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Diterpene Lactones from *Andrographis paniculata* and *In Vitro* Studies on α-Amylase and α-Glucosidase Inhibitory Properties

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ABSTRACT

Plants contain chemical compounds that have medicinal properties. *Andrographis paniculata* (bitter herb) is commonly used as a traditional medicine, including treating hyperglycemia. *A. paniculata* was known to contain major compounds, terpenoids, and flavonoids. The phytochemical investigation aimed to isolate and identify chemical compounds from the aerial part of the bitter herb and then evaluate the antihyperglycemic properties. *A. paniculata* was extracted using 96% ethanol. The extract was partitioned with three organic solvents successively to obtain the chloroform fraction, ethyl acetate fraction, and methanol fraction. Isolation was carried out on the ethyl acetate fraction using column chromatography with a silica stationary phase and an organic solvent mobile phase in various ratios. The isolate in the form of white powder was found in subfraction E8 with the mobile phase chloroform-ethyl acetate (1-2). The structure of the isolated compounds was characterized using spectroscopic methods, including 1D NMR (^{1}H and ^{13}C), 2D NMR (HSQC and HMBC), and LCMS. The isolated compounds were evaluated *in vitro* with α-amylase and α-glucosidase tests. The results of the data analysis indicated the identification of 14-deoxy-11,12-didehydroandrographolide and 14-deoxyandrographolide as the isolated compounds. The isolated compounds showed α-amylase inhibitory activity with an inhibitory concentration (IC_{50}) of 167.31±4.92 µg/ml and α-glucosidase inhibitory activity with an IC_{50} of 267.10±6.72 µg/ml. The results of this study indicate that paniculata contains diterpene lactones, which are active in hyperglycemia therapy.

1. Introduction

In Indonesia, bitter herbs have been empirically used as a traditional Indonesian herbal medicine (jamu) for health disorders such as diabetes mellitus (Ministry of Health of the Republic of Indonesia 2012). Bitter herb is a well-known medicinal plant that has been widely used in traditional Asian medicine for centuries. This plant was also found in formulas in medicinal systems in China (Traditional Chinese Medicine) and India (Ayurveda) (Abas *et al.* 2016).

Water extract from *A. paniculata* leaves showed anti-type 2 diabetes activity *in vivo* study in white rats based on their metabolic profile (Abas *et al.* 2016). Studies show that the ethanol extract of *A. paniculata* and the andrographolide compound have potent activity towards inhibiting the enzymes α-amylase and α-glucosidase. Alpha-amylase and alpha-glucosidase are enzymes that play a role in carbohydrate digestion. By inhibiting the activity of these two enzymes, the absorption of glucose from food becomes slower, thereby preventing spikes in blood glucose levels after eating. *In vivo* studies show that ethanol extract of *A. paniculata* and andrographolide compounds can significantly reduce blood glucose levels (antihyperglycemia) in rats (Subramanian *et al.*

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2008). Improper handling of hyperglycemia can cause complications associated with coronary artery disease, cerebrovascular disease, renal failure, blindness, limb amputation, and neurological disorders (Sunil *et al.* 2019).

Phytochemical investigations were carried out to determine the chemical compounds contained in the extract and determine their activity. In previous research, diterpene lactones were found in *A. paniculata* extracts such as andropaniosides A, andropaniosides B, two new ent-labdane diterpenoid glucosides (My *et al.* 2020), andrograpanin, andrographolide, andropanolide, andrographidine A, andrographidine F (Hong Hanh *et al.* 2020), 15-spiro diterpenoid dimer (Sun *et al.* 2020), and ent-labdane diterpenoid dimers (Gao *et al.* 2019). Chemical compounds play an important role in therapeutic activity because they have structures and properties that enable specific interactions with biological targets in the body, such as enzymes, receptors, or proteins.

This study aims to discover diterpene lactone compounds and their biological activity. The stages of this study were isolation, structural elucidation from the aerial parts of *A. paniculata* growing in Indonesia, and in vitro study about the inhibition activity of α-amylase and α-glucosidase effects from the isolate. Inhibition of these enzymes has become an important strategy in controlling type 2 diabetes mellitus.

2. Materials and Methods

2.1. Plant Material

The selected dried aerial parts of *A. paniculata* Nees were collected from Yogyakarta, Indonesia, and identified by the Environmental Biology Laboratory, Faculty of Biology, Jenderal Soedirman University, Indonesia. A voucher specimen (No. 2020–2021) has been deposited in the herbarium of the Department of Pharmacy, Jenderal Soedirman University.

2.2. Extraction and Fractionation

1000 g *A. paniculata* powder was extracted by maceration using 96% ethanol for 3 × 24 hours. The extracted sample was evaporated to obtain the ethanolic extract (115.75 g). The crude extract was defatted with hexane. The defatted extract was fractionated by silica gel vacuum column chromatography with 500 ml chloroform, 500 ml ethyl acetate, and 500 ml methanol as solvents. Fractionation produced a chloroform fraction of 33.24 g, an ethyl acetate fraction of 40.37 g, and a methanol fraction of 28.44 g.

2.3. Isolation

The ethyl acetate fraction was subjected to silica gel column chromatography and eluted with increasing solvent polarity. The solvents used were chloroform, chloroform-ethyl acetate (7-3, 5-5, 3-7), ethyl acetate, ethyl acetate-methanol (7-3, 5-5, 3-7), methanol to yield 18 subfractions (E1-E15). Fraction E8 was purified by silica gel column chromatography eluted with chloroform-ethyl acetate (1-2) to obtain a white powder compound (74.2 mg).

2.4. Identification

The structural formula characterized by the isolate uses the method of spectroscopy, which includes 1D NMR (^{1}H and ^{13}C), 2D NMR (HSQC and HMBC), and LCMS. 1H NMR data ($CDCl_3$, 500 MHz) and 13C NMR data ($CDCl^3$, 125 MHz). The LCMS analysis was measured on Waters Xevo-G2 XS QTof. Separation was performed using Waters BEH C18 column as a stationary phase, and the mobile phases consisted of acetonitrile (B) and Milli-Q water (A) supplemented with 0.1% formic acid.

2.5. *In Vitro* Assay of α-amylase and α-glucosidase Inhibition

In vitro is a test conducted outside of living organisms. In this study, α-amylase and α-glucosidase tests were conducted in buffer media that resemble the physiological conditions of the body (especially pH and temperature of the digestive tract).

The inhibitory activity of diterpene lactones isolate (14-deoxyandrographolide and compound 2 and 14-deoxy-11,12-didehydroandrographolide) on α-amylase and α-glucosidase were investigated.

2.5.1. Determination *In Vitro* Assay of of α-amylase Inhibition

In vitro, testing of α-amylase was carried out in a phosphate buffer pH 6.9 that approximates the physiological conditions where the α-amylase enzyme works in the oral cavity and small intestine.

Twenty-five µL of 2 U/ml α-amylase solution in phosphate buffer pH 6.9 was put into a microplate, then 25 µL of FFBA solution of various concentrations was added and incubated at 25°C for 10 minutes. Then 25 µL of 0.5% starch solution was added and again incubated at 25°C for 10 minutes. The reaction was stopped by adding 50 µL of DNS (dinitro salicylic acid) solution, and then the microplate was incubated in boiling water for 10 minutes. If the solution is at room temperature,

absorbance readings using spectrophotometry are carried out at a wavelength of 540 nm.

The absorbance value was then calculated to determine the percentage value of enzyme inhibitory activity.

$$\text{Percentage of inhibitory activity} = \frac{\left(\text{Abs control} - \text{Abs diterpene lactones}\right)}{\text{Abs control}} \times 100$$

The IC_{50} values were determined from plots of percent inhibition versus log concentration. This is calculated by linear regression analysis of the average inhibitor value. Acarbose was used as a standard inhibitor of alpha-amylase. Tests were performed in triplicate.

2.5.2. Determination *In Vitro* Assay of α-glucosidase Inhibition

In vitro, testing of α-glucosidase was carried out in a phosphate buffer pH 6.9 that approximates the physiological conditions where the α-amylase enzyme works in the small intestine.

Sixty µL of 0.2 UI/ml α-glucosidase solution in 0.1 M phosphate buffer pH 6.9 was put into a microplate. Then 60 µL of FFBA solution with various concentrations and acarbose as a comparison were added. 30 µL of 0.5 mM p-nitrophenyl-α-D-glucopyranoside (pNPG) in 0.02 M sodium phosphate buffer pH 6.9 as substrate was added into the microplate. The solution was incubated at 37°C for 30 minutes. The reaction was stopped by adding 40 µL of 0.2 M sodium carbonate solution. The solution was cooled to room temperature, and the absorbance was read at a wavelength of 410 nm.

The absorbance value was then calculated to determine the percentage value of enzyme inhibitory activity.

$$\text{Percentage of inhibitory activity} = \frac{\left(\text{Abs control} - \text{Abs diterpene lactones}\right)}{\text{Abs control}} \times 100$$

The IC_{50} values were determined from plots of percent inhibition versus log concentration. This is calculated by linear regression analysis of the average inhibitor value. Acarbose was used as a standard inhibitor of alpha-glucosidase. Tests were performed in triplicate.

3. Results

3.1. Identification of Diterpene Lactone Isolates

The results of the identification of isolated compounds from *A. paniculata* based on spectroscopy include 1D NMR (1H and 13C), 2D NMR (HSQC and HMBC), and LCMS.

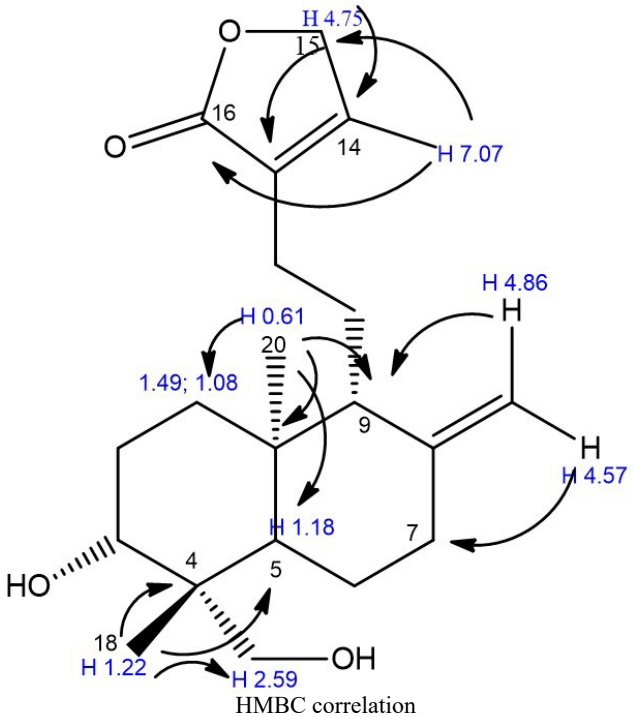
Compound 1 was obtained as 14-deoxyandrographolide. Its LCMS analysis (m/z 333.94 $[M^+]$; 316.92 $([M\text{-}H_2O]^+$; , 298.65 $[M\text{-}2H_2O]^+$). The ^{1}H-NMR spectrum of 1 showed one angular methyl at δ_H 0.61 (3H, s), one tertiary methyl at δ_H 1.18 (1H, td), one hydroxymethyl at δ_H 3.45 (1H, m), two double bonds including eco-cyclic methylene δ_H 4.57 (1H, s) and δ_H 4.86 (1H, s), trisubstituted double bond δ_H 7.07 (1H, t), one oxymethylene group at δ_H 3.45 (1H), one oxymethylene group δ_H 1.22 (1H). Analysis of ^{13}C-NMR and heteronuclear single quantum coherence (HSQC) presence of five sp^2 carbons including one carbonyl carbon δ_C 174.57 and four olefinic carbons δ_C 109.39; 134.87; 144.26 and 146.97, 15 sp^3 carbons which one oxymethyne at δ_C 80.81, one oxymethylene at δ_C 22.90 two methylenes, two methyls, and nine methynes indicated the molecular formula $C_{20}H_{30}O_4$. This is shown in Table 1, compound 1. Compound 1 was elucidated as an ent-labdane monoterpenoid featuring a tricyclic system comprised of spiroketal moiety, and this structure is named 14-deoxyandrographolide. Heteronuclear multiple bond connectivity confirmed this feature (HMBC) correlation, shown in Figure 1A.

Compound 2 was 14-deoxy-11,12-didehydroandrographplide (m/z 331.29 $([M\text{-}1]^+$); 313.33 $[M\text{-}H_2O]^+$; 295.56 $[M\text{-}2H_2O]$). The ^{1}H-NMR spectrum of 1 showed one angular methyl at δ_H 0.79 (3H), one tertiary methyl at δ_H 1.14 (1H, dd), one hydroxymethyl at δ_H 3.45 (1H, m), three double bonds including exocyclic methylene δ_H 4.50 (1H, d) and δ_H 4.77 (1H, dd), trisubstituted double bond δ_H 7.15 (1H, t) and a trans- disubstituted olefin δ_H 6.84 (1H, dd) and δ_H 6.09 (1H, d), one oxymethyne group at δ_H 3.45 (1H), one oxymethylene group δ_H 1.27 (1H). Analysis of 13C-NMR and heteronuclear single quantum coherence (HSQC) presence of seven sp^2 carbons including one carbonyl carbon δ_C

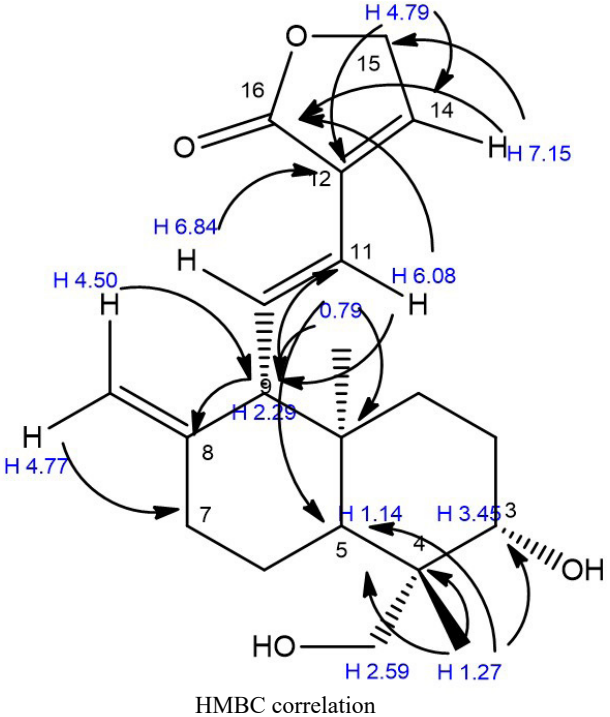
Table 1. Data HNMR and CNMR of compound 1 and compound 2

14-deoxyandrographolide			14-deoxy-11,12-didehydroandrographolide		
Position	δ_C [(a,b)]	δ_C [(a,c,d)] mult. (J in Hz)	Position	δ_C [(a,b)]	δ_C [(a,c,d)] mult. (J in Hz)
1	36.76	1.08 dd 4, 10 1.49 tt 3,5; 7	1	36.76	1.91 td 5,8 2.29 d 10
2	28.30	1.73	2	28.30	1.73
3	80.81	3.45	3	80.81	3.45
4	43.07	-	4	43.07	-
5	55.46	1.18 td 2,5; 5	5	55.46	1.14 dd 2; 5,5
6	24.17	1.29 t 4 1.73	6	24.17	2.05
7	38.36	2.05 2.41	7	38.36	2.41 1.91 td
8	146.97	-	8	146.97	2.59 s
9	56.21	1.57 m	9	56.21	-
10	39.23	-	10	39.23	2.29 d
11	22.11	1.57 1.73	11	22.11	- 6,84 dd 5,5;
12	23.16	1.32 dd 4;8,5 1.73	12	23.16	10 6.09 d 6,75
13	134.87	-	13	134.87	-
14	144.26	7.07 t	14	144.26	7.15 t
15	70.34	4.75 d	15	70.34	4.79 d
16	174.57	-	16	174.57	-
17	109.39	4.57 s 4.86 s	17	109.39	4.50 d 4.77 dd
18	64.40	2.59 s	18	64.40	2.59 s
19	22.90	1,22	19	22.90	1.27
20	15.42	0.61 s	20	15.42	0.79

[a]Recorded in CDCl3, [b]125MHz, [c]500MHz, [d]overlapped signal are shown without multiplicity



HMBC correlation
A



HMBC correlation
B

Figure 1. (A) HMBC correlation of compound 1 (14-deoxy andrographolide), (B) HMBC correlation of compound 2 (14-deoxy-11,12-didehydroandrographplide)

172.5 and six olefinic carbons δ_C 107.59; 121.30, 129.46; 136.20 and 143.17, 13 sp^3 carbons which one oxymethyne at δ_C 81.04, one oxymethylene at δ_C 22.85, two methyls and nine methynes indicated the molecular formula $C_{20}H_{28}O_4$. This is shown in Table 1, compound 2. Compound 1 was elucidated as an ent-labdane monoterpenoid featuring a tricyclic system comprised of spiroketal moiety, and this structure is named 14-deoxyandrographolide. This feature was confirmed by a heteronuclear multiple bond connectivity (HMBC) correlation, shown in Figure 1B.

3.2. *In Vitro* Assay of α-amylase and α-glucosidase Inhibition

There, the result inhibitory activity of diterpene lactones isolates (14-deoxyandrographolide and compound 2 and 14-deoxy-11,12-didehydroandrographolide) on α-amylase and α-glucosidase. The α-amylase is shown in Figure 2, and α-glucosidase is shown in Figure 3.

The α-amylase inhibition of diterpene lactones isolate (14-deoxyandrographolide and compound 2 and 14-deoxy-11,12-didehydroandrographolide) was compared with acarbose. The diterpene lactones isolate had an IC_{50} of 167.31±4.92 µg/ml. The α-amylase inhibition of the diterpene lactones isolate was lower than compared to acarbose, which had an IC_{50} of 55.44±3.23 µg/ml.

Alpha-glucosidase inhibition of diterpene lactones isolate (14-deoxyandrographolide and compound 2 and 14-deoxy-11,12-didehydroandrographolide) was compared with acarbose. The diterpene lactones isolate had an IC_{50} of 267.10±6.72 µg/ml. The

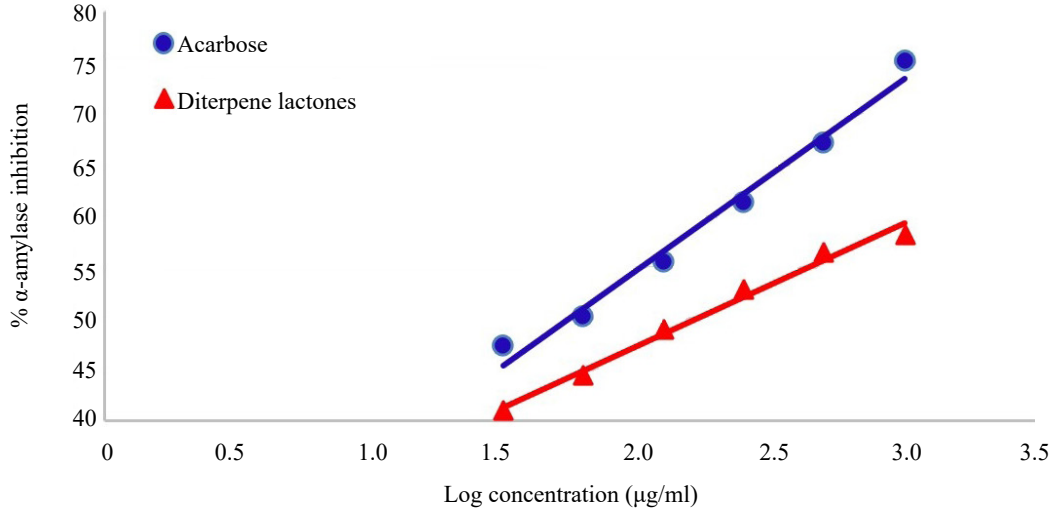


Figure 2. % α-amylase inhibition diterpene lactones compared to acarbose

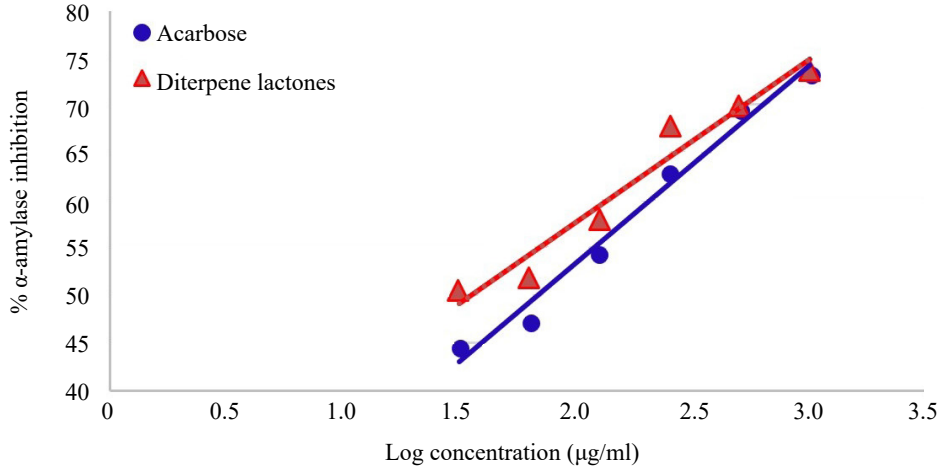


Figure 3. % α-glucosidase inhibition diterpene lactones compared to acarbose

α-glucosidase inhibition of the diterpene lactones isolates was higher than compared to acarbose, which had an IC_{50} of 363.92±6.66 µg/ml.

4. Discussion

The results of the isolation and identification of the *A. paniculata* ethanol extract in this study were 14-deoxyandrographolide and 14-deoxy-11,12-didehydroandrografolide, which are later referred to as diterpene lactones isolate. Previous research has identified both compounds. In previous research, several methods have been applied to obtain isolation of this compound. The compounds 14-deoxyandrographolide and 14-deoxy-11,12-didehydroandrographolide were obtained from the ethanol extract of *A. paniculata*, which was fractionated using a chromatography column with silica and the eluent CH2CL2/MeOH (70:1 to 0:1) preparative HPLC (MeOH/H_2O, 45:55) (Wen *et al.* 2020). Hexane was used to defatten the crude ethanol extract, which was subsequently separated into methanol and chloroform. To create different fractions, the methanol fraction was subjected to silica gel column chromatography and eluted with chloroform-methanol. To obtain these two compounds, all fractions were subsequently submitted to repeated column chromatography on silica gel and eluted with varying amounts of the chloroform-methanol solvent system (Shaikh *et al.* 2019). Isolation 14-deoxy-11,12-didehydroandrographolide using two-dimensional High-speed counter-current chromatography (HSCCC) from extract *A. paniculata* (Sun *et al.* 2019). Cold extraction of powdered dry leaves and stem of *A. paniculata* was done with dichloromethane: methanol (1:1). The extract was subjected to column chromatography with increasing polarity of eluting solvent-major phytochemicals of the plant 14-deoxy-11,12-di-dehydroandrographolide (Sharma *et al.* 2018).

One of the mechanisms of antihyperglycaemic action was the inhibition of α-amylase and α-glucosidase. The research was carried out to determine how much of the activity mechanism of inhibitory α-amylase and α-glucosidase of diterpene lactones is isolated *in vitro* by means of spectroscopic photometry. *In vitro* tests on the inhibitory activity of α-amylase and α-glucosidase enzymes were carried out in pH 6.9 buffer media, which were similar to the physiological conditions of the oral and small intestine digestive tracts.

The α-amylase and α-glucosidase enzyme belongs to the hydrolase enzyme group. The α-amylase enzyme is an enzyme that has the activity of breaking down the bonds in starch to form maltose. Ptyalin amylase enzyme, which is an amylase enzyme produced by the salivary glands, is tasked with breaking down carbohydrates or starch into simple sugars in the mouth. The amylase enzyme that the pancreas produces is known as pancreatic amylase. The α-glucosidase is an enzyme that catalyzes the conversion of complex carbohydrates, oligosaccharides, and disaccharides into monosaccharides and glucose, which then enter the intestinal lumen and bloodstream. This enzyme can increase blood sugar levels (Nair *et al.* 2013; DiPiro *et al.* 2020; Magaji *et al.* 2020).

The results of the correlation coefficient values of alpha-amylase from acarbose and diterpene lactone isolate showed that the higher the concentration of the isolate, the higher its inhibitory activity. Based on the slope value, the increase in acarbose concentration against alpha-amylase inhibitory activity was sharper than in diterpene lactones isolate inhibiting activity. The diterpene lactones isolate compound showed lower α-amylase inhibitory activity with an inhibitory concentration (IC_{50}) of 167.31±5.92 µg/ml compared to acarbose, a standard reference drug with an IC_{50} value of 55.44±3.23 µg/ml. This suggests that the inhibitory activity of alpha-amylase on acarbose is still better than diterpene lactones isolate.

The results of the correlation coefficient values of alpha-glucosidase from acarbose and diterpene lactone isolate showed that the higher the concentration of the isolate, the higher its inhibitory activity. Based on slope values, increases in acarbose concentration against alpha-glucosidase inhibitory activity were sharper than in diterpene lactones isolate inhibiting activity. The isolated compound showed stronger α-glucosidase inhibitory activity with an IC_{50} of 267.10±39.72 µg/ml compared to acarbose, a standard drug with an IC_{50} value of 363.92±30.66 µg/ml. It suggests that the inhibitory activity of alpha-glucosidase of diterpene lactones isolate is better than acarbose. The acarbose slope sharpness of both graphs indicates that with the addition of acarbose concentrations, there will be a significant increase in the inhibitory activity of alpha-amylase and alpha-glucosidase.

The previous study carried out the activity of alpha-amylase and alpha glycosidase on ethanol extracts of *A. paniculata* and andrographolide isolates. The

A. paniculata extract showed α-amylase inhibitory effect on concentration IC_{50} = 50.9±0.17 mg/ml and α-glucosidase inhibitory activity IC_{50} = 17.2±0.15 mg/ml. Andrographolide isolate showed alpha-amylase inhibitory activity with an IC_{50} value of 11.3±0.29 mg/ml and α-glucosidase inhibitory activity with an IC_{50} value of 11.0±0.28 mg/ml (Subramanian *et al.* 2008).

14-deoxy-11,12-didehydro-15-(4-ethoxybenzylidene) andrographolide was found to have the highest potential as an α-glucosidase inhibitor with an IC_{50} value of 160±5.1 µM. This is a significant improvement over the clinical dose of acarbose, which had an IC_{50} value of 390±8.1 µM. 15-benzylidene derivative of 14-deoxy-11,12-didehydroandrographolide with a 1,3-dioxane moiety at C(3) and C(19), 14-deoxy-11,12-didehydro-3,19-(2′-hydroxybenzylidene)-15-(2-hydroxybenzylidene) andrographolide, also showed good inhibition with IC_{50} 260±13 µM. These isolates from *A. paniculata* have the potential for antihyperglycemic (Ly *et al.* 2020).

Diterpene lactones isolate, compared to standard acarbose drugs, showed the mechanism of alpha-glucosidase inhibition activity was better than alpha-amylase. The inhibitory activity of alfa glucosidases of diterpene lactone isolates was stronger than acarbose. This suggests that the diterpene lactones isolate has a good antihyperglycemic potential through the inhibitions of alpha-glucosidase.

The mechanism of action of diterpene lactones isolate on alpha-amylase was to inhibit the enzyme α-amylase in the hydrolysis of the α-1,4 internal glucoside binding of the polysaccharide (Ajayi *et al.* 2021). The mechanism of the diterpene lactones isolate action on the activity of α-glucosidase was to inhibit the enzyme α-glucosidase in the hydrolysis of p-nitrophenyl-α-D-glucopiranoside into p- nitrophenol and glucose. By inhibiting this enzyme, it will decrease the rate of glucose absorption, thus reducing the postprandial glucose surge. Reducing postprandial hyperglycemia was one of the therapeutic strategies for diabetes in the early stages (Kashtoh and Baek 2022). This finding represents an opportunity for the development of diabetes mellitus drugs through the mechanisms of inhibition of alpha-amylase and alpha-glucosidase. The inhibitory activity of alpha-amylase and alpha-glucosidase of lactone-filled isolates is likely due to the presence of the chemical structure of unsaturated lactones. These diterpene lactones isolated from *A. paniculata* results provide new possibilities for the optimization of antidiabetic medications in the future.

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