

Bioactive chromatographic fractions from *Uncaria sclerophylla* (W.Hunter) Roxb. leaves on dipeptidyl peptidase-4 inhibition and antioxidant capacity, phytochemicals, and compound profiling using UPLC-ESI-QToF-MS/MS

[Fracciones cromatográficas bioactivas de hojas de *Uncaria sclerophylla* Roxb sobre la inhibición de la dipeptidil peptidasa-4 y la capacidad antioxidante, fitoquímicos y perfiles de compuestos utilizando UPLC-ESI-QToF-MS/MS]

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Abstract

Context: *Uncaria sclerophylla* (W.Hunter) Roxb., a traditional antidiabetic drug for Kalimantan people, has never been scientifically reported as an antidiabetic agent, especially in dipeptidyl peptidase-4 (DPP-4) inhibition and as an antioxidant.

Aims: To identify the leaf's extract most active chromatographic fraction in DPP-4 inhibition and as an antioxidant and determine the compounds contained.

Methods: Extraction was carried out using the four-grade maceration method, fractionation used column chromatography techniques, and bioassay used spectrofluorometric principles. Compound profiling was performed using liquid chromatography combined with electrospray ionization and quadrupole time-of-flight mass spectrometry (UPLC-ESI-QToF-MS/MS), and molecular docking was used to investigate interactions between major compounds and DPP-4.

Results: This study highlights the potential of methanol leaf extract as an antidiabetic agent with a DPP-4 inhibition mechanism (IC_{50} : 79.67 ± 2.95 μ g/mL) and antioxidant mechanism with EC_{50} value of 9.50 ± 0.319 μ g/mL (FRAP method) and 9.94 ± 0.1572 μ g/mL (DPPH method). The methanol extract of the leaves has been proven to produce the best chromatographic fraction (FMet5) as a DPP-4 enzyme inhibitor agent, with an IC_{50} value of 50.71 ± 1.22 μ g/mL, and as an antioxidant, with an EC_{50} value of 8.89 ± 0.1701 μ g/mL (FRAP method) and 6.07 ± 0.125 μ g/mL (DPPH method). UPLC-ESI-QToF-MS/MS profiling of the compounds in FMet5 revealed the presence of various alkaloid and flavonoid compounds like dehydrosilybin, 19-epi-3-isoajmalicine, procyanidin A2, cinchonain I b, maokonine, and cuscohygrine.

Conclusions: Extracts and chromatographic fractions derived from the leaves of *U. sclerophylla* have potent antioxidant and DPP-4 inhibitory properties, indicating their potential as an antidiabetic agent with DPP-4 inhibition and antioxidant mechanisms.

Keywords: antioxidant; dipeptidyl-peptidase-4; *Uncaria sclerophylla*; UPLC-ESI-QToF-MS/MS compound profiling.

Resumen

Contexto: *Uncaria sclerophylla* (W.Hunter) Roxb., un fármaco antidiabético tradicional para los habitantes de Kalimantan, nunca ha sido reportado científicamente como agente antidiabético, especialmente en la inhibición de la dipeptidil peptidasa-4 (DPP-4) y como antioxidante.

Objetivos: Identificar la fracción cromatográfica del extracto de la hoja más activa en la inhibición de DPP-4 y como antioxidante y determinar los compuestos que contiene.

Métodos: La extracción se realizó mediante el método de maceración de cuatro grados, el fraccionamiento utilizó técnicas de cromatografía en columna y el bioensayo utilizó principios espectrofluorométricos. El perfilado de compuestos se realizó mediante cromatografía líquida combinada con ionización por electropulverización y espectrometría de masas de tiempo de vuelo de cuadrupolo (UPLC-ESI-QToF-MS/MS), y se utilizó acoplamiento molecular para investigar las interacciones entre los compuestos principales y DPP-4.

Resultados: Este estudio destaca el potencial del extracto metanólico de hojas de como agente antidiabético con un mecanismo de inhibición de DPP-4 (IC_{50} : 79.67 ± 2.95 μ g/mL) y mecanismo antioxidante con un valor de CE_{50} de 9.50 ± 0.319 μ g/mL (método FRAP) y 9.94 ± 0.1572 μ g/mL (método DPPH). Se ha demostrado que el extracto metanólico de las hojas produce la mejor fracción cromatográfica (FMet5) como agente inhibidor de la enzima DPP-4, con un valor de IC_{50} de 50.71 ± 1.22 μ g/mL, y como antioxidante, con un valor de EC_{50} de 8.89 ± 0.1701 μ g/mL (método FRAP) y 6.07 ± 0.125 μ g/mL (método DPPH). El perfil UPLC-ESI-QToF-MS/MS de los compuestos en FMet5 reveló la presencia de varios compuestos alcaloides y flavonoides como deshidrosilibina, 19-epi-3-isoajmalicina, procianidina A2, quinaína I b, maokonina y cuscohigrina.

Conclusiones: Los extractos y fracciones cromatográficas derivadas de las hojas de *U. sclerophylla* tienen potentes propiedades antioxidantes e inhibitoras de la DPP-4, lo que indica su potencial como agente antidiabético con mecanismos antioxidantes y de inhibición de la DPP-4.

Palabras Clave: antioxidante; dipeptidil-peptidasa-4; perfil de compuestos UPLC-ESI-QToF-MS/MS; *Uncaria sclerophylla*.

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INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a widespread health issue that is expected to become more prevalent in the future (Khan et al., 2020). The prevalence of T2DM varies substantially between urban and rural areas across the globe. According to the World Health Organization (WHO) and the 9th edition of the International Diabetes Federation (IDF), diabetes is the seventh leading cause of death worldwide, with a person dying from diabetes every six seconds. In 2019, over 463 million people across the world were affected by diabetes mellitus, a significantly higher number than the previous estimate of 382 million people in 2013. The number of individuals affected by diabetes is expected to increase to 700 million by 2045 (Boyko et al., 2021; Pinchevsky et al., 2020; Singh et al., 2021).

T2DM is characterized by insulin resistance and inadequate insulin production by pancreatic beta cells, resulting in reduced glucose transport to the liver, muscles, and adipose tissue (Galicía-García et al., 2020). This condition is a chronic metabolic disease that results in both microvascular and macrovascular complications, which gradually reduce the overall quality of life (Mansour et al., 2023). Type 2 diabetes is linked to several risk factors, including high blood sugar levels, insulin resistance, dyslipidemia, obesity, renal failure, low-density lipoprotein decline, and high triglycerides (Bodaghi et al., 2023; Packard et al., 2020). T2DM, if not appropriately managed, can lead to several complications, one of which is diabetic cardiomyopathy (DCM). This condition is a leading cause of end-stage heart failure (HF) and can result in mortality and morbidity in T2DM patients. DCM is identified by abnormal cardiac structure and function in diabetic patients with no known causes, such as significant valvular and congenital heart disease, uncontrolled hypertension, and coronary artery disease (Paolillo et al., 2019; Pham et al., 2023; Seferović et al., 2018).

Medications like sulfonylurea, metformin, sodium-glucose co-transporter-2 inhibitors, and thiazolidinedione are used in type 2 diabetes mellitus medication. However, they have drawbacks, such as lack of efficacy, hypoglycemia, weight gain, obesity, abdominal pain, and atherosclerosis (Singh et al., 2021; Taylor et al., 2021). Dipeptidyl peptidase-4 (DPP-4) inhibitors offer a unique therapeutic approach for treating T2DM (Saini et al., 2023), which does not cause hypoglycemia, weight gain, or indigestion (Taylor and Lam, 2020) and can be safely given to patients with renal impairment. There are several clinical trials that suggest dipeptidyl peptidase-4 (DPP-4) inhibitors

are safe and effective treatments for diabetic patients with chronic kidney disease (Daza-Arnedo et al., 2021).

DPP-4 inhibitors improve the half-life of incretin hormones by inhibiting DPP-4 and prolonging their circulation in the blood. This hormone plays a crucial role in regulating insulin secretion and inhibiting glucagon secretion, thereby improving glucose control. This is a beneficial management strategy for T2DM by preventing the breakdown of gut incretins GLP-1 and GIP. DPP-4 inhibitors are the first class of antidiabetic drugs based on incretin that have been approved for treating T2DM (Deacon, 2018; Singh et al., 2021). DPP-4 inhibitors work in a glucose-dependent manner, which means that they carry a low risk of hypoglycemia (Florentin et al., 2022; Li et al., 2018). Although synthetic DPP-4 inhibitors are generally well-tolerated, there have been reports of side effects like nasopharyngitis, headaches, and urinary infections (Makrilakis, 2019; Saini et al., 2023). Therefore, it is essential to search for natural products that contain DPP-4 inhibitors and explore their potential as a diabetes treatment.

In type 2 diabetes, the high levels of glucose in the blood occur due to poor glucose uptake, abnormal hormonal regulation of glucose metabolism, and impaired β -cell function. The development of vascular complications and progression of diabetes are caused by damage to β -cells and blood vessels due to high glucose levels (Berbudi et al., 2020; Galicía-García et al., 2020). Reactive oxygen and nitrogen species are known to be associated with this damage (Akpoveso et al., 2023). Reactive oxygen species (ROS) play a significant role in the development of T2DM and worsen its pathology and complications by interfering with regulatory pathways involved in β -cell dysfunction and insulin resistance. High levels of ROS are associated with hyperglycemia, inflammation, and dyslipidemia (Akpoveso et al., 2023; Caturano et al., 2023; Fatima et al., 2023). Antioxidant supplementation improves diabetic status by regulating glucose metabolism, insulin secretion, insulin resistance, and levels of glycated hemoglobin (HbA1c) (Fatima et al., 2023; Shrivastav et al., 2023). Recent clinical data has shown that supplementing antioxidants can improve the glycemic status of diabetic patients, as evidenced by reduced HbA1c and random blood sugar levels (Balbi et al., 2018; Shrivastav et al., 2023).

Research suggests that antidiabetic plants contain compounds like phenolics, flavonoids, terpenoids, alkaloids, and glycosides that improve insulin sensitivity and lower blood sugar levels (Salleh et al., 2021). The *Uncaria* genus has scientific data on antidiabetic and antioxidant activity. Several species of this

genus, including *U. gambier*, *U. tomentosa*, *U. longiflora*, *U. calophylla*, *U. cordata*, *U. lucida*, and *U. acida*, have been reported to possess such activity (Ahmad et al., 2011; Aprely et al., 2021; Araujo et al., 2018; Arundita et al., 2020; Domingues et al., 2011; Pane et al., 2018; Zebua et al., 2018). This demonstrates the potential of the *Uncaria* genus in the management of diabetes.

Uncaria sclerophylla (W.Hunter) Roxb. (family *Rubiaceae*) is a synonym of *Uncaria cordata* (Lour.) Merr. and has been used for generations as a traditional antidiabetic medicine among the people of Kalimantan, Indonesia. However, no scientific evidence supports its use as an antidiabetic drug. This has encouraged scientific investigations to reveal scientific data about this plant as an antidiabetic through inhibiting dipeptidyl peptidase-4, including its antioxidant capacity. Not only from the extract but bioassay-guided fractionation was also carried out to obtain the chromatography fraction with the best activity, including revealing the major compounds contained in the most active fraction via UPLC-ESI-QToF-MS/MS and compound's interaction mechanism through molecular docking.

MATERIAL AND METHODS

Chemicals and instruments

Chemicals: n-hexane, dichloromethane, ethanol 96%, ethyl acetate, methanol, silica gel 70-230 mesh, thin-layer chromatography (TLC) plate 254GF, and glacial acetic acid were purchased from Merck (Darmstadt, Germany). Dragendorff spray reagents, AlCl_3 , Folin-Ciocalteu reagent, 5% H_2SO_4 spray reagent, gelatin, NaCl-gelatin, Bontrager reagent, Molisch reagent, anhydrous acetic acid, quercetin, DPP-4 enzyme, glycine-proline-paranitroaniline, Trizma Base, 2,4,6-tripiridyl-s-triazine (TPTZ) were acquired from Sigma-Aldrich (St. Louis, Missouri, USA). Distilled water was purchased from SmartLab (Jakarta, Indonesia). UPLC-ESI-QToF-MS/MS from Acquity UPLC H-Class System; Xevo G2-S QToF, Waters (Milford, USA). Microplate reader from Glomax, Promega (Southampton, UK).

Plant material

The leaves of *U. sclerophylla* were collected from Meratus Mountain Forest in Kalimantan, Indonesia. Voucher specimens with verified authenticity have been deposited at the Pharmacognosy-Phytochemistry Laboratory, Faculty of Pharmacy, University of Indonesia, under voucher specimen number 237/LB/XI/2021. The leaves were cleaned with running water and dried at low temperatures. After drying, the leaves were ground into powder using a grinder and sieved through a 40-mesh sieve.

Extraction

The leaves of *U. sclerophylla* were extracted using a four-stage maceration process, which was adopted from the method described by Triadisti et al. (2017), with some modifications made to the number of solvent types used. Organic solvents with increasing polarity were used for the extraction process, and these included n-hexane, dichloromethane, ethyl acetate, and methanol. The ratio of dry material to solvent was maintained at 1:20 to obtain n-hexane extract, dichloromethane extract, ethyl acetate extract, and methanol extract. The extracts were evaporated using a rotary evaporator, and the process was completed with a dehydrator.

Phytochemical screening

Thin-layer chromatography was used for phytochemical analysis to detect alkaloids, phenols, and flavonoids. Dragendorff spray reagent was used for alkaloid detection, AlCl_3 for flavonoid detection, Folin-Ciocalteu and sodium carbonate for phenol detection (Dinakaran et al., 2018; Pobłocka-Olech et al., 2018; Zhang et al., 2021). The screening for saponins, tannins, anthraquinones, and glycosides was conducted following the methods described in Harborne (1998).

Determination of total phenol content

The method for determining total phenol is based on the Folin-Ciocalteu method using a microplate with slight modifications (Sembiring et al., 2018). A total of 25 μL of the extract solution was mixed with 100 μL of Folin-Ciocalteu reagent diluted (1:4) and shaken for 60 seconds in a microplate. The mixture was left for 4 minutes, then 75 μL of sodium carbonate solution was added and shaken at medium speed for 1 minute. After 2 hours, absorbance was measured at 765 nm using a microplate reader at room temperature. The assay was carried out in triplicate, and each sample's standard deviation was calculated. The gallic acid solution served as a calibration standard, and the phenol content was expressed as gallic acid equivalent (GAE) in mg/g extract.

Determination of total flavonoid content

Total flavonoids were determined using a colorimetric assay using AlCl_3 with slight modifications (Sembiring et al., 2018). A standard quercetin solution was prepared in 96% ethanol. 50 μL of extract (1 mg/mL) or quercetin was added to 10 μL of AlCl_3 10% solution then 150 μL of 96% ethanol was added. 10 μL of CH_3COONa solution (1 M) was added to the solution mixture in microplates of 96 wells. Ethanol 96% was used as a blank. All the above solutions were mixed and incubated for 40 minutes and protected

from light at room temperature then absorbance was measured at 415 nm using a microplate reader) at room temperature. The assay was carried out in triplicate, and each sample's standard deviation was calculated. The total flavonoid content was expressed as quercetin equivalent (QE) in mg/g extract.

Fractionation

The column chromatography fractionation method was used with slight modifications (Khan et al., 2022). In this method, extracts (15 g for ethyl acetate extract and 40 g for methanol extract of leaves) were fractionated by column chromatography using silica gel (70-230 mesh) as the stationary phase with a ratio of 1:15. To obtain a gradient elution system with different polarities, a mixture of solvents with specific ratios (n-hexane, ethyl acetate, and methanol) was used as mobile phase. The elution was performed with a ratio of n-hexane and ethyl acetate of 9:1, 8:2, and so on up to 0:10, followed by eluent ethyl acetate and methanol in a ratio of 9:1, 8:2, 7:3 and so on up to 0:10. The elution results were collected in 100 mL volumes, and their chromatogram patterns were determined using TLC. Collections with the same or similar chromatogram patterns were then combined. Finally, the solvent was evaporated at a temperature of 16-19°C.

Dipeptidyl peptidase-4 inhibition assay

DPP-4 inhibitory activity was performed *in vitro* using spectrophotometry to assay extracts, fractions, and positive standard (sitagliptin) with slight modifications to the adopted method (Al-Masri et al., 2009). 35 µL of either samples or positive controls were added to 96 microplate wells. Then, 15 µL of DPP-4 enzyme (0.1 units/mL) was added, and the mixture was incubated at 37°C for 10 minutes to enhance the inhibitor binding capacity. After that, 50 µL of Gly-Pro-p-nitroanilid substrate (GPPN) 1.25 mM was added, and the mixture was again incubated at 37°C for 30 minutes. 25 µL of 30% glacial acetic acid was added to terminate the reaction. The absorbance was then measured at 405 nm using a microplate reader at room temperature. The assay was carried out in triplicate, and each sample's standard deviation was calculated. Negative controls (enzyme reaction absorbance without inhibitors) were measured at each assay time to ensure consistency of the measurement results between assay times. DPP-4 inhibition activity of the sample and sitagliptin as positive control was expressed as a percentage of inhibition, which is defined as equation [1].

$$\text{DPP-4 inhibition (\%)} = \frac{\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Sample}}}{\text{Abs}_{\text{Control}}} \times 100 \quad [1]$$

Where $\text{Abs}_{\text{Control}}$ is the negative control absorbance, and $\text{Abs}_{\text{Sample}}$ is the sample absorbance.

IC_{50} was calculated using the regression equation $y = a + bx$, where the x-axis represented the sample's final concentration, and the y-axis represented the % inhibition. The IC_{50} value was obtained using the equation [2] (in µg/mL).

$$\text{IC}_{50} (\mu\text{g/mL}) = \frac{50 - a}{b} \quad [2]$$

Antioxidant capacity

Two separate antioxidant assays, ferric reducing antioxidant power (FRAP) and 1,2-diphenyl-2-picrylhydrazyl (DPPH) were used to measure antioxidant capacity.

FRAP method

The method used for assaying antioxidant capacity involves the use of reagents FRAP, with a slight modification to the procedure adopted (Pereira et al., 2017). To carry out the assay, 30 µL of the sample was pipetted into a microplate well, followed by the addition of 270 µL of FRAP reagent. The mixture was then incubated for 5 minutes, and the absorbance was measured at 593 nm using a microplate reader at room temperature. The assay was conducted thrice, and the standard deviation of each sample was calculated. The blank in the assay was a mixture of 30 µL of methanol and 270 µL of FRAP reagent. The EC_{50} value was calculated based on the percent antioxidant capacity of each sample solution concentration using the following equation [3].

$$\text{Antioxidant capacity (\%)} = \frac{\text{Abs}_{\text{Sample}} - \text{Abs}_{\text{Blank}}}{\text{Abs}_{\text{Sample}}} \times 100 \quad [3]$$

After obtaining the percent antioxidant capacity for each concentration, equation [2] was determined by using regression calculations. In this equation, x represents the final concentration (in µg/mL), and y represents the percent antioxidant capacity (%).

DPPH method

The DPPH method was used to measure antioxidant capacity, with some methodology modifications applied (Bobo-García et al., 2014). Sample solution (20 µL) mixed with 150 µmol/L DPPH solution (180 µL) into a microwell plate. The control solution for the DPPH assay was made up of methanol (20 µL) and 150 µmol/L DPPH solution (180 µL). The blank solution consisted of methanol (200 µL). The mixture was shaken for one minute, incubated in the dark at room temperature for 40 minutes, and then the absorbance was measured at 516 nm using a microplate reader at room temperature. The assay was conducted thrice, and the standard deviation of each sample was calculated. The EC_{50} value is determined by measuring the scavenging of DPPH free radicals from different con-

centrations of sample solutions. Antioxidant capacity is presented as a percentage of DPPH scavenging calculated using equation [1].

Once the percentage of DPPH scavenging is obtained, equation [2] was calculated using regression analysis, where x is the sample concentration (in $\mu\text{g/mL}$), and y is the percentage of DPPH scavenging (in %). The antioxidant capacity of the sample is expressed as an effective concentration of 50% (EC_{50}), which is the sample concentration that can scavenge DPPH radicals by 50%.

UPLC-ESI-QToF-MS/MS

Compound profiling was carried out using a combination of Ultra-Performance Liquid Chromatography (UPLC) equipment and a mass spectrometer. The UPLC equipment used was the Acquity UPLC® by Waters, USA, while the mass spectrometer used was the Xevo G2-S QToF, by Waters, USA. A C18 column (Acquity UPLC®, Waters, USA) was used for liquid chromatography separation, with the column temperature set to 40°C and the room temperature at 25°C. The eluent comprised a mixture of 0.1% formic acid in water and acetonitrile with 0.1% formic acid. The flow rate was 0.6 mL/minute, and a step gradient was used. Electrospray ionization (ESI) was used as an ionizer in positive mode, and a Quadrupole Time-of-Flight (QToF) as a mass analyzer. The low energy collision used was 6 Ev, while the high energy ranged between 25-60 Volts. Data acquisition and compound analysis were carried out using Masslynx software and the Unifi Database.

Docking molecular

Ligand structure retrieval: 3D structure of compounds procyanidin A2 (CID 124025), 19-epi-3-isojalmaric acid (CID 179461), cuscohygrine (CID 1201543), dehydrosilybin (CID 5467200), cinchonin Ib (CID 10456516) and maakonine (CID 54704413) was downloaded from the NCBI PubChem database. Sitagliptin, as a control compound, was downloaded with access code 4369359 in the NCBI PubChem database. Protein structure retrieval, the 3D structure of the target protein, namely dipeptidyl peptidase-4, was downloaded from the Protein Data Bank database with access code 3G0B (Zhang et al., 2011). Docking simulation: The active site of the protein structure is predicted by importing it into the Molegro virtual Docker 5.0 program with a maximum molecular surface van der Waals parameter of 5 (Bitencourt-Ferreira and de Azevedo, 2019), while the active site of the dipeptidyl peptidase-4 protein is on grid X= 44.79 Å; Y= 32.64 Å; Z=18.74 Å; this nine grid radius is used for the docking process. The target compounds and proteins have interacted with the Molegro virtual docker program

with a specific grid, namely the active site grid (Bitencourt-Ferreira and de Azevedo, 2019). Docking parameters with Molegro virtual docker are score function moldock score [Grid]; grid resolution 0.30; algorithm MolDock SE; number of runs 10, max iterations 1500; max population size 50; pose generation energy threshold 100, tries 10 – 30; simplex evolution max steps 300; neighbor distance factor 1.00; multiple poses number of poses 5; energy threshold 0.00; cluster similar poses RMSD threshold 1.

Docking data were visualized with PyMol 2.3 and Discovery Studio version 21.1.1. Interaction analysis was also carried out with the Discovery Studio program version 21.1.1.

Statistical analysis

The data from each assay (total phenol and flavonoid content, percentage of antioxidant capacity, and DPP-4 inhibition activity) was analyzed for standard deviation, and the data represents the mean $\pm$ standard deviation. Data correlation between antioxidant (FRAP and DPPH method) and DPP-4 inhibition activity of methanol extract fractions were analyzed using Spearman correlation by Minitab 21 version. The significance of each bioassay result was analyzed through an independent T-test and one-way ANOVA using the Minitab 21 version with significant differences of $p < 0.05$.

RESULTS

Extraction and phytochemical screening

The leaves of *U. sclerophylla* were extracted using a four-stage maceration method with solvents of different polarities, namely n-hexane, dichloromethane, ethyl acetate, and methanol. The research findings indicated that the highest yield was obtained from the methanol extract, which reached 20.82%. Further details can be seen in Table 1 for the complete yield of *U. sclerophylla* leaf extract.

Phytochemical screening revealed the presence of various compounds in *U. sclerophylla* leaves, including alkaloids, phenols, flavonoids, tannins, saponins, anthraquinones, and glycosides (Table 2). This is the first report of phytoconstituent detection in *U. sclerophylla* leaves.

Total phenol and flavonoid content

Determination of total phenols and flavonoids in *U. sclerophylla* leaves showed that all extracts had phenol content ranging from 64.31 ± 1.6131 to 409.97 ± 12.2067 mg GAE/g extract, and flavonoid content ranged from 20.29 ± 3.9601 to 86.74 ± 5.3284 mg QE/g extract.

Table 1. *U. sclerophylla* leaves extraction yield with four-graded maceration.

Solvent	Dry material weight (g)	Extract weight (g)	Yield (%)
n-Hexane	1000	20.506	2.05
Dichloromethane		22.252	2.23
Ethyl acetate		16.388	1.64
Methanol		208.215	20.82

Table 2. Phytochemical screening in various *U. sclerophylla* leaves extract.

Phytoconstituent	Methanol extract	Ethyl acetate extract	Dichloromethane extract	n-Hexane extract
Alkaloids	Positive (+)	Negative (-)	Positive (+)	Negative (-)
Phenol	Positive (+)	Positive (+)	Positive (+)	Positive (+)
Flavonoids	Positive (+)	Positive (+)	Positive (+)	Positive (+)
Tannin	Positive (+)	Positive (+)	Positive (+)	Negative (-)
Saponins	Positive (+)	Positive (+)	Negative (-)	Negative (-)
Anthraquinone	Positive (+)	Positive (+)	Negative (-)	Negative (-)
Glycosides	Positive (+)	Positive (+)	Negative (-)	Negative (-)

Table 3. Determination of total phenols and flavonoids in various *U. sclerophylla* leaf extracts.

Solvent	Total phenols (mg GAE /g extract)	Total phenols (%)	Total flavonoids (mg QE/g extract)	Total flavonoids (%)
n-Hexane	64.31 ± 1.6131*	6.43 ± 0.1613*	20.29 ± 3.9601 ^{bcd}	2.03 ± 0.3960 ^{bcd}
Dichloromethane	93.77 ± 0.9123*	9.38 ± 0.0912*	76.37 ± 8.2611 ^a	7.64 ± 0.8261 ^a
Ethyl acetate	381.40 ± 16.4249*	38.14 ± 1.6425*	79.91 ± 4.4451 ^a	7.99 ± 0.4445 ^a
Methanol	409.97 ± 12.2067*	41.00 ± 1.2207*	86.74 ± 5.3284 ^a	8.67 ± 0.5328 ^a

Data are mean ± SD for triplicate measurements. *Statistically significant differences among the total phenol content (One-way ANOVA, Tukey's post hoc test, $p < 0.05$, $n = 3$). ^{a,b,c,d} Different letters in superscript, in the same column, indicate significant differences (One-way ANOVA, Tukey's post hoc test, $p < 0.05$, $n = 3$).

Methanol extract of *U. sclerophylla* leaves showed the highest total phenol and flavonoid content compared to other extracts. The complete results of determining total phenols and flavonoids can be seen in Table 3. Determination of total phenols and flavonoids of various *U. sclerophylla* leaf extracts using gallic acid and quercetin for the calibration curve (Fig. 1).

The DPP-4 inhibitory activity assay of extract

The DPP-4 inhibitory activity assay was conducted on four *U. sclerophylla* extracts: n-hexane, dichloromethane, ethyl acetate, and methanol. The assay results can be seen in Fig. 2, and it is known that all extracts have inhibitory activity with an activity range of 5.66 ± 0.6779 to $69.26 \pm 0.9372\%$. Methanol demonstrates the most potent inhibitory activity against DPP-4 compared to the other extracts, with an IC_{50} of

$79.67 \pm 2.95 \mu\text{g/mL}$, as depicted in Fig. 3.

Antioxidant capacity of extract

Extracts from *U. sclerophylla* leaves were assayed for their antioxidant capacity using the FRAP and DPPH methods. The results showed that the methanol extract of the leaves had the highest potential as an antioxidant, with an EC_{50} value of $9.50 \pm 0.319 \mu\text{g/mL}$ (FRAP method) and $9.94 \pm 0.1572 \mu\text{g/mL}$ (DPPH method). The ethyl acetate extract also showed good antioxidant potential, with an EC_{50} value of $19.38 \pm 0.326 \mu\text{g/mL}$ (FRAP method) and $21.45 \pm 6.32 \mu\text{g/mL}$ (DPPH method). The detailed test results are available in Fig. 4. The total phenol and flavonoid content of the leaves aligns with the results of the capacity activity, where the methanol extract shows the highest phenol and flavonoid content compared to other leaf extracts.

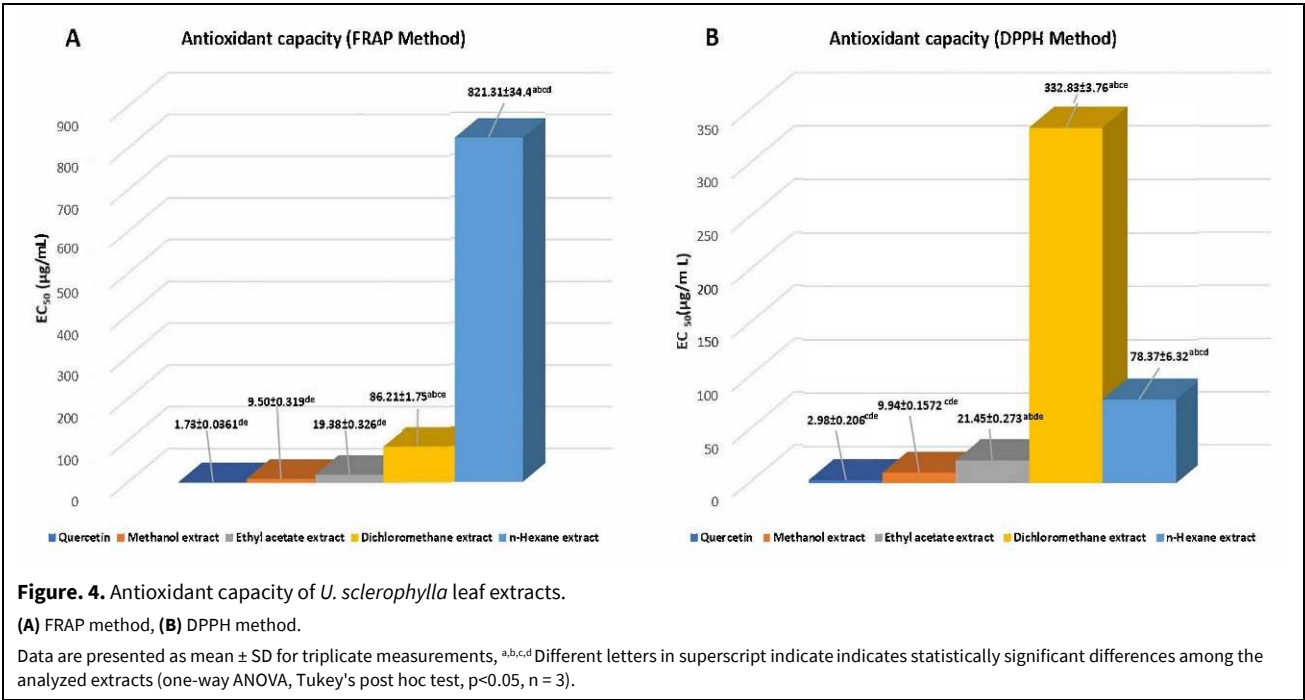
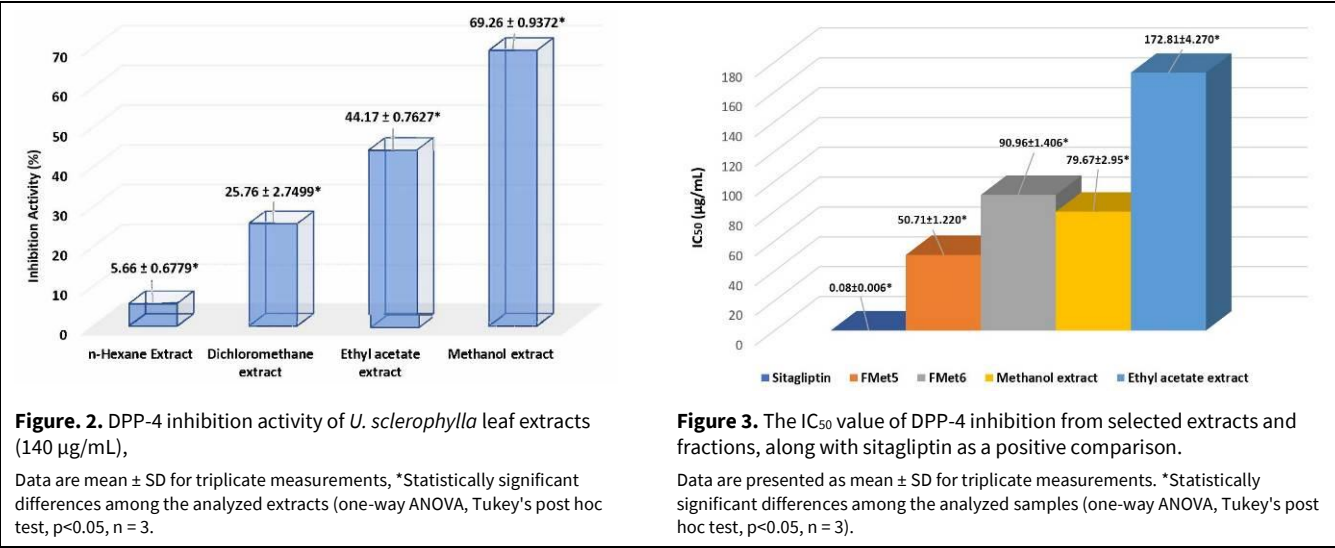
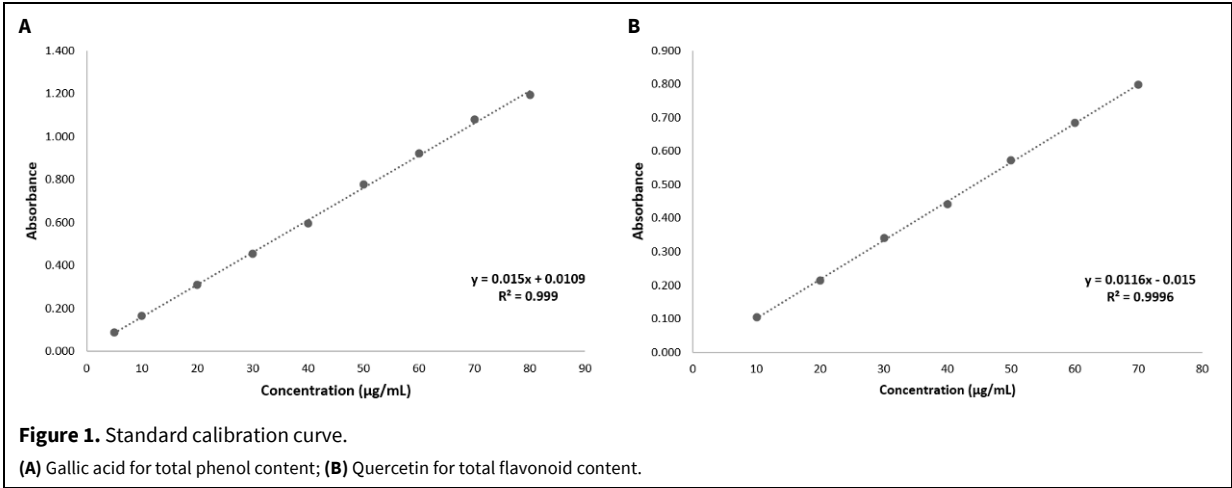


Table 4. The eluent and weight of fractions obtained from (A) methanol and (B) ethyl acetate leaf extracts.

A (fractions of methanolic extracts)		
Fraction	Eluent of column	Fraction weight (g)
FMet1	n-Hexane/ethyl acetate = 9:1 – 6:4	0.029
FMet2	n-Hexane/ethyl acetate = 6:4 – 4:6	0.031
FMet3	n-Hexane/ethyl acetate = 3:7 – 2:8	0.077
FMet4	n-Hexane/ethyl acetate = 1:9 – Ethyl acetate/methanol = 9:1	0.173
FMet5	Ethyl acetate/methanol = 9:1 – 8:2	2.654
FMet6	Ethyl acetate/methanol = 8:2 – 7:3	15.192
FMet7	Ethyl acetate/methanol = 7:3 – 6:4	4.487
FMet8	Ethyl acetate/methanol = 6:4	3.091
FMet9	Ethyl acetate/methanol = 6:4 – 3:7	6.261
FMet10	Ethyl acetate/methanol = 3:7	0.060
FMet11	Ethyl acetate/methanol = 2:8 – 1:9	0.236
FMet12	Ethyl acetate/methanol = 1:9 – 0:10	0.646
B (fractions of ethyl acetate extracts)		
Fraction	Eluent of column	Fraction weight (g)
FEa1	n-Hexane/ethyl acetate = 9:1	0.1910
FEa2	n-Hexane/ethyl acetate = 9:1 – 8:2	0.3990
FEa3	n-Hexane/ethyl acetate = 8:2 – 7:3	0.5470
FEa4	n-Hexane/ethyl acetate = 7:3	0.6440
FEa5	n-Hexane/ethyl acetate = 6:4	0.1580
FEa6	n-Hexane/ethyl acetate = 6:4	0.1740
FEa7	n-Hexane/ethyl acetate = 6:4 – 4:6	0.6720
FEa8	n-Hexane/ethyl acetate = 3:7 – 0:10	1.3710
FEa9	n-Hexane/ethyl acetate = 0:10 – Ethyl acetate/methanol = 9:1	0.5920
FEa10	Ethyl acetate/methanol = 9:1	2.2250
FEa11	Ethyl acetate/methanol = 8:2 – 7:3	0.6820
FEa12	Ethyl acetate/methanol = 7:3 – 0:10	0.1450

Fractionation

Fractionation was performed on the ethyl acetate and methanol extracts obtained from the leaves of *U. sclerophylla*. The methanol extract exhibited the best DPP-4 enzyme inhibition activity, and both extracts displayed superior antioxidant capacity compared to other extracts. In addition, the total phenol and flavonoid content of the two extracts were higher compared to other extracts obtained from *U. sclerophylla*. The fractionation results are shown in Table 4A-B, which show the eluent of each fraction so that it can be explained the polarity conditions of each fraction and the weight of each fraction obtained. These fractions were then assayed for their inhibitory activity

against the DPP-4 and its activity as an antioxidant. The best fraction was then analyzed for its compound content using UPLC-ESI-QToF-MS/MS.

The DPP-4 inhibitory activity assay of fractions

Fractions obtained from the *U. sclerophylla* leaf extract were assayed for their ability to inhibit the DPP-4 enzyme. The assay involved determining the percentage of inhibition of the DPP-4 enzyme in fractions at a concentration of 140 µg/mL. The assay was conducted only on fractions of methanol extract of leaves, as this extract showed an IC₅₀ value for DPP-4 inhibition below 100 µg/mL, making it the most suitable for further assay. The DPP-4 inhibitory activity assay

results for the fractions are shown in Table 5. The results of the DPP-4 inhibition assay from 12 leaf methanol extract fractions showed that all fractions had DPP-4 inhibitory activity, ranging from $4.14 \pm 0.7172\%$ to $72.29 \pm 1.9354\%$. The potent activity was possessed by FMet5 and FMet6, with inhibition percentages of $72.29 \pm 1.9354\%$ and $60.48 \pm 1.2156\%$, respectively. The IC_{50} values were then determined for these two fractions, and values of $50.71 \pm 1.220 \mu\text{g/mL}$ for FMet5 and $90.96 \pm 1.406 \mu\text{g/mL}$ for FMet6 were obtained (Fig. 3).

Antioxidant capacity of fractions

The study evaluated the antioxidant capacity of fractions obtained from the ethyl acetate and methanol extracts of *U. sclerophylla* leaves using the FRAP and DPPH methods. The findings showed that several fractions exhibited significant potential as antioxidants. The fractions displayed potent antioxidant capacity at a $15 \mu\text{g/mL}$ sample concentration for the FRAP method and $50 \mu\text{g/mL}$ for the DPPH method. Table 6A-B presents the results of the antioxidant assay conducted on the fractions.

Twelve fractions of the methanol extract obtained from the leaves were assayed for their antioxidant capacity. The results indicated that fractions FMet4-9 and FMet12 exhibited antioxidant capacity, with their EC_{50} ranging from $7.06 \pm 0.0751 \mu\text{g/mL}$ to $16.98 \pm 0.411 \mu\text{g/mL}$ (FRAP method) and $6.07 \pm 0.1250 \mu\text{g/mL}$ to $12.04 \pm 0.333 \mu\text{g/mL}$ (DPPH method). Although some fractions showed strong antioxidant capacity, only FMet5 and FMet6 exhibited inhibitory activity against DPP-4. Twelve fractions of the ethyl

acetate extract obtained from the leaves were assayed for their antioxidant capacity. The results indicated that FEa8, Fea9, and FEa10 exhibited potent antioxidant capacity with their EC_{50} ranging from 6.89 ± 0.0569 to $24.35 \pm 0.272 \mu\text{g/mL}$ (FRAP method) and 6.85 ± 0.0794 to $17.57 \pm 0.1097 \mu\text{g/mL}$ (DPPH method). Detailed fractions' antioxidant EC_{50} values can be seen in Table 6.

Correlation between antioxidant and DPP-4 inhibition activity of methanol extract fractions

A Spearman correlation analysis was conducted on the assay results of leaf methanol fractions to determine the correlation between antioxidant capacity (measured by FRAP and DPPH methods) and DPP-4 inhibitory activity. The results of the analysis are displayed in the correlation coefficient matrix plot (Fig. 5). A strong correlation ($p < 0.05$) was found between the activity test results. The correlation was observed between the antioxidant capacity of the FRAP and DPPH methods, and also between the antioxidant capacity (FRAP and DPPH methods) and the inhibitory activity of DPP-4. The correlation value obtained between the antioxidant assay results using the FRAP and DPPH methods shows a very strong correlation, reaching 88.1 % with a positive value ($r: 0.881$). It was found that there is a strong correlation between DPP-4 inhibitory activity and antioxidant capacity as measured by the FRAP (reaching 72.7%) and DPPH methods (reaching 66.4%). The correlation coefficient (r) was 0.727 for FRAP and 0.664 for DPPH, indicating that the greater the DPP-4 inhibitory activity, the greater the antioxidant capacity.

Table 5. DPP-4 inhibition activity from methanol extract fractions of *U. sclerophylla* leaves.

Fraction (140 $\mu\text{g/mL}$)	DPP-4 inhibition activity (%)
FMet1	$4.14 \pm 0.7172^{defghijkl}$
FMet2	$7.66 \pm 1.1741^{defghijkl}$
FMet3	$4.02 \pm 1.6496^{defghijkl}$
FMet4	$19.22 \pm 1.1973^{abcefg hijkl}$
FMet5	$72.29 \pm 1.9354^{abcd fghijkl}$
FMet6	$60.48 \pm 1.2156^{abcde ghijkl}$
FMet7	$40.25 \pm 1.2747^{abcde fijkl}$
FMet8	$40.33 \pm 0.9109^{abcde fijkl}$
FMet9	$35.64 \pm 0.6684^{abcde fghijkl}$
FMet10	$23.69 \pm 1.2192^{abcde fghikl}$
FMet11	$12.66 \pm 1.4681^{abcde fghijl}$
FMet12	$31.07 \pm 1.4764^{abcde fghijk}$

Data are indicated as mean $\pm$ SD for triplicate measurements. ^{a,b,c,d,e,f,g,h,i,j,k,l} Different letters in superscript indicate statistically significant differences among the analyzed extracts (one-way ANOVA and Tukey's post hoc test, $p < 0.05$, $n = 3$).

Table 6. Antioxidant capacity from (A) methanol and (B) ethyl acetate extract extract fractions of *U. sclerophylla* leaves.

A (fractions of methanolic extracts)				
Fraction	FRAP method		DPPH method	
	% Antioxidant capacity (15 µg/mL)	EC ₅₀ (µg/mL)	% Antioxidant capacity (50 µg/mL)	EC ₅₀ (µg/mL)
FMet1	2.23 ± 0.4619 ^{cdefghijkl}	ND	4.92 ± 0.4419 ^{cdefghijkl}	ND
FMet2	0.63 ± 0.2082 ^{cdefghijkl}	ND	6.23 ± 0.6124 ^{cdefghijkl}	ND
FMet3	17.90 ± 0.4583 ^{abdefghijkl}	ND	39.15 ± 0.8838 ^{abdefghijkl}	ND
FMet4	93.57 ± 5.3966 ^{abcdeghijkl}	7.06 ± 0.0751 ^{bcdefgh}	89.01 ± 0.0964 ^{abcghijkl}	7.01 ± 0.0306 ^{bcdefgh}
FMet5	80.87 ± 3.3307 ^{abcdfghijkl}	8.89 ± 0.1701 ^{acdefgh}	88.43 ± 0.2551 ^{abcdeghijkl}	6.07 ± 0.1250 ^{acdefgh}
FMet6	73.37 ± 3.3292 ^{abcdeghijkl}	11.14 ± 0.4890 ^{abdefgh}	88.11 ± 0.1114 ^{abcdeghijkl}	8.59 ± 0.1060 ^{abdefgh}
FMet7	45.43 ± 1.2014 ^{abcdeghijkl}	16.98 ± 0.4110 ^{abcefg}	85.15 ± 0.3857 ^{abcdeghijkl}	11.19 ± 0.3560 ^{abcgh}
FMet8	56.57 ± 0.7638 ^{abcdeghijkl}	13.17 ± 0.1185 ^{abcdegh}	86.60 ± 0.3477 ^{abcdeghijkl}	11.73 ± 0.1550 ^{abch}
FMet9	49.63 ± 0.8505 ^{abcdeghijkl}	15.49 ± 0.1041 ^{abcdegh}	89.04 ± 0.2946 ^{abcghijkl}	11.33 ± 0.0115 ^{abcgh}
FMet10	22.97 ± 0.6506 ^{abdefghijkl}	ND	37.00 ± 0.2227 ^{abcdeghijkl}	ND
FMet11	10.43 ± 1.1719 ^{abcdeghijkl}	ND	16.88 ± 1.5155 ^{abcdeghijkl}	ND
FMet12	52.23 ± 2.4379 ^{abcdeghijkl}	14.23 ± 0.0503 ^{abcdegh}	86.15 ± 0.0557 ^{abcdeghijkl}	12.04 ± 0.3330 ^{abcdfh}
Quercetin	ND	1.73 ± 0.0361 ^{abcdegh}	ND	2.98 ± 0.2060 ^{abcdegh}

B (fractions of ethyl acetate extracts)				
Fraction	FRAP method		DPPH method	
	% Antioxidant capacity (15 µg/mL)	EC ₅₀ (µg/mL)	% Antioxidant capacity (50 µg/mL)	EC ₅₀ (µg/mL)
FEa1	< 10	ND	< 10	ND
FEa2	< 10	ND	< 10	ND
FEa3	< 10	ND	< 10	ND
FEa4	< 10	ND	< 10	ND
FEa5	< 10	ND	< 10	ND
FEa6	< 10	ND	< 10	ND
FEa7	< 10	ND	< 10	ND
FEa8	65.90 ± 1.7521 ^{bcde}	11.29 ± 0.1553 ^{bcd}	88.50 ± 0.1151 ^{de}	12.06 ± 0.3250 ^{bcd}
FEa9	92.03 ± 1.2702 ^{acde}	6.89 ± 0.0569 ^{acd}	89.00 ± 0.3205 ^{cde}	6.85 ± 0.0794 ^{acd}
FEa10	30.13 ± 2.4987 ^{abde}	24.35 ± 0.2720 ^{abd}	86.54 ± 0.0997 ^{bde}	17.57 ± 0.1097 ^{abd}
FEa11	20.17 ± 1.3317 ^{abc}	ND	64.01 ± 1.5669 ^{abce}	ND
FEa12	19.43 ± 1.9732 ^{abc}	ND	66.93 ± 0.4496 ^{abcd}	ND
Quercetin	ND	1.73 ± 0.0361 ^{abc}	ND	2.98 ± 0.2060 ^{abc}

Data are presented as mean ± SD for triplicate measurements. ND: Not determined. ^{a,b,c,d,e} Different letters in superscript indicate statistically significant differences among the analyzed fractions (one-way ANOVA and Tukey's post hoc test, p<0.05, n = 3).

Compound profiling using UPLC-ESI-QToF-MS/MS

Compounds found in the active fraction of FMet5 were identified using UPLC-ESI-QToF-MS/MS. The data obtained was processed using Masslynx software and the Unifi database. This database provided

a general description of the major compounds found in FMet5, which is composed of six major compounds: Dehydrosilybin, 19-epi-3-isoajmalicine, procyanidin A2, cinchonain Ib, maokonine, and cuscohygrine (Fig. 6 and Figs. S1-S7). Table 7 presents the results of UPLC-ESI-QToF-MS/MS analysis, which

includes retention time (rt), precursor ion mass (m/z, positive mode), the formula of each compound, and their phytochemical class.

Molecular docking

The study investigated the major compounds found in FMet5 and their effectiveness in inhibiting DPP-4 *in silico*. The interaction mechanism between these compounds and the DPP-4 enzyme protein was determined using molecular docking, and the visuali-

zation of the interaction between compounds and the DPP-4 enzyme protein can be seen in Figs. 7-8. The bond energy of the complexes showed a range of bond energy values of -263.6–313.6 kJ/mol, with the types of bonds detected in the complexes being hydrogen bonds, electrostatic, hydrophobic, halogen, halogen hydrogen, and unfavorable bonds. The detailed interaction, including binding energy, type of binding, and the amino acid residues involved, can be seen in Table 8.

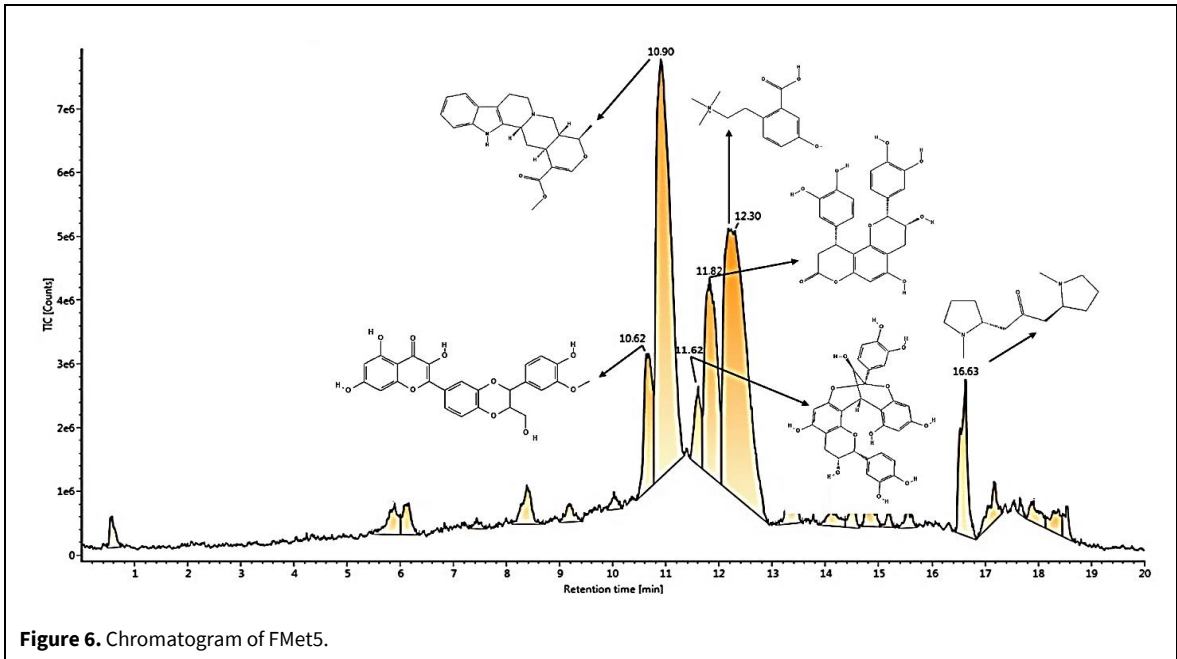
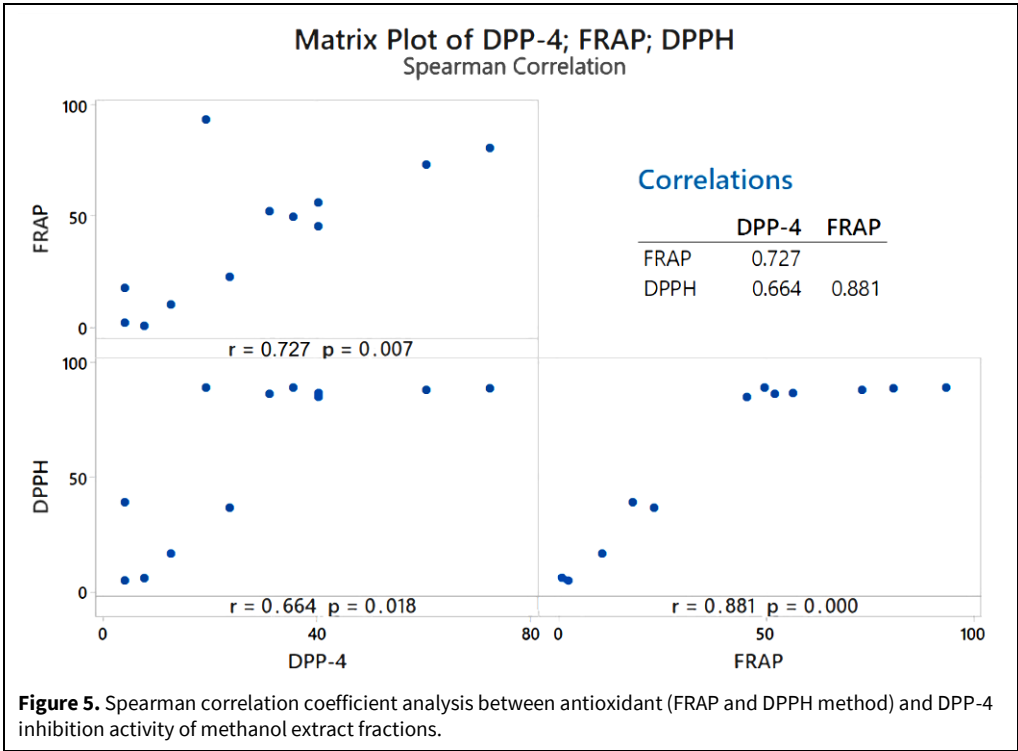


Table 7. Compounds detected in FMet5.

No	Retention time (min)	Ion mass (m/z) [M+H] ⁺	Formula [M+H] ⁺	Compound	Phytochemical class
1.	10.62	481.1120	C ₂₅ H ₂₀ O ₁₀	Dehydrosilybin	Flavonoid
2.	10.90	353.1853	C ₂₁ H ₂₄ N ₂ O ₃	19-epi-3-Isoajmalicine	Alkaloid
3.	11.62	577.1352	C ₃₀ H ₂₄ O ₁₂	Procyanidin A2	Flavonoid
4.	11.82	453.1197	C ₂₄ H ₂₀ O ₉	Cinchonain I b	Flavonoid
5.	12.30	224.1290	C ₁₂ H ₁₇ NO ₃	Maokonine	Alkaloid
6.	16.63	225.1953	C ₁₃ H ₂₄ N ₂ O	Cuscohygrine	Alkaloid

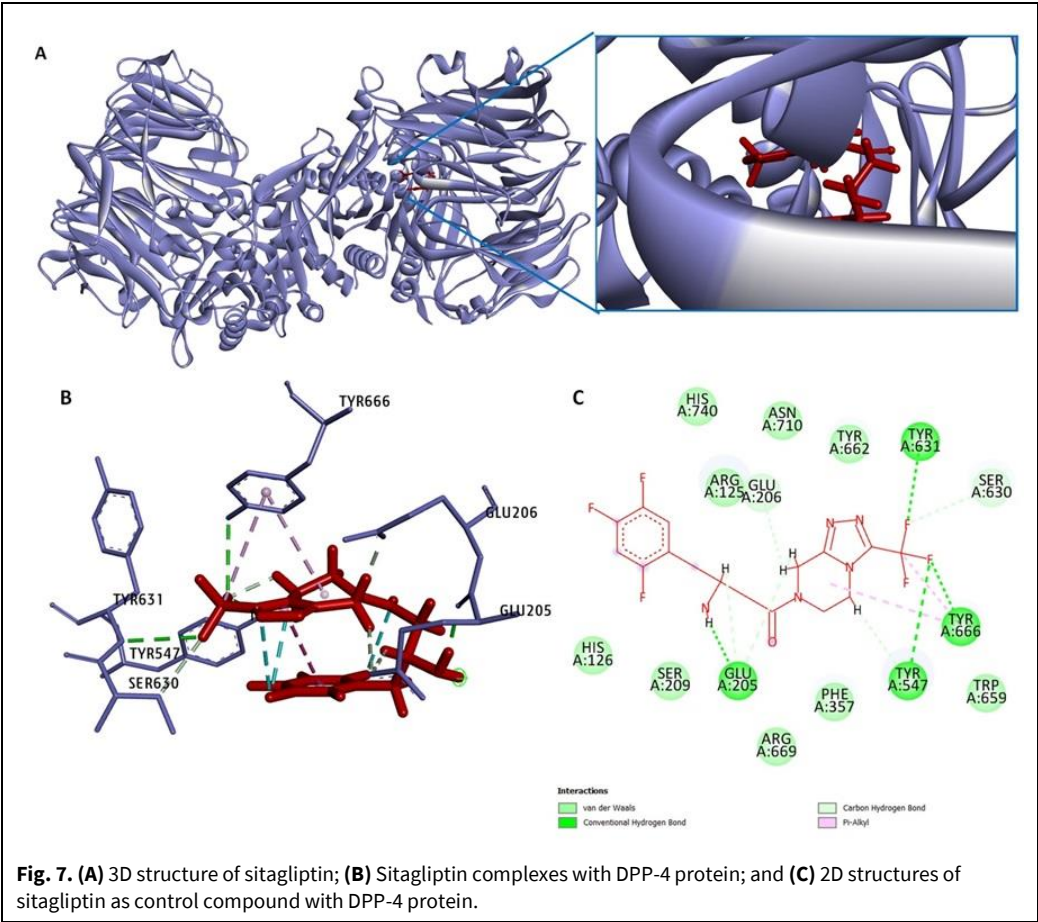


Fig. 7. (A) 3D structure of sitagliptin; (B) Sitagliptin complexes with DPP-4 protein; and (C) 2D structures of sitagliptin as control compound with DPP-4 protein.

DISCUSSION

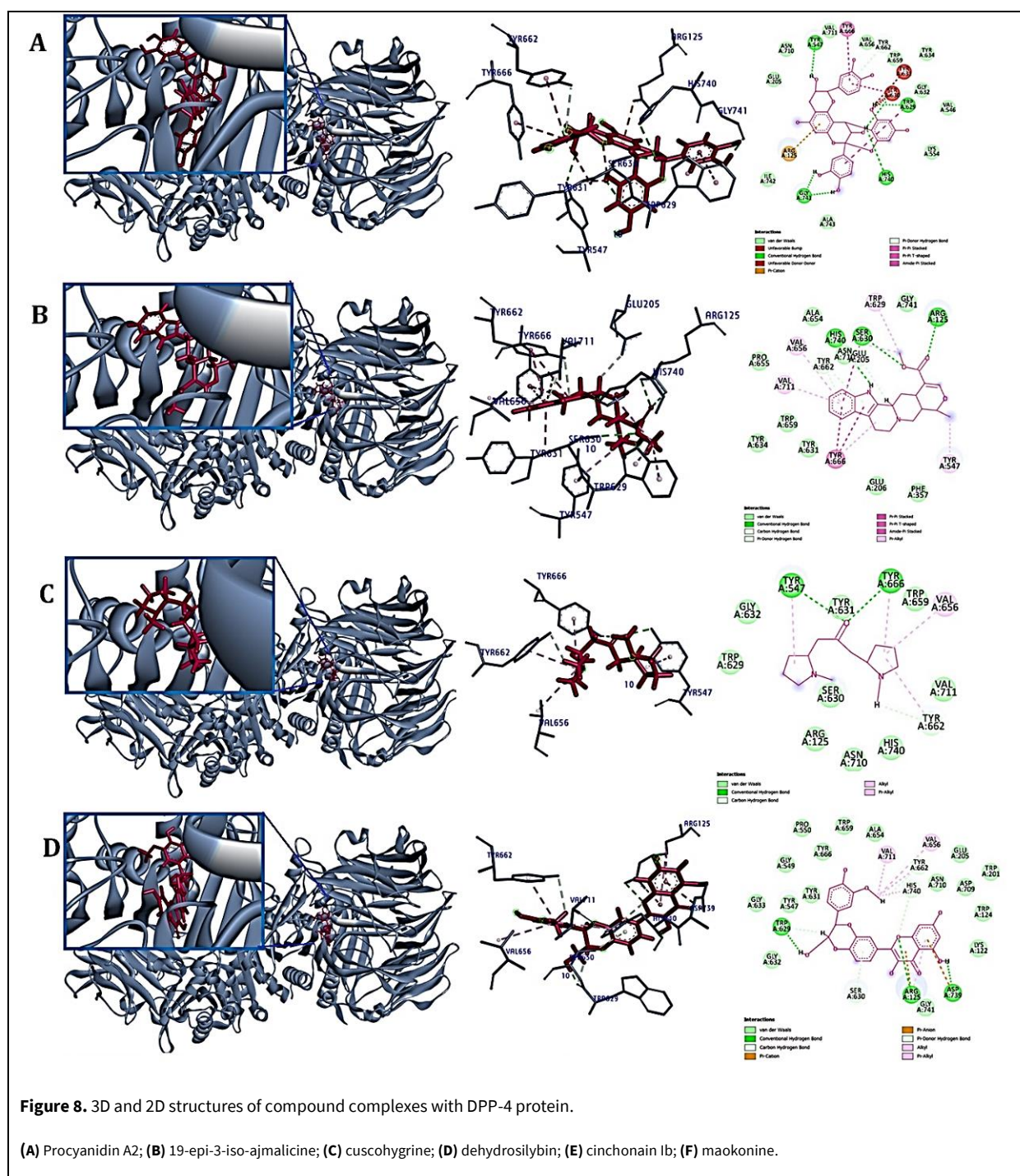
U. sclerophylla is a medicinal plant widely used in traditional diabetes treatment by the people of Kalimantan, Indonesia. This study has revealed scientific data that strengthened the use of this plant as an anti-diabetic through investigation of this plant to inhibit dipeptidyl peptidase-4 and also its activity as an antioxidant both in the form of extracts and chromatographic fractions along with identification of the compounds contained in the most active fraction using UPLC-ESI-QToF-MS/MS.

The results of the DPP-4 inhibition and antioxidant assay indicate that *U. sclerophylla* leaves have the po-

tential to be antidiabetic agents. This is especially evident in the ethyl acetate and methanol extracts that were assayed compared to extracts from other solvents. DPP-4 inhibitory activity has never been reported from the *Uncaria* genus, so as far as we know, this study is the first report of DPP-4 inhibitory activity from this genus. The methanol extract of *U. sclerophylla* leaves is the most active extract in inhibiting DPP-4 (IC₅₀ 79.67 ± 2.95 µg/mL) and has a very strong antioxidant capacity with EC₅₀ 9.50 ± 0.319 µg/mL (FRAP method) and 9.94 ± 0.1572 µg/mL (DPPH method). Besides that, the methanol extract also shows the highest total phenol (409.97 ± 12.2067 mg GAE/g extract) and flavonoid content (86.74 ± 5.3284

mg QE/g extract) compared to other extracts. The methanol extract likely exhibits the best antioxidant and DPP-4 inhibitory activities due to the presence of total phenols and flavonoids, compared to extracts using other solvents. Phenols and flavonoids play an important role in antidiabetic activity due to their structural features supporting DPP-4 inhibitory and antioxidant capacity, including the total number and configuration of hydroxyl groups found in phenol and flavonoid compounds that can increase the antioxidant and antidiabetic effects (Praparatana et al., 2022).

Several species of the *Uncaria* genus have been reported for their antioxidant capacity, including *U. lucida*, *U. longiflora*, *U. calophylla*, *U. cordata*, *U. acida* (Ahmad et al., 2011), *U. rhynchophylla* (Kim et al., 2012), *U. tomentosa* (Azevedo et al., 2019; Navarro et al., 2019), and *U. gambir* (Ersa et al., 2021; Musdja et al., 2018). Scientific data on the antioxidant capacity of various species of the *Uncaria* genus have been reported. Still, scientific data on the antioxidant capacity of the species *U. sclerophylla* has never existed before, and this study is the first investigation to reveal the antioxidant capacity of this species.



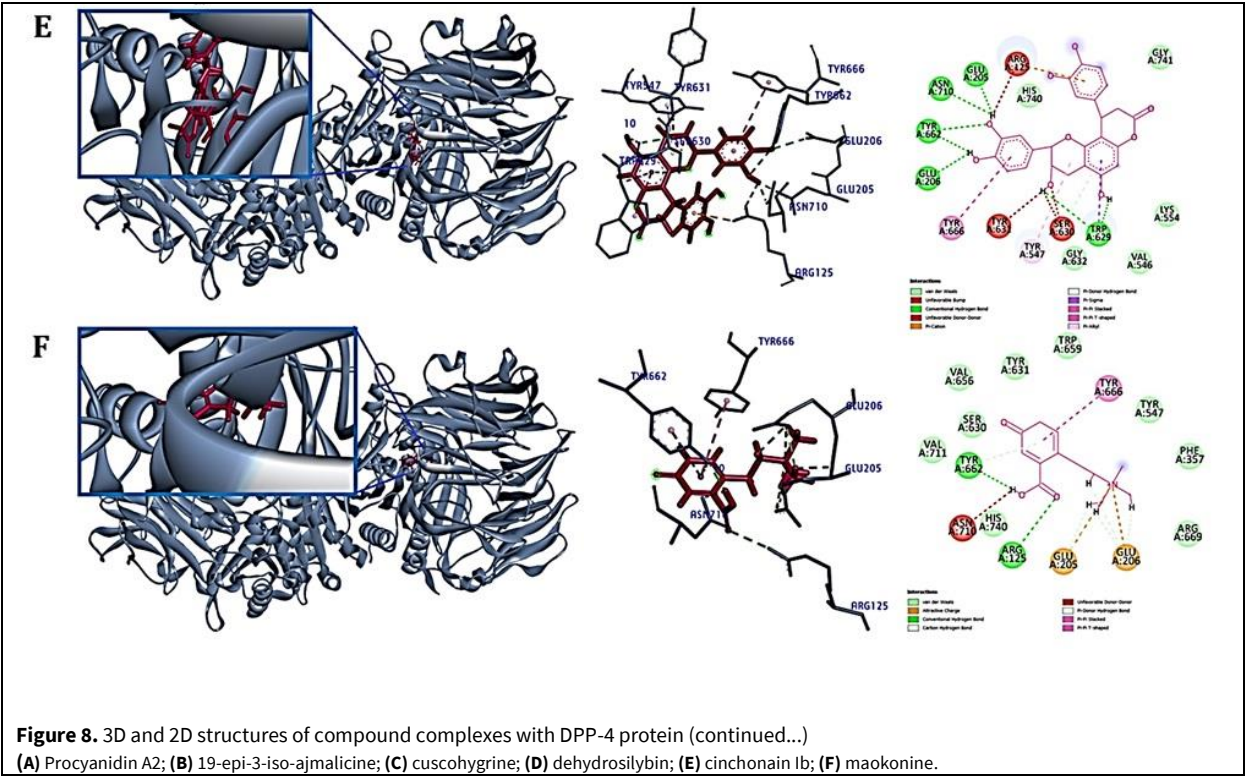


Figure 8. 3D and 2D structures of compound complexes with DPP-4 protein (continued...) (A) Procyanidin A2; (B) 19-epi-3-iso-ajmalicine; (C) cuscohygrine; (D) dehydrosilybin; (E) cinchonain Ib; (F) maokoniine.

Table 8. Interaction between compound and DPP-4 protein.

Compound	Binding energy (kJ/mol)	Interaction	Distance (Å)	Binding type
Procyanidin A2	-275.6	:10:H8 - A:SER630:OG	1.97434	Hydrogen bond
		:10:H8 - A:HIS740:NE2	3.0158	Hydrogen bond
		:10:H13 - A:TYR547:OH	2.43554	Hydrogen bond
		:10:H16 - A:TRP629:O	2.34435	Hydrogen bond
		:10:H21 - A:GLY741:O	1.73981	Hydrogen bond
		:10:H22 - A:GLY741:O	2.37906	Hydrogen bond
		A:ARG125:NH2 - :10	3.37005	Electrostatic
		A:TYR662:OH - :10	4.08012	Hydrogen bond
		:10 - A:TYR662	4.79228	Hydrophobic
		:10 - A:TRP629	5.55089	Hydrophobic
		:10 - A:TYR666	4.69666	Hydrophobic
		A:TRP629 - :10	5.39132	Hydrophobic
		A:SER630:C,O;TYR631:N - :10	4.3189	Hydrophobic
		:10:O7 - A:SER630:CB	2.14426	Unfavorable
		:10:H16 - A:SER630:CB	1.72744	Unfavorable
		:10:H16 - A:TYR631:N	2.37126	Unfavorable
		:10:O3 - :10:O7	2.82155	Unfavorable
19-epi-3-iso-ajmalicine	-282.6	:10:H13 - A:HIS740:NE2	2.35441	Hydrogen bond
		A:ARG125:NH1 - :10:O3	3.14074	Hydrogen bond
		A:ARG125:NH2 - :10:O3	3.0447	Hydrogen bond

Table 8. Interaction between compound and DPP-4 protein (continued...)

Compound	Binding energy (kJ/mol)	Interaction	Distance (Å)	Binding type
Cuscohygrine	-278.6	A:SER630:OG - :10:O2	2.96479	Hydrogen bond
		:10:H3 - A:GLU205:OE1	2.58874	Hydrogen bond
		A:TYR662:OH - :10	3.18309	Hydrogen bond
		A:TYR662:OH - :10	4.043	Hydrogen bond
		:10 - A:TYR662	4.80318	Hydrophobic
		:10 - A:TYR662	4.29437	Hydrophobic
		:10 - A:TYR666	5.4647	Hydrophobic
		:10 - A:TYR666	5.7556	Hydrophobic
		A:SER630:C,O;TYR631:N - :10	3.86227	Hydrophobic
		:10 - A:VAL656	4.68539	Hydrophobic
		:10 - A:VAL711	4.92571	Hydrophobic
		A:TYR547 - :10:C13	4.6231	Hydrophobic
		A:TRP629 - :10:C21	3.56589	Hydrophobic
		A:TRP629 - :10:C21	4.72102	Hydrophobic
		A:TYR666 - :10	4.36293	Hydrophobic
		A:HIS740 - :10:C21	4.7466	Hydrophobic
		A:TYR547:OH - :10:O1	2.88756	Hydrogen bond
		A:TYR666:OH - :10:O1	2.84849	Hydrogen bond
		:10:H23 - A:TYR662:OH	2.47592	Hydrogen bond
		A:VAL656 - :10	4.80443	Hydrophobic
Dehydrosilybin	-313.6	A:TYR547 - :10	4.11872	Hydrophobic
		A:TYR662 - :10	4.34119	Hydrophobic
		A:TYR666 - :10	4.8191	Hydrophobic
		:10:H11 - A:TRP629:O	1.71824	Hydrogen bond
		:10:H19 - A:ASP739:OD1	2.61809	Hydrogen bond
		A:ARG125:NH2 - :10:O4	2.73887	Hydrogen bond
		:10:H2 - A:TRP629:O	2.77572	Hydrogen bond
		:10:H17 - A:TYR662:OH	3.09072	Hydrogen bond
		A:HIS740:CD2 - :10:O4	2.98607	Hydrogen bond
		A:ARG125:NH1 - :10	3.37326	Electrostatic
		A:ASP739:OD2 - :10	4.64335	Electrostatic
		A:SER630:OG - :10	3.5489	Hydrogen bond
		:10:C25 - A:VAL656	4.96591	Hydrophobic
		:10:C25 - A:VAL711	3.87566	Hydrophobic
		:10 - A:ARG125	5.07481	Hydrophobic
		:10 - A:ARG125	4.23809	Hydrophobic
		A:TYR662 - :10:C25	4.23081	Hydrophobic
		A:HIS740 - :10:C25	4.72745	Hydrophobic

Table 8. Interaction between compound and DPP-4 protein (continued...)

Compound	Binding energy (kJ/mol)	Interaction	Distance (Å)	Binding type
Cinchonain Ib	-302	:10:H9 - A:TRP629:O	2.58131	Hydrogen bond
		:10:H14 - A:TRP629:O	2.82744	Hydrogen bond
		:10:H18 - A:GLU205:OE1	1.98369	Hydrogen bond
		:10:H18 - A:ASN710:OD1	2.22423	Hydrogen bond
		:10:H20 - A:GLU206:OE2	2.63114	Hydrogen bond
		:10:H20 - A:TYR662:OH	1.96093	Hydrogen bond
		A:TYR662:OH - :10:O7	3.19884	Hydrogen bond
		A:ARG125:NH1 - :10	3.10311	Hydrogen bond; electrostatic
		A:SER630:OG - :10	3.79588	Hydrogen bond
		A:TRP629:CB - :10	3.66407	Hydrophobic
		A:TRP629 - :10	5.04711	Hydrophobic
		:10 - A:TYR666	5.77769	Hydrophobic
		A:TYR547 - :10	4.30566	Hydrophobic
		:10:O3 - A:SER630:CB	2.12665	Unfavorable
		:10:H9 - A:SER630:CB	1.84024	Unfavorable
		:10:H9 - A:TYR631:N	2.27737	Unfavorable
		:10:H18 - A:ARG125:NH2	2.6703	Unfavorable
Maokonine	-263.6	:10:N1 - A:GLU205:OE1	4.75527	Electrostatic
		:10:N1 - A:GLU206:OE1	3.55277	Electrostatic
		:10:H17 - A:TYR662:OH	2.00507	Hydrogen bond
		A:ARG125:NH2 - :10:O3	2.94449	Hydrogen bond
		:10:H2 - A:GLU206:OE1	2.88922	Hydrogen bond
		:10:H7 - A:GLU206:OE1	1.92336	Hydrogen bond
		:10:H12 - A:GLU205:OE1	2.87358	Hydrogen bond
		:10:H13 - A:GLU205:O	2.73939	Hydrogen bond
		:10:H13 - A:GLU206:OE1	2.65625	Hydrogen bond
		A:TYR662:OH - :10	3.56329	Hydrogen bond
		:10 - A:TYR662	4.99102	Hydrophobic
		:10 - A:TYR666	4.88503	Hydrophobic
		:10:H17 - A:ASN710:ND2	2.65202	Unfavorable
Sitagliptin	-313.4	A:TYR547:OH - :10:F2	3.39074	Hydrogen bond; halogen
		A:TYR631:N - :10:F1	3.0779	Hydrogen bond; halogen
		A:TYR666:OH - :10:F2	3.15522	Hydrogen bond; halogen
		:10:H12 - A:GLU205:O	1.90715	Hydrogen bond
		A:SER630:CB - :10:F1	2.77218	Hydrogen bond
		:10:H3 - :10:F2	2.08046	Hydrogen bond
		:10:H4 - A:TYR547:OH	2.85691	Hydrogen bond
		:10:H5 - A:GLU206:OE1	1.87929	Hydrogen bond
		:10:H6 - A:GLU205:OE1	2.78961	Hydrogen bond

Table 8. Interaction between compound and DPP-4 protein (continued...)

Compound	Binding energy (kJ/mol)	Interaction	Distance (Å)	Binding type
		:10:H9 - A:GLU205:OE1	2.71157	Hydrogen bond
		:10:O1 - :10:F4	3.26191	Halogen
		:10:N2 - :10:F5	3.60239	Halogen
		:10:N5 - :10:F5	3.32007	Halogen
		:10 - :10	4.15482	Hydrophobic
		A:TYR666 - :10	4.41485	Hydrophobic
		A:TYR666 - :10:C11	4.53062	Hydrophobic

The antioxidant potential of these leaf extracts further strengthens the potential of *U. sclerophylla* leaves to be developed in treating diabetes because of the need for antioxidants in diabetic conditions. Several studies show that type 2 diabetes mellitus is significantly modulated by oxidative stress (Bhatti et al., 2022). ROS, such as hydrogen peroxide and superoxide anion, can cause β -cell damage, affecting insulin production (Dinić et al., 2022). Hyperglycemia-induced oxidative stress plays an essential role in complications, including diabetic nephropathy (Singh et al., 2022).

Fractionation uses a column chromatography method, where a gradient system is applied to this fractionation to improve separation using a combination of n-hexane, ethyl acetate, and ethyl acetate-methanol eluents. The collected elution results were analyzed for chromatogram patterns using thin-layer chromatography to determine the fraction. Fractionation using a silica gel stationary phase separates groups of compounds in an extract based on their polarity, where this separation depends on the eluent system used (Srivastava et al., 2021). A gradient system is applied in this fractionation process to increase the separation resolution so that it is hoped that groups of active compounds can be separated from groups of compounds that are not or less active (Larson and Bou-Assaf, 2023). According to several studies, the most active chromatographic fraction has better activity than the extract (Mahayasih et al., 2017; Manyawi et al., 2023). However, silica gel-column chromatography is limited in recovering polar compounds when bound to the polar stationary phase (Zhang et al., 2022).

The assay for DPP-4 inhibitory activity of fractions revealed that FMet5 showed the strongest activity with an IC_{50} of $50.71 \pm 1.220 \mu\text{g/mL}$ compared to the other fractions. Sitagliptin, as a positive standard, had activity with an IC_{50} value of $0.08 \pm 0.006 \mu\text{g/mL}$; this shows that sitagliptin is still superior to the most active fraction in inhibiting DPP-4. Furthermore, FMet5

has the potential to be a potent antioxidant with an EC_{50} of $8.89 \pm 0.1702 \mu\text{g/mL}$ (FRAP method) and $6.07 \pm 0.125 \mu\text{g/mL}$ (DPPH method) when quercetin, as a positive standard, still showed superior antioxidant capacity with IC_{50} : $1.73 \pm 0.0361 \mu\text{g/mL}$ (FRAP method) and $2.98 \pm 0.206 \mu\text{g/mL}$ (DPPH method). This is because sitagliptin and quercetin are single compounds, while FMet5 is still a fraction, a mixture of active and inactive compounds. Compared to other fractions, FMet5 has the advantage of being a promising candidate for further development and investigation of its antidiabetic properties. The compounds contained in FMet were profiled using the UPLC-ESI-QToF-MS/MS technique to reveal compounds that may have an antidiabetic role, both as DPP-4 inhibitors and as antioxidants.

UPLC-ESI-QToF-MS/MS has been trusted and widely used in compound content profiling because this method has superior sensitivity and selectivity compared to other chromatographic techniques (Almeida, 2021; Yang et al., 2023). Ultra-high performance liquid chromatography (UPLC) combined with Quadrupole and Time-of-Flight (QToF) as a mass analyzer can provide various spectral information, including compound ion fragmentation and compound mass, to accurately identify compounds in a sample (Li et al., 2021; Shu et al., 2016). In this study, six major compounds have been identified from FMet5, which includes three alkaloid and three flavonoid compounds. The identification of these compounds using UPLC-ESI-QToF-MS/MS has not been reported from *U. sclerophylla* before. The compounds revealed from this species are reported for the first time in this study. The flavonoid compounds in the FMet5 fraction cause this fraction's potent antioxidant and DPP-4 inhibition activity. The presence of a flavonoid structure feature is thought to indicate the possibility of DPP-4 inhibitory activity and antioxidants, specifically the presence of a C-2-C-3 double bond and the ketone group at C-4 in ring C can increase the inhibitory activity of DPP-4 enzymes (Dewi

et al., 2024; Pan et al., 2022). The existence of the catechol system in ring B is not significant enough without the C-2-C-3 double bond and ketonic group at position C-4 in antioxidant and antidiabetic activity; thus, the existence of the catechol system accompanied by double bonds and ketone groups in ring B can increase the inhibitory activity of DPP-4 enzymes, besides that, the catechol system itself plays a vital role in antioxidant activity (Sarian et al., 2017). Apart from playing a role in antioxidant activity (Shen et al., 2022; Wang et al., 2023; Zheng et al., 2022), flavonoids have been widely reported to inhibit DPP-4 (Pan et al., 2022; Parmar et al., 2012). The alkaloid compounds detected may have a role in the DPP-4 inhibitory activity in the FMet5 fraction, as it has been widely reported that alkaloids have the potential to inhibit DPP-4 and as antidiabetic (Mkabayi et al., 2024; Rahman et al., 2021; Rasouli et al., 2020; Shukla and Srinivasan, 2012; Zhu et al., 2021).

Molecular docking showed that the target compound interacted with the DPP4 enzyme protein in the same binding region as the control compound (sitagliptin). Residues that are the same as the control include GLU205, GLU206, TYR662, TYR547, and SER630. The GLU205 residue was identified in the active site of maokonine, cinchonain Ib, and 19-epi-3-iso-ajmalicine, while the GLU206 residue was identified in the active site of maokonine and cinchonain Ib. TYR662 residue was identified in all active sites of the compound. Residue TYR547 was identified in the active site of procyanidin A2, 19-epi-3-iso-ajmalicine, cuscohygrine, and cinchonain Ib. Residue SER630 was detected in the binding region of procyanidin A2, 19-epi-3-iso-ajmalicine, dehydrosilybin and cinchonain Ib. These same active site residues indicate that the target compounds bind to DPP-4 on the inhibitor site and have the same mechanism as the control compound. Based on the binding energy, five target compounds (19-epi-3-Isoajmalicine, procyanidin A2, cinchonain Ib, maokonine, cuscohygrine) had binding energy values close to the control. Dehydrosilybin showed a lower binding energy value than sitagliptin as a control. This shows the potential of this compound as an antidiabetic agent with a DPP-4 inhibition mechanism. The study by Kempegowda et al. (2018) reported the molecular docking of the cuscohygrine against the DPP-4 enzyme using a different docking method, which shows the activity of this compound *in silico*. However, the docking data for other major compounds found in the most active fraction of *U. sclerophylla* against the DPP-4 enzyme has never been reported. Nonetheless, two major compounds (19-epi-3-isoajmalicine and procyanidin A2) are active against other enzymes related to antidiabetic activity (α -glucosidase, α -amylase, and PTP-IB) *in silico* (Anandari et al., 2021; Nguyen-Vo et al., 2017).

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CONCLUSION

The methanol extract of *U. sclerophylla* leaves has been shown to have potential as an antidiabetic agent. This is evidenced by its IC₅₀ of DPP-4 inhibitory activity, which reached 79.67 ± 2.95 μ g/mL, and its EC₅₀ of antioxidant capacity, which also reached 9.50 ± 0.319 μ g/mL (FRAP method) and 9.94 ± 0.1572 μ g/mL (DPPH method). Moreover, this extract produced FMet5, which is more active than the original extract, with its IC₅₀ for DPP-4 inhibitory activity reaching 50.71 ± 1.22 μ g/mL and its EC₅₀ for antioxidant capacity reaching 8.89 ± 0.1701 μ g/mL (FRAP method) and 6.07 ± 0.125 μ g/mL (DPPH method). The potential of *U. sclerophylla* leaves in antidiabetic treatment is further strengthened by the disclosure of the alkaloid and flavonoid compounds contained (dehydrosilybin, 19-epi-3-isoajmalicine, procyanidin A2, cinchonain Ib, maokonine, and cuscohygrine) in FMet5 as the most active fraction through UPLC-ESI-QToF-MS/MS analysis. The molecular docking of compounds with the DPP-4 enzyme highlights the inhibitory potential of DPP-4 from *U. sclerophylla* leaves, particularly FMet5. This research shows that the potential of *U. sclerophylla* leaves as an antidiabetic agent is limited to its DPP-4 inhibitory and antioxidant activities. Thus, further investigation is needed to uncover other antidiabetic mechanisms to establish this plant as an effective treatment for diabetes.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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AUTHOR CONTRIBUTION:

Contribution	Triadisti N	Elya B	Hanafi M	Mohd Hashim N
Concepts or ideas	x	x	x	x
Design	x	x	x	x
Definition of intellectual content	x	x	x	x
Literature search	x			
Experimental studies	x	x	x	x
Data acquisition	x	x	x	x
Data analysis	x	x	x	x
Statistical analysis	x			
Manuscript preparation	x	x	x	x
Manuscript editing	x	x	x	x
Manuscript review	x	x	x	x

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Supplementary data

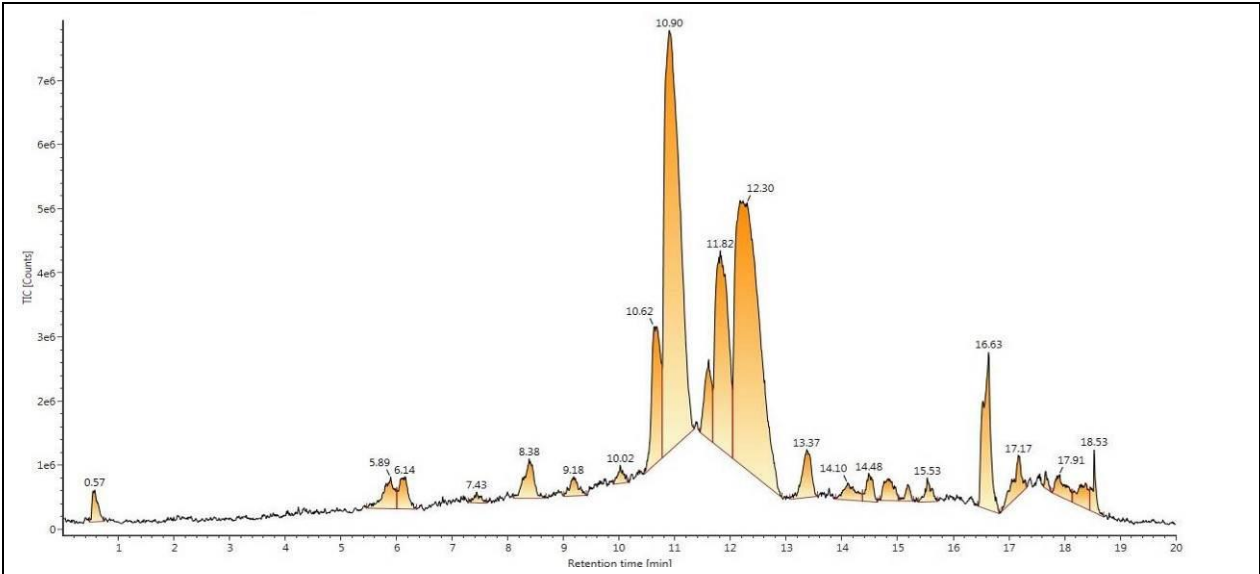


Figure S1. Chromatogram of FMet5.

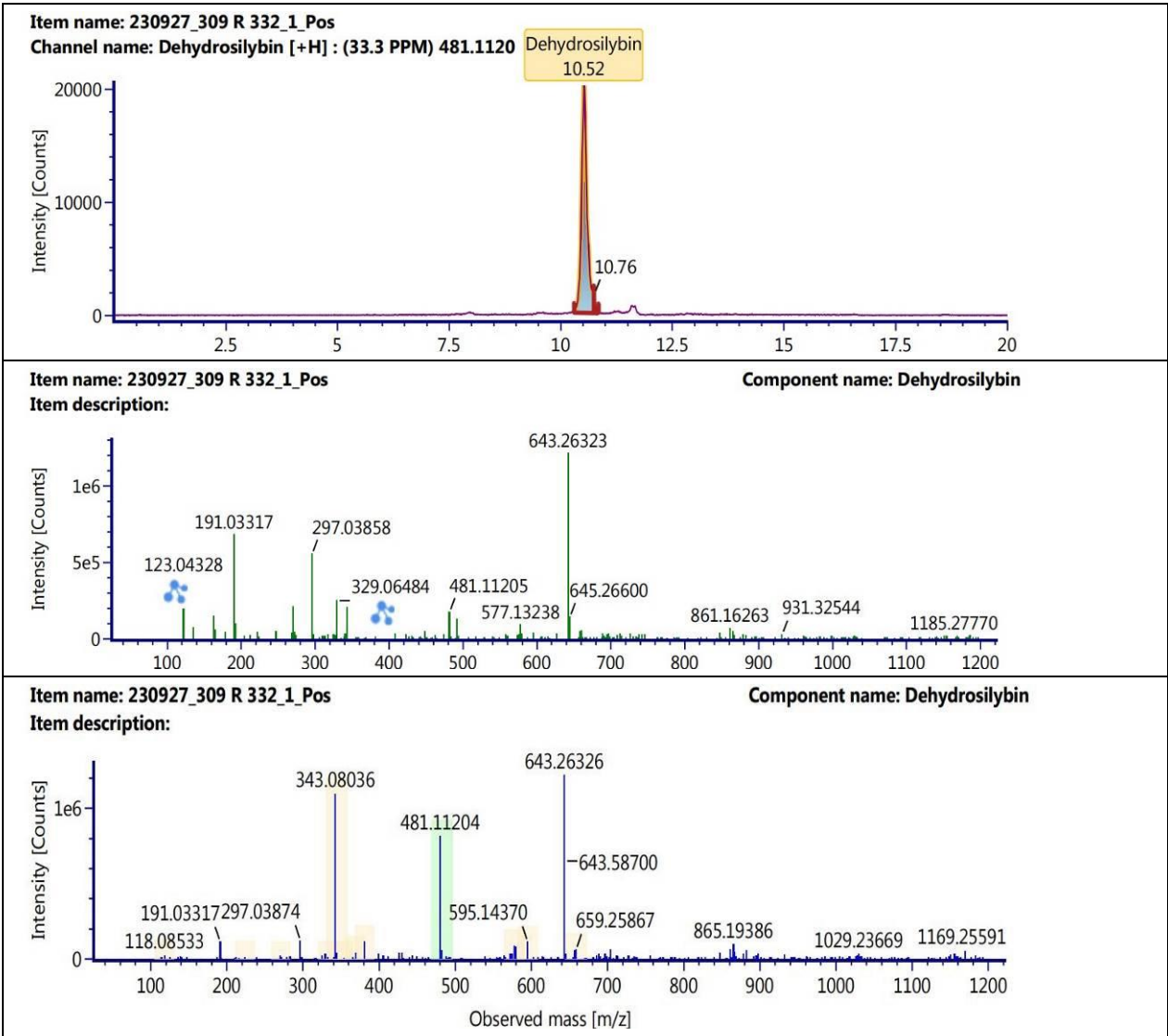


Figure S2. Chromatogram of dihydrosilybin isolated from FMet5.

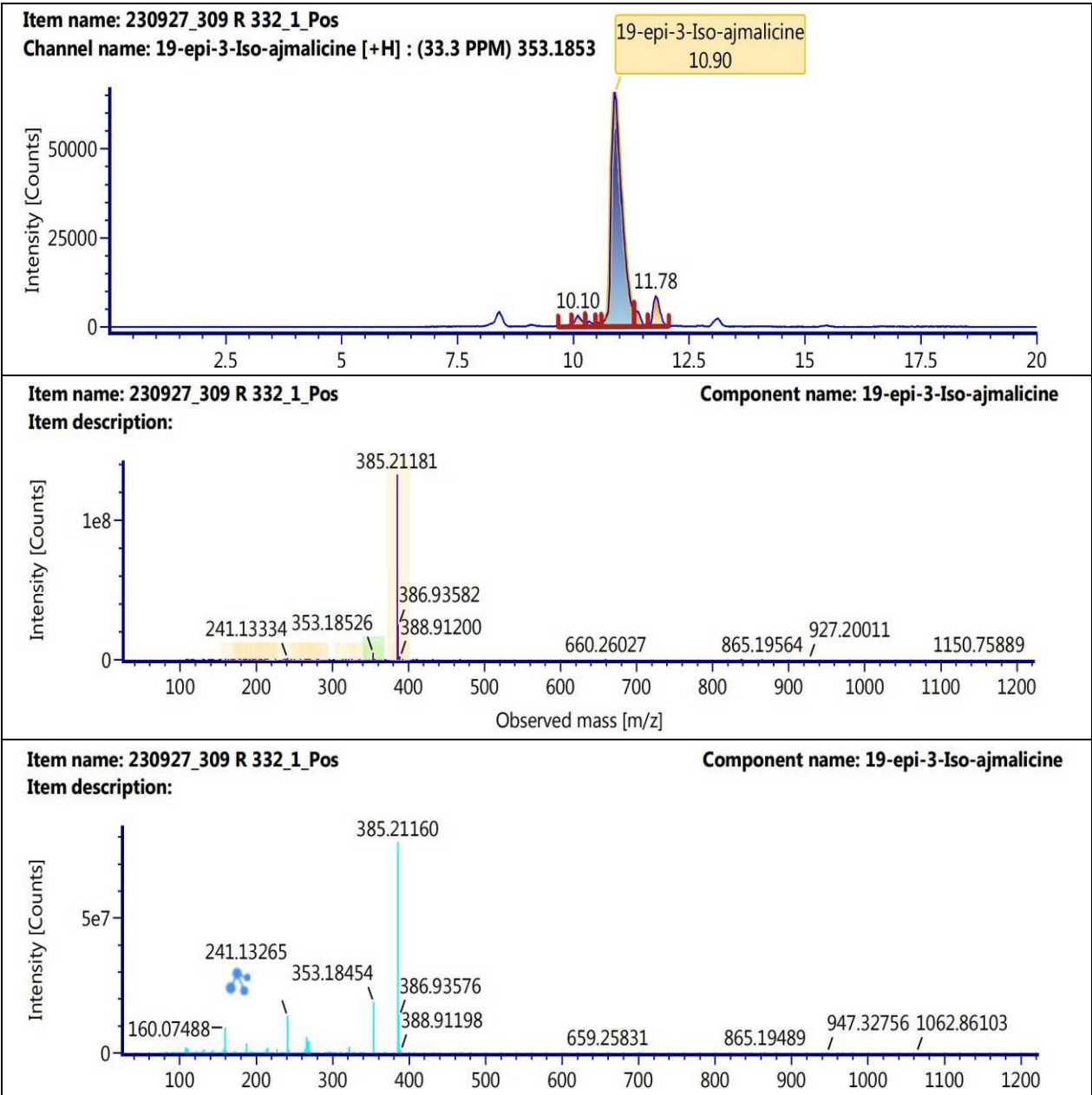


Figure S3. Chromatogram of 19-epi-3-Iso-ajmalicine isolated from FMet5.

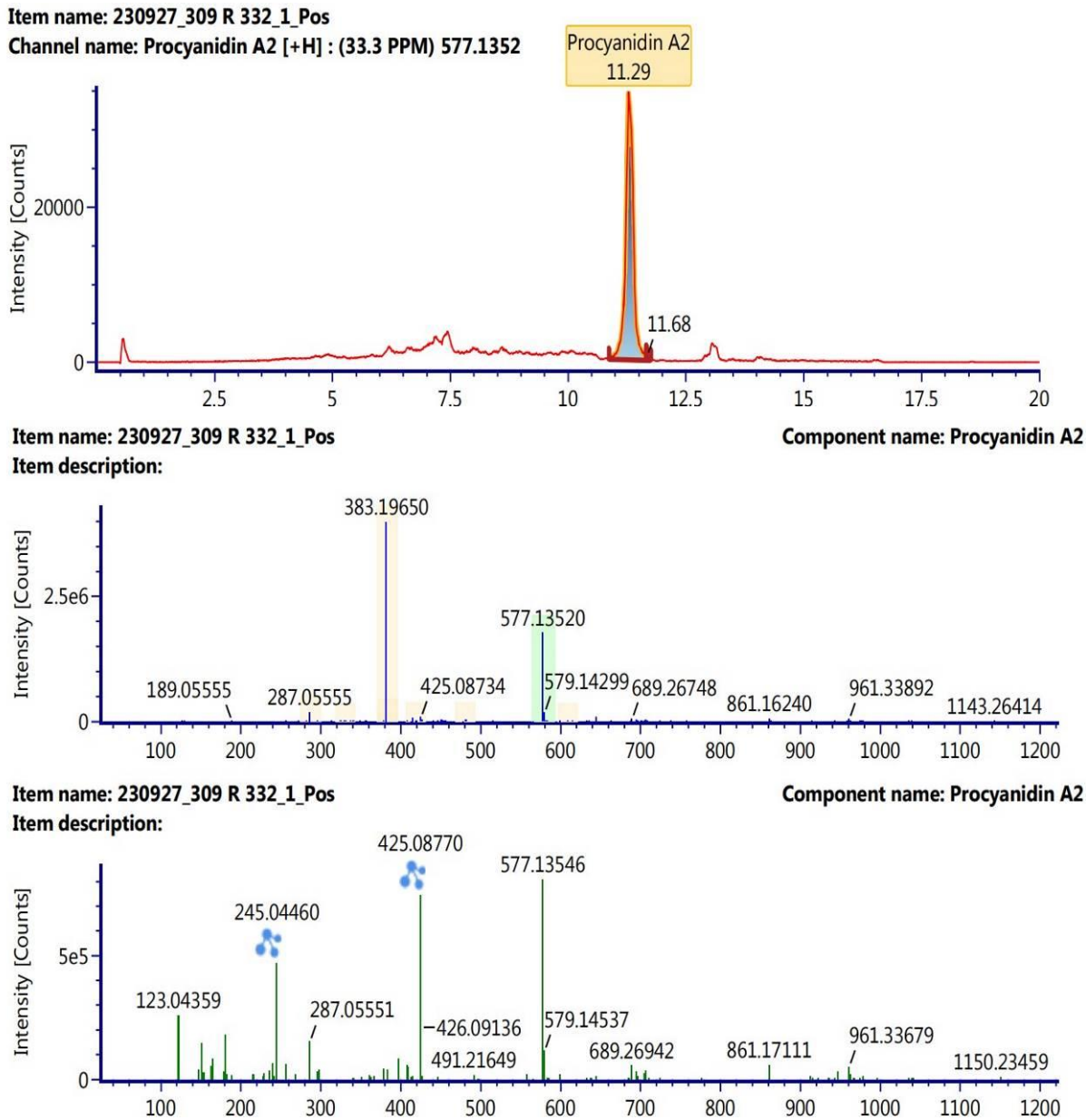


Figure S4. Chromatogram of procyanidin A2 isolated from FMet5.

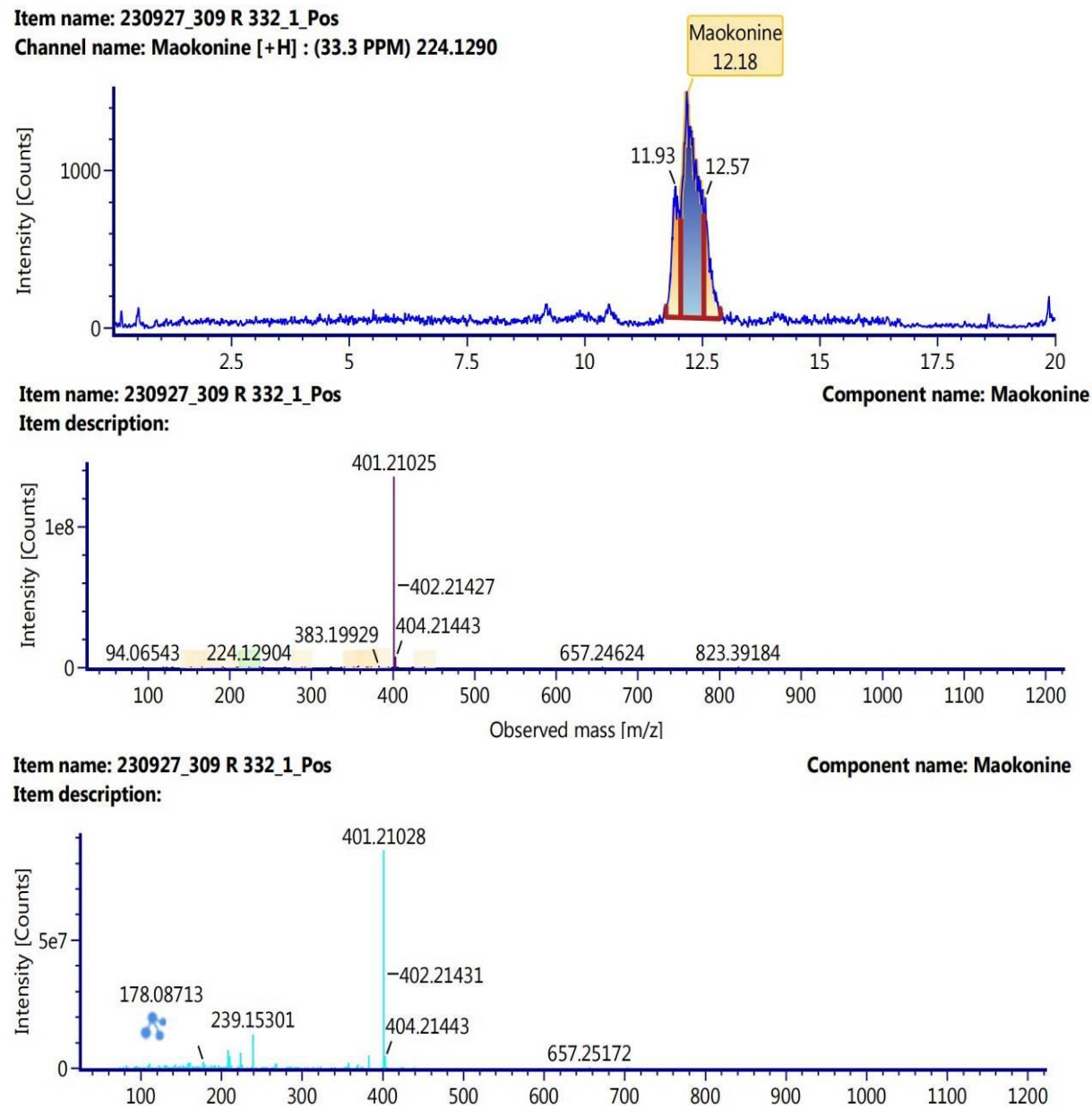


Figure S5. Chromatogram of maokonine isolated from FMet5.

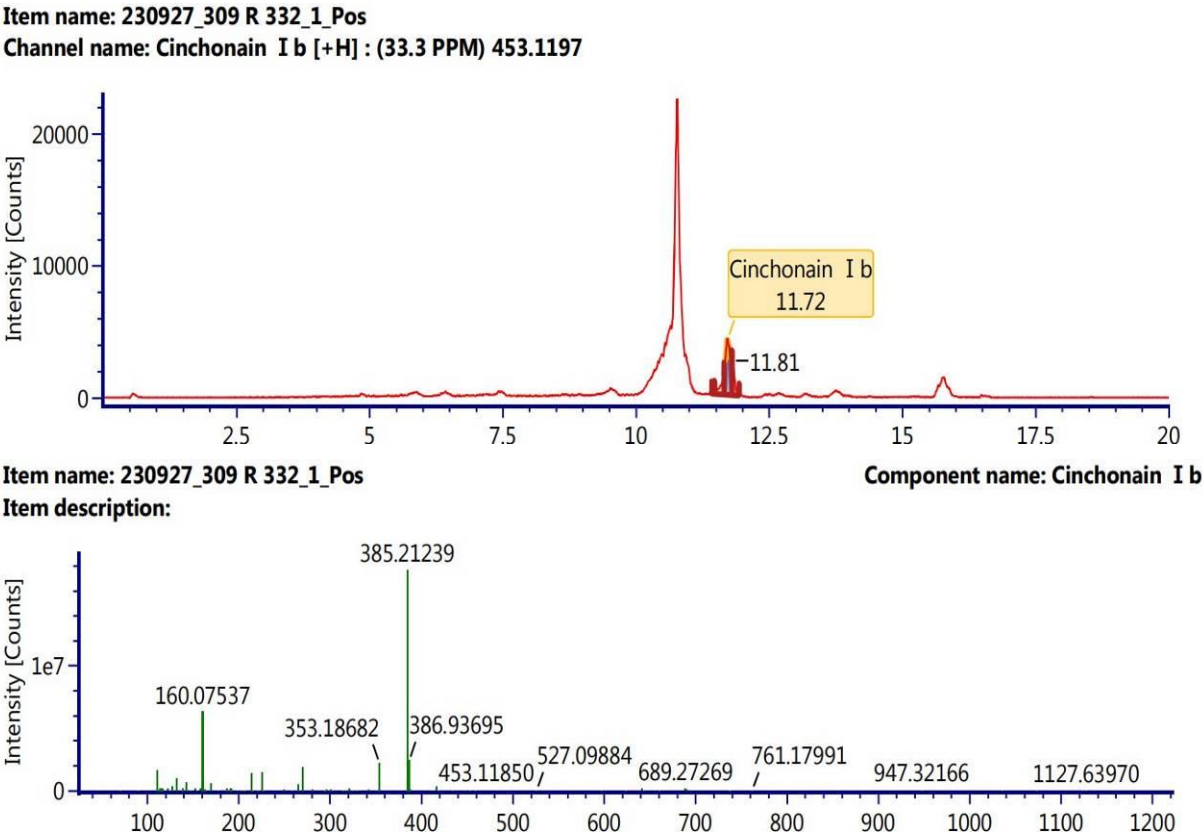


Figure S6. Chromatogram of cinchonain I b isolated from FMet5.

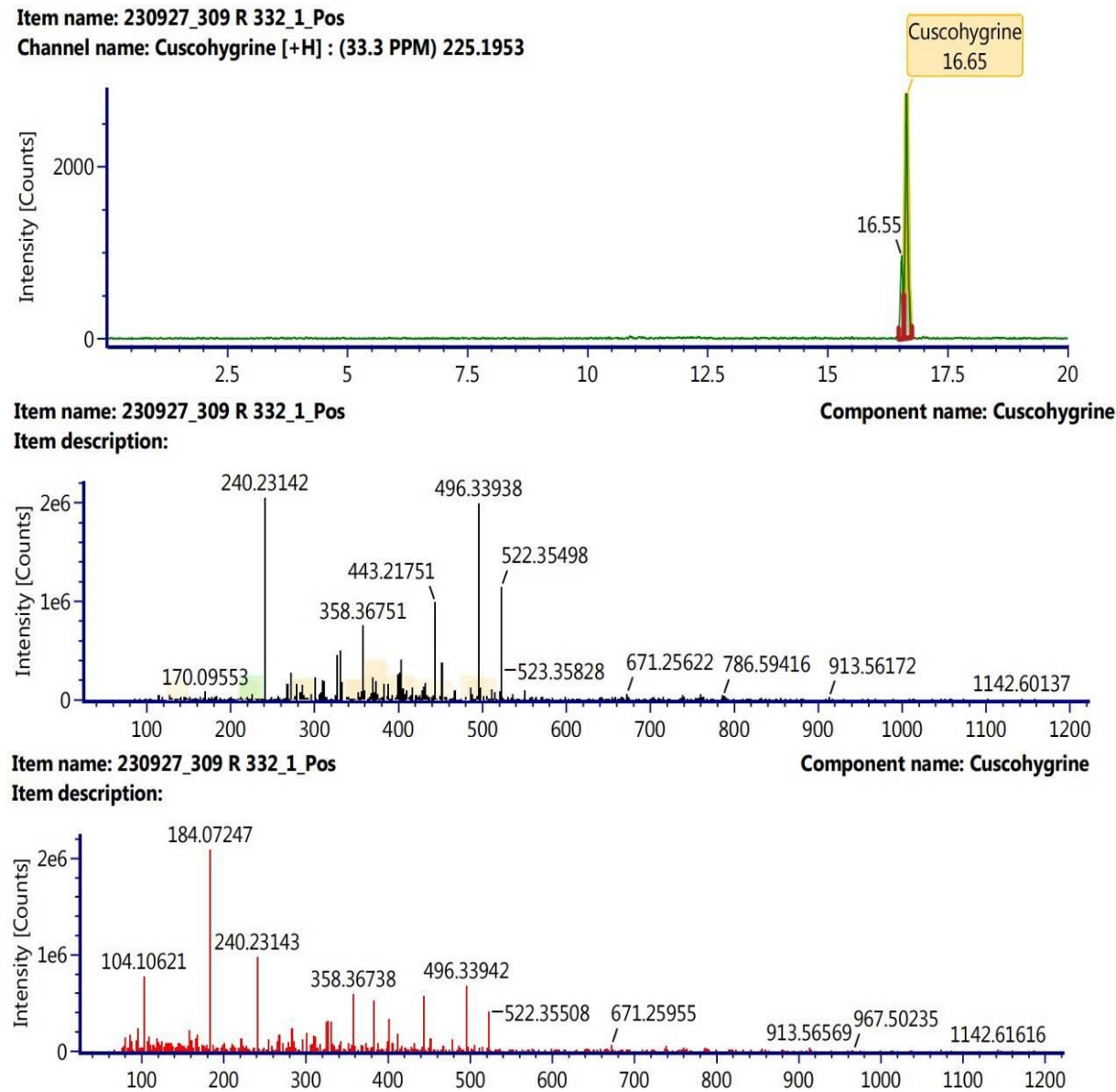


Figure S7. Chromatogram of cuscohygrine isolated from FMet5.