rf values and the corresponding color stains formed on the TLC plates. The Rf 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 Rf 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.

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	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)					Blackish	
		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	-

Notes:

(+) = Contains tested compounds

(-) = Does not contain the tested compounds

3.3. Determination of total phenolic and total flavonoid content

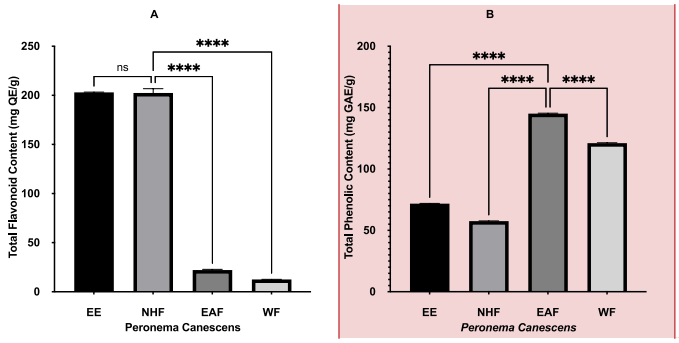


Figure 1. Total Flavonoid Content (A) and Total Phenolic Content (B). EE: Ethanolic Extract, NHF: n-Hexane Fraction, EAF: Ethyl Acetate Fraction, WF: Water Fraction. ns: Non-significant; ****: Significant difference (P > 0.05)

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

Figure 1 shows that the n-hexane fraction of PC leaves exhibits 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 on the analysis of PC leaves' active fraction (ethyl acetate fraction), the compounds identified are listed in Table 3.

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

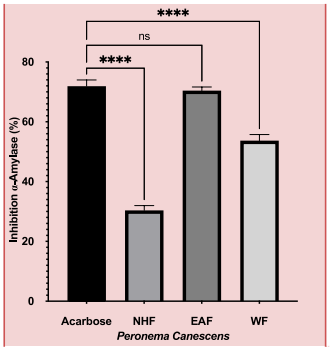
Peak	Retention Time	Percent Area(%)	Molecular Weight	Base Peak	Similarity Index	Molecular Formula	Compound
1	20,208	1,70	390	149	97	C24 H38 O4	Bis(2-ethylhexyl) phthalate
2	21,656	0,58	478	57	63	C32 H62 O2	Myristyl oleate
3	21,942	0,57	593	57	59	C39 H76 O3	Oleic acid
4	22,825	2,12	288	57	64	C21 H36	14-Beta-H-Pregna
5	22,902	8,77	723	57	77	C45 H86 O6	Tetradecanoic acid
6	23,021	16,46	639	57	75	C39 H74 O6	Dodecanoic acid
7	23,142	9,25	723	57	68	C45 H86 O6	Tetradecanoic acid
8	23,255	27,05	751	57	77	C47 H90 O6	Hexadecanoic acid
9	23,301	19,04	639	57	83	C39 H74 O6	Dodecanoic acid
10	23,367	14,46	639	183	71	C39 H74 O6	Dodecanoic acid

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

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 dinitrosalicylic acid (DNS) method, with starch as the substrate. DNS 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.

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



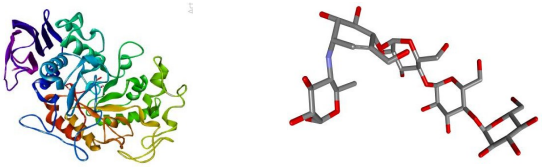
Comment[DS14]: Idem.

Figure 3. 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).

Based on Figure 3, 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±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 (Ki) of the same magnitude obtained during the fourth docking run.



A B

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

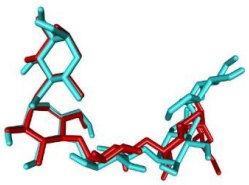


Figure 5. The overlay of the acarbose confirmation before (blue) and after (red) redocking

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

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. Results of ADME Analysis of Active Fraction Compounds of PC Leaves

No	Compound	Absorption (Caco2 permeability) (log Papp in 10^{-6} cm/s)	Distribution (BBB permeability y) (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

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

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

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 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).

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 $FeCl_3$ 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 (593) and its retention time further suggests its bioactive potential. The diversity of compounds, 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%, points to the complex nature of the phytochemical constituents of PC. 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 anti diabetic 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 (Ki) 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 (Ki) 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 Ki 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 Ki 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 Ki 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 Ki 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 Ki 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.

5. Acknowledgements

This research was supported by Hibah Penelitian dan Pengabdian Kepada Masyarakat Akademik Pendidikan Tinggi Vokasi under Contract Number: 103/SPK/D.D4/PPK.01.APTV/III/2024 and Derivative Contract Number: 018/LL6/PPM-V/AL.04/2024.

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Manuscript revision request

Manuscript revision request

HAYATI
Journal of Biosciences

Vol. XX No. X, XXXXXXXX XXXX XXX-XXX
DOI:10.4308/hjb.XX.X.XXX-XXX
ISSN: 1978-3019
EISSN: 2086-4094

Research Article

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

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ARTICLE INFO

Article history:
Received October 14, 2024
Received in revised form January 3, 2024
Accepted January 13, 2025

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

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±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.

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 It 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, $FeCl_3$, $MgSO_4$, 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/pkcsm/).

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 ($FeCl_3$) 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 (CH_3COOH) and heated, then cooled and treated with sulfuric acid (H_2SO_4). 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 $FeCl_3$, 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 ($AlCl_3$) 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 Likeliness 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. $CaCO_2$ 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 ± 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 Rf values and the corresponding color stains formed on the TLC plates. The Rf 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 Rf 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.

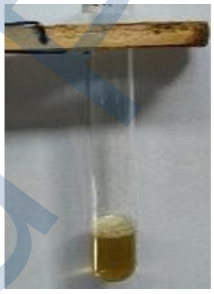
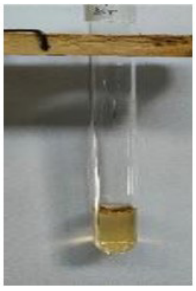
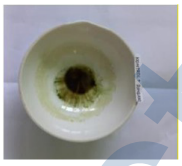
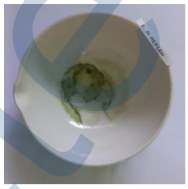
3.4. Active Fraction of PC Leaves Using GC-MS

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	*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				

Table 1. Continued

Compound group	Reagent	Ethanol extract	*n*-hexane fraction	Ethyl acetate fraction	Water fraction
	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_2SO_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	*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, 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	*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	-

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

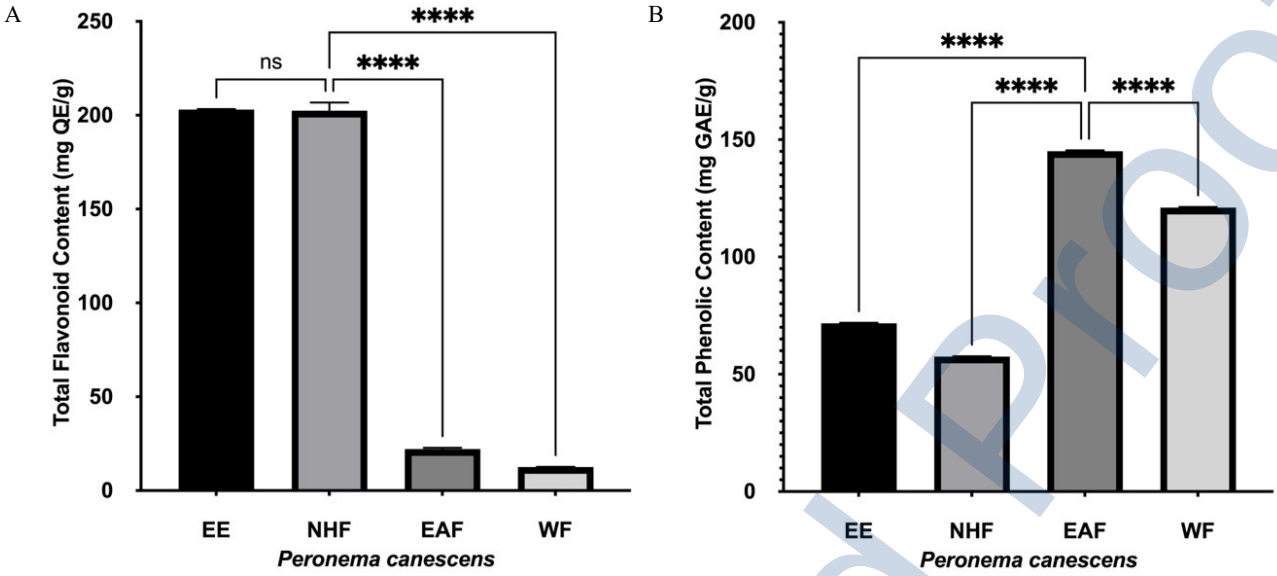


Figure 1. (A) Total flavonoid content and (B) total phenolic content. EE: ethanolic extract, NHF: *n*-hexane fraction, EAF: ethyl acetate fraction, WF: water fraction. ns: non-significant; ****: significant difference (P>0.05)

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±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 (Ki) 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

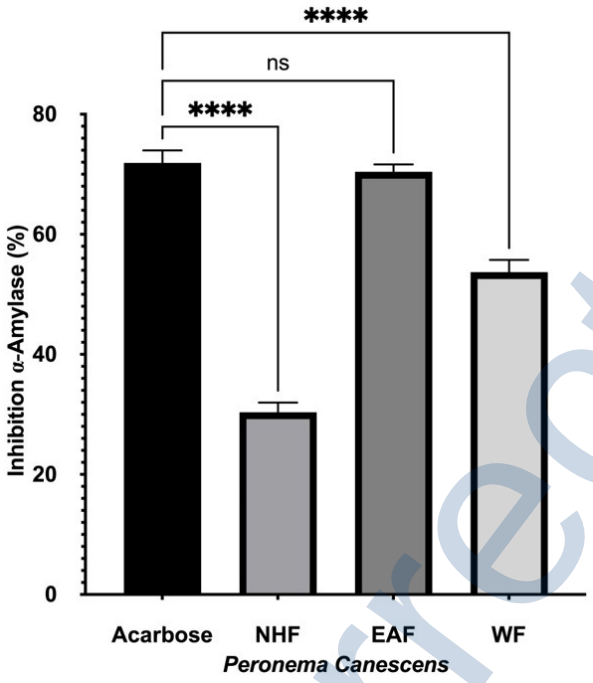


Figure 2. Percentage inhibition of α-amylase enzyme activity. NHF: *n*-hexane fraction, EAF: ethyl acetate fraction, WF: water fraction, ns: non-significant, ****: Significant difference (P>0.05)

Table 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

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 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

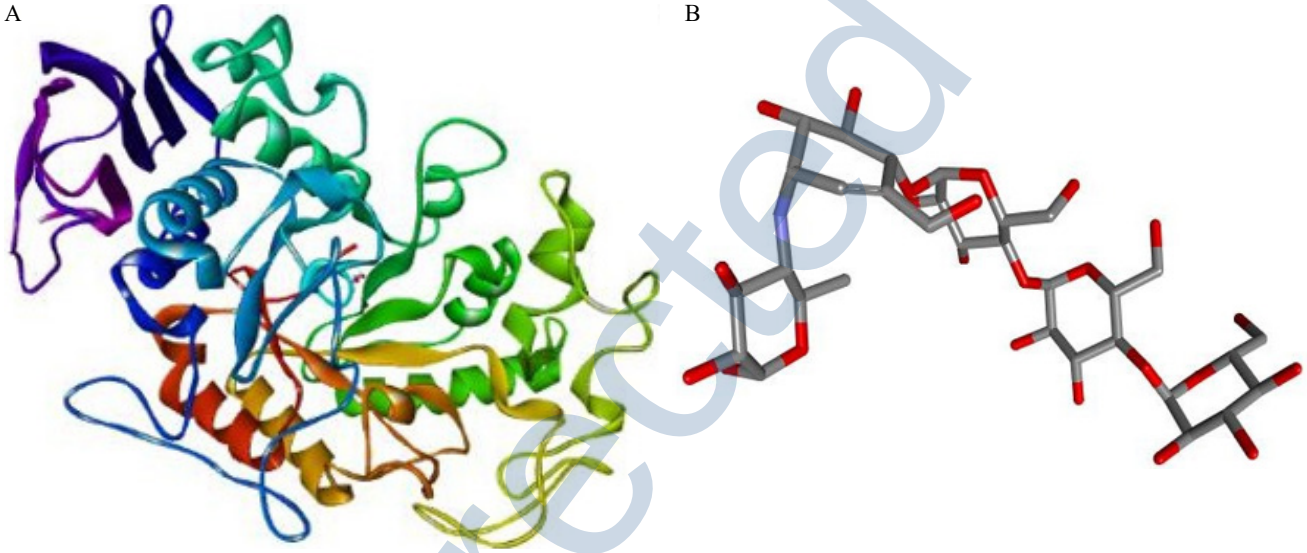


Figure 3. (A) The three-dimensional structure of the α-amylase enzyme and (B) its natural ligands

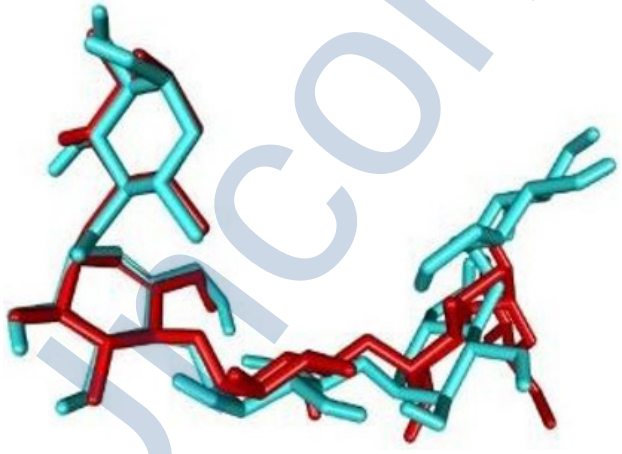


Figure 4. The overlay of the acarbose confirmation before (blue) and after (red) redocking

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 $FeCl_3$ 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 (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 ($C_{32}H_{62}O_2$) 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 (Ki) 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 (Ki) 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 Ki 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 Ki 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 Ki 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 Ki 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 Ki 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.

Acknowledgements

This research was supported by Hibah Penelitian dan Pengabdian Kepada Masyarakat Akademik Pendidikan Tinggi Vokasi under contract number 103/SPK/D.D4/PPK.01.APTV/III/2024 and derivative contract number: 018/LL6/PPM-V/AL.04/2024.

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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±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.

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 It 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/pkcsm/).

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 colours 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 (CH_3COOH) and heated, then cooled and treated with sulfuric acid (H_2SO_4). 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 ($AlCl_3$) 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.

$$\text{Inhibition of } \alpha - \text{amylase } (\%) = \frac{((Ac - Ac') - (As - As'))}{(Ac' - As')} x100$$

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 mL/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 x 40 x 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 Likeliness 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 to further assess the compounds' suitability for drug development. $CaCO_2$ 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 ± 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.

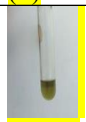
3. Results

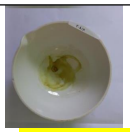
3.1. Phytochemical Screening

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.

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_2SO_4.	 Green color (+)	 Green color (+)	 Green color (+)	 No Green color (+)

Description

(+) = Contains the compound

(-) = Does not contain the compound

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 Rf values and the corresponding color stains formed on the TLC plates. The Rf 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 Rf 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.

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	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 Blackish	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	-

Notes:

(+) = Contains tested compounds

(-) = Does not contain the tested compounds

3.3. Determination of total phenolic and total flavonoid content

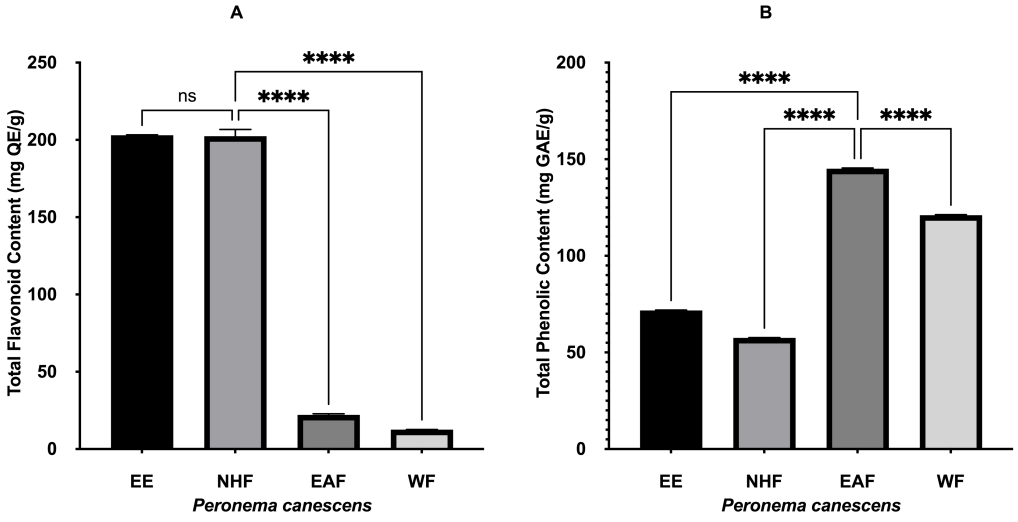


Figure 1. Total Flavonoid Content (A) and Total Phenolic Content (B). EE: Ethanolic Extract, NHF: n-Hexane Fraction, EAF: Ethyl Acetate Fraction, WF: Water Fraction. ns: Non-significant; ****: Significant difference (P > 0.05)

Figure 1 shows that the n-hexane fraction of PC leaves exhibits 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 on the analysis of PC leaves' active fraction (ethyl acetate fraction), the compounds identified are listed in Table 3.

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

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.

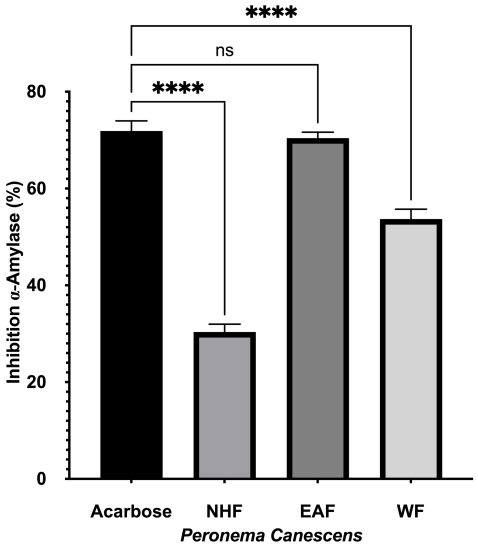
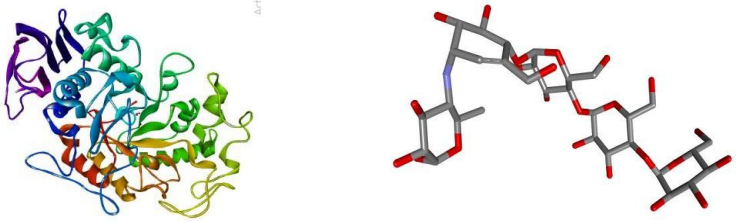


Figure 3. Percentage Inhibition of α-Amylase Enzyme Activity. NHF: n-hexane fraction, EAF: Ethyl acetate fraction, WF: Water fraction, ns: non-significant, ****: Significant difference (P > 0.05).

Based on Figure 3, 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±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 (Ki) of the same magnitude obtained during the fourth docking run.



A B

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

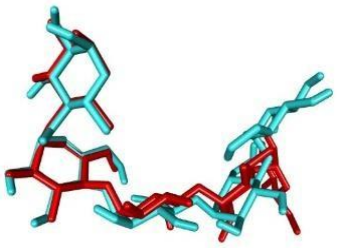


Figure 5. The overlay of the acarbose confirmation before (blue) and after (red) redocking

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

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. Results of ADME Analysis of Active Fraction Compounds of PC Leaves

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

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

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

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 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).

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 $FeCl_3$ 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.

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

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 (Ki) 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 (Ki) 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 Ki 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 Ki 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

519 significantly lower Ki 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 Ki 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 Ki 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,

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.

5. Acknowledgements

This research was supported by Hibah Penelitian dan Pengabdian Kepada Masyarakat Akademik Pendidikan Tinggi Vokasi under contract number: 103/SPK/D.D4/PPK.01.APTV/III/2024 and derivative contract number: 018/LL6/PPM-V/AL.04/2024.

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Revised Manuscript

Revised Manuscript

HAYATI
Journal of Biosciences

Vol. 32 No. 3, May 2025 712-726
DOI:10.4308/hjb.32.3.712-726
ISSN: 1978-3019
EISSN: 2086-4094

Research Article

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

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ARTICLE INFO

Article history:
Received October 14, 2024
Received in revised form January 3, 2025
Accepted January 13, 2025

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

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±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.

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 It 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, $FeCl_3$, $MgSO_4$, 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/pkcsm/).

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 ($FeCl_3$) 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 (CH_3COOH) and heated, then cooled and treated with sulfuric acid (H_2SO_4). 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 $FeCl_3$, 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 ($AlCl_3$) 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 Likeliness 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. $CaCO_2$ 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 ± 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 Rf values and the corresponding color stains formed on the TLC plates. The Rf 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 Rf 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.

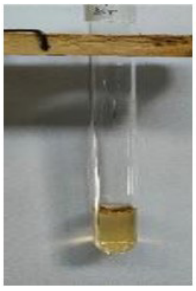
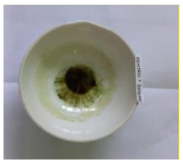
3.4. Active Fraction of PC Leaves Using GC-MS

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	*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				

Table 1. Continued

Compound group	Reagent	Ethanol extract	*n*-hexane fraction	Ethyl acetate fraction	Water fraction
	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_2SO_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	*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, 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	*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	-

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

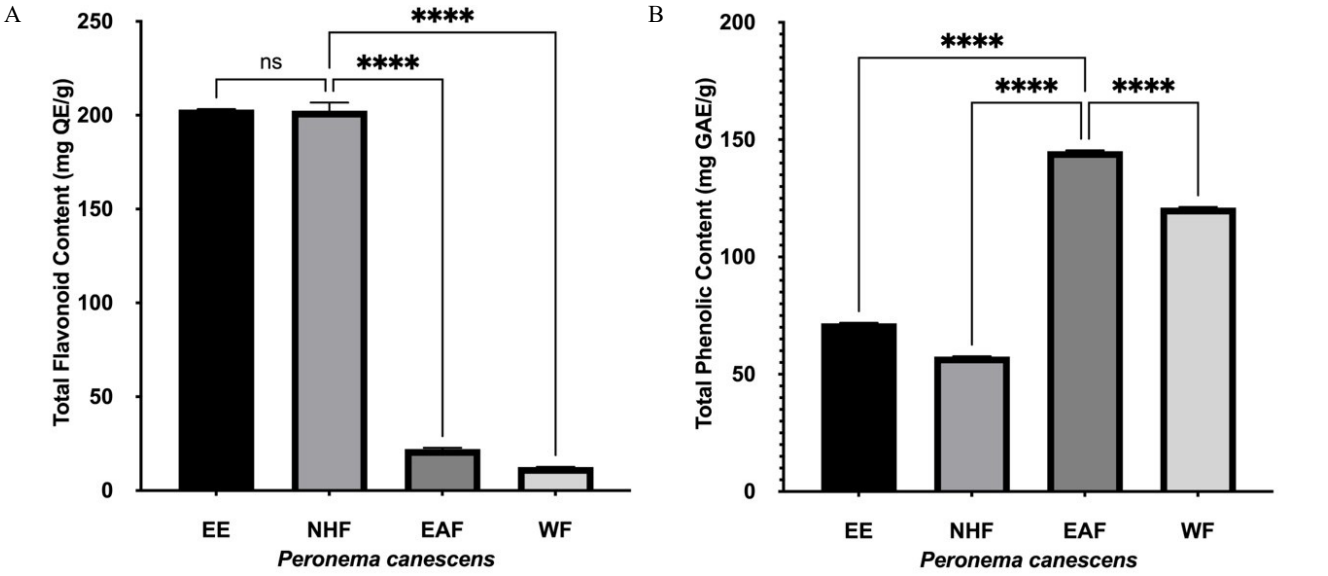


Figure 1. (A) Total flavonoid content and (B) total phenolic content. EE: ethanolic extract, NHF: *n*-hexane fraction, EAF: ethyl acetate fraction, WF: water fraction. ns: non-significant; ****: significant difference (P>0.05)

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±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 (Ki) 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

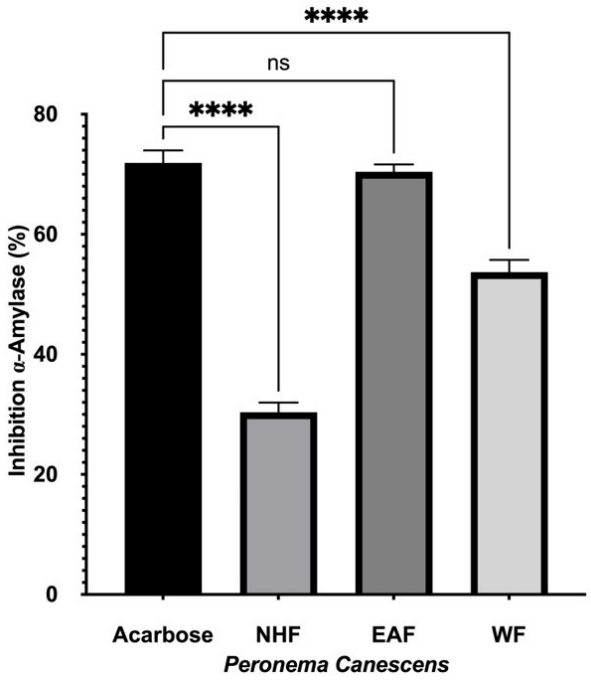


Figure 2. Percentage inhibition of α-amylase enzyme activity. NHF: *n*-hexane fraction, EAF: ethyl acetate fraction, WF: water fraction, ns: non-significant, ****: Significant difference (P>0.05)

Table 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

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 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 $FeCl_3$ 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 (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 ($C_{32}H_{62}O_2$) 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 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 (Ki) 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 (Ki) 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 Ki value suggests that this compound may not be a potent inhibitor of α-amylase.

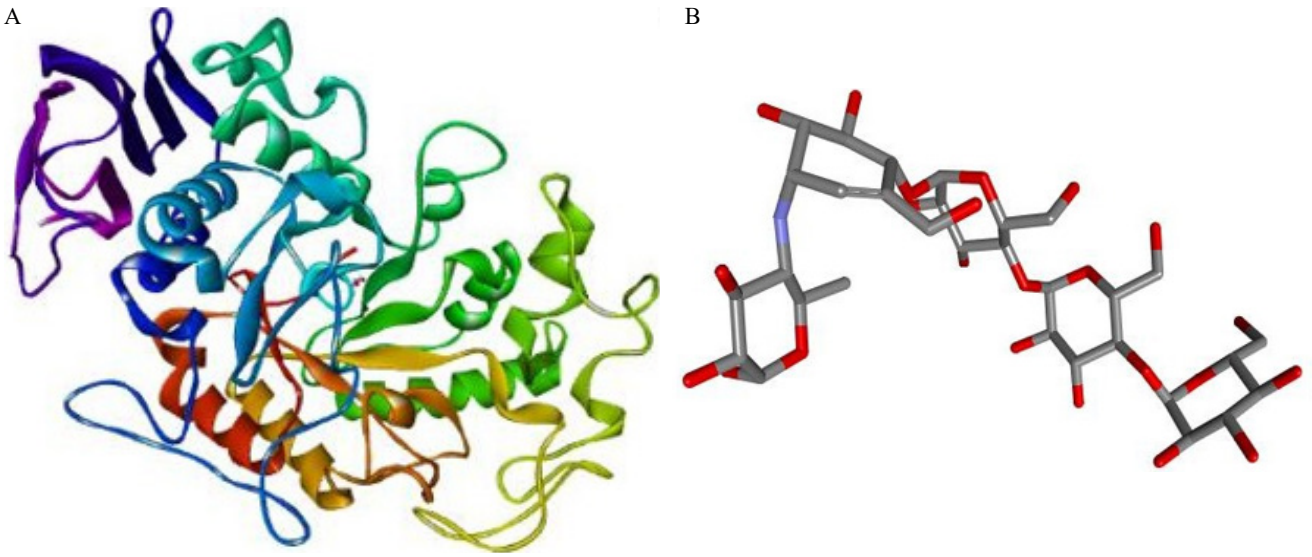


Figure 3. (A) The three-dimensional structure of the α-amylase enzyme and (B) its natural ligands

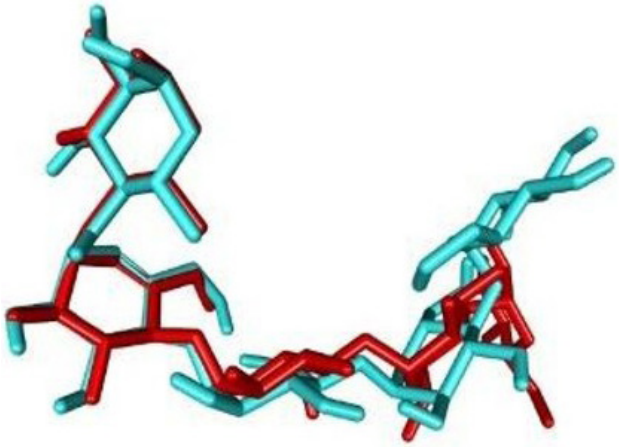


Figure 4. The overlay of the acarbose confirmation before (blue) and after (red) redocking

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 Ki 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 Ki 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 Ki 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 Ki 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.

Acknowledgements

This research was supported by Hibah Penelitian dan Pengabdian Kepada Masyarakat Akademik Pendidikan Tinggi Vokasi under contract number 103/SPK/D.D4/PPK.01.APTV/III/2024 and derivative contract number: 018/LL6/PPM-V/AL.04/2024.

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