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Antidiabetic Properties of *Uncaria sclerophylla* Roxb: *In Vitro*, Metabolite Profiling, and Molecular Docking

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Abstract

Uncaria sclerophylla Roxb is a traditional medicinal plant used to treat diabetes mellitus in Kalimantan, Indonesia, and the antidiabetic properties of its stem bark have not been previously investigated. This research will focus on investigating the potential of *U. sclerophylla* stem bark as an antidiabetic with the mechanism of inhibiting dipeptidyl peptidase-4, α-glucosidase, and antioxidants from extracts to chromatographic fractions, including the exploration of the major compounds contained in the most active chromatographic fraction. Extraction using a four-grade maceration technique, bioassays were carried out using spectrophotometric methods, fractionation using gradient column chromatography, and compound profiling using UHPLC-Q-ToF-MS/MS. The profiled compounds were predicted for their bioactivity *in silico*. The stem bark of *U. sclerophylla* demonstrated antidiabetic potential, and the methanol extract showed superior antidiabetic potential compared with the other extracts. From the extract, the most active chromatographic fraction, FUS2, was successfully obtained, which had the best activity with DPP-4 inhibition IC_{50} of 83.07 ± 6.3393 µg/mL, α-glucosidase inhibition IC_{50} of 58.06 ± 1.6226 µg/mL, and antioxidant IC_{50} of 8.47 ± 0.0443 (DPPH method) and 8.47 ± 0.0234 µg/mL (FRAP method). Compound profiling of FUS2 and *in silico* bioassays revealed potential antidiabetic compounds, including rhynchophyllic acid, arecatannin A2, silydianin, and procyanidin A2.

Keywords:

Uncaria sclerophylla Roxb;
Chromatographic Fractionation;
Dipeptidyl Peptidase-4;
α-Glucosidase;
Antioxidant;
UHPLC-QToF-MS/MS.

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1- Introduction

Diabetes mellitus is a chronic metabolic disease that progresses over time and affects the majority of the population worldwide, with considerable morbidity and mortality rates caused by various risk factors such as genetic predisposition, obesity, aging, and lack of physical activity [1, 2]. The prevalence of this worldwide health issue is on the rise, with 537 cases currently, and is projected to surge to 784 million cases by 2045 [3]. Chronic diabetes presents with gradually developing severity, leading to both microvascular and macrovascular complications. Diabetes complications can lead to increased medical expenses, diminished quality of life, and heightened risk of patient mortality. Diabetes complications incur medical costs, worsen the quality of life of patients, and increase the risk of mortality [4].

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Several therapeutic options are available for the treatment of diabetes mellitus, including dipeptidyl peptidase-4 (DPP-4) and α-glucosidase inhibitors. DPP-4 inhibitors used clinically, such as vildagliptin, sitagliptin, and saxagliptin, have been reported to cause headaches, dizziness, urinary tract infections, increased blood pressure, and joint pain [5–7]. α-Glucosidase inhibitors, such as voglibose, miglitol, and acarbose, are clinically used. These medications cause mild digestive side effects and do not lead to hypoglycemia or weight gain [8]. Thus, efforts are continuing to explore DPP-4 and α-glucosidase inhibitors, which are expected to have better efficacy and lower costs with minimal side effects.

Numerous studies have reported that individuals with diabetes exhibit lower plasma antioxidant levels [9, 10]. This leads to the body's inherent antioxidant scavenging system being unable to neutralize free radicals, resulting in oxidative stress and cellular damage. Oxidative stress plays a significant role in the pathogenesis and complications of several diseases, including diabetes. These findings highlight the importance of supplementing antioxidant deficiency [11, 12]. Recent studies have established a strong association between antioxidant consumption and diabetes. Previous studies have suggested that supplementation with antioxidants can protect beta cells against apoptosis caused by oxidative stress, thereby improving the prognosis of diabetes [9, 13].

Medicinal plants are essential sources of therapeutic bioactive chemicals that are widely used to discover and develop drugs for diabetes [14]. Medicinal plants are considered more economical and accessible and are believed to have potential in treatment, where various medicinal plants have long been used as traditional medicine to manage diabetes in many countries [15, 16]. One promising genus with antidiabetic potential is Uncaria. This genus has been reported to have antidiabetic activity through various mechanisms, such as inhibition of α-glucosidase [17–19] and amylase [20]. In vivo assays have also proven its activity in increasing insulin sensitivity [21] and reducing glycemic levels [22, 23]. *Uncaria sclerophylla*, a species from this genus, has been reported to have antidiabetic potential derived from its twigs, stems [24], and leaves, which have been investigated for their antidiabetic mechanisms as α-glucosidase inhibitors, dipeptidyl peptidase-4 inhibitors, and antioxidants [25, 26]. The stems, twigs, and leaves of *Uncaria sclerophylla* have been studied for their antidiabetic potential; however, the antidiabetic properties of the stem bark have not been reported, even though the stem bark of this plant has traditionally been used by the people of Kalimantan to treat diabetes. The stem bark is usually cleaned, cut into pieces, boiled, and the decoction is consumed two to three times daily. The use of this plant has become widespread in the community, but there are no scientific data to support the effectiveness of the stem bark as an antidiabetic agent. The Uncaria genus is a medicinal plant that has long been used as a traditional remedy, including for the treatment of diabetes [27]. Therefore, research exploring the potential of the stem bark of this plant is important to complement scientific data and confirm its antidiabetic potential. The study will begin with four-graded maceration of *U. sclerophylla* stem bark to obtain its extract, which will then be analyzed for its phytochemical properties. Bioassays were conducted to determine the antidiabetic mechanism, including α-glucosidase and dipeptidyl peptidase-4 inhibition and antioxidant activities. The most active extract will be fractionated to obtain the most active chromatographic fraction, in which the compound profile will be revealed using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), accompanied by prediction of DPP-4 and α-glucosidase inhibition activities and their activity profiles *in silico*.

2- Material and Methods

Research exploring the potential of *U. sclerophylla* stem bark involves a series of methods to obtain extracts with various polarities, phytochemical profiles, and antidiabetic activity profiles through DPP-4 and α-glucosidase inhibition assays. This was followed by obtaining the most active chromatographic fractions and profiling the compounds contained therein. Through molecular docking, we identified the compounds with the best activity in inhibiting DPP-4 and α-glucosidase activities in silico. The research process is further clarified through the flowchart (Figure 1).

2-1- Chemical and Instrumentation

Chemicals: dichloromethane, n-hexane, ethanol 96%, ethyl acetate, glacial acetic acid, methanol, thin-layer chromatography plate 254GF, silica gel 70-230 mesh were obtained from Merck, Germany. Bontrager reagent, Dragendorff spray reagents, Molisch reagent, aluminum chloride, sodium chloride, gelatin, Folin-Ciocalteu reagent, anhydrous acetic acid, 5% sulfuric acid spray reagent, quercetin, gallic acid, trizma base, glycine-proline p-nitroanilide (GPPN), dipeptidyl peptidase-4 (DPP-4) enzyme, TPTZ (2,4,6-tripiridyl-s-triazine), sodium carbonate, p-nitrophenyl-α-d-glucoside (pNP-G), α-glucosidase from *Saccharomyces cerevisiae*, and DPPH (1,2-diphenyl-2-picrylhydrazyl) were obtained from Sigma-Aldrich, USA. Instrumentation: Microplate reader (Glomax, Promega, UK) and UHPLC-QToF-MS/MS (Acquity UPLC I-Class System; Xevo G2-S QToF, Waters, USA).

2-2- Plant Material, Extraction, and Phytochemical Screening

U. sclerophylla Roxb plants were collected from the Meratus forest, South Kalimantan, Indonesia. The authenticity of this plant was confirmed, and the voucher specimen was stored in the Pharmacognosy-Phytochemistry Laboratory,

Faculty of Pharmacy, Universitas Indonesia, with access number 237/LB/XI/2021. The stem bark of this plant was washed carefully, dried at a temperature of 16 °C, and then processed into simplicia by powdering and sifting with a 40-mesh size. Four solvents with different polarities (n-hexane, dichloromethane, ethyl acetate, and methanol) were used for extraction using a four-grade maceration technique. The powdered simplicia was combined with a solvent in a 1:20 ratio, beginning with a non-polar solvent and moving to a polar one, ranging from n-hexane to methanol. The mixture was stirred for 20 min, four times a day, allowed to macerate for two days, and subsequently filtered. The filtrate was concentrated using a rotary evaporator and stored at 8 °C for further analysis. The phytoconstituent content of the extract, such as alkaloids, flavonoids, and phenols, was detected using thin-layer chromatography with spray reagents. Dragendorff for alkaloids, 5 % $AlCl_3$ for flavonoids, and Folin-Ciocalteu for phenols [24]. Other phytoconstituents, such as tannins, saponins, glycosides, and anthraquinones, were detected using color reactions and precipitation methods [28].

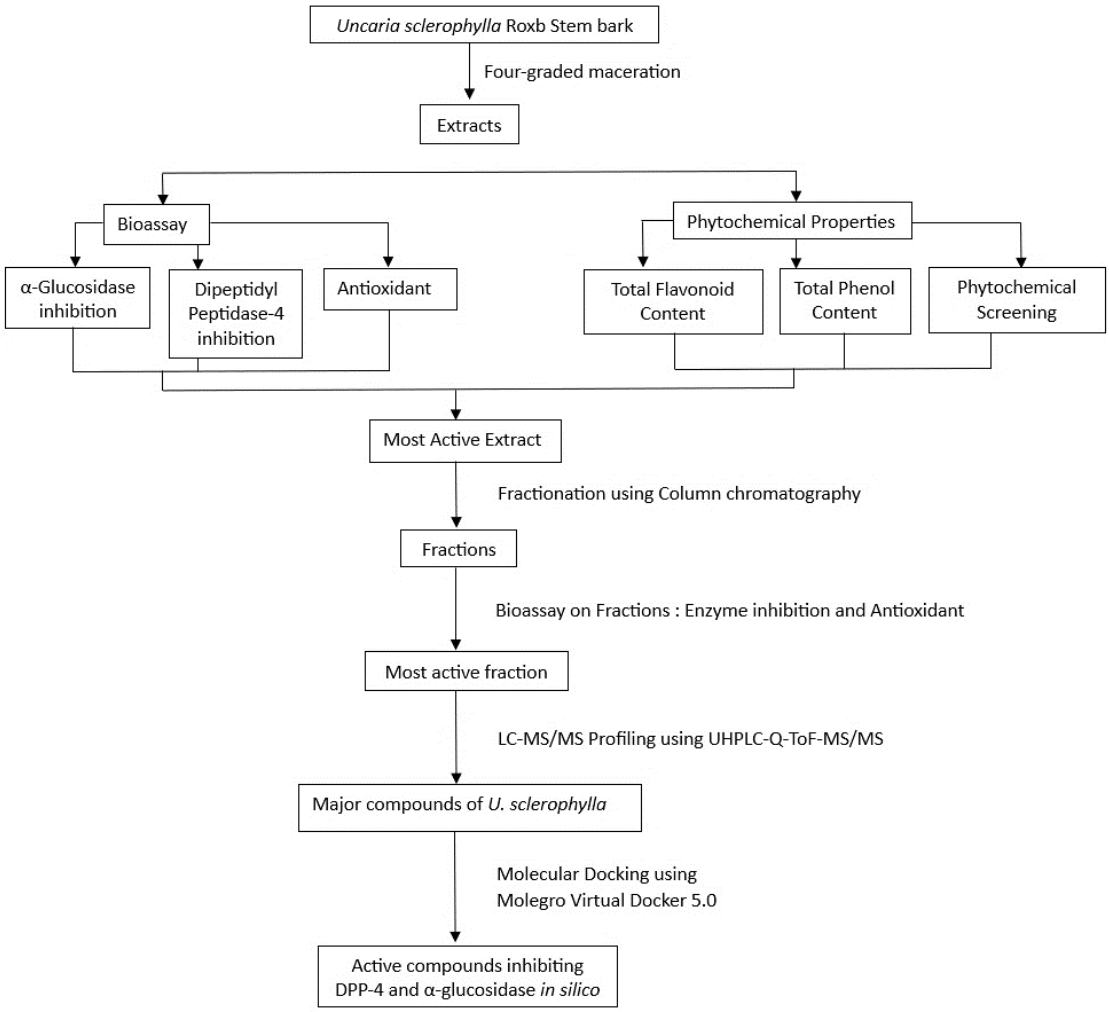

Figure 1. Research process flowchart

2-3- Microplate Assay for Total Phenol Content and Total Flavonoid Content

The Folin-Ciocalteu method was used to determine the total phenolic content using a microplate, adopting a slightly modified method [29], using gallic acid as the phenol standard for calibration. In the microplate, 100 µL of diluted Folin-Ciocalteu solution (1:4) was mixed with 25 µL of extract, shaken for 1 min, and left for 5 min. The solution mixture was then added to 75 µL of 7 % Na_2CO_3 solution and shaken for 1 min. After 120 min in the dark, the absorbance was measured at 765 nm and 25 °C using a microplate reader. The total phenolic content was expressed as gallic acid equivalents (GAE) in mg/g of extract.

The aluminum chloride colorimetric method was used to determine the total flavonoid content using a microplate, adopting a slightly modified method [30] using quercetin as the flavonoid standard for calibration. In the microplate, 10 µL of a 10 % $AlCl_3$ solution (in methanol) was mixed with 50 µL of the extract and combined with 150 µL of 96 % ethanol. The mixture was then mixed with 10 µL of 1 M CH_3COONa and incubated in the dark at 25 °C for 40 min. The absorbance was measured at 415 nm using a microplate reader. The blank contained 96 % ethanol, and the total flavonoid content was expressed as quercetin equivalent (QE) in mg/g extract.

2-4- Fractionation

The most active extract was fractionated using column chromatography with a gradient system. In this study, silica gel (70-230 mesh) was used as the stationary phase in a ratio of 1:15. The polarity of the eluent was adjusted from non-polar to more polar by using varying ratios of n-hexane and ethyl acetate at ratios of 8:2, 7:3, 6:4, and polarity continuing up to 0:10, followed by ethyl acetate and methanol in ratios of 9:1,8:2, 7:3, 6:4, and progressing up to 0:10. The filtrate was collected every 100 mL, evaporated at 16 ˚C, and analyzed for its chromatogram pattern using thin-layer chromatography. Fractions were obtained by combining the filtrates with the same or similar patterns.

2-5- DPP-4 Inhibition Assay

The DPP-4 inhibition activity of the sample was determined by spectrophotometry using a microplate reader and a slightly modified method [31]. In the microplate, 35 µL of sample was mixed with 15 µL of DPP-4 enzyme solution (0.1 units/mL) in pH 7.6 trizma-HCl buffer, shaken for 1 min, incubated at 37 ˚C for 10 min, mixed with 50 µL GPPN 1.25 mM (in pH 7.6 trizma-HCl buffer) as a substrate, shaken again for 1 min, and incubated at 37 ˚C for 30 min. The enzymatic reaction was terminated by adding 25 µL glacial acetic acid (30 %) as a stopper. The absorption of p-nitroaniline resulting from the enzymatic reaction was measured at 405 nm. The negative control consisted of an enzyme reaction without an inhibitor. The DPP-4 inhibition activity was expressed as a percentage of DPP-4 inhibition, which was used to obtain the IC_{50} value from the regression equation y = a + bx. The x-axis represents the final concentration of the sample and the y-axis represents the percentage of DPP-4 inhibition.

$$\text{Percentage of DPP-4 inhibition (\%)} = \frac{(X-Y)-(Z-Y)}{(X-Y)} \times 100\ \% \quad (1)$$

where, X = absorbance of the enzyme reaction without an inhibitor; Z = absorbance of the sample; and Y = absorbance of the control.

2-6- α-Glucosidase Inhibition Assay

α-Glucosidase inhibition activity was determined based on the principle of spectrophotometry using a microplate reader [32]. In the microplate, 30 µL of sample dissolved in phosphate buffer (pH 6.8) was mixed with 17 µL of 5 mM pNP-G substrate (in CO_2-free demineralized water) and 36 µL of phosphate buffer (pH 6.8) in a microplate. The mixture was preincubated at 37 °C for 5 min, then mixed with 17 µL of 0.12 Units/mL α-glucosidase enzyme (in pH 6.8 phosphate buffer, in which 0.2 % bovine serum albumin was dissolved) and incubated at 37 °C for 15 min, then 100 µL of 267 mM Na_2CO3 stopper solution was added to stop the enzyme reaction, and the absorbance of p-nitrophenol due to the enzyme reaction was read at a wavelength of 405 nm. The negative control for the α-glucosidase inhibition assay was an enzyme reaction without an inhibitor. The α-glucosidase inhibition activity was expressed as a percentage of the α-glucosidase inhibition, which was used to obtain the IC_{50} value from the regression equation y = a + bx. The x-axis represents the final concentration of the sample and the y-axis represents the percentage of α-glucosidase inhibition.

$$\text{Percentage of α-glucosidase inhibition (\%)} = \frac{(X-Y)-(Z-Y)}{(X-Y)} \times 100\ \% \quad (2)$$

where, X = absorbance of the enzyme reaction without an inhibitor; Z = absorbance of the sample; and Y = absorbance of the control.

2-7- Antioxidant Activity

Two methods with different mechanisms were applied to evaluate the antioxidant activity of the samples, including the method using 1,2-diphenyl-2-picrylhydrazyl (DPPH) reagent and ferric reducing antioxidant power (FRAP) reagent, and analysis using a microplate reader by adopting a slightly modified method [33, 34].

DPPH Method. The assay was initiated by adding 20 µL of the sample to a microplate, followed by the addition of 180 µL of 150 µmol/L DPPH reagent. The solution mixture was shaken for 1 min and incubated in the dark (25 °C) for 40 min. The absorbance was measured at a wavelength of 517 nm. In this assay, 200 µL of methanol was used as a blank, and a mixture of solutions consisting of 180 µL of 150 µmol/L DPPH reagent and 20 µL of methanol was used as the DPPH control solution. Antioxidant activity was expressed as the EC_{50} value, which was determined by measuring the percentage of DPPH scavenging, where the x-axis represents the sample concentration (µg/mL) and the y-axis represents the percentage of DPPH scavenging (%), using linear regression.

$$\text{Percentage of DPPH scavenging (\%)} = \frac{(X-Y)}{X} \times 100\ \% \quad (3)$$

where, X = absorbance of DPPH control solution; and Z = absorbance of the sample solution.

FRAP Method. The FRAP solution consists of a mixture of the following components: 10 mM TPTZ in 40 mM HCl, 20 mM $FeCl_3$, and an acetate buffer in a ratio of 1:1:10. The acetate buffer was prepared by mixing sodium acetate (300 mM) with glacial acetic acid to achieve a pH of 3.6. This assay was initiated by adding 30 µL of the sample to a

microplate, mixed with 270 µL of FRAP solution, shaken for 1 min, and incubated in a dark room (25 °C) for 5 min. Absorbance of the mixed solution was measured at a wavelength of 595 nm. A mixture of 270 µL of FRAP solution and 30 µL of methanol was used as the FRAP solution. Antioxidant activity was expressed as the EC_{50} value obtained based on the percentage of antioxidant activity used in the EC_{50} value calculation from the regression analysis, where the x-axis is the sample concentration (µg/mL) and the y-axis is the percentage of antioxidant activity (%).

$$\text{Percentage of antioxidant activity (\%)} = = (X - Y) \times 100 \quad (4)$$

where, X = absorbance of the sample solution; and Z = absorbance of the FRAP solution.

2-8- LC-MS/MS

The compound profiles were analyzed using UHPLC-Q-ToF-MS/MS (Acquity UPLC by Waters, USA). The column used was the C18 column (Acquity UPLC, Waters, USA). The most active fraction, FUS2, was dissolved in methanol, sonicated for 30 min, filtered using a PTFE syringe filter, injected into the liquid chromatography (LC) column, and eluted using 0.1 % formic acid in water (A) and 0.1 % formic acid in acetonitrile (B) using a step gradient system with a flow rate of 0.6 mL/min. Mobile phase B was set at 1 % in 0.5 min, increased to 35 % in 16 min, increased to 100 % in 18 min, and returned to 1 % until minute 20. The column temperature was set at 40 °C with an autosampler temperature of 15 °C. The mass spectrometer used was the Xevo G2-S QToF (Waters, USA), the ionization source used is electrospray ionization (ESI) with positive ionization modes, and Quadrupole Time-of-Flight (QToF) used as a mass analyzer. MS conditions: analysis range at 50 – 1200 m/z, collision energy was set at 6 eV (low) and 15-40 eV (high), desolvation temperature at 500 °C, source temperature at 120 °C, desolvation gas flow at 1000 L/h, cone gas flow at 30 L/h, cone voltage at 100 V, capillary voltage at 3 kV. Compound data analysis was performed using the UNIFI software and database.

2-9- Docking Molecular

The 3D structures of DPP-4 and α-glucosidase enzyme proteins obtained from the PDB database are shown in Table 1 [35, 36]. The proteins were then prepared by removing the solvent and native ligands using Discovery Studio 21.1.1. The docking process began by predicting the active sites of DPP-4 and α-glucosidase enzyme proteins using Molegro Virtual Docker 5.0, with a molecular surface van der Waals parameter of 5. Subsequent docking using the active site grid allowed the DPP-4 and α-glucosidase enzyme proteins and the compound to interact according to the specific active site grid [37].

Table 1. Target protein

Protein	PDB ID	X	Y	Z	Radius	Native ligand
Dipeptidyl peptidase-4	3G0B	53.56	27.88	22.08	22	2-({6-[(3R)-3-aminopiperidin-1-yl]-3-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl}methyl)benzonitrile
α-Glucosidase	3A4A	19.17	-5.47	21.38	11	α-D-glucopyranose

The structures of the target compounds (Table 2) were obtained from the NCBI PubChem database. Canonical smiles of dihydro-N-methylisopelletierine, rhynchophyllic acid, and 3,8-dihydroxy-4,10-dimethoxy-7-oxo-[2] benzopyrano [4,3-b] [1] benzopyran-7-(5H)-one were predicted using the Cheminfo program (https://www.cheminfo.org/). The 3D structures of arecatannin A2, dihydro-N-methylisopelletierine, rhynchophyllic acid, and 3,8-dihydroxy-4,10-dimethoxy-7-oxo-[2] benzopyrano[4,3-b] [1] benzopyran-7-(5H)-one were modeled using the Corina program (https://demos.mn-am.com/corina.html).

Table 2. Target compound and access codes

No.	Compound	PubChem CID
1	19-epi-3-isoajmalicine	179461
2	Silydianin	11982272
3	Procyanidin A2	124025
4	Nobiletin	72344
5	Arecatannin A2	16162335
6	Leucopelargonidin	3286789
7	Procyanidin B7	13990892
8	Dihydro-N-methylisopelletierine	-
9	Rhynchophyllic acid	-
10	3,8-dihydroxy-4,10-dimethoxy-7-oxo-[2] benzopyrano[4,3-b] [1] benzopyran-7-(5H)-one	-
11	Acarbose	41774
12	Sitagliptin	4369359

MolDock scoring was used for docking simulation using a grid resolution of 0.30, maximum iterations of 1500 with a minimum of 10 runs for each ligand, simplex evolution size set for steps 300, and neighbor distance factor 1.00. It was performed with an energy threshold of 100, a maximum population size of 50, tries 10-30, poses a number of poses, and an RMSD threshold of 1. The results were analyzed using Molegro Virtual Docker 5.0, combined with protein (superimposed) using PyMol 2.2. Visualization of docking results was performed using Discovery Studio 21.1.1, in 2D and 3D views, accompanying their interactions.

2-10-Statistical Analysis

Bioassay result data is the mean ± standard deviation, and its significance was analyzed statistically using the independent T-test and One-way ANOVA using Minitab version 21 with a significant difference of <0.05. The correlation test was performed using Pearson's correlation in Minitab version 21.

3- Results

3-1-Extraction and Phytochemical Screening

Extraction of *U. sclerophylla* stem bark revealed that a four-grade maceration technique effectively separated compounds with different polarities. The methanol extract produced the best results at 14.02 %, and the extracts with different solvents showed varying phytoconstituent contents (Table 3). In previous studies, the methanol extract of *U. sclerophylla* leaves provided the best yield (20.82%) and showed the presence of phytoconstituents also found in the stem bark [25]. The stems and twigs of *U. sclerophylla* have been reported to exhibit extraction yields of 21.36% and 10.80%, respectively [24]. This study and previous studies have shown that methanol is the most effective solvent for extraction compared to other solvents used in four-grade maceration.

Table 3. Phytochemical screening of various *U. sclerophylla* stem bark extracts

Solvent	Yield (%)	The presence of phytoconstituents						
		Alkaloid	Phenol	Flavonoid	Tannin	Saponin	Glycoside	Terpenoid
n-Hexane	0.24	(-)	(+)	(+)	(+)	(-)	(-)	(+)
Dichloromethane	1.22	(+)	(+)	(+)	(+)	(-)	(+)	(+)
Ethyl acetate	1.60	(+)	(+)	(+)	(+)	(-)	(+)	(+)
Methanol	14.02	(+)	(+)	(+)	(+)	(+)	(+)	(-)

3-2-Total Phenol and Flavonoid Content

The phenol and flavonoid contents of the stem bark extracts of *U. sclerophylla* assayed in various solvents ranged from 97.43 ± 2.4779 to 405.84 ± 25.3888 mg GAE/g of extract and 7.46 ± 1.7520 to 123.91 ± 8.1117 mg QE/g of extract, respectively (Table 4). The total flavonoid content in the stem bark of the ethyl acetate and methanol extracts of *U. sclerophylla* was superior to that in the ethyl acetate and methanol extracts of its leaves, which contained total flavonoids of 79.91 ± 4.4451 and 86.74 ± 5.3284 mg QE/g, respectively [25].

Table 4. Determination of total phenols and flavonoids in various *U. sclerophylla* stem bark extracts

Solvent	Total Phenol (mg GAE /g Extract) ± SD	Total Flavonoid (mg QE /g Extract) ± SD
n-Hexane	171.13 ± 1.6777^{a}	43.62 ± 0.6570^{a}
Dichloromethane	97.43 ± 2.477	7.46 ± 1.7520
Ethyl acetate	405.84 ± 25.3888^{c}	111.52 ± 2.7352^{c}
Methanol	400.05 ± 7.3901	123.91 ± 8.1117^{d}

Data are presented as mean ± SD for triplicate measurements. Different superscript letters indicate statistically significant differences among the analyzed extracts (One-way ANOVA, Tukey's post hoc test, P<0.05, n=3).

3-3-The DPP-4 and α-Glucosidase Inhibition Activity of Extracts

Table 5 shows the DPP-4 and α-glucosidase inhibition activities of each extract obtained from the stem bark of *U. sclerophylla*. The ethyl acetate extract of the stem bark showed the highest DPP-4 inhibition activity, with a percentage inhibition of 63.51 ± 1.0143 %. In contrast, the methanol extract of the stem bark displayed the best α-glucosidase inhibition activity, with a percentage inhibition of 75.85 ± 3.0387 %. In a previous study, the ethyl acetate and methanol extracts of *U. sclerophylla* leaves exhibited DPP-4 inhibitory activities of 44.17 ± 0.7627% and 69.26 ± 0.9372%, respectively [25]. These results indicate that both leaf and stem bark extracts of *U. sclerophylla* have the potential to inhibit DPP-4. The α-glucosidase inhibitory potential of the stem bark methanol extract was demonstrated by its activity (IC_{50}:50.97 ± 0.8347 µg/mL), which was better than that of the reference compound acarbose (IC_{50}:66.01 ± 3.3602 µg/mL).

Table 5. DPP-4 and α-glucosidase inhibition activity (%) of *U. sclerophylla* stem bark extract

Sample	DPP-4		α-Glucosidase	
	% Inhibition (140 µg/mL)	IC_{50} (µg/mL)	% Inhibition (75 µg/mL)	IC_{50} (µg/mL)
n-Hexane extract	15.57 ± 0.8998[a]	-	22.76 ± 1.0230[a]	-
Dichloromethane extract	6.62 ± 1.0497[b]	-	8.13 ± 3.9575[b]	-
Ethyl acetate extract	63.51 ± 1.0143[c]	-	68.23 ± 3.1271[c]	-
Methanol extract	58.02 ± 0.9776[c]	91.32 ± 1.6400	75.85 ± 3.0387[c]	50.97 ± 0.8347
Sitagliptin	-	0.09 ± 0.0086	-	-
Acarbose	-	-	-	66.01 ± 3.3602

Data are presented as mean ± SD for triplicate measurements. Different superscript letters indicate statistically significant differences among the analyzed extracts (One-way ANOVA, Tukey's post hoc test, P<0.05, n=3).

3-4- Antioxidant Activity of Extracts

The stem bark extract of *U. sclerophylla* exhibited varying levels of antioxidant activities. However, the methanol extract demonstrated superior antioxidant activity when assessed using the DPPH and FRAP methods, with EC_{50} values of 12.73 ± 0.4020 and 9.35 ± 0.1580 µg/mL, respectively. Detailed data are shown in Table 6. These results indicate a similarity in antioxidant activity between the methanol extract of the stem bark in this study and the activity of the methanol extract of *U. sclerophylla* leaves that has been previously studied, with EC_{50} values of 9.50 ± 0.3190 µg/mL (FRAP method) and 9.94 ± 0.1572 µg/mL (DPPH method), respectively [25]. This similarity in antioxidant activity may be due to the comparable total phenol and flavonoid content between the leaves and stem bark of this plant.

Table 6. Antioxidant activity (DPPH and FRAP methods) of *U. sclerophylla* stem bark extract

Sample	DPPH Method (EC_{50} in µg/mL)	FRAP Method (EC_{50} in µg/mL)
n-Hexane extract	25.31 ± 0.4596[a]	28.65 ± 0.8466[a]
Dichloromethane extract	221.47 ± 1.8080[b]	236.70 ± 9.2738[b]
Ethyl acetate extract	11.33 ± 0.1120[c]	14.87 ± 0.0986[c]
Methanol extract	12.73 ± 0.4020[c]	9.35 ± 0.1580[c,d]
Quercetin	2.98 ± 0.2588[d]	1.73 ± 0.0475[d]

Data are presented as mean ± SD for triplicate measurements. Different superscript letters indicate statistically significant differences among the analyzed extracts (One-way ANOVA, Tukey's post hoc test, P<0.05, n=3).

3-5- Fractionation and Bioassay of Fractions

The methanol extract of *U. sclerophylla* stem bark was selected for fractionation using column chromatography because of its high enzyme inhibition and antioxidant activities. Fractionation of the methanol extract of *U. sclerophylla* resulted in ten fractions labeled FUS1-10 (Table 7). Among the fractions, FUS2 exhibited significant potential as an inhibitor of DPP-4 and α-glucosidase, as well as an antioxidant. Therefore, this fraction was selected for compound analysis using LC-MS/MS. The IC_{50} and EC_{50} of the FUS2 fraction as DPP-4 and α-glucosidase inhibitors, as well as an antioxidant, were compared with standard compounds (sitagliptin, acarbose, and quercetin), and the results are presented in Tables 8 and 9. Fractionation of selected extracts yielded the most active fraction, which exhibited better activity than the original extract. As observed with FUS2, which was more active than the methanol stem bark extract, the most active fraction obtained in a previous study [25] was more active than its original extract. FUS2, the most active fraction, exhibited a more comprehensive activity than the other fractions, with FUS2 showing greater activity than acarbose and the highest percentage of DPP-4 inhibition among all fractions.

Table 7. Eluent and weight of fractions obtained from the methanol extract of the stem bark

Fraction	Eluent of Column	Fraction Weight (g)
FUS1	n-Hexane/Ethyl acetate = 8 : 2 – 1 : 9	0.622
FUS2	n-Hexane/Ethyl acetate = 0 : 10 – Ethyl acetate/Methanol = 8 : 2	6.136
FUS3	Ethyl acetate/Methanol = 8 : 2	4.199
FUS4	Ethyl acetate/Methanol = 8 : 2 – 7 : 3	3.504
FUS5	Ethyl acetate/Methanol = 7 : 3	0.938
FUS6	Ethyl acetate/Methanol = 6 : 4	5.716
FUS7	Ethyl acetate/Methanol = 6 : 4	0.399
FUS8	Ethyl acetate/Methanol = 6 : 4 – 5 : 5	8.172
FUS9	Ethyl acetate/Methanol = 5 : 5 – 4 : 6	1.091
FUS10	Ethyl acetate/Methanol = 4 : 6 – 0 : 10	1.185

Table 8. DPP-4 and α-glucosidase inhibition activity of methanol extract fractions of *U. sclerophylla* stem bark

Fraction	DPP-4		α-Glucosidase	
	% Inhibition activity (140 µg/mL)	IC_{50} (µg/mL)	% Inhibition activity (75 µg/mL)	IC_{50} (µg/mL)
FUS1	36.54 ± 1.1619[a]	-	58.47 ± 0.5599[a]	-
FUS2	60.21 ± 0.8110[b]	83.07 ± 6.3392	52.44 ± 0.3896[b]	58.06 ± 1.6226
FUS3	54.93 ± 0.2436[c]	-	58.51 ± 1.9024[a]	-
FUS4	52.42 ± 0.3065[c,d]	-	51.60 ± 1.5586[b]	-
FUS5	50.71 ± 0.6131[d]	-	72.57 ± 2.0331[c]	-
FUS6	31.36 ± 0.7442[e]	-	38.63 ± 1.7746[d]	-
FUS7	50.77 ± 0.7308[d]	-	63.19 ± 0.9258[e]	-
FUS8	27.94 ± 2.0301[e]	-	26.83 ± 1.4812[f]	-
FUS9	18.24 ± 2.2430[f]	-	25.62 ± 1.3352[f]	-
FUS10	18.10 ± 2.4777[f]	-	58.10 ± 1.6457[a]	-
Sitagliptin	-	0.09 ± 0.0086	-	-
Acarbose	-	-	-	66.01 ± 3.3602

Data are presented as mean ± SD for triplicate measurements. Different superscript letters indicate statistically significant differences among the analyzed extracts (One-way ANOVA, Tukey's post hoc test, P<0.05, n=3).

Table 9. Antioxidant activity of methanol extract fractions of *U. sclerophylla* stem bark

Fraction	DPPH		FRAP	
	% Inhibition activity (50 µg/mL)	EC_{50} (µg/mL)	% Inhibition activity (15 µg/mL)	EC_{50} (µg/mL)
FUS1	82.91 ± 0.0558[a]	-	92.07 ± 3.3292[a]	-
FUS2	82.72 ± 1.1295[a,b]	8.47 ± 0.0443	91.47 ± 4.6285[a]	8.47 ± 0.0234
FUS3	82.14 ± 0.5667[a,b]	-	70.03 ± 0.8505[b]	-
FUS4	80.46 ± 0.1675[a,b]	-	49.87 ± 0.9074[c]	-
FUS5	78.92 ± 0.3350[b]	-	55.57 ± 0.2887[c]	-
FUS6	70.37 ± 1.6018[c]	-	21.40 ± 0.5568[d,f]	-
FUS7	78.95 ± 0.0558[b]	-	62.27 ± 1.6503[e]	-
FUS8	73.66 ± 2.6421[d]	-	21.27 ± 0.5132[f]	-
FUS9	70.76 ± 0.8053[c,d]	-	26.13 ± 1.5177[f]	-
FUS10	79.69 ± 0.2559[b]	-	43.33 ± 1.4295[g]	-
Quercetin	-	2.98 ± 0.2588	-	1.73 ± 0.0475

Data are presented as mean ± SD for triplicate measurements. Different superscript letters indicate statistically significant differences among the analyzed extracts (One-way ANOVA, Tukey's post hoc test, P<0.05, n=3).

3-6- Correlation

The results of the Pearson correlation analysis are shown in the correlation coefficient matrix plot in Figure 2. Pearson correlation analysis was performed on the results of the *U. sclerophylla* stem bark extract assay to determine the correlation between all extract assay results, including the correlation between total phenol, total flavonoids, and enzyme inhibition activity, which showed a robust correlation (0.976 – 0.996). Pearson correlation analysis was also performed on the assay results of the methanol extract fractions (FUS1–10) to determine the correlation between antioxidant and enzyme inhibition activity, which showed a strong correlation (0.642 – 0.758). The correlation analysis between DPP-4 and α-glucosidase inhibition activities showed a moderate correlation (0.588), whereas the antioxidant activities of the FRAP and DPPH methods showed a robust correlation (0.892).

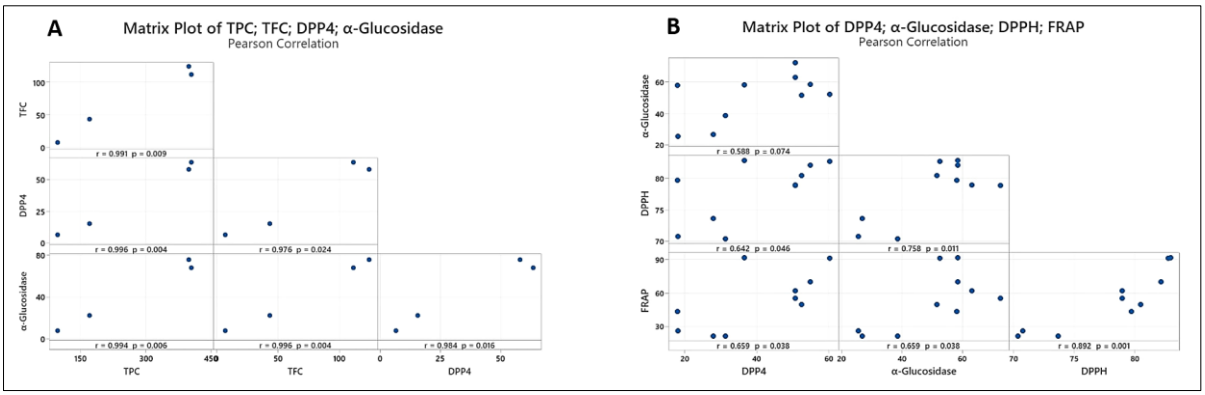

Figure 2. Pearson correlation coefficient analysis, (A) between total phenol content, total flavonoid content, DPP-4 inhibition activity, and α-glucosidase inhibition activity of the extracts, (B) between DPP-4 inhibition activity, α-glucosidase inhibition activity, and antioxidant activity (DPPH and FRAP methods) of the methanol extract fractions of *U. sclerophylla* stem bark.

3-7- LC-MS/MS

Fraction FUS2 exhibited superior enzyme inhibition and antioxidant activities, prompting profiling analysis by LC-MS/MS to identify its compounds. Profiling analysis revealed the presence of 10 major compounds, including flavonoids, tannins, and alkaloids. The major compounds identified were 19-epi-3-isoajmalicine (1), 3,8-dihydro-4,10-dimethoxy-7-oxo-[2]benzopyrano[4,3-b][1]benzopyran-7-(5H)-one (2), silydianin (3), procyanidin A2 (4), nobiletin (5), dihydro-N-methylisopelletierine (6), rhynchophyllic acid (7), arecatannin A2 (8), leucopelargonidin (9) and procyanidin B7 (10), as shown in Figure 3. The detailed analysis results, including the compound formula, ion mass (m/z, positive mode), retention time (min), and phytochemical class, are presented in Table 10. Compound profiling of the most active fraction of *U. sclerophylla* leaves revealed the presence of compounds that were also detected in the most active fraction of *U. sclerophylla* stem bark, namely 19-epi-3-isoajmalicine and Procyanidin A2 [25] . Procyanidin A2 is the major compound detected in both the methanol extract of the leaves and the methanol extract of the stem bark of *U. sclerophylla*. Alkaloid and tannin compounds dominate the content of FUS2 as the most active fraction, indicating that these groups of compounds play a role in the activity of FUS2.

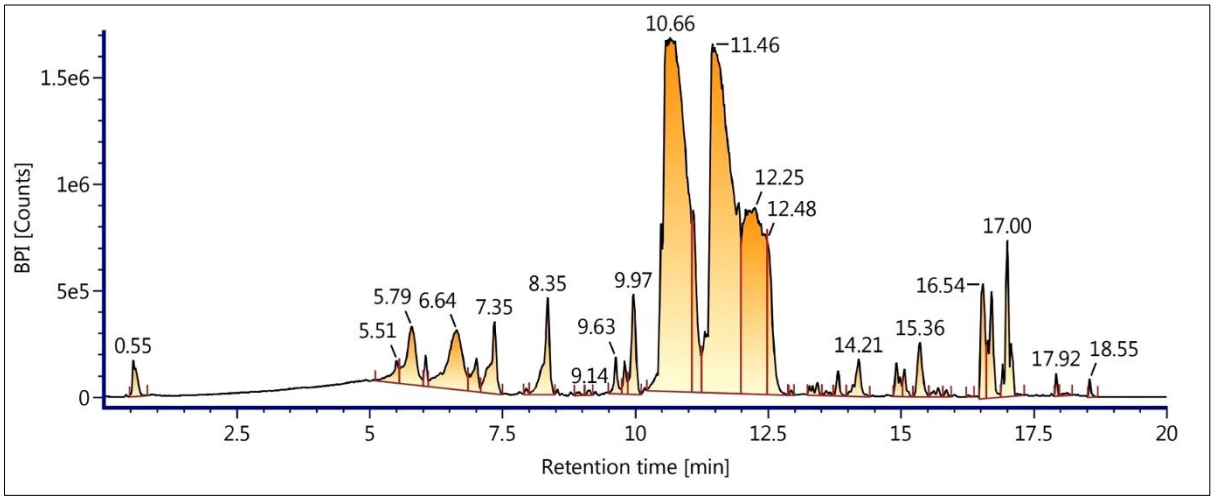

Figure 3. Chromatogram of FUS2

Table 10. Major compounds detected in FUS2

No.	Compound	Formula $[M+H]^+$	Ion mass (m/z) $[M+H]^+$	Retention Time (min)	Phytochemical Classes
1	19-epi-3-isoajmalicine	$C_{21}H_{24}N_2O_3$	353.1861	10.66	Alkaloid
2	3,8-dihydro-4, 10-dimethoxy-7-oxo-[2] benzopyrano [4, 3-b] [1] benzopyran-7-(5H) -one	$C_{18}H_{14}O_7$	343.0813	11.46	Flavonoid
3	Silydianin	$C_{25}H_{22}O_{10}$	483.1288	12.25	Flavonoid
4	Procyanidin A2	$C_{30}H_{24}O_{12}$	577.1343	12.48	Flavonoid
5	Nobiletin	$C_{21}H_{22}O_8$	403.1385	17.00	Flavonoid
6	Dihydro-N-methylisopelletierine	$C_9H_{19}NO$	158.1544	16.54	Alkaloid
7	Rhynchophyllic acid	$C_{21}H_{26}N_2O_4$	371.1964	9.97	Alkaloid
8	Arecatannin A2	$C_{60}H_{50}O_{24}$	1155.2764	8.35	Tannin
9	Leucopelargonidin	$C_{15}H_{14}O_6$	291.0862	7.35	Flavonoid
10	Procyanidin B7	$C_{30}H_{26}O_{12}$	579.1496	6.64	Flavonoid

3-8- Docking Molecular

The major compounds in FUS2 were identified by LC-MS/MS, as outlined in the molecular docking analysis. The binding energy data revealed a range of binding energy values for each enzyme, with values spanning from -181.2 to -498.5 kJ/mol for binding to the DPP-4 enzyme protein and -145.8 to -371.4 kJ/mol for binding to the α-glucosidase enzyme protein. Figures 4 to 6 show a visual representation of the interaction between the native ligand of each enzyme and the reference drugs (sitagliptin and acarbose) on the DPP-4 and α-glucosidase enzymes, along with the four best interactions of the compounds with each enzyme, with a comprehensive summary of the molecular docking between DPP-4 and α-glucosidase proteins and all ligands listed in Table 11.

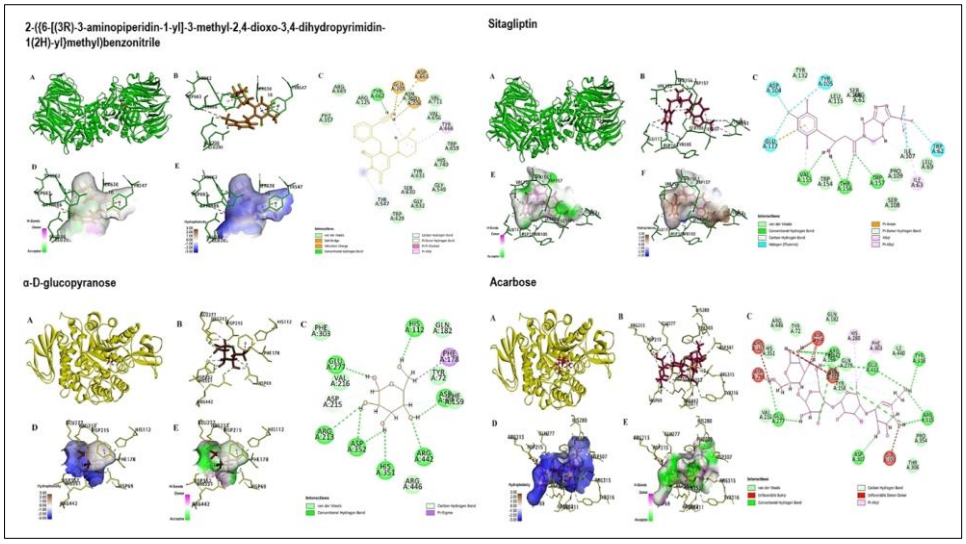

Figure 4. Interactions between the native ligand of each enzyme and the reference drugs (sitagliptin and acarbose) on DPP-4 and α-glucosidase enzymes. A-B. 3D structure, C. 2D structure, D. Hydrophobic interaction, E. Hydrogen bond

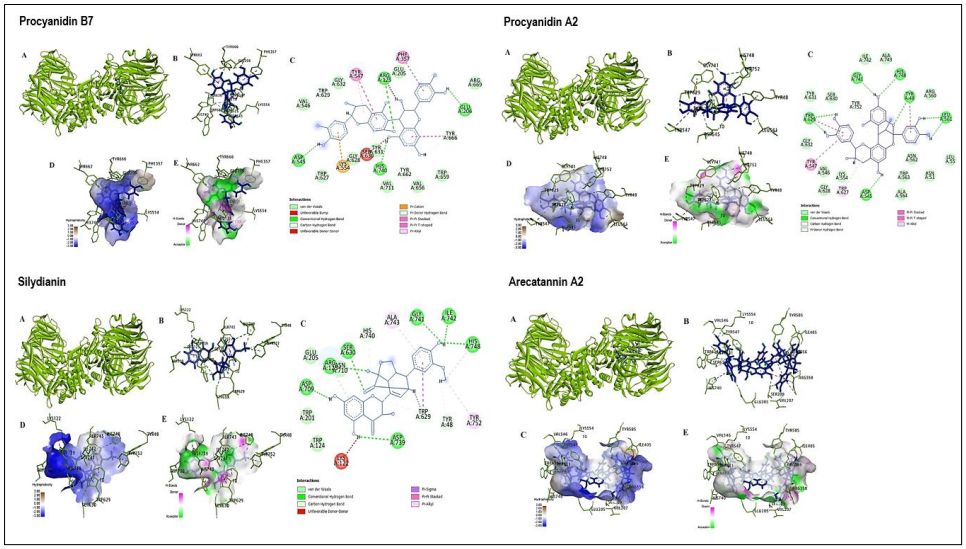

Figure 5. Interactions between procyanidin B7, procyanidin A2, silydianin, and arecatannin A2 and DPP-4 protein. A – B. 3D structure, C. 2D structure, D. Hydrophobic interaction, E. Hydrogen bond

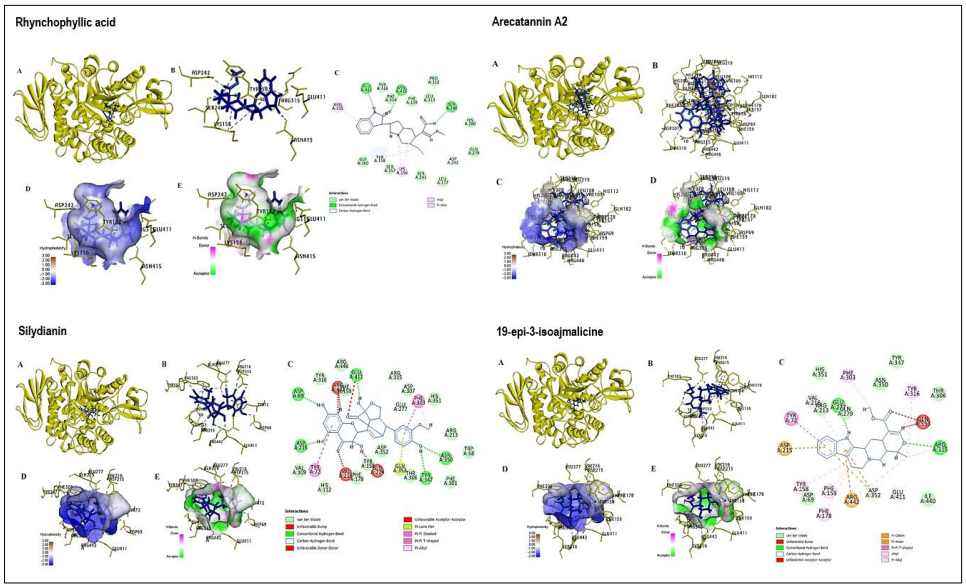

Figure 6. Interaction between rhynchophyllic acid, arecatannin A2, silydianin, and 19-epi-3-isoajmalicine against the α-glucosidase protein. A – B. 3D structure, C. 2D structure, D. Hydrophobic interaction, E. Hydrogen bond

Table 11. Molecular docking of the enzyme proteins and compounds contained in FUS2

Compounds	Dipeptidyl Peptidase-4 (3G0B)		α-Glucosidase (3A4A)	
	Binding Energy (kJ/mol)	Interaction	Binding Energy (kJ/mol)	Interaction
2-({6-[(3R)-3-aminopiperidin-1-yl]-3-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl}methyl) benzonitrile (Native ligand)	-316.7	H-bond (GluA205, GluA206, TyrA662, TyrA547, SerA630), phi-phi stacked (TyrA547), phi-alkyl (TyrA547, TyrA662, TyrA666)	-	-
Sitagliptin (Drug control)	-301.0	H-bond (ThrA156, ValA155, TrpA157, IleA107, SerA106), fluorine-bond (TrpA62, AspA104, TyrA105, IleA107, GluA117), phi-anion (GluA117), alkyl bond (IleA163, IleA107), phi-alkyl (ValA155)	-	-
19-epi-3-isoajmalicine	-283.8	H-bond (SerA460, LysA463, PheA461), phi-sigma bond (IleA63), Alkyl bond (ArgA61)	-288.3	H-bond (GluA277, ArgA315, GluA411, AspA352), phi-cation (ArgA442), phi-anion (AspA215, AspA352), phi-phi T-shaped (TyrA72), alkyl bond (ArgA315, ArgA442), phi-alkyl (ValA216, TyrA158, PheA159, PheA178, PheA303, TyrA316)
3,8-dihydro-4, 10-dimethoxy-7-oxo-[2] benzopyrano [4, 3-b] [1] benzopyran-7-(5H) -one	-270.0	H-bond (AspA739, GluA205, ArgA125), phi-cation (ArgA125), phi-anion (AspA709, phi-phi stacked (TrpA629), alkyl bond (ArgA125), phi-alkyl (ArgA125, TrpA124, TrpA201, TrpA629)	-268.2	H-bond (SerA157, AspA307, SerA241, LysA156, ThrA310, AspA307), phi-phi stacked (TyrA158, HisA280), alkyl bond (LeuA177, ArgA315), phi-alkyl (LysA156, TyrA158)
Silydianin	-343.0	H-bond (GlyA741, IleA742, AspA739, AspA709, ArgA125, SerA630, HisA748, TyrA48, TrpA629, HisA740), phi-sigma bond (TrpA629), phi-phi stacked (TrpA629), phi-alkyl (AlaA743, ArgA125, HisA748, TyrA752	-313.2	H-bond (GluA411, TyrA347, AspA215, AspA69, AsnA350, GluA277, ArgA315), phi-phi stacked (PheA303), phi-phi T-shaped (TyrA72), phi-alkyl (PheA303)
Procyanidin A2	-344.6	H-bond (AspA545, GlyA741, LeuA561, TrpA629, TyrA48, HisA748, AspA545, TyrA752), phi-phi stacked (TrpA629), phi-phi T-shaped (TyrA752, TyrA48, TyrA547), phi-alkyl (TrpA627)	-213.0	H-bond (GluA277, GluA411, TyrA316, TyrA347, AspA242, PheA178), phi-cation (ArgA442), phi-phi stacked (PheA303, TyrA158), alkyl bond (ArgA315), phi-alkyl (ArgA315, ValA216)
Nobiletin	-265.5	H-bond (TrpA216, IleA107, TrpA62, LeuA214, ThrA156, TrpA157, ProA159), phi-sigma bond (ProA109), phi-phi T-shaped (TrpA216), alkyl bond (IleA163, ProA109, LysA463), phi-alkyl (ProA109, ProA159, TrpA215)	-233.4	H-bond (AspA307, TyrA158, GluA411, HisA351, AspA352, HisA112, ArgA315), phi-anion (AspA215, GluA277, GluA411), phi-phi stacked (PheA303), phi-phi T-shaped (TyrA72), alkyl bond (ArgA315, ValA109), phi-alkyl (ValA216, TyrA72, HisA112, TyrA158, PheA159, PheA303, HisA351)
Dihydro-N-methylisopelletierine	-181.2	H-bond (GluA205), alkyl bond (ValA656), phi-alkyl (TyrA662, TyrA666, HisA740)	-179.8	H-bond (SerA241, SerA240), alkyl bond (LeuA177, Lys A156), phi-alkyl (TyrA158)
Rhynchophyllic acid	-309.0	H-bond (HisA740, ArgA125, SerA630, TyrA662), phi-phi stacked (TyrA662), phi-phi T-shaped (TyrA666), Amide-phi stacked (SerA630, TyrA631), phi-alkyl (ValA656, ValA711, TyrA547)	-371.4	H-bond (GluA411, SerA240, AsnA415, AspA242, TyrA158), alkyl bond (Lys156), phi-alkyl (ArgA315, TyrA158)
Arecatannin A2	-498.5	H-bond (SerA209, TyrA547, GluA205, ValA546, TyrA585, IleA405, ValA207, ArgA358, SerA209, LysA554, TyrA631, SerA630, phi-cation (ArgA358), phi-sigma bond (ArgA356), phi-phi stacked (TyrA547), phi-phi T-shaped (HisA740), alkyl bond (ArgA358), phi-alkyl bond (ArgA356, ArgA358)	-371.2	H-bond (AspA69, GluA277, AspA352, ArgA213, AspA215, LeuA108, GlnA182, GlnA279, MetA278, SerA218, ThrA310, SerA157, GluA411, ArgA442, HisA112, TyrA158), phi-cation (ArgA213, ArgA442, ArgA446), phi-anion (AspA215, GluA277), phi-sigma ValA109, ValA216), phi-phi stacked (TyrA72, TyrA158, HisA351), phi-phi T shaped (TrpA58, HisA280), alkyl bond (ArgA215), phi-alkyl (ValA216, ProA61, ArgA213, LeuA219)
Leucopelargonidin	-274.0	H-bond (AspA739, AspA709, ArgA125), phi-anion (AspA709), phi-phi stacked (TrpA629, phi-phi T-shaped (HisA740), phi-alkyl (ArgA125)	-255.2	H-bond (GluA277, GlnA353, AspA215, GlnA279), phi-cation (ArgA442), phi-anion (GluA411), phi-phi T-shaped (PheA303, TyrA72), phi-alkyl (ValA216)
Procyanidin B7	-356.6	H-bond (HisA740, GluA206, AspA545, ArgA125, TrpA629, TyrA666, SerA630, TyrA662), phi-cation (LysA554), phi-phi stacked (TyrA662, PheA357), phi-phi T-shaped (TyrA547, TyrA666), phi-alkyl (TyrA547)	-145.8	H-bond (GluA277, AspA352, GlnA353, TyrA316, GluA411, AspA242, GlnA22, GlnA279, AsnA350), phi-cation (ArgA315, phi-anion (AspA352, GluA411), phi-phi stacked (PheA303, PheA301), phi-phi T-shaped (TyrA158), phi-alkyl (ArgA315, ArgA442, PheA303)
α-D-glucopyranose (Native ligand)	-	-	-217.6	H-bond (HisA112, AspA69, HisA351, AspA352, GluA277, ArgA213, ArgA442, AspA215), phi-sigma (PheA178)
Acarbose (Drug control)	-	-	-204.8	H-bond (GluA277, AspA307, AspA352, GluA411, TyrA316, ArgA315, ArgA442, AspA215, GlnA353), phi-alkyl (HisA280, PheA303)

Some compounds have lower binding energies than sitagliptin for DPP-4. These compounds also exhibited lower binding energies than acarbose when binding to α-glucosidase. These compounds were rhynchophyllic acid, silydianin, procyanidin A2, and arecatannin A2, whose chemical structures are shown in Figure 7.

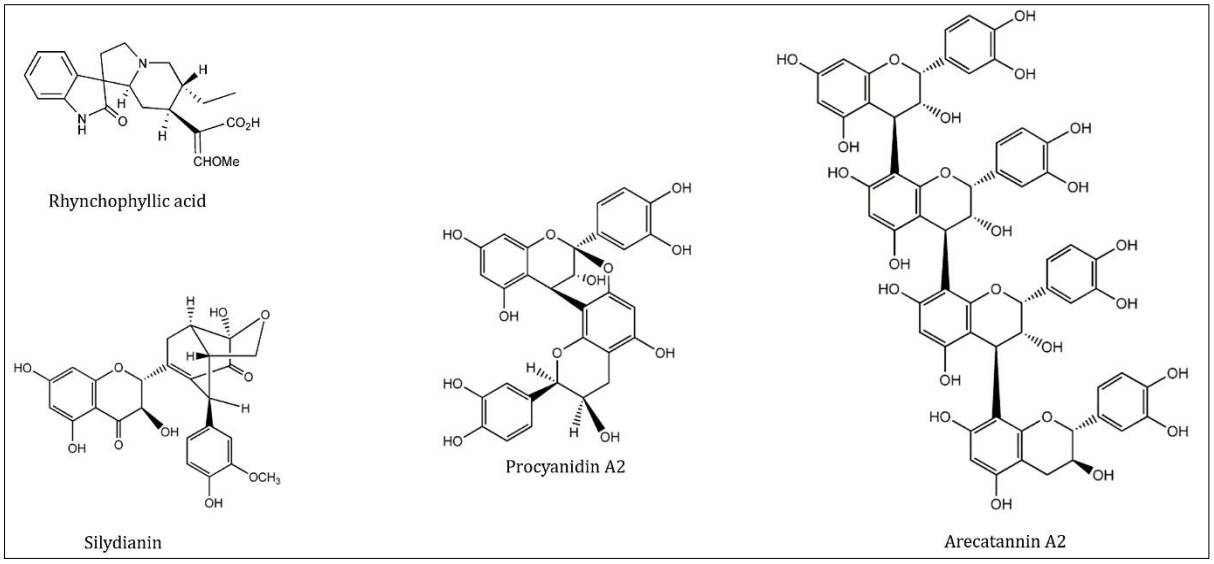

Figure 7. Chemical structures of compounds with lower binding energies than sitagliptin and acarbose

4- Discussion

The stem bark of *U. sclerophylla* was extracted using various solvents with different polarities, resulting in varying levels of phenolic and flavonoid content, antioxidant activity, and enzyme inhibition. Owing to their higher phenol and flavonoid contents, the ethyl acetate and methanol extracts exhibited higher activity than the n-hexane and dichloromethane extracts. The presence of these compounds in the extracts may contribute to their antioxidant and inhibition activities against DPP-4 and α-glucosidase enzymes. The configuration and total number of hydroxyl groups in phenols and flavonoids play significant roles in these activities [38]. Correlation analysis revealed a strong relationship (0.976–0.996) between the flavonoid-phenolic content and enzyme inhibition activity. Phenolic compounds, including flavonoids, have been widely reported to play an essential role in the inhibition of DPP-4 [39–41] and α-glucosidase [42, 43] and as antioxidants [44, 45]. Phenols and flavonoids have been proven, *in vitro*, *in vivo*, and in pre-clinical trials, to be effective in treating diabetes and its complications. These compounds have great potential for future development as antidiabetic agents [46, 47].

The methanol extract was selected for fractionation using column chromatography, resulting in 10 fractions (FUS1-10). Fractionation allows the separation of more active compounds from less active or inactive compounds [48, 49], yielding a more promising fraction for further development than the original extract. The resulting fractions displayed different levels of antioxidant and inhibition activities against DPP-4 and α-glucosidase. FUS2 exhibited the highest antioxidant and potent DPP-4 inhibition activities among the fractions. It also showed a strong α-glucosidase inhibition activity. Pearson correlation analysis revealed a moderate correlation (0.588) between the results of the DPP-4 and α-glucosidase inhibition activity assays. Additionally, the correlation between the enzyme inhibition and antioxidant activity assays was moderate to strong, ranging from 0.642 to 0.758. These results suggest that the strong antioxidant compounds in FUS2 may play a role in inhibiting DPP-4 and α-glucosidase. Various studies have shown that compounds, especially phenolic compounds and flavonoids, are potent antioxidants and have promising activities in inhibiting enzymes [38, 50]. The combination of the inhibition activity of DPP-4 and α-glucosidase enzymes and the antioxidant activity of FUS2 makes this fraction have great potential as an antidiabetic. In diabetes, antioxidants are required to overcome oxidative stress and prevent the production of advanced glycation end products (AGEs), which worsen diabetic complications [11, 12, 51]. Additionally, DPP-4 inhibition increases insulin secretion [7, 52], whereas α-glucosidase inhibition helps overcome hyperglycemia [53, 54]. Therefore, FUS2 is a potential candidate for use as an antidiabetic agent.

Ten major compounds were identified in FUS2, which was the most potent fraction from the stem bark of *U. sclerophylla*. These compounds were identified using UPLC-ESI-QToF-MS/MS profiling analysis. Among them are alkaloid compounds, such as 19-epi-3-isoajmalicine, dihydro-N-methylisopelletierine, and rhynchophyllic acid. Interestingly, the activity of these compounds in inhibiting DPP-4 and α-glucosidase or as antidiabetic agents has not been previously reported. *In vitro* and *in vivo* studies have identified several alkaloid compounds with potential antidiabetic properties. These compounds function through different mechanisms, including the inhibition of digestive enzymes, suppression of aldose reductase and protein tyrosine phosphatase-1B, increased insulin release, inhibition of AGEs production, and enhancement of glucose uptake [55]. Several flavonoids have been identified in FUS2, including 3,8-dihydro-4, 10-dimethoxy-7-oxo-[2] benzopyrano[4, 3-b][1] benzopyran-7-(5H)-one, silydianin, procyanidin A2, procyanidin B7, nobiletin, and leucopelargonidin. Silydianin, as part of a flavonoid complex, has been reported to have

antidiabetic activity by lowering blood glucose levels and providing nephroprotective effects in type 2 diabetes [56]. *In vitro*, *in vivo*, and clinical trials have reported the potential of natural ingredients rich in silydianin for preventive and antidiabetic therapy through various mechanisms [57–60]. Procyanidins, such as procyanidin A2 and procyanidin B7, also contribute to the antidiabetic effect of FUS2, and natural ingredients containing procyanidins have been reported to exhibit antidiabetic properties [61, 62], including the inhibition of dipeptidyl peptidase-4 [63]. Nobiletin has been reported to have antidiabetic activity [64], including a protective effect on human islet survival and function, and in overcoming oxidative stress [65], which also affects insulin resistance [66]. Leucopelargonidin exhibits antidiabetic activity by enhancing insulin secretion, and *in vivo* studies have shown that it exerts hypoglycemic effects [67–69]. Flavonoid compounds possess structural features that contribute to their antidiabetic and antioxidant properties, such as hydroxyl groups at positions C3', C4', C5, and C7 on rings A and B. Additionally, catechol groups on the structure of ring B, C4 ketone groups on ring C, and double bonds at C2 and C3 of ring C support their beneficial activities [46, 70]. Many studies have reported the potential of various flavonoid compounds as antidiabetic agents [71–73]. Natural ingredients containing arecatannin A2 have been reported to possess hypoglycemic potential [74, 75]. However, antidiabetic activity of arecatannin A2 has not been reported. Tannins are phytoconstituents with the potential as antidiabetic agents, both isolated tannin compounds and tannin-rich plants can aid in the prevention and treatment of diabetes [76, 77] as well as in alleviating symptoms of diabetes complications [78, 79].

The docking results against the DPP-4 enzyme showed that compounds 19-epi-3-isoajmalicine and nobiletin showed a similar binding area to sitagliptin, whereas other compounds bound in a similar area as the native ligand. Some compounds bind to DPP-4 in a similar area as the native ligand protein DPP-4, namely arecatannin A2 (GluA205) and procyanidin B7 (GluA206 and TyrA662). Silydianin, procyanidin B7, and arecatannin A2 also showed identical residues as the native ligand, namely at SerA630. Rhynchophyllic acid has a similar binding site as the native ligand, including SerA630, TyrA662, TyrA666, and TyrA547. The compound that has a similar binding site to sitagliptin is nobiletin, which binds to residues ThrA156, TrpA157, IleA107, and IleA63. Several target compounds inhibit the activity of the α-glucosidase protein in the similar binding area of the α-D-glucopyranose substrate of α-glucosidase, and in the similar area as acarbose, except for the compound dihydro-N-methylisopelletierine. The target compounds: nobiletin, procyanidin A2, procyanidin B7, 19-epi-3-isoajmalicine, leucopelargonidin, arecatannin A2, silydianin, 3,8-dihydroxy-4,10-dimethoxy-7-oxo-[2] benzopyrano[4,3-b] [1] benzopyran-7-(5H)-one, and rhynchophyllic acid showed the similar binding site with acarbose and with α-D-glucopyranose as a native ligand, including at residues: GluA277, AspA307, AspA352, GluA411, GluA277, TyrA316, ArgA315, ArgA442, AspA215, HisA280, PheA303, GlnA353, and AspA69. The interaction between the target compound and DPP-4 protein and α-glucosidase produced varying binding energy values. The binding energies of several compounds were lower than those of the control compounds. The lower the bond energy in the complex, the stronger the interaction between the compound and the protein. The magnitude of the bond energy is influenced by the number of hydrogen bonds, hydrophobic interactions, van der Waals forces, type of bond, and the complexity of the structure of the compound and protein [80, 81]. Based on the binding energy, the compounds that bind stronger than the control against DPP-4 protein are rhynchophyllic acid, silydianin, procyanidin B7, procyanidin A2, and arecatannin A2, while against the α-glucosidase protein are silydianin, rhynchophyllic acid, nobiletin, leucopelargonidin, arecatannin A2, 3,8-Dihydroxy-4,10-dimethoxy-7-oxo-[2] benzopyrano[4,3-b] [1] benzopyran-7-(5H)-one, procyanidin A2, and 19-epi-3-isoajmalicine. The compounds rhynchophyllic acid, silydianin, procyanidin A2, and arecatannin A2 demonstrated stronger binding than the control drugs (sitagliptin and acarbose), indicating that these four compounds possess significant potential for inhibiting DPP-4 and α-glucosidase.

5- Conclusion

This study explored the stem bark of *U. sclerophylla*, particularly its potential antidiabetic activity. The antidiabetic mechanisms of the ethyl acetate and methanol extracts of *U. sclerophylla* stem bark involve the inhibition of DPP-4 and α-glucosidase. The antioxidant activity present in both the ethyl acetate and methanol extracts of this plant further strengthens its antidiabetic potential, considering the significant role of antioxidants in addressing oxidative stress and diabetic complications. The methanol extract of *U. sclerophylla* stem bark is a better α-glucosidase inhibitor than the standard drug, acarbose. This extract also exhibited promising DPP-4 inhibitory activity and was highly active as an antioxidant.

Fractionation of the methanol extract of the stem bark successfully separated the inactive compound groups, resulting in FUS2 as the most active chromatographic fraction, with better activity than the methanol extract. FUS2 showed the best activity, with an IC_{50} value for DPP-4 inhibition of 83.07 ± 6.3393 µg/mL, an IC_{50} for α-glucosidase inhibition of 58.06 ± 1.6226 µg/mL, and antioxidant EC_{50} values of 8.47 ± 0.0443 (DPPH method) and 8.47 ± 0.0234 µg/mL (FRAP method). Further exploration of FUS2, including compound profiling, revealed several major compounds that are likely to play a role in DPP-4 and α-glucosidase enzyme inhibition. Compound profiling of FUS2 and *in silico* bioactivity assays indicated that these compounds have potential as antidiabetic agents (via DPP-4 and α-glucosidase inhibition), particularly rhyncophyllic acid, arecatannin A2, silydianin, and procyanidin A2.

6- Declarations

6-1- Author Contributions

Conceptualization, N.T. and B.E.; methodology, B.E.; software, N.T.; validation, N.M.H. and M.H.; formal analysis, M.H.; investigation, N.T.; resources, B.E.; data curation, M.H.; writing—original draft preparation, N.T.; writing—review and editing, B.E.; visualization, N.M.H.; supervision, B.E.; project administration, N.M.H.; funding acquisition, B.E. All authors have read and agreed to the published version of this manuscript.

6-2- Data Availability Statement

The data presented in this study are available in the article.

6-3- Funding

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6-5- Institutional Review Board Statement

Not applicable.

6-6- Informed Consent Statement

Not applicable.

6-7- Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this manuscript. In addition, the ethical issues, including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancies have been completely observed by the authors.

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