Determination $\alpha$-amylase inhibitor activity of methanol extract of coffee leaves using UV-Vis spectrophotometric method and validation

L Wulandari$^1$, Nuri$^1$, D K Pratoko$^1$, P Khairunnisa$^1$, L Muyasaroh$^{1*}$
$^1$Faculty of Pharmacy, University of Jember, Jember, Indonesia

*lestyowulandari@unej.ac.id

Abstract. Diabetes mellitus (DM) is a chronic condition caused by the decrease of insulin. Some studies have shown that coffee leaves act as antidiabetic agents. DM can be characterized by high blood glucose levels. To maintain blood glucose levels in normal conditions can be through the inhibition of carbohydrate catabolism such as inhibition of $\alpha$-amylase. Assay of $\alpha$-amylase inhibition of coffee leaves extracts in vitro using UV-Vis Spectrophotometric method has been developed to obtain optimum condition. The optimum condition of measurement wavelength, incubation time, substrate concentration, enzyme concentration, and DNS concentration was 540 nm, 15 min, 0.5 mg/mL, 0.5 U/mL, and 4.38 ppm respectively. The inhibitor concentration of acarbose and extract was 25 ppm and 500 ppm. Evaluation of the validation method showed linear result $r = 0.9979$ (acarbose); $r = 0.997$ (extract). Detection limit and quantitation limits of acarbose and extract were 4.7377 ppm; 14.2133 ppm and 108.3539 ppm; 325.0618 ppm. RSD (%) of repeatability precision and intermediate precision test for day 1, day 2, and day 3 were 4.899; 4.899; 6.502 and 4.566 for acarbose and 0.394; 0.394; 0.377 and 0.238 for the extract. The accuracy test showed that the IC50 profile decreased as the number of acarbose additions increased. The IC50 of arabica coffee leaves was 286.804 $\mu$g/mL.

1. Introduction
Diabetes is a chronic disease caused by insulin which decreases relatively well or is balanced [1]. Reduced insulin occurs because the pancreas is not enough to produce insulin, causing an increase in blood sugar levels [2]. Diabetes Mellitus is classified into 2 types, namely, type 1 and type 2. Diabetes mellitus (DM) type 2 is a more common type of diabetes, more sufferers than type 1 diabetes. Type 2 DM gets 90% with insulin resistance and insulin deficiency relative [3]. All types of DM can cause complications in parts of the body and overall can increase the risk before the end [2].

The global prevalence of diabetes according to the International Diabetes Federation (IDF) is estimated at 151 million in 2000 [4] and 285 million in 2010 [5]. While in 2011 it reached 336 million people and it was estimated that it reached 552 million in 2030 [6]. In 2050, the adult population of the United States is expected to increase in the number of people with type 2 Diabetes Mellitus reaching 21% to 33% [7]. The high prevalence of diabetes is followed by an increase in the use of oral diabetes drugs and insulin as pharmacological therapies in diabetics. Most oral anti-diabetes drugs have
undesirable side effects, so experts have developed traditional treatment systems for DM that are relatively safe and easy to make [8].

Several studies have shown that coffee leaves plants have compounds that act as antidiabetic agents. There are several compounds contained phytochemistry contained in the methanol extract of Arabica coffee leaves such as alkaloids, flavonoids, saponins, and tannins. One of the flavonoid content in methanolic extracts of Arabica and Robusta coffee leaves contain is chlorogenic acid compounds [9,10]. Chlorogenic acid is a specific competitive inhibitor of glucose-phosphate translocation in rat liver microsomes. Resulting in an inhibition of glucose absorption in the intestine [11].

α-Amylase is the main product released by the pancreas gland (5-6%) and saliva [12]. In addition, the α-amylase enzyme can also be found in microorganisms, animals, plants, fungi (ascomycetes and basidiomycetes), and bacteria (Bacillus) [13].

Some researchers have tested the α-amylase inhibition of various plant extracts in vitro using UV-Vis Spectrophotometry and there are variations in the analysis conditions and test concentrations [14]. The mechanism of α-amylase inhibition in vitro using DNS is based on reducing the amount of reducing sugar resulting from the hydrolysis reaction of dissolved starch by the α-amylase enzyme in the presence of inhibitor compounds. Dissolved starch will be hydrolyzed by the enzyme α-amylase to a shorter oligosaccharides reducing sugar due to the α-D- (1-4) glycosidic disconnection. The end product of the α-amylase hydrolysis reaction is still a mixture of oligosaccharides, such as maltose, maltotriose, and branched oligosaccharides with 6-8 glucose units containing α-1,4 or α-1-6 bonds [12]. Validation, as well as development or modification, needs to be done to adjust to the laboratory conditions used. Due to modification of references analytical method, we validated the new method prior to determination α-amylase inhibitor activity of methanol extract of coffee leaves using uv-vis spectrophotometric method.

2. Methods

The type of research conducted was True Experimental Laboratories. The ingredients used were methanol extract of arabica and robusta coffee leaves; acarbose (Sigma); the enzyme α-amylase (from porcine, Sigma-Aldrich); starch (Merck KGaA); 3,5-dinitrosalicylic acid (Sigma-Aldrich); sodium potassium tartrate; sodium hydroxide; sodium dihydrogen phosphate; sodium hydrogen phosphate; and aquabidest.

The tools used in this study were UV-Vis (Shimadzu) spectrophotometers; micropipette (Socorex); tip; pH meter (Denver Instrument); hot plate; disposable cuvette; digital analytical scales; stirring rod; funnel; and glassware.

The research consisted of several stages, namely the first stage was carried out optimization of analysis conditions to obtain optimum conditions in the analysis (wavelength optimization, optimization of incubation time, optimization of dye reagents, optimization of substrate and enzyme concentrations, and optimization of test concentrations).

2.1. Optimization of Observation Wavelength

Optimization of observation wavelength was done to determine the wavelength that gives a significant signal. The optimum wavelength assessment was done by mixing 25 µl of the α-amylase enzyme solution, and 475 µl of phosphate buffer pH 6.9. Furthermore, pre-incubation at 25°C for 10 minutes was added, 100 µl of starch substrate and incubated for 10 minutes at 25°C. The reaction was stopped by the addition of 400 µl of DNS solution, heated in boiling water for 15 minutes, and then cooled. The resulting solution is read for its absorbance at a wavelength of 400-800 nm.

2.2. Optimization of Incubation Time

Incubation time optimization was done to determine the incubation time needed for amylase inhibition reaction. The Optimization of incubation time was done by making a mixture in accordance with those listed above. Optimization was done when the enzyme mixture, the substrate is added with DNS. The
absorbance was observed starting from minute 0 (after adding DNS), then heating and monitoring were absorbed every 5 minutes for 30 minutes.

2.3. Optimization of the Substrate and Enzyme Concentration
In the optimization of the substrate concentration used was a starch solution with a concentration of 0.1 mg/mL; 0.5 mg/mL; 1 mg/mL; 1.5 mg/mL; and 2 mg/mL. An enzyme with a concentration of 3 U/mL was added to phosphate buffer and incubated. Then the substrate was added with various concentrations above and re-incubated, added DNS and incubated in boiling water. Then, absorbance was observed with UV-Vis Spectrophotometry.

Furthermore, the enzyme concentration was optimized by mixing phosphate buffer and α-amylase enzyme solution at a concentration of 0.1; 0.5; 1; 1.5; 2 and 3 U/mL. The mixture was incubated and 100 µL of starch was added according to the optimized concentration then incubated. 400 µL of DNS was added and incubated in boiling water. Then, absorbance was observed.

2.4. Optimization of Dye Reagent
Optimization of dye reagents was done by adding DNS to the mixture of enzymes and starch substrates with different DNS volumes, namely 200 µL, 400 µL, 600 µL, and 800 µL. The mixture of the resulting solution was read absorbance.

2.5. Optimization of Test Concentration
The validation step of the analytical method starts from the linearity test by making a standard solution of Acarbose and Robusta coffee leaves extract dissolved in phosphate buffer pH 6.9 with 10 levels of concentration in the range of 25-200% of the concentration of the optimized test results. The standard solution was reacted with an enzyme solution, substrate, and coloring reagent according to the previous procedure. Furthermore, absorbance was measured and % inhibition was calculated. Linearity and range parameter values are calculated with a validation program.

Detection limit test (DL) and quantitation limit (QL) were done by making a standard solution of Acarbose and samples of Robusta coffee leaves extract were dissolved in the buffer and made 10 levels of concentration below the linearity concentration, then reacted with enzymes, substrates and coloring reagents. Then the absorbance was measured at the wavelength of the results of optimization. The absorbance value obtained was used to calculate the % inhibition and see the value of the detection limit and the limit of quantitation with the validation program, then determined the value of DL and QL.

Selectivity test was done by comparing the standard response to the response of blank. The test solution was made by reacting the enzyme and phosphate buffer, then incubated and added the DNS and heated. The absorbance was observed and compared between the absorbance of the standard test solution and the absorbance of the blank solution. The blank solution was test solution without enzymes.

The precision tests included repeatability and intermediate precision. The standard curve was made at 6 levels of concentration between 80% -180% of the target concentration. Repeatability test was evaluated by weighing a number of standard Acarbose and Robusta coffee leaves extract samples (three replication) and dissolved it in the buffer then reacted with enzymes, substrates and coloring reagents. Absorbance was measured at 540nm.

The procedure was carried out three times on three different days to determine intermediate precision. The results obtained were then calculated% inhibition, IC50 values, standard deviation (SD) and relative standard deviation (RSD).

Accuracy testing was performed in two stages, preparation of additional samples and determination of inhibition activity. Additional samples consist of samples with standard addition 10%, 20%, 30% and 40%. Determination of inhibition activity of additional samples was made in 3 replications.

Additional samples were reacted with enzymes, substrates and coloring reagents. Then the absorbance was measured at the wavelength of 540 nm. The absorbance value obtained was used to
calculate the % inhibition and IC50. The accuracy parameter value was determined using profile of IC50 value of each accuracy sample.

Testing of α-amylase inhibitor activity in samples was carried out on negative controls (without sample or standard solution), positive control solution (with acarbose solution), and sample solution (with arabica coffee leaves extract). A total of 375 µL of phosphate buffer pH 6.9 and 25 µL of enzyme solution were put into a test tube with a negative control label. 100 µL of acarbose and 25 µL of enzyme solution were put into a test tube with a positive control label. As much as 100 µL of extract sample and 25 µL of enzyme solution were added to the test tube with the sample label (S).

Each mixture of solutions with different labels was incubated for 10 minutes. After the first incubation, the next process can be carried out in determining the optimum concentration of enzymes and substrates. The α-amylase enzyme inhibitory value in each extract was calculated by the following equation:

\[
\text{% inhibition} = \left( \frac{K-S}{K} \right) \times 100\%
\]

Information:

\[K = \text{Absorption of negative controls}\]
\[S = \text{Absorbance of sample / Absorbance of positive control}\]

IC50 was calculated using a linear regression equation with sample concentration as the x-axis and % inhibition as the y-axis. The linear regression equation \( y = bx + a \), used to determine IC50.

\[ IC50 = \frac{(50-a)}{b} \]

3. Results

3.1. Optimization of Wavelength

The results of determination the maximum wavelength used in this study was 540 nm according to the previous research conducted by Subramanian et al [14] that using a wavelength of 540 nm in testing the activity of the α-amylase enzyme.

3.2. Optimization of Incubation Time

The results of determination of the optimum incubation time was 15 minutes because after 15 minutes, the absorbance did not show significant change and give steady state results. It means that the enzymatic reaction finished at the 15 minutes, so that 15 minutes was chosen as the third incubation time in determination of the inhibitory activity of the α-amylase enzyme.

3.3. Optimization of Substrate and Enzyme Concentration

The determination of the substrate concentration used in this study was 0.5 mg/mL because the substrate concentration of 0.05 mg/mL to 1.5 mg/mL gave absorbance that did not change significantly. The result of determining the concentration of the enzyme used was 0.5 U/mL.

3.4. Optimization of Dye Reagent

For the results of the determination of the concentration of DNS as a coloring reagent was at a volume of 400 µL containing 3,5-dinitro salicylic acid as much as 4.38 ppm. And for the results of determining the concentration of test data optimization results of the test concentration can be seen in table 1.

3.5. Optimization of Test Concentration

Based on these data concentrations of 22.643 ppm for acarbose and 506.785 ppm for Robusta coffee leaves extract can inhibit 50% of activity, so the concentration used in this study is 25 ppm for acarbose and 500 ppm for Robusta coffee leaves extract.

Linearity data was evaluated using the Software Validation Method of Analysis. The acceptance criteria of linearity test was correlation coefficient \((r) \geq 0.99\); The coefficient of function variation
(Vxo) <5\% for compounds in metabolites and biological materials and the value of Xp was smaller than the smallest concentration used [15].

The linearity test results for the Acarbose Standard were shown in table 2 and the linearity test results for Robusta coffee leaves extract were shown in table 3.

In DL and QL testing, the value of linearity parameters such as r, Vx0 and Xp must be met first through the Software Validation Method of Analysis, then the DL and QL values can be determined using the same software. DL and QL test results can be seen in Figure 1 dan 2.

| Table 1. Optimization of Acarbose Standard Test Concentration and Robusta Coffee Leaves Extract |
|-----------------------------------------------|----------|---------|---------|
| C (ppm) | IC50 (ppm) | SD     | CV      |
|        |            |        |         |
| 5      |            |        |         |
| 10     |            |        |         |
| 12.5   |            |        |         |
| 15     |            |        |         |
| 17.5   | 22.643     | 1.412  | 6.237   |
| 20     |            |        |         |
| 22.5   |            |        |         |
| 25     |            |        |         |
| 27.5   |            |        |         |
| 30     |            |        |         |
| 100    |            |        |         |
| 200    |            |        |         |
| 300    |            |        |         |
| 400    | 506.785    | 12.399 | 2.446   |
| 500    |            |        |         |
| 600    |            |        |         |
| 700    |            |        |         |
| 800    |            |        |         |

Note:  
C = Concentration  
IC50 = Inhibitory concentration of 50%  
SD = Standard deviation  
CV = Coefficient correlation

| Table 2. Acarbose Standard Linearity Test Results. |
|-----------------------------------------------|----------|
| C(ppm) | %Inhibition |
|        |            |
| 5.122  | 20.676     |
| 10.245 | 28.792     |
| 15.366 | 39.517     |
| 20.489 | 48.213     |
25.612  53.430
30.734  60.386
40.978  78.551
46.101  84.734

**Linear Regression Equations:**
\[ y = 13.9398 + 1.5555x \]
\[ r = 0.9979 \]
\[ V_{xo} = 4.1551\% \]
\[ X_p = 4.7377 \]

**Table 3. Linearity Test Results of Robusta Coffee Leaves Extract**

| C (ppm) | %Inhibition |
|---------|-------------|
| 300.720 | 37.512      |
| 400.960 | 45.167      |
| 501.260 | 52.536      |
| 601.440 | 57.129      |
| 701.680 | 65.933      |
| 801.920 | 75.694      |
| 902.160 | 81.244      |

**Linear Regression Equations:**
\[ y = 15.2475 + 0.0732x \]
\[ r = 0.9970 \]
\[ V_{xo} = 3.0162\% \]
\[ X_p = 108.3539 \]

**Figure 1.** Standard linearity curve of concentration vs % inhibition in DL and QL tests
Figure 2. Linearity curve of sample concentration vs % inhibition in DL and QL tests

From the DL and QL test results, the DL and QL values from the test using acarbose standards and samples of young Robusta coffee leaves extract using the UV-Vis Spectrophotometry method were 4.733 ppm; 108.3539 ppm and 14.2133 ppm; 325.0618 ppm respectively. The DL value indicates the lowest inhibitor concentration that can still be detected using this method. QL value was the lowest inhibitor concentration that can still be quantified in the form of inhibitory values [16].

Figure 3. Selectivity/Specificity Spectra Test

Based on figure 3 it can be shown that the spectra between the test solution (blue curve) and blank solution (red curve). Blank solution was test solution without coffee extracts. It can be concluded that
the wavelength used in this research method was selective and specific because blank solution gave no response of detector that means the response of detector observed was response of test solution.

The RSD value of repeatability test of acarbose and coffe extract were 4.899%; 0.394% respectively. While the RSD value of the intermediated precision test in three days observation between acarbose and coffe extract were 4.899%; 6.502%; 4.566% and 0.394%; 0.377%; 0.238% respectively. RSD values obtained fulfilled the requirements of acceptance criteria of precision tests with analytes content in the sample has concentration ≥0.001% is ≤7.3% and for the concentration ≥0.01% is ≤3.7% [16].

Accuracy test using the standard addition method was done by determination of inhibition profile in the Robusta coffee leaves extract with the addition of acarbose standard of 10%, 20%, 30% and 40%. Based on table 4 and figure 4, it can be shown that the inhibition activity increase while the concentration of additional standard increase [16].

The decrease in IC50 value from the test was due to the addition of the standard acarbose to the extract sample, with the addition of the standard it was able to increase the inhibitory activity in the test so that the IC50 value decreased with the addition of addition.

![Figure 4. Profile of Addition vs IC50](image)

**Table 4. Accuracy Test Results**

| C(ppm) | IC50 Addition |
|---|---|
| 100 | 297.131 | 199.712 | 151.312 | 55.781 |
| 200 | 300 | 297.131 | 199.712 | 151.312 | 55.781 |
| 300 | 300 | 297.131 | 199.712 | 151.312 | 55.781 |
| 400 | 300 | 297.131 | 199.712 | 151.312 | 55.781 |
| 500 | 300 | 297.131 | 199.712 | 151.312 | 55.781 |
| 600 | 300 | 297.131 | 199.712 | 151.312 | 55.781 |
| 700 | 300 | 297.131 | 199.712 | 151.312 | 55.781 |
| 800 | 300 | 297.131 | 199.712 | 151.312 | 55.781 |
The results of the α-amylase enzyme inhibition activity test of Arabica coffee leaves extract were indicated by IC50 values. So, the smaller the IC50 value, the greater the inhibitory activity of the α-amylase enzyme. Figure 5 shows the inhibitory activity profile of the α-amylase enzyme extract of Arabica coffee leaves where IC50 of Arabica coffee leaves extract is at a concentration of 286,804 µg/mL.

**Figure 5. %α-Amylase Inhibition Curve of Arabica Coffee Leaves Extract**

4. Conclusion
Validation tests performed provide valid analysis results that were linear, sensitive, selective/specific precise and accurate. The results of inhibitory activity (IC50) of Arabic coffee leaves extract was 286.804 µg/mL.

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