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Research Article

The Potential of Stem Bark of Kayu Sarampa (*Xylocarpus moluccensis* (Lam.) M. Roen)) as α-glucosidase Inhibitor

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ABSTRACT

Introduction: The prevalence of diabetes mellitus type 2 in the world is more than 230 million people, increases about 3% in a year. Kayu Sarampa or Nyirih batu (*Xylocarpus moluccensis* (Lam.) M. Roen) has traditionally been used to treat diabetic patient by native people in Ratahan, North Celebes, Indonesia. Therefore, this research was sequentially extracted bioactive component from stem bark of kayu sarampa showed alpha glucosidase inhibitor. **Objective:** To assess antioxidants and alpha glucosidase inhibitory activity of hexane, ethyl acetate, and methanol extract from stem bark of Kayu Sarampa. **Method:** The Stem bark was extracted with Reflux method using hexane, ethyl acetate, and methanol as mobile phae/solvent. The Hexane Extract (HE), Ethyl Acetic Extract (EAE) and Methanol Extract (ME) were subjected to the antioxidant activity assay by the 2.2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and the ferric-reducing antioxidant power (FRAP) method. Antidiabetic activity was determined by enzymatic alpha-glucosidase inhibitor. **Results:** The extract which had the highest activity based on the DPPH test and FRAP test was the ME compared with EAE, and HE with IC_{50} values of 16.51 µg/mL, 34.10 51 µg/mL, and 38.82 51 µg/mL , respectively. Ferrous equivalent antioxidant capacity (FeEAC) method, methanolic extract had a higher reduction capacity than the EH and EEA which were 148.96 µmol/gr, 48.96 µmol/gr, and 148.96 µmol/gr, respectively. The result showed that kayu sarampa stem bark exhibited antidiabetic activity due to its high inhibition compared with control (acarbose). ME showed inhibition of 53,11% followed with EAE 49,7%, HE 44,53%, and acarbose as control 29,32%.Conclusion: stem bark of kayu sarampa have bioactive component as alpha glucosidase inhibitor

Key words: Kayu Sarampa, Antidiabetic, Alpha-glucosidase inhibitor, Antioxidant.

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INTRODUCTION

The prevalence of diabetes mellitus type 2 in the world is more than 230 million people, increases about 3% in a year.[1] Hyperglycemia accelerates the formation of reactive oxygen species that increases lipid, DNA and protein modification in human tissue.[2] Molecular modification in some tissues causes an imbalance between protective antioxidant and free radical production. That was the beginning of oxidative damage which is known as oxidative stress.[3]

Antioxidant was used to inhibit or minimalize oxidative damage. One common method which is used to evaluate antioxidant activity is 1,1-diphenyl-2-pilcrylhidrazyl (DPPH).[4] This method measures synthetic radicals DPPH in a polar solute such as ethanol or methanol at room temperature which is scavenged by an antioxidant compound.[5]

Native people in Ratahan, Sulawesi Utara use batu Nyirih (*Xylocarpus moluccensis*) to treat diabetic patients. This plant is known as Kayu sarampa**.** *Xylocarpus* genus spread from India beach, Ceylon, Burma, Malaysia and Indonesia. Many researchers study about active components in Kayu Sarampa. It contains antibacterial, antidiabetes, antioxidant, antifilarial, antidiarrhea, antidepressant and cytotoxic activity.[6] Kayu sarampa can be used to treat fever, joint pain, headache, and disorders such as cholerae, constipation, and diarrhea[7].

Methanol extract of *X. moluccensis* was found to be significantly effective in scavenging DPPH method.[8] The fruits of *X. moluccensis* contain limonoid which can change enzyme activity to metabolize glucose and increase glucose absorption by muscle tissue.[9] In humans, alpha-glucosidase enzymes aid the digestion of dietary carbohydrates and starches to produce glucose for intestinal absorption, which in turn, leads to an increase in blood glucose levels. Elbakyan (1998) reported that a compound obtained from an isolate of ethyl acetic fraction was 2-methoxy-5-(1-propenyl) phenol and 2-methoxy-4-(1-propenyl) phenol with m/z 180 and 164, respectively.[10]

Extraction is an initial step to separate bioactive from plants. Solvent extraction is the most common technique to extract natural products from plants.[11] Secondary metabolites in plants have different polarity. Therefore, extraction with various solvent polarities was preferable.[12] The use of solvent-based on its polarity can be applied to dry powder of plant tissue using reflux. It was done at discontinued high temperature condition although soxhlet was done under continued high temperature. The advantages of reflux compared with soxhlet was the less use of solvent, and compared to creation, it only needs a short time of extraction.[13] Therefore, this research uses sequential reflux extraction in hexane, ethyl

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acetate, and methanol solvent. Kayu sarampa plant consists of bioactive components used to cure diabetic patients. However, there is no evidence of the use of stem bark of *X. moluccensis* to inhibit α-glucosidase. This research was done to sequentially extract bioactive component from stem bark of *X. moluccensis* and asses their α-glucosidase inhibitory activity.

MATERIAL

Plant material

Sample used in this study was the stem bark of *X. moluccensis* which was obtained from Ratatotok district, North Sulawesi and was identified by Herbarium Bogoriensis, Biological research centre, Indonesian Institute of Science.

Chemicals

Chemicals used in this study were Phosphate buffer pH 7, dichloromethane, dimethyl sulphoxide (Merck cat. 3.17275, Germany), Alpha-glucosidase (Wako Pure Chemical Industries Ltd., Japan), *Acarbose*, ethanol, ethyl acetate, methanol, n-hexane, para nitrophenyl alpha-D-glucopyranoside (Wako Pure Chemical Industries Ltd. EC 3.2.1.20, Jepang), Sodium carbonate (Merck cat. 1.09940, Germany), 1.1-diphenyl-2-picrylhidrazyl (DPPH), ascorbic acid (Sigma-Aldrich, A5960), Ferrous sulfate heptahydrate (MERCK, Germany)

Sample preparation

The stem bark of *X. moluccensis*. from Ratatotok, North Sulawesi, was freshly picked, collected, sorted, and dried. The Stem bark were then crushed until they became smaller.

Microscopic observations by scanning electron microscope (SEM) and light microscope

Microscopic analysis of Leaf and Stembark of *X. moluccensis* was performed using SEM Model: JSM – IT 200 in the Zoology Field of the Biology Research Centre – Indonesian Institute of Sciences (LIPI), Cibinong, and the observations were also made using a light microscope.

Extraction and Fractionation

Four kilograms of stem bark powder of dried *X. moluccensis* was extracted using hexane. Extraction was initially done using reflux apparatus to obtain hexane extract. Hexane extract then was evaporated using a rotary evaporator to obtain a crude hexane extract (HE). It was stored under room temperature. The same way was done for ethyl acetate solvent to obtain ethyl acetate extract (EAE). Then, methanol was used to extract the residue to obtain methanolic extract (ME).

Determination of free radical 1.1-diphenyl-2-picrylhidrazyl (DPPH) scavenging activity

DPPH radical scavenging activity was done according to the procedure declared by Burda & Oleszek (2001) with slight modification.[14] Five hundred microliter extracts were added into 1.5 mL DPPH solution, mixed for 2 minutes, and incubated in dark room for 30 minutes. The absorbance of the solution was measured for 5 minutes before incubation time ends. Color change from purple to yellow means free radical scavenging efficiency. Free radical scavenging activity was calculated as the percentages of color decreasing of DPPH solution using the following equation:

$$\textit{Free radical scavenging activity (\%)} = \left(1 - \frac{\textit{sample absorbance}}{\textit{control absorbance}}\right) \times 100\%$$

After the inhibition percentage of each concentration was obtained, linear regression was made so that the equation y = a + bx was obtained, where x is the concentration (µg/mL) and y is the percentage of inhibition (%). Antioxidant activity is expressed by 50% Inhibitory Concentration or IC_{50}, which is the concentration of the sample that can reduce DPPH radicals by 50% from the initial concentration.

FRAP assay test

This tes was conducted using the method described by Bhagat et al. Acetate buffer (300 mM, pH 3.6), TPTZ (2,4,6-tripyridyl-s-triazine) 10 mM in 40 mM HCl, FeCl3•6H2O (20 mM). The working FRAP reagent was prepared by mixing the three solutions in the ratio of 10:1:1. Extract of stem bark of X. moluccensis (100 µL) was mixed with 3 mL of working FRAP reagent and the absorbance was measured at 598 nm after vertexing. Methanol solutions of FeSO4•7H2O ranging from 100 to 2000 µM were prepared and used for the preparation of the calibration curve of known Fe2+ concentration. The parameter equivalent concentration was defined as the concentration of antioxidant have a Ferric-TPTZ reducing ability equivalent to that of 1 mM FeSO4•7H2O.

Standard and sample examination of Alpha-glucosidase Inhibitor

Acarbose as a standard and sample was weighed and dissolved in phosphate buffer solution pH 6.8. Sample with low solubility in phosphate buffer was dissolved in maximum 10% DMSO. Then standard and sample were diluted into some concentrations.

Thirty microliter standard and sample solution were added to 17 µL PNPG substrate. The solution was incubated for 5 minutes at 37°C, and added to 17 µL alpha-glucosidase solution. Solution was incubated again at 37°C for 15 minutes. After that, 100 µL sodium carbonate 267 mM was added. Solution absorbance was measured using a microplate reader at 405 nm wavelength.

Alpha-glucosidase inhibitory activity was calculated by the following equation[16]:

$$\%\ \textit{Alpha} - \textit{glucosidase Inhibition} = \frac{\textit{blank control absorbance} - \textit{sample absorbance}}{\textit{blank control absorbance}}\ X100\%$$

Statistical analysis

All the experimental data do triplicates and the results are expressed as mean ± SD. IC_{50} was analyzed using Probit. Antioxidant power and alpha-glucosidase inhibitory activity were analyzed using One way ANOVA analysis followed by Duncan Multiple Range Test. Analysis was performed using SPSS software version 20.

RESULTS AND DISCUSSIONS

Extraction

X. moluccensis was extracted sequentially using reflux with hexane, ethyl acetate, and methanol as the solvent. Relux is an extraction using heat. Miller (1975) stated that heating under reflux process can increase the possibility to damage tissue membrane of plant material, so that solvent can penetrate into tissue void which contains active components.[7] Active components will dissolve in because of its different concentration between active component inside and outside the tissue, so concentrated solution is pulled outside. The yield of reflux sequential extraction was displayed in the following table 1.

Table 1 shows that methanolic extract exhibits the highest yield followed with EEA and EH. A polar bioactive component from kayu sarampa extract is dissolved in methanol. The yield of the extract is depending on the effectivity of solvent used for extraction. High yield of extract means high extracted component.

Microscopic observations by scanning electron

microscope (SEM) and light microscope

Antioxidant activity

Antioxidant activity of kayu sarampa was evaluated using DPPH and FRAP methods.

Free Radicals DPPH Scavenging Activity

The evaluated *X. moluccensis* concentration were 25, 50, 75, 100, and 125 mg/L, respectively. Free radical DPPH commonly used as substrate to evaluate antioxidant activity. Antioxidant properties of *X. moluccensis* extract reacted with DPPH, the solution color changed from purple to yellow. The change in solution color affected to DPPH absorbance. The higher concentration of antioxidant component in solution, the lower the absorbance of DPPH.

The change in solution color is caused by a component that donates hydrogen atom to DPPH radical. Antioxidant reducted DPPH radical to more stable form namely DPPH-H (2,2-diphenyl-1-picrylhidrazine).[4] Evaluation of antioxidant activity using DPPH method is presented in Figure 4.

Figure 4 showed that antioxidant activity of *X. moluccensis* through DPPH method is increased in a concentration-dependent manner. Methanol extract exhibited the highest free radical DPPH scavenging activity 82,93%, followed with ethyl acetate extract 74,75%, and Hexane extract 27,83%, respectively. Ascorbic acid as antioxidant standard exhibited 88,09% activity. Lai *et al.* (2001) stated that antioxidant activity using radical DPPH mostly increase in a concentration-dependent manner.[17]

IC_{50} value represents the ability of *X. moluccensis* extract to scavenge 50% of DPPH free radical. The lowest IC_{50} value of an extract represents high antioxidant activity.[18] IC_{50} was obtained from a regression linear equation in Figure 1 which plotted x axis (concentration) with y axis (free radical DPPH scavenging activity), so a regression equation and IC_{50} obtained is presented in Table 3.

Table 3 showed that ME IC_{50} of *X. moluccensis* exhibits the highest activity compared with EAE, and HE. IC_{50} value of an extract represents its potential to inhibit free radicals. A strong level IC_{50} categorized with IC_{50} value 50-100 mg/L, medium range category 100-250 mg/L, and low level category was 250-500 mg/L.[19]

High antioxidant activity of methanolic extract of kayu sarampa is caused by polar secondary metabolite such as flavonoid. Interaction of flavonoid with nitric oxide synthases (NOS) activity may modulate the NO production. Xanthine oxidase (XO) is considered as a key source of free radicals, and some flavonoids such as quercetin, silibin, and luteolin have been shown to inhibit such activity. Flavonoids may also reduce the activity of peroxidase and may inhibit the release of free radicals by neutrophils and activation of these cells by α1-antitrypsin.[20]

Ascorbic acid used as a control in this study is a strong antioxidant. IC_{50} of ascorbic acid exhibits IC_{50} value 10,49 mg/L. It was caused possibly by 2 groups of hydroxy (-OH) on its double bond which is easily oxidized by free radicals. In addition, vitamin C used in this study is a pure substance so it exhibits strong IC_{50} or high antioxidant activity.[21]

Ferric reduction antioxidant power

The FRAP method is a method of testing antioxidant activity through the ability of antioxidant compounds to reduce Fe^{3+} ions to Fe^{2+} in the presence of 2.4,6-tri (2-pyridyl)-s-triazine (TPTZ) in an acidic atmosphere which produces intensive blue colour from the Fe^{2+}-TPTZ complex and causes an increase in absorbance when it is measured using a microplate reader at a maximum absorption of 593 nm. In the test using the FRAP method, ferrous sulfate heptahydrate (FSH) is used as a standard. First, the standard FSH calibration curve is made. The antioxidant activity is measured based on the sample equality with the AFS standard. The linear regression equation of FSH obtained y = 0.00257 x + 0.04715. Antioxidant power of sarampa extract is shown in following table 4.

Table 4 shows that ME from *X. moluccensis* stembark exhibits higher antioxidant power compared to HE and EAE which are 148.96 µmol/gr, 48.96 µmol/gr, and 148.96 µmol/gr, respectively. Statistical analysis reveals that antioxidant power of samples extracted with different solvent are significantly different ($p<0.05$). Based on the result presented in Table 4, samples extracted with methanol and ethyl acetic show higher antioxidant power compared to n-hexane extract. This is due to the high polarity of methanol and ethyl acetic, whilst hexane is non polar. These results are influenced by the polarity of solvent because methanol is the most polar solvent used in this experiment, while ethyl acetate and hexane in descending order were less polar than methanol. The higher total antioxidant content in extract means more compounds can reduce Fe^{3}+ to Fe^{2}+ (blue), and compounds that reduce Fe^{3}+ is an

Table 1: The yield of *X. moluccensis* extraction.

	HE	EAE	ME
Extract weight (g)	24.3	63.38	625.56
Yield %	1.215	3.169	31.278

Note : hexane extract (HE), ethyl acetate extract (EAE), methanolic extract (ME)

Table 2: A summary of the *X. moluccensis* leaf side section.

Leaf Surface	Distribution of stomata on leaf surface	Morphological type of mature stomata	Stomatal Measurements		
			Lenght (µm)	Breadth (µm)	Pore size(µm)
Upper	Hypostomatic	Paracytic	0	0	30 ± 0.5*
Lower	Hypostomatic	Paracytic	20 ± 0.2	10 ± 05	10 ± 12

*SEM value

Table 3: Regression equation and IC_{50} of *X. moluccens* is extracted with different solvents.

	HE	EAE	ME	Ascorbic acid
Regression Equation	y = 0.2158x + 0.9182	y = 0.3007x + 39.77	y = 0.3197x + 44.72	y = 0.3788x + 46.026
IC_{50} (µg/L)	38.82	34.10	16.51	10.49

Note : hexane extract (HE), ethyl acetate extract (EAE), methanolic extract (ME)

antioxidant compound. Prior *et al* 2005 states that methanol is a polar compound which is easy to position the hydrogen atoms of a compound or hydroxyl groups to form hydrogen bonds because the bonds would facilitate the transfer of protons (hydrogen atoms antioxidants).[22]

Alpha-glucosidase inhibitory activity

Concentration of stem bark kayu sarampa extract used in this test is 100 ppm. Alpha-glucosidase inhibitory activity of stem bark *X. moluccensis* extract was presented in Table 5.

The result shows that kayu sarampa stem bark exhibits antidiabetic activity due to its high inhibition compared with control. Methanolic extract shows inhibition 53,11% followed with ethyl acetate extract 49,7%, Hexane extract 44,53%, and acarbose as control 29,32%.

Srivastava *et al* reported that antihyperglycemia and anti dyslipidemia activity of ethyl acetate fraction of *X. moluccensis* (EAXm) effectively increase glucose recovery, decrease blood glucose level and fructosamin serum dose in diabetic mice induced by streptozotocin. EAXm also decreases cholesterol serum, triglyceride, LDL cholesterol, but increases HDL cholesterol, liver function, and kidney fuction in HFD/HSD-STZ mice for 10 consecutive days. Moreover, EAXm increases glucose absorption by muscle tissue L-6 and inhibites alpha glucosidase enzyme *in vitro* with IC_{50} 28,4 µg/ml. Although there is no research about secondary metabolite in the stem bark of *X. moluccensis* as α-glucosidase inhibitor. [8]

The secondary metabolite which possibly exhibites antidiabetic activity through α-glucosidase inhibition in kayu sarampa is limonoid. Das *et*

Table 4: Antioxidant power using FRAP.

Sample	Antioxidant Power (FRAP) (µmol FeII/g)	% Scavenging (DPPH)
Control (Ascorbic Acid)	340.48 ± 0.02	85.67 ± 0.03
n-Hexane	48.96 ± 1.01	22.68 ± 0.02
Ethyl Acetate	131.84 ± 0.03	72.06 ± 0.05
MeOH	148.96 ± 0.02	75.94 ± 0.65

Data is presented as the mean ± standard deviation (N=3). Means with different letters are significantly different (p<0,05).

Table 5: Alpha-glucosidase inhibition activity at concentration 100 ppm.

	HE	EAE	ME	Acarbose
Percent Inhibition (%)	44.53	49.70	53.11	29.32

Note : hexane extract (HE), ethyl acetate extract (EAE), methanolic extract (ME)

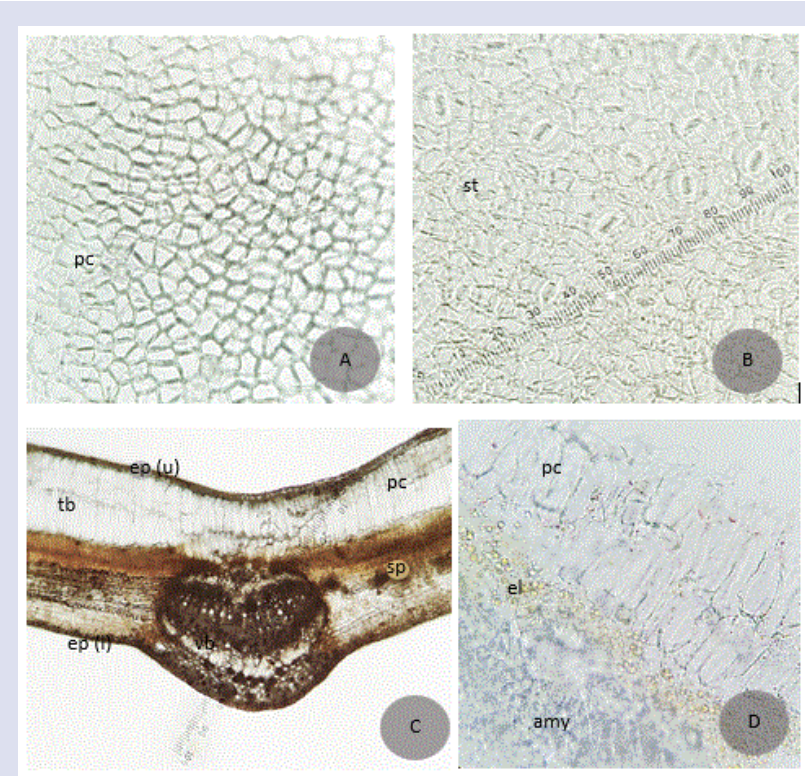

Figure 1: *X. moluccensis's* leaf side section A: upper side (pc: parenchyma) with angular parenchyma , B: lower side (st: stomata) with paracytic type of stoma, C: tangential side (ep-u: epidermal upper side, tb: tubular palisade, pc: parenchyma, sp: sponge palisade, ep-l: lower epidermal, vb: vesicular bundle), D: parenchymal tissue x 1000 (amy: amyloplast, el: elailoplast, pc: parenchyma).

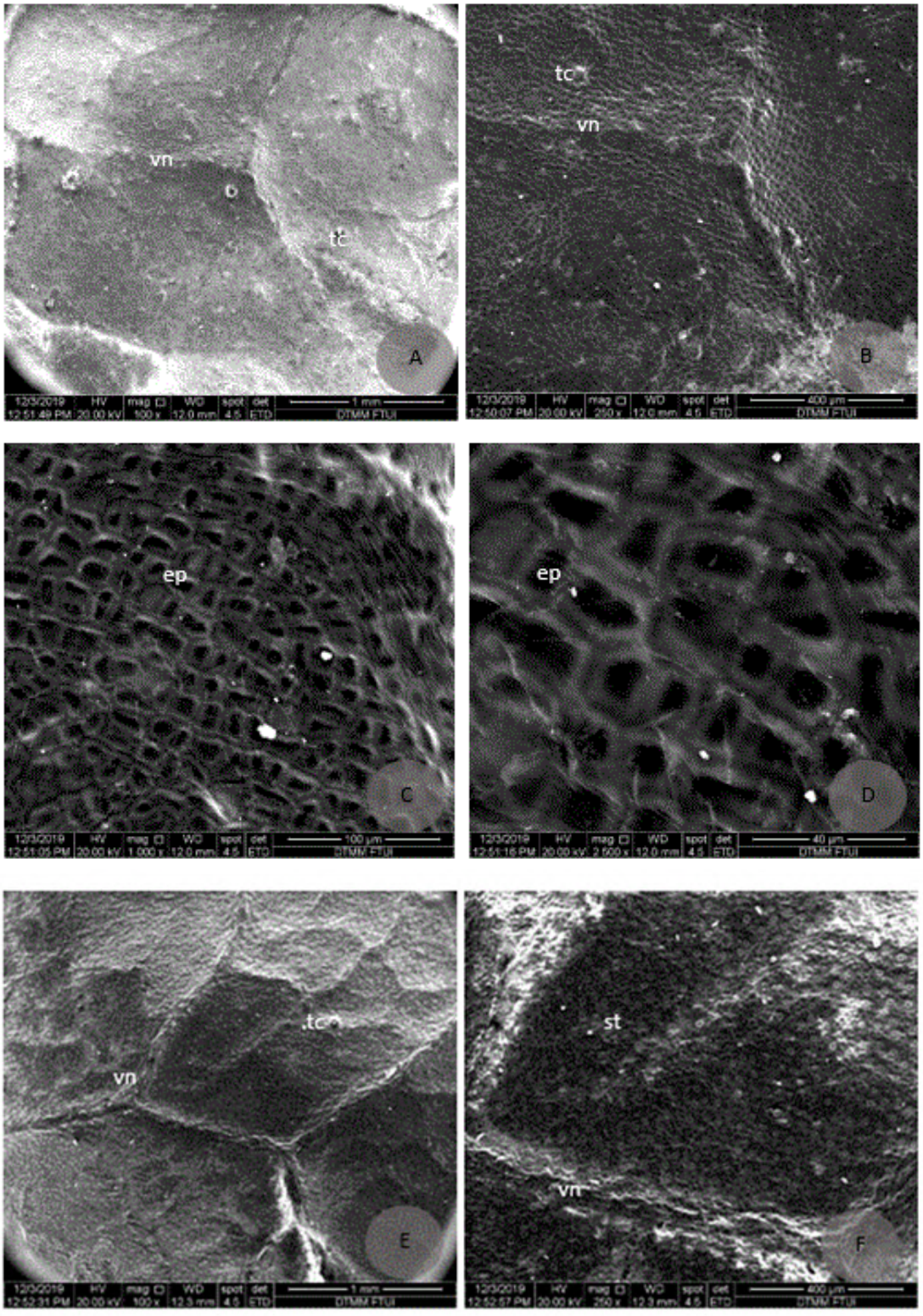

Figure 2: *X. moluccensis's* leaf side (SEM) A: upper side (vn: leaf's venular, tc: trichome) 100x, B: upper side 250x, C: upper side 100 x (ep: epidermal tissue with angular parenchyma) , D: upper side 2.500x magnification, E: lower side 100x (vn: leaf's venular, tc: trichome), F: lower side 100x (vn: leaf's venular, st: stoma).

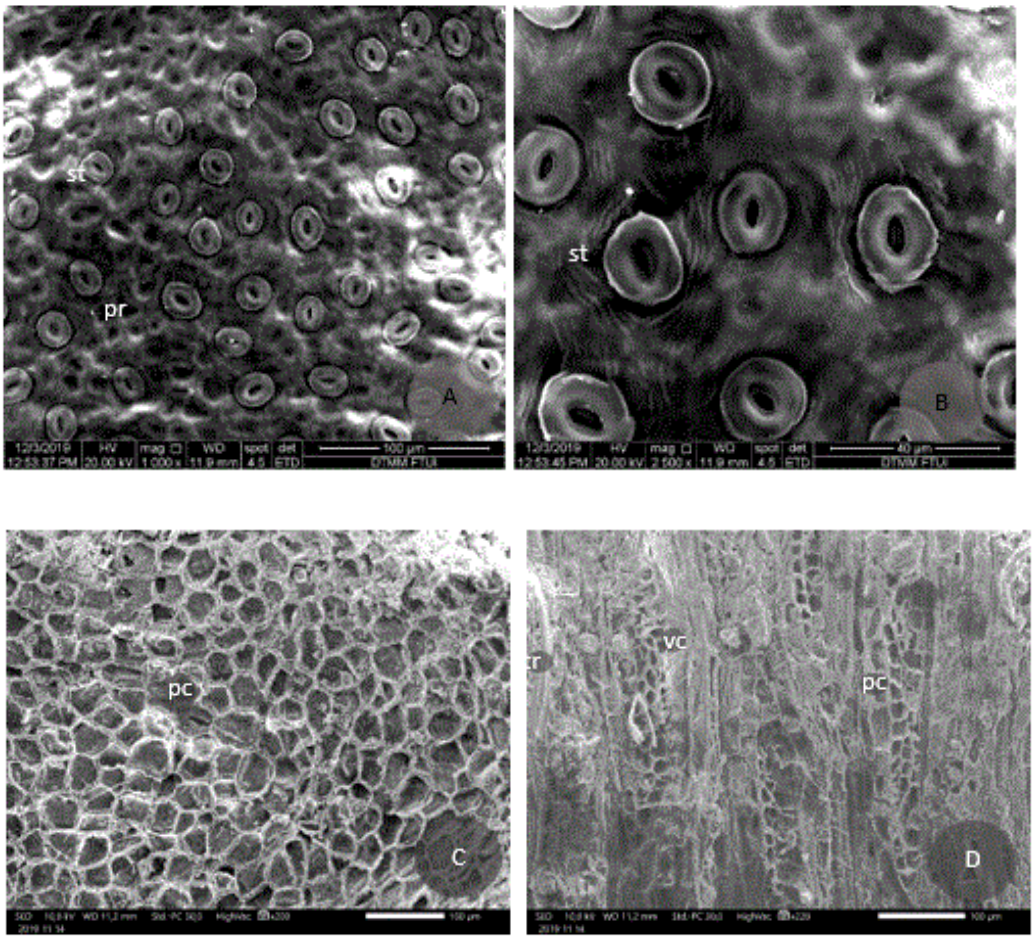

Figure 3: *X. moluccensis's* lower leaf side (A-B) and stem (C-D) (SEM) A: lower side leaf (pr: leaf's phore, st: stoma) 1000x magnification, B: lower side leaf 2.500 x (st: stoma paracytic type with with distinct subsidiaries), C: tangential slice section of stem 100 x magnification (pc: parenchyma cell of stem, irregular angular type), D: longitudinal side section of stem (vascular side, xylem) tr: tracheal tissue of xylem, vc: vascular, pc: xylem's parenchyma.

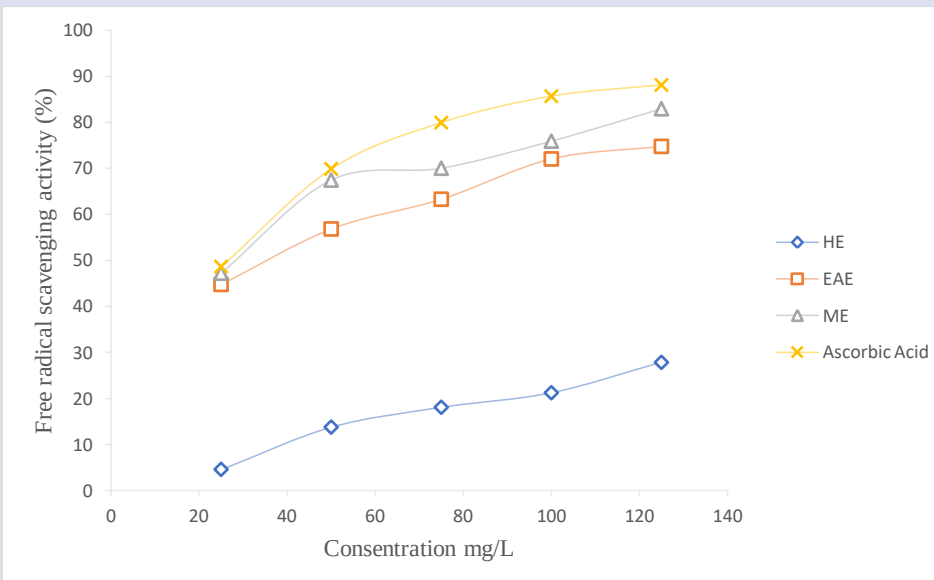

Figure 4: DPPH radicals scavenging activity from *X. moluccensis* extract and Ascorbic acid.

al reported that xyloccensin-L contains interaction and can bond when it is close to the target then confirm the possibility antidiabetic action mode by isolated compound from X. *granatum*. xyloccensin-L is a novel limonoid from X. *granatum*.[23]

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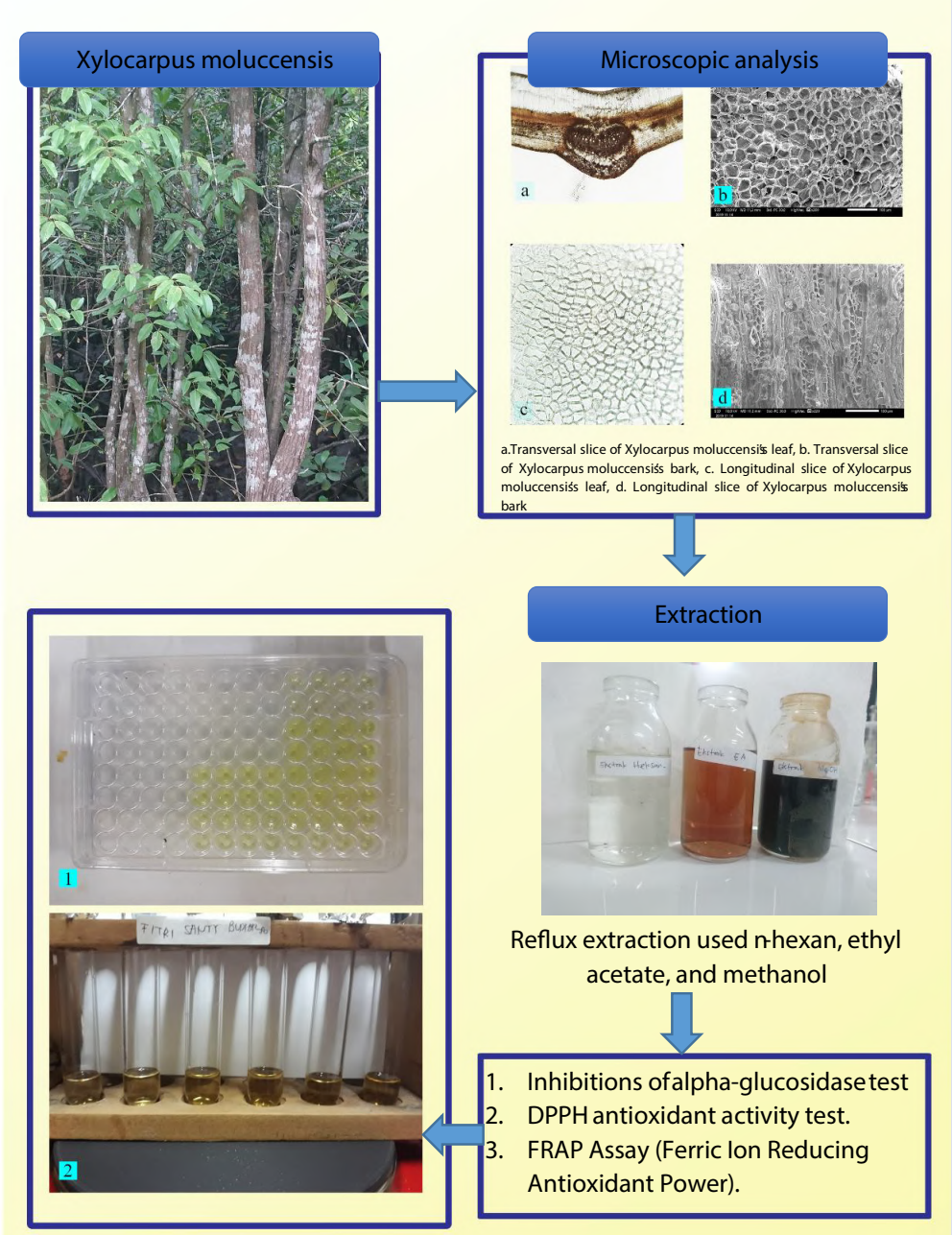
GRAPHICAL ABSTRACT



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