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Inhibition of Alpha-Glucosidase and Antioxidant Test Using DPPH Method of Leaf Extracts of *Garcinia fruticosa* Lauterb., and Phytochemical Screening on the Most Active Extract

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Abstract : Diabetes mellitus is a chronic endocrine disorder characterized by hyperglycemia, which blood sugar levels rise due to the pancreas unable to produce enough insulin or the body's cells can't respond to the insulin that is produced. Hyperglycemic conditions can also generate free radicals which can cause oxidative damage to biomolecules such as proteins, lipids, and DNA which can significantly cause diabetes or worsen the complications. Therefore, it is necessary to find drug compounds that can give an effect in lowering blood glucose levels while giving antioxidant benefits at the same time. This study aims to test the in-vitro inhibitory effect of α-glucosidase, an enzyme involved in the digestion of carbohydrates, and determine the antioxidant activity using DPPH method of *Garcinia fruticosa* Lauterb leaves n-hexane, ethyl acetate, and methonal extract. Both tests were done by using the Microplate Reader. The test results showed that the ethyl acetate extract had the most actuve IC_{50} values, ie 25.314 mg/mL of α-glucosidase inhibition test and 12.369 mg/mL on the antioxidant activity test. Furthermore, the phytochemical screening was done on the ethyl acetate extract of *Garcinia fruticosa* leaves and several some classes of phytochemical compounds were found, which were alkaloids, flavonoids, glycosides, tannins and saponins.

Keywords : *α-glucosidase; antioxidant; antidiabetic; Garcinia fruticosa leaves; phytochemical screening.*

Introduction

Diabetes mellitus (DM) is a chronic endocrine disorder characterized by hyperglycemia and is a serious health problem because of the complications[1]. Reactive oxygen species (ROS) overproduction was considered as a factor that significantly leads to other degenerative diseases, including DM. Hyperglycemic condition of DM patients also will produce ROS from glucose auto-oxidation and protein glycosylation thus lead to cell disturbance and secondary complications in the sufferer. Therefore, it is necessary to find drug compounds that is effective in managing diabetes and also works as antioxidant[2,3,4,5]. A pharmacological therapy for DM patients is by decreasing the postprandial hyperglycemia using α-glucosidase inhibitor that will inhibit the carbohydrate digestion[6].

Plant from Garcinia genus had been widely studied and showed various pharmacological activity, some of them were α-glucosidase inhibitor and antioxidant[7,8]. Study about α-glucosidase inhibitor activity was showed in vitro using *Garcinia daedalanthera* Pierre ethanolic extract with IC_{50} value obtained was 2.33 μg/mL which much smaller than IC_{50} standard value, IC_{50} value of acarbose was 117.20 μg/mL[7]. However the IC_{50} value of antioxidant activity by using in vitro DPPH method of *Garcinia lateriflora* Blume leaves metanolic extract was 6.18 μg/mL, while in quercetin standard was 2.4 μg/mL[9].

In this study, we performed a test using *Garcinia fruticosa* Lauterb leaves which until now there was no study had reported about its chemical content and the pharmacological activity. According to the taxonomy, *G. fruticosa* had a phylogenetic relationship with *G. daedalanthera* and *G. lateriflora*, thus it was expected that this plant would show a good α-glucosidase inhibition and antioxidant activity. In this study, we performed α-glucosidase inhibition and antioxidant test using in vitro DPPH method in microplate reader in n-hexane, ethyl acetate, and methanol extract of *G. fruticosa* obtained by gradual maceration, and also conduct a phytochemical determination to determine the chemical groups obtained in the most active extract.

Materials & Method

Materials. The study material is *G.fruticosa* leaves obtained from Kebun Raya Bogor and had been determined in Plant Conservation Center Indonesia, Bogor, WestJava, Indonesia. The chemical materials such as α-glucosidase enzyme which was obtained from *Saccharomyces cerevisae* (Sigma Aldrich, German), and DPPH (Sigma Aldrich, USA). The standard materials were Acarbose (Sigma Aldrich, USA) and Quercetin (Sigma Aldrich, India),

Extraction. 910 gram simplicial powder were extracted by gradual macerations using solvent with an increase polarity, which were non polar solvent (n-hexane), semi polar (ethyl acetate), and polar (methanol). Each of the extract solvent then evaporated in the solvent using rotary vacuum evaporator or by using water bath to obtain a thick extract.

Preliminary test of α-glucosidase. The preliminary test performed was to determine the maximum wavelength of p-nitrophenol and optimize enzyme activity using microplate reader. Enzyme activity optimization was including optimization of pH, incubation temperature, enzyme and substrate concentration. The maximum wavelength was determined by measuring the test solutions in 390, 395, 400, 403, 405, 408, and 410 nm wavelengths. pH optimization was performed using various pH in 6.6, 6.8, and 7.0. Incubation temperature optimization was performed in various temperatures in 35, 37, 38, 39, and 40°C. Enzyme concentration optimization was performed in various concentrations of 0.025, 0.035, 0.045, 0.055, 0.065, 0.075 U/mL and also the substrate optimization concentration was performed using various substrate concentration which were 1,2,3,4,5 and 6 mM. Each test was conducted three times (triplo).

α-glucosidase inhibition test in standard and extract. Acarbose standard solutions were made in 200, 500, 800, 1100, and 1400 µg/mL. The n-hexane extract was made in 1000, 1500, 2000, 2500, and 3000 µg/mL with the final concentrations of each well were, 150, 225, 300, 375, and 450 µg/mL.The ethyl acetate and methanol extract solutions were prepared in 100, 150, 200, 250, and 300 µg/mL with the final concentrations of each well was 15, 22.5, 30, 37.5, and 45 µg/mL. Then, each acarbose standard solutions and extract solutions were collected 30 µL, then added 36 µL phosphate buffer pH 6.8 and 17 µLpNPG 5 mM, then incubated for 5 minutes in 39°C. Then added 17µL α-glucoside 0.045 U/mL and incubated for 15 minutes. After incubated, added 100 µL sodium carbonate 200 mM solution. In control standard or sample, sodium carbonate 200 mM solutions were added first before enzyme addition. The absorption then measured using microplate reader in 400 nm. The tests were conducted three times.

Activity of α-glucosidase standard and extract could be defined in %inhibition which obtained using the following formula:

$$\%\,inhibition = \frac{(\mathrm{A-B})-(\mathrm{C-D})}{\mathrm{A-B}} \times 100\%$$

With:

A = blank solution absorption

B = blank control solution absorption
C = sample solution absorption
D = sample control solution absorption

DPPH wavelength optimization. DPPH solutions in 150 μmol/L were measured to obtain the absorption using microplate reader in 501, 503, 505, 507, 509, 511, 513, 515, 517, 519, 521, 523, 525, 527, 529, and 531 nm to obtain wavelength with a maximum absorbance.

Antioxidant test using DPPH method. Quercetin standard solutions were prepared in the concentrations of 15, 20, 25, 30, and 35 μg/mL with the final concentrations for each well were 1.5, 2, 2.5, 3 and 3.5 μg/mL.n-hexane extract was prepared in 160, 200, 240, 280, and 320 μg/mL with the final concentrations for each well were 16, 20, 24, 28, and 32 μg/mL. Ethyl acetate extract solutions were prepared to obtain 80, 100, 120, 140, and 160 μg/mL with the final concentrations for each well were 8, 10, 12, 14, and 16 μg/mL. Methanol extract solutions were prepared in 100, 140, 180, 220, and 260 μg/mL with the final concentrations for each well were 10, 14, 18, 22, and 26 μg/mL. Then, each of quercetin standard solutions and extract were collected for 20 μL, then added 180 μL DPPH 150 μmol/L solutions. The mixtures then stirred for 60 seconds and incubated in room temperature in a dark room for 40 minutes. After incubated, the solutions then measured to obtain the absorption using microplate reader in 519 nm wavelength. The samples were tested three times (triplo). For the control solutions, sample were replaced with 20 μL water. While the blank well was containing 20 μL water and 190 μL methanol-water (80:20, v/v). DPPH reduction or inhibition percentage then determined using the following formula:

$$\%\, inhibition = \frac{control\ absorbance - sample\ absorbance}{control\ absobance - blank\ absorbance} \times 100\%$$

Phytochemical determination. Phytochemical determination was including alkaloids identification using Mayer, Bouchardat, and Dragendorff reaction. The flavonoids was identified using Shinoda using Mg and Zn powder. Glycosides identification using Molisch, terpenoidswith Liebermann-Burchard reaction, tannins with $FeCl_3$ and Pb acetate reagent, saponin with bubbles reaction, and antraquinone with $FeCl_3$ 10%-HCl.

Results and Discussion

Extraction. Extraction method used was gradual macerations. Gradual macerations performed with an increase polarity of the solvent. Solvents used, respectively, were n-hexane (non-polar), ethyl acetate (semi-polar), and methanol (polar). The percentages of each extract yield were shown below:

Table 1. Thick extract weight and % yield extract value

Solvent	Simplicial weight extracted (gram)	Thick extract weight (gram)	Yield (%)
n-hexane	910	42	4,62
Ethyl acetate	910	82	9,01
Methanol	910	104	11,43

Preliminary Test of α-glucoside activity. The determination of maximum wavelength and pH optimization and also incubation temperature was performed in enzyme solutions 0.025 U/mL and substrate concentration 5 mM. According to the measurement result, we obtained the maximum absorption was in 400 nm wavelength.

Enzyme activity optimization with the various pH was needed because pH will affect the ionization active site of the enzyme so that they could interact with the substrate to affect the enzymatic reaction rate[10]. pH variation used in some literature were pH 6.8 and 7.0 therefore the optimization was performed in those pH value, by adding pH 6.6 variation to complete the curve. According to the data obtained, the maximum absorption was in pH 6.8 and decreased in 7.0. This was happen because in extreme pH, enzyme will experience denaturation[10].

Then, performing optimum incubation temperature determination. Temperature optimization was needed because the enzyme reaction rate will increase with the increased temperature because of the kinetic energy and frequency of molecule collisions react also increased[11]. Temperature used in the product information from Sigma Aldrich was 37°C, however the optimum temperature of human enzyme was ranged from 35 to

40°C, and would be denaturized in above 40°C[10]. Therefore, the various temperatures were used in this optimization test, which were 35, 37, 38, 39, and 40°C. According to the data obtained, pH 6.8 of the maximum absorption was found in 39°C. The absorption increase in 35°C to maximum in 39°C and decreased in 40°C which showed that the enzyme had been denaturized.

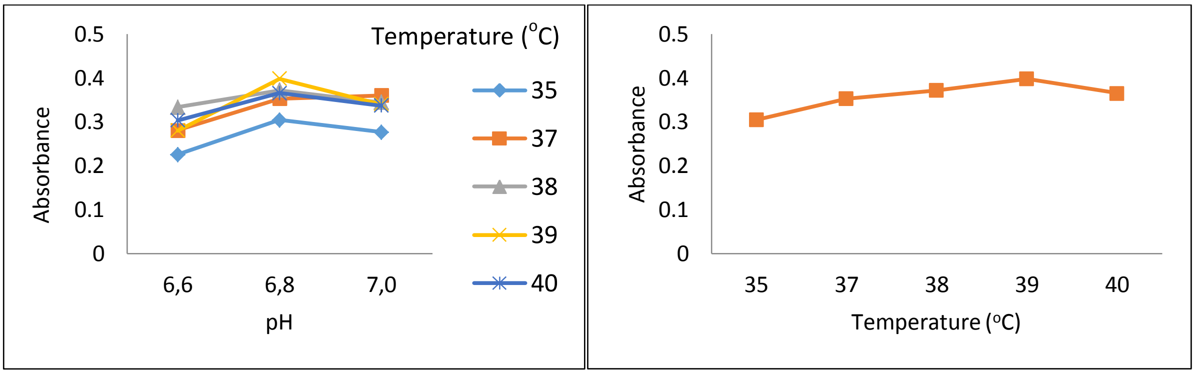


Figure 1. pH (left) and temperature (right)optimization curve

After obtaining the wavelength, pH, and optimum incubation temperature, then we performed an enzyme optimization. This was performed to determine the enzyme unit needed to obtain product with the maximum absorption which absorption ranged from 0.2-0.8. In this optimization, we used substrate concentration of 5 mM in various enzyme units, such as 0.025, 0.035, 0.045, 0.055, 0.065 dan 0.075 U/mL. Those variations were chosen because the enzymatic product of 5 mM substratewith 0.025 U/mL enzyme showed a low absorption, so that the concentration of 0.025 U/mL become the lower limit and 0.075 U/mL as the upper limit of concentration selection. According to the data obtained, 0.045 U/mL enzyme unit provide the maximum absorbance which meet the reading requirement, which was 0.797. Then the substrate concentration was optimized to determine the appropriate substrate concentration to react with enzyme unit used. Substrate concentration was said optimum if all active site in the enzyme had been bound with the substrate, so that there were no free enzyme which would produce products[10]. In substrate optimization, we used enzyme unit of optimization which was 0.045 U/mL with substrate variations were 1, 2, 3, 4, 5, and 6 mM. According to the data obtained, pNPG optimum concentration was showed in 5 mM because it showed the highest absorption in the curve. Besides that, the substrate addition in 6 mM, provide a similar results with 5 mM, and tend to be constant because the enzyme had reach its saturated state.

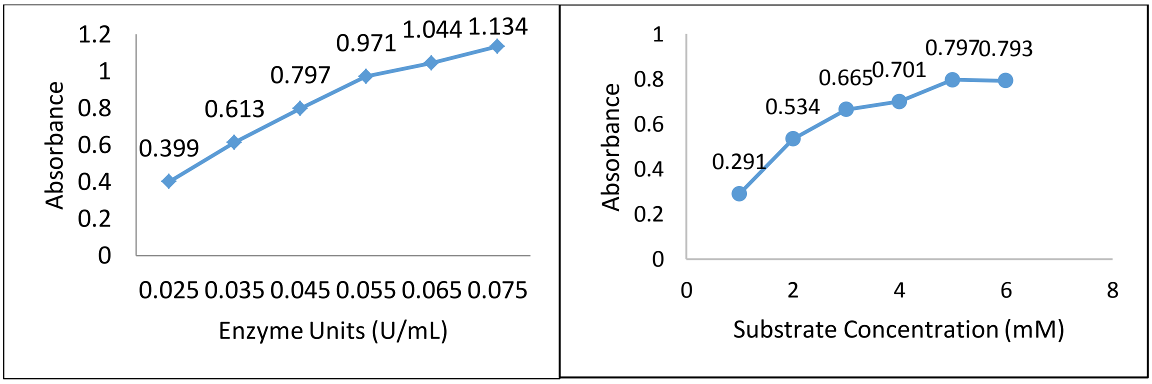


Figure 2. Optimization curve of enzyme unit (left) and substrate concentration (right)

Inhibition test of α-glucosidase in standard and extract. Standard used was acarbose. According to the data obtained, IC_{50} value of acarbose was 141.53 μg/mL. In vitro study about IC_{50} value of acarbose before showed IC_{50} value as 117.20 μg/mL[7]. This difference could be caused by several factors, such as different instrument used in the study, different acarbose and different of the reagent used. IC_{50} value of acarbose obtained then compared with IC_{50} value of extract in α-glucoside activity. Concentration and inhibition percentage in each standard solution of acarbose could be found in Table 2. The graph of acarbose standard testing could be found in the following below:

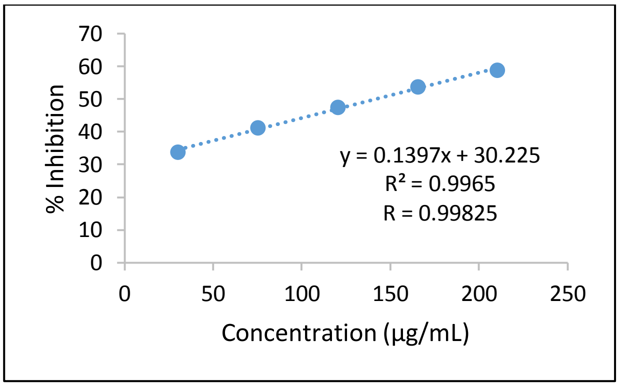
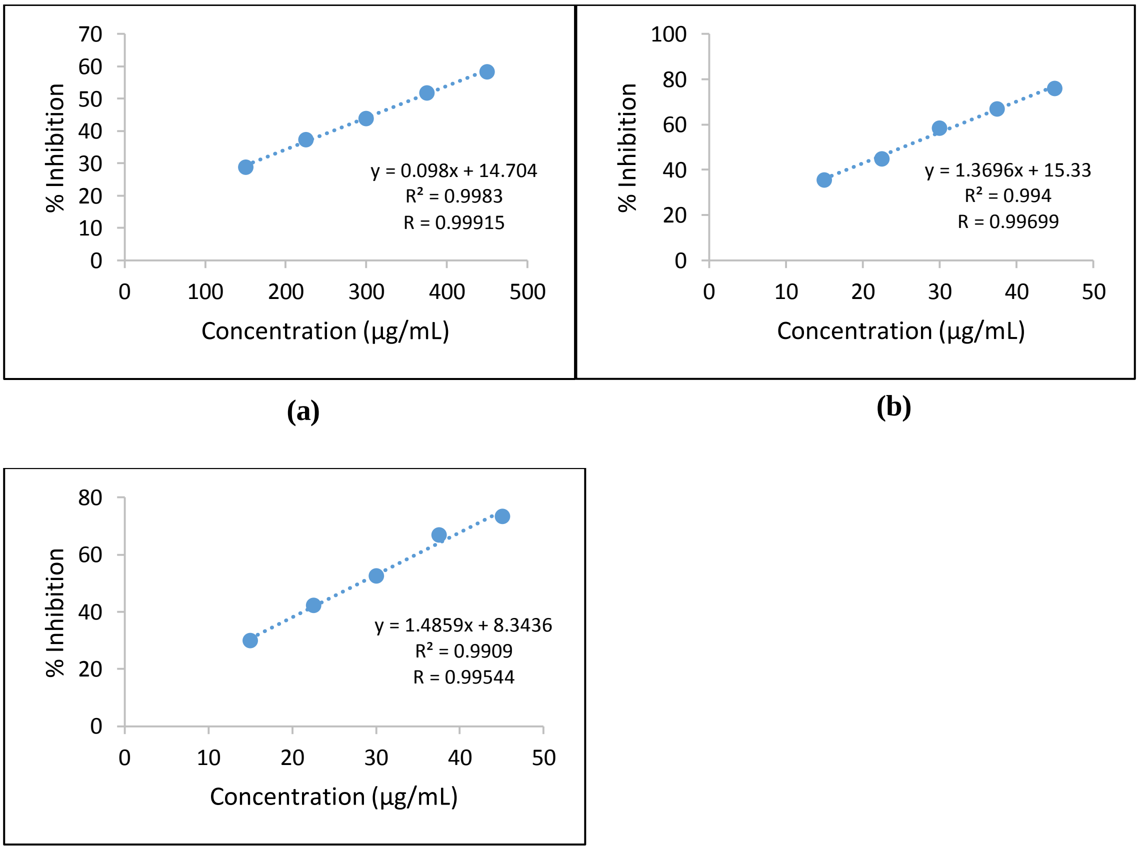


Figure 3. Inhibition test of α-glucosidase by Acarbose graph

Then the three extracts, such as n-hexane, ethyl acetate, and methanol extract were tested. According to the data obtained, ethyl acetate showed the lowest IC_{50} value so that ethyl acetate extract was the most active extract in inhibiting α-glucosidase. This could happen because the chemical substance in ethyl acetate extract work synergically in inhibiting α-glucosidase activity so that the IC_{50} value obtained lower than acarbose. In this IC_{50} value inhibition test, concentration range used for three extract was prepared differently and without repetition, thus the most reactive extract did not fully representative. Therefore, in the further study the concentration variations should be prepared in the same concentrations and in some repetitions to compare the activity of the three extract. Results in the graph, inhibition percentage and IC_{50} value each can be found in Figure and Table below:



(a)

(b)

(c)

Figure 4. Inhibition test of α-glucosidase in (a) n-hexane; (b) ethyl acetate; (c) methanol extract graph

Table 2. % Inhibition and IC$_{50}$ value standard and extract against α-glucosidase

Test Solution	Concentration (μg/mL)	% Inhibition	IC$_{50}$ (μg/mL)
Acarbose	30,084	33,767	141,553
	75,210	41,172	
	120,336	47,478	
	165,462	53,743	
	210,588	58,991	
n-hexane extract	150,06	28,969	360,163
	225,09	37,339	
	300,12	43,948	
	375,15	51,931	
	450,18	58,455	
Ethyl acetate extract	15,002	35,683	25,314
	22,502	44,978	
	30,003	58,414	
	37,504	67,004	
	45,005	76,035	
Methanol extract	15,019	29,912	28,034
	22,529	42,247	
	30,039	52,511	
	37,549	66,784	
	45,059	73,436	

DPPH Wavelength Optimization. Maximum wavelength was obtained by measuring the absorption of control solutions in 501-531 nm to define the maximum absorption. According to the data obtained, the maximum absorption was in 519 nm wavelength.

Antioxidant Test Using DPPH Method. Quercetin and sample standard were tested by measuring the absorption in each concentration in 519 nm wavelength using microplate reader. From the test results, we could determine the % inhibition of each concentration. According to the test, the IC$_{50}$ value of quercetin was 2.505 μg/mL. Study about IC$_{50}$ value of quercetin in in vitro had been performed before and showed a value as 2.4 μg/mL[9]. Although did not differ too much from the IC$_{50}$ value obtained in this study, the different IC$_{50}$ value could be caused by several factors, including different instrument used. IC$_{50}$ value then compared with IC$_{50}$ value extract. Concentrations and % inhibition of quercetin standard solutions of each concentration could be found in Table 3. The graph of quercetin testing result is showed below:

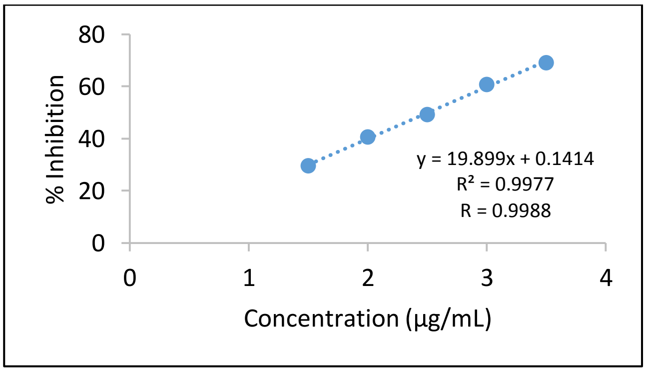
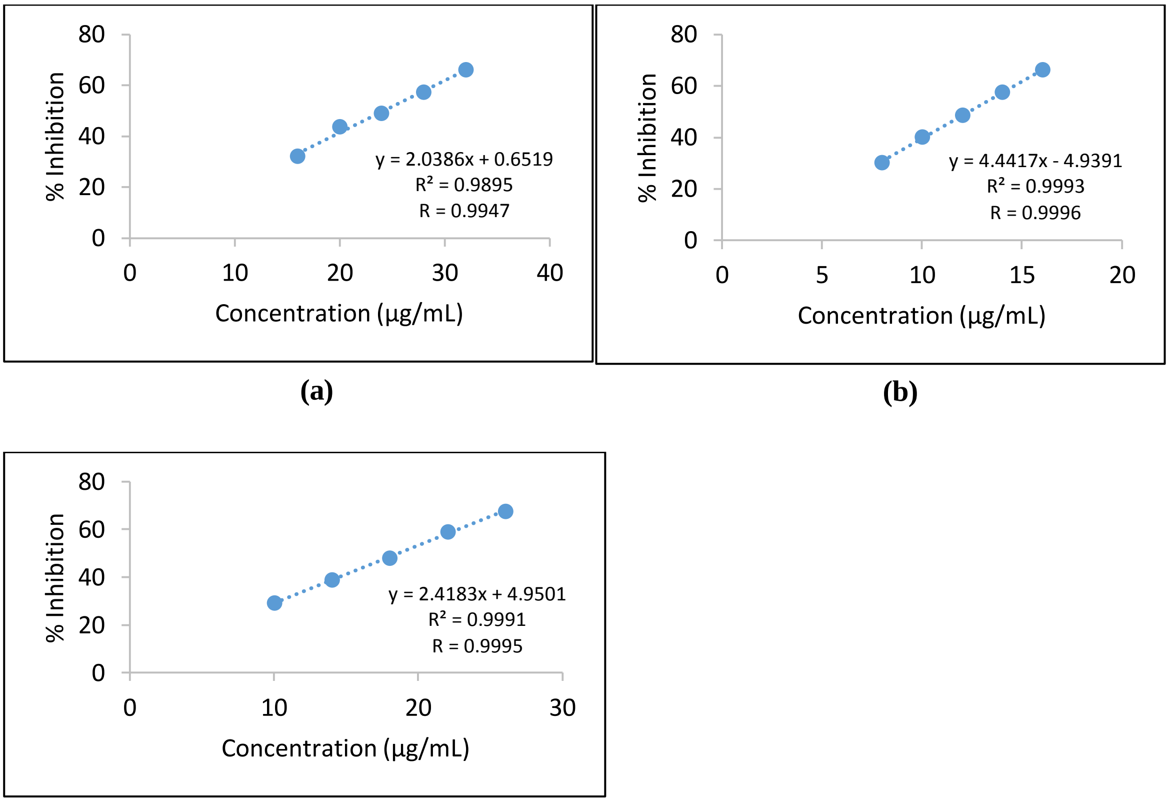


Figure 5. Antioxidant activity test of quercertin graph

Then in the sample testing, we obtained results that could be found in the graph below:



(a) **(b)**

(c)

Figure 6. Antioxidant activity test of (a) n-hexane; (b) ethyl acetate; (c) methanol extract graph

Table 3. % inhibition and IC_{50} value standard and extract against DPPH

Test Solution	Concentration (µg/mL)	% Inhibition	IC_{50} (µg/mL)
Quersetin	1,5	29,495	2,505
	2,0	40,657	
	2,5	49,343	
	3,0	60,758	
	3,5	69,192	
n-hexane extract	16	30,071	24,207
	20	43,638	
	24	48,896	
	28	57,255	
	32	66,036	
Ethyl acetate extract	8,022	30,259	12,369
	10,028	40,169	
	12,034	48,489	
	14,039	57,498	
	16,045	66,137	
Methanol extract	10,028	29,232	18,629
	14,039	38,959	
	18,050	48,107	
	22,062	58,991	
	26,073	67,718	

According to the data obtained from the three extract test, the lowest IC_{50} value was obtained in ethyl acetate extract which showed that this extract had the most active antioxidant activity in the three extract. This was happen because the chemical substances in ethyl acetate extract which provide better antioxidant activity than other extract. However, if this result compared with IC_{50} value of quercetin, the IC_{50} value still was higher. This could happen because quercetin is a pure substance. However, the IC_{50} value showed in ethyl acetate still showed a very high antioxidant activity because it was lower than 50 µg/mL[12]. Similar with the α-glucosidase inhibition test, variations of the three extract to test the antioxidant activity was different and did not prepared in repetition, thus the most active extract was not representative. Therefore, in the further study, the concentration variations should be prepared the same and in some repetitions to define the most reactive extract between the three extract.

Phytochemical Determination. After performing α-glucosidase and antioxidant inhibition activity test, we obtained extract with the most active based on the IC_{50} value which was ethyl acetate extract. Therefore, the phytochemical determination was performed in ethyl acetate extract to define the chemical group obtained in this extract. Result of the study could be found in the following table

Table 4. Phytochemical determination in ethyl acetate extract

Compound	**Reagents**	**Ethyl acetate extract**	**Conclusion**
Alkaloids	Mayer LP Bouchardat LP Dragendorff LP	+ + +	+
Flavonoids	Shinoda Mg Shinoda Zn	+ +	+
Glycosides	Molisch LP	+	+
Terpenoids	Liebermann-Burchard	-	-
Tannins	$FeCl_3$ 3% Pb (II) asetat	+ +	+
Saponin	Hot Water + HCL	+	+
Anthraquinone	Diethyl eter + Ammonia	-	-

Conclusions

Ethyl acetate extract of *Garciniafruticosa*Lauterb. leaves was the most active extract with the lowest IC_{50} value in α-glucosidase (25.314 µg/mL) and antioxidant (12.369 µg/mL) inhibition test. Ethyl acetate extract provide several chemical compound from the phytochemical determination, such as flavonoid, glycoside, tannin, and saponin.

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