



## $\alpha$ -Amylase Inhibition Activities by Flavonoid Compounds from Panda Plants (*Kalanchoe tomentosa*)

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### Abstract

Diabetes mellitus (DM) is one of the major health problems. One way to deal with diabetes mellitus is by inhibiting the work of enzymes that hydrolyze carbohydrates, thereby reducing glucose absorption. The enzyme that plays a role in the breakdown of starch into simple sugars is the  $\alpha$ -amylase. The genus *Kalanchoe* or Panda plant has been widely reported to contain a variety of secondary metabolites that have several activities such as antimalarial, antibacterial, antidiabetic, and anticancer potential. This study aims to isolate, determine the chemical structure, and test the inhibitory activity of  $\alpha$ -amylase from the ethyl acetate fraction of *K. tomentosa* leaves. The isolation stage began with the maceration process with *n*-hexane and fractionated with ethyl acetate. Ethyl acetate extract was refracted using a gradient liquid vacuum chromatography (KCV) method (*n*-hexane: ethyl acetate: methanol). Repeated gravity columns separated the fraction of KCV results and preparative thin-layer chromatography (TLC-P) to obtain isolate pure. Isolate pure was characterized using several spectroscopic methods, including UV-Vis, IR, NMR. Determination of the structure of the compound against isolate pure shows that this isolate is a 3,7,4'-trihydroxyflavonol or kaempferol compound. The results of the test activity of 3,7,4'-trihydroxyflavonol compounds in inhibiting the enzyme  $\alpha$ -amylase in vitro showed an IC<sub>50</sub> value of 34.6  $\mu$ U/mL. The results obtained indicate that the IC<sub>50</sub> value is higher than that of the Acarbose, which was 39.3  $\mu$ U/mL.

### 1. Introduction

Diabetes mellitus is a disease caused by abnormalities in insulin secretion, insulin action, or both. It is also a metabolic disease characterized by hyperglycemia or an increase in blood sugar levels. Normal fasting blood sugar levels range from 70 to 105 mg/dL [1], while fasting blood sugar levels that reach 126 mg/dL are categorized as a hyperglycemic state. This situation can induce tissue damage and excessive free radical production. If the amount of antioxidants in the body is not sufficient, the body will experience oxidative stress and cause complications of diabetes [2, 3]. Complications in the form of disorders in other organs that cause the emergence of new diseases such as hypertension, neuropathy, coronary heart disease, and peripheral vascular. Acarbose, metformin, voglibose are drugs that have been used to control blood sugar by

inhibiting the work of digestive enzymes. Several studies [4, 5] show that the use of antidiabetic drugs such as acarbose, metformin, or voglibose has side effects such as gastrointestinal disorders (diarrhea and flatulence). Therefore, the development of antidiabetic drugs that have low side effects is needed.

One alternative that can be done is the use of natural ingredients and the development of antidiabetic drugs from active compounds of natural ingredients using plant extractive substances as antidiabetic drugs that work to inhibit the activity of digestive enzymes, one of which is  $\alpha$ -amylase. Inhibition of the  $\alpha$  amylase enzyme is one strategy that can be used to reduce blood sugar levels during (postprandial glucose) in people with diabetes in preventing disease complications resulting from hyperglycemic conditions. Several studies have shown that the bioactive component of the flavonoid compound

in the genus *Kalanchoe* is able to inhibit the action of the  $\alpha$ -amylase enzyme [6]. The problem to be revealed in this study is to characterize the flavonoid compounds from *K. tomentosa* extract and the activity of the resulting flavonoid compounds to inhibit the action of the  $\alpha$ -amylase enzyme.

## 2. Methodology

### 2.1. Equipment

Laboratory glassware, a set of maceration tools, blenders, chambers, capillary pipes, vial bottles, analytical balance, tweezers, Ultraviolet lamps of Vilber Lourmat brand VL-8.LC, rotary evaporator brands of Heidolph Laborota 4000, UV lamps of Vilbert Luomart ( $\lambda$  254 nm and 365 nm), vacuum liquid chromatography, Hewlett Packard 8453 Ultraviolet spectrophotometer, 500 MHz  $^1\text{H-NMR}$  spectrometer

### 2.2. Material

*Kalanchoe tomentosa* leaves, distilled water, various organic solvents for separation (acetone, ethyl acetate, dichloromethane, methanol, *n*-hexane) with technical grade redistillation, chloroform pa (Merck), silica GF<sub>254</sub> (Merck), GF<sub>254</sub> Thin Layer Chromatography plates (Merck), silica gel 60 (0.2–0.5 mm) (Merck), silica gel 60 G (Merck), 5% AlCl<sub>3</sub> reagent in ethanol, kaempferol as standard in isolate pure and acarbose as a control in the  $\alpha$ -amylase test

### 2.3. Material preparation

20 kg of fresh *K. tomentosa* leaves were cleaned of impurities and mashed with a blender until a thick pulp of *K. tomentosa* leaves was obtained.

### 2.4. Extraction and isolation

The thick pulp of the *K. tomentosa* plant was extracted using *n*-hexane solvent for 3x24 hours at room temperature. The *n*-hexane extract obtained was filtered, and the solvent evaporated using a vacuum rotary evaporator at 40°C so that the concentrated *n*-hexane extract (9 g) was obtained. Then the concentrated *n*-hexane extract was macerated with ethyl acetate for 3x24 hours at room temperature. The ethyl acetate extract was evaporated using a vacuum rotary evaporator at 40°C to obtain concentrated ethyl acetate extract (13 g). The yield produced was 0.065%.

Concentrated ethyl acetate extract was separated by its chemical composition using the column chromatography method with the stationary phase is silica gel G<sub>60</sub> (70–230 mesh), with a mobile phase eluent variation of *n*-hexane: ethyl acetate which has a gradient to produce 25 fractions. The obtained fraction was then separated using TLC with the eluent of *n*-hexane: ethyl acetate (6: 4) to then proceed with the merging of the fractions, resulting in 10 merging fractions, i.e., fraction A – J. The H fraction formed a precipitate, the precipitate was recrystallized using *n*-hexane slowly, then decantation was carried out on the precipitate. Next, the precipitate obtained was separated using TLC with various solvents to test the purity of the isolates. The

purity test was then performed and compared with kaempferol standards.

### 2.5. Structure Determination

The chemical structure of pure compounds was determined using spectroscopic methods, which include Ultraviolet (UV), Infrared (IR), and  $^1\text{H-Nuclear Magnetic Resonance}$  ( $^1\text{H-NMR}$ ) as well as comparison of spectra data obtained with various literature.

### 2.6. Enzyme Inhibition Test [7]

A total of 1 mL of extract was made with a concentration of 25 and 50 ppm and then added 1 mL (1% w/v) of starch solution. Next, pre-incubation with 1 mL  $\alpha$ -amylase enzyme (100x dilution) was carried out. The mixture was incubated at 37°C for 10 minutes, and the reaction was stopped with the addition of 1 mL DNS. The mixture is then heated in boiling water for 20 minutes, followed by cooling at 25°C. After that, an additional 1 mL of arsenic molybdate was added, followed by 7 mL of distilled water. Blanks were prepared without extracts and the  $\alpha$ -amylase enzyme in phosphate buffer pH 7. Acarbose (an anti-diabetic drug) was used as a positive control (1 tablet dose of 50 mg dissolved in 10 mL of distilled water. Absorbance measurements were carried out at a wavelength of 540 nm.

## 3. Results and Discussion

Isolate 1 obtained was a yellow solid which dissolve entirely in methanol. Thin-layer chromatography treatment showed that isolate 1 glowed under UV light at  $\lambda$  of 254 and 365 nm. This shows the existence of conjugated double bonds. Whereas measurements by UV spectroscopy showed that isolate 1 glowed under UV light.

UV spectrum of isolate 1 gave maximum absorption at  $\lambda_{\text{max}}$  of 264 nm ( $\log \epsilon$  3.81) and 364 nm ( $\log \epsilon$  3.80), which showed the presence of a conjugated alkene system and the presence of an aromatic system. The maximum UV absorption of the flavonoid compound at 257 nm (Band II), 377 nm (Band I), is the main framework in the form of flavones or substituted 3-OH flavonols [8]. Next, to determine the functional group of isolate 1, an FTIR analysis was performed. Based on the IR spectra, isolate 1 has an absorption peak at wave number 3412  $\text{cm}^{-1}$  indicating the presence of OH phenol functional groups that have hydrogen bonds. Sharp absorption of about 1606  $\text{cm}^{-1}$  and 1498  $\text{cm}^{-1}$ , which is a stretch of the aromatic C=C functional group and is supported by absorption bands at 833  $\text{cm}^{-1}$  (substituted benzene) in the fingerprint area. While the absorption at wavenumbers 1606  $\text{cm}^{-1}$  to 1710  $\text{cm}^{-1}$  indicates the presence of a carbonyl group C = O.

Furthermore, isolate 1 was analyzed by  $^1\text{H-NMR}$  spectroscopy giving absorption as follows,  $^1\text{H-NMR}$  i.e. (500 MHz, Acetone-*d*<sub>6</sub>)  $\delta$ 12.18 (s, 1n), 8.15 (dd, *J* = 6.9, 2.3 Hz, 2n), 7.02 (dd, *J* = 6.9, 1.7 Hz, 2n), 6.54 (d, *J* = 1.7 Hz, 1n), 6.27 (d, *J* = 2.3 Hz, 1n), 3.31 (s, 0n), 1.29 (d, *J* = 8.6 Hz, 2n). In the  $^1\text{H-NMR}$  spectra, isolate 1 showed a singlet signal at  $\delta$ H 12.179 ppm, indicating the presence of a -OH group in C-5 ring A adjacent to C=O carbonyl in the C-8 ring (Figure 1). This can show the characteristic that isolate 1

belongs to the group of flavonoid compounds. According to Syah [9], a common feature of flavonoid compounds is the presence of -OH groups at C-5 in the <sup>1</sup>H-NMR spectrum, which is indicated by the presence of a singlet signal at a shift of around 11-14 ppm. Furthermore, there is a 2H signal at δH 6.272 and 6.543 ppm with a value of J = 1.7 Hz and J = 2.3 Hz coupled with meta. This is an aromatic proton signal from C-6 and C-8, so in C-7, there is an -OH group. Then two aromatic proton signals of 2H were detected at δH 7.022 and δH 8.162 ppm, which coupled ortho with a value of J = 6.9 Hz with double doublet multiplicity (dd). This shows the presence of monosubstituted and H benzene signals in C-3' and C-6' in ring B, and there is an -OH group at C-4'. Syah [9] states that the proton signal H-2/H-6 appears as dd due to ortho coupling from H-3 and H-5 and meta coupling from H-4. So, from these results, it can be interpreted that the main skeleton of isolate A is identical to the skeleton of dihydroflavonol compounds.

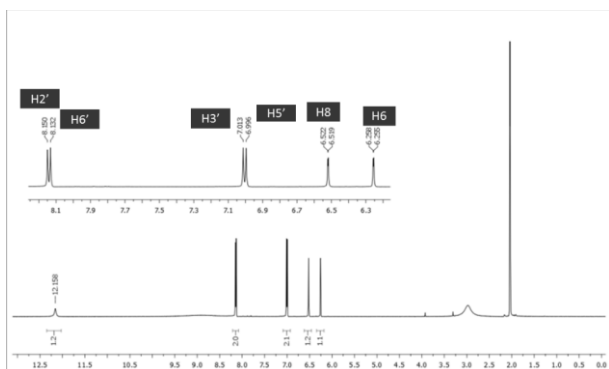


Figure 1. <sup>1</sup>H-NMR of isolate 1

According to Syah [9], the dihydroflavonol group is characterized by a pair of doublet signals for H-2 and H-3. The H-2 signal appears at δH around 5-6 ppm and H-3 at δH around 4.8 ppm, followed by the -OH signal not widening and coupling with the H-3 signal so that the signal will appear as a double doublet (dd) with small J values. Also, Syah [9] also states that benzene aromatic proton signals typically appear in the 6-8 ppm range, where these signals overlap with alkenes at δH 5-7 ppm. Then, if it is seen from the value of J, that the highest J value for aromatic derivatives is 7-9 Hz for ortho couplings, 1-3 Hz for meta couplings, and 0-2 Hz for para couplings.

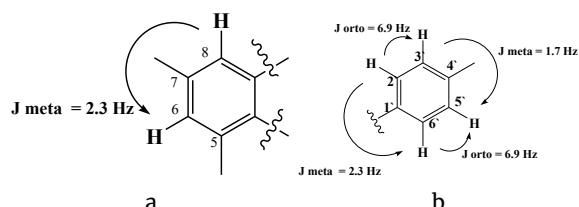


Figure 2. Prediction of the structure of rings A (a) and Ring B (b)

The presence of δH at 3.31 ppm and δH 1.29 ppm indicates an alkyl group bound to an aromatic alkene. Syah [9] states if other hereditary functional groups are in the area of 0.8-1.6 ppm, 1-4 ppm, and 2-3 ppm with details: The alkane derivative methyl group is at δH 1 ppm,

methylene group at δH 1-1.5 ppm, methyl group at δH 1.6 ppm, and alkyl groups attached to aromatic alkene at δH 2-3 ppm.

Furthermore, isolates 1 performed <sup>1</sup>H-NMR data comparisons of compounds 5,7,4'-trihydroxyflavonols or kaempferol referring to Aisyah *et al.* [10] as presented in Table 1 and the TLC comparison analysis as shown in Figure 2.

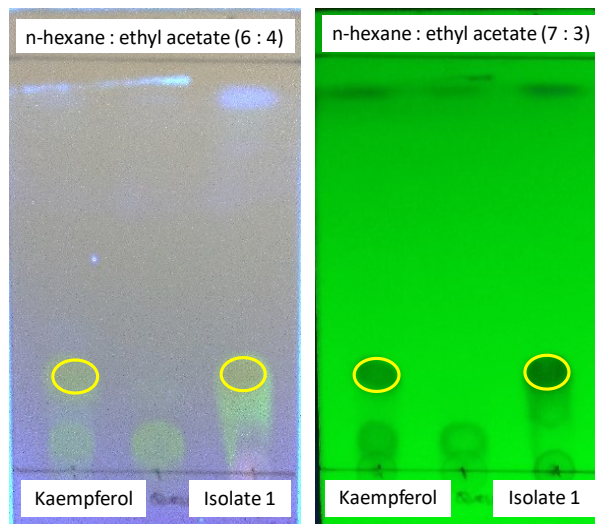


Figure 3. Comparison of TLC between isolate 1 with standard Kaempferol.

Table 1. Comparison of <sup>1</sup>H-NMR spectroscopy data between isolate 1 and kaempferol [10]

Position	Pure Isolate 1	Kaempferol
	δ <sub>H</sub> (ppm)(H, mult, J= Hz)	δ <sub>H</sub> (ppm)(H, mult, J= Hz)
2' 6'	8.15 (2H, dd, J = 6.9, 2.3 Hz)	8.17 (2H, d, J = 6,8 Hz, H-2' and H-6')
3' 5'	7.02 (2H, dd, J = 6.9, 1.7 Hz)	7.01 (2H, d, J = 6,8 Hz, H-3' and H-5')
8	6.54 (1H, d, J = 1.7 Hz, 1n)	6.52 (1H, d, J = 1.95 Hz, H-8),
6	6.27 (1H, d, J = 2.3 Hz,)	6.28 (1H, d, J = 1.95 Hz, H-6)
	3.31 (s, 0n)	-
	1.29 (1H, d, J = 8.6 Hz)	-

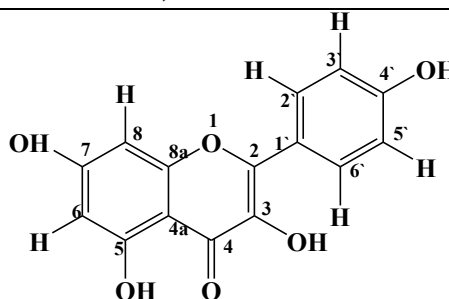


Figure 4. Structure of kaempferol compound

The α-amylase inhibition test was performed to determine the decrease in the activity of the α-amylase enzyme in the breakdown of starch. If more maltose is produced from a starch means that more starch is hydrolyzed into maltose and glucose. The higher the

absorbance value produced, the more glucose in the sample [11]. This means, if the enzyme activity is inhibited, less sugar is formed and observed with a decrease in the measured absorbance value. The method used in the  $\alpha$ -amylase enzyme inhibition test is to refer to Bhutkar and Bhise [7] with a slight modification. The test results are shown in Table 2.

Table 2. Results of  $\alpha$ -amylase inhibition test

Control Test		$\alpha$ -amylase inhibition		IC <sub>50</sub> value ( $\mu$ U/mL)
Sample	Concentration	Content	unit	
Enzyme *	-	319.35	mU/mL	-
Acarbose **	25 ppm	163.63	mU/mL	39.2
	50 ppm	145.01	mU/mL	
Extract	25 ppm	300.23	mU/mL	346
	50 ppm	298.73	mU/mL	
Isolate	25 ppm	349.18	mU/mL	379.8
	50 ppm	298.26	mU/mL	

Description \*: Negative control, \*\*: Positive control

In this study,  $\alpha$ -amylase enzyme activity was observed as a decrease in blue color intensity in the iodine-starch complex due to reduced starch substrate concentration due to hydrolysis by the  $\alpha$ -amylase enzyme [12] and increase of glucose or maltose content formed.

Then to find out the linear regression equation from the data, a standard curve was made between the % inhibition of the amylase enzyme to each concentration of extract, isolate, and acarbose. Linear regression graphs of extracts, isolates, and acarbose are presented in Figure 4.

The results showed an increase in the value of  $\alpha$ -amylase inhibition, along with the increase in concentration. This showed the influence of the concentration of the enzyme  $\alpha$ -amylase on extracts, isolates, and acarbose. The %inhibition value of extracts and isolates is lower than the % inhibition of acarbose, so the IC<sub>50</sub> value of extracts and isolates is greater than the IC<sub>50</sub> value of acarbose. This shows that extracts and isolates can inhibit enzymes with weaker inhibition when compared to acarbose, where the greater the IC<sub>50</sub> value, the weaker the inhibitory power. In flavonoid compounds such as quercetin, myricetin, luteolin, and kaempferol, IC<sub>50</sub> values are smaller than 500  $\mu$ M. According to Tadera *et al.* [13], if extracts and isolates were compared, it can be seen that the IC<sub>50</sub> value in extracts is lower than the IC<sub>50</sub> value in isolates. This happens because there are still many other compounds in the extract, whereas in isolates, there is only one pure compound.

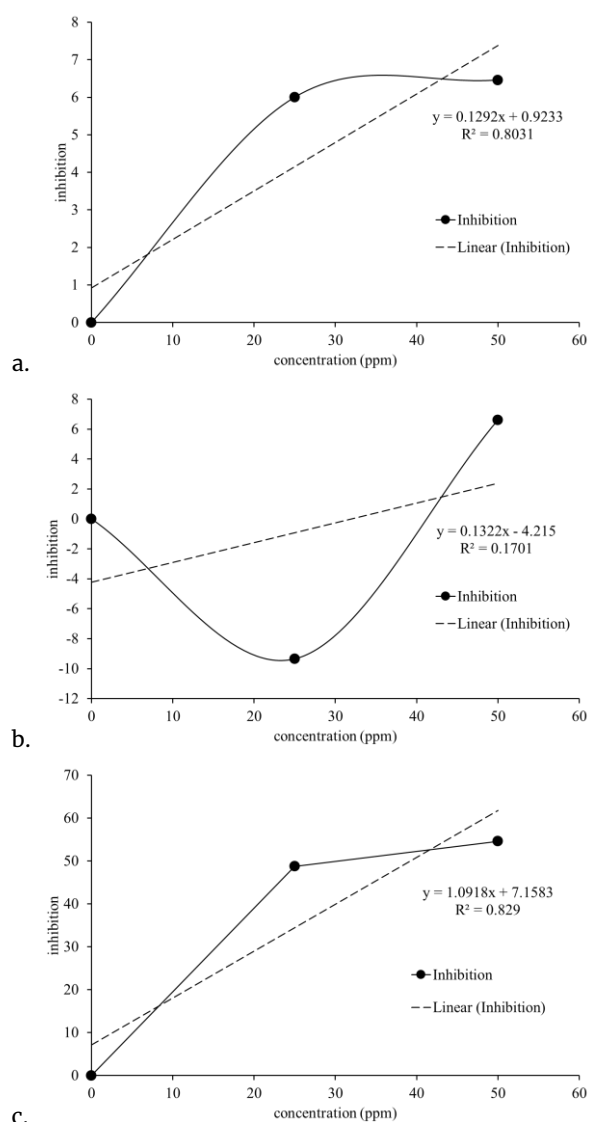


Figure 4. The  $\alpha$ -amylase inhibition curve (a) extract (b) isolate 1 (c) acarbose

The mechanism of action of  $\alpha$ -amylase consists of two stages: the first stage in the form of amylose degradation to maltose and maltotetraose, which occurs randomly, followed by a decrease in viscosity. The second stage is the formation of glucose and maltose as the final result. The  $\alpha$ -amylase has carboxyl and nitrogen groups on the active side. If the action of this enzyme is not inhibited, the enzyme will form an adsorption complex with the substrate in which the glycosidic binding position of the substrate is in a position facing each other with the carboxyl groups of the substrate and the imidazole group. This carboxyl group attacks the nucleophile C-1 of the starch substrate to neutralize the imidazole chain. The neutralizing of the imidazole group is the basis for breaking the polysaccharide chain in starch. If the action of the  $\alpha$ -amylase enzyme is inhibited by a compound or acarbose (a drug commonly used in people with diabetes), the process does not take place that way. The C-1 side of the substrate does not bind to the carboxyl group of enzymes but is bound by unsaturated cyclohexane rings and glycosidic nitrogen found in the acarbose structure. This causes the active site of the enzyme to be difficult to reach the breaking region,



namely the 1,4  $\alpha$  chain and the OH group in the inhibitor that interacts with the carboxyl group from the active site of the enzyme. The binding process of the substrate is hypothesized in Figure 5.

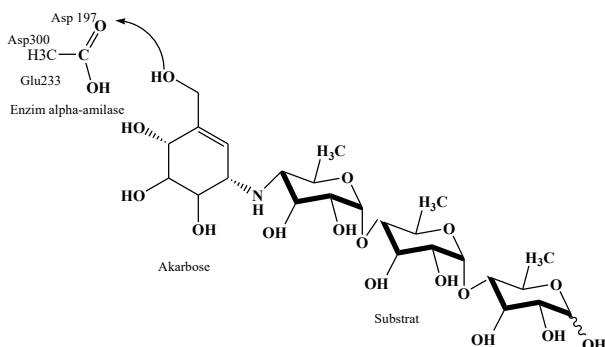


Figure 5. Binding of the substrate by acarbose

The similarity of the structure of acarbose with the structure of kaempferol compounds, which contain many hydroxy groups, is one of the factors that can increase the inhibition of  $\alpha$ -amylase. This is reinforced by the idea of Daud AK *et al.* [14], which states that flavonoids of the group of dihydroxy flavonols can inhibit the activity of  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes. In the process of enzyme inhibition, flavonoids act as  $\alpha$ -amylase inhibitors through the interaction of OH groups on C3' and C4' on the B ring with the active site of the enzyme [14]. According to Ariani *et al.* [15], hydroxy groups in ring A and ring B in flavonoid compounds increase the ability of enzyme inhibition. Hydroxy groups C3' and C4' are the main factors in the inhibition of the  $\alpha$ -amylase enzyme. The dihydroxy groups C3' and C4' on ring B play a role in interactions with the active site of the  $\alpha$ -amylase enzyme through hydrogen bonds.

Meanwhile, the presence of OH groups in C3' serves to maintain the flavonoids to be bound to the active site of the enzyme appropriately. The oxygen present in the hydroxyl group in C3', C4' in ring B, and C3 in ring C plays a role in binding with hydrogen from the active site of the enzyme. In general, the molecular interactions of flavonoids with proteins are divided into two types: first, the Van der Waals interaction type, in which nonpolar aromatic rings can disperse interactions with amino acid residues, and secondly, electrostatic interactions. Then the type of hydrogen bonds is the most important interaction with the role of OH groups that act as hydrogen donors as well as hydrogen acceptors of amino acids and peptide bonds [16].

The interaction process that occurs in isolate 1 is the interaction of hydrogen bonds in the presence of OH groups in the structure of kaempferol compounds. Then in isolate 1 there was also a deviation in the activity value where the activity of isolate 1 at 25 ppm was higher than the value of enzyme activity without the addition of extracts. This is likely because the carboxyl group on the active site of the enzyme is still able to reach the glycosidic bonds and attack the nucleophile C on the substrate, causing a deglycosylation reaction or the breaking of the sugar chain. This occurs because the nitrogen group of the inhibitor does not bound the

nucleophile on the C-1 substrate. After all, it is known that in the kaempferol structure, there is no nitrogen group as in acarbose. The binding process of kaempferol compounds to the substrate and enzymes can be seen in Figure 6.

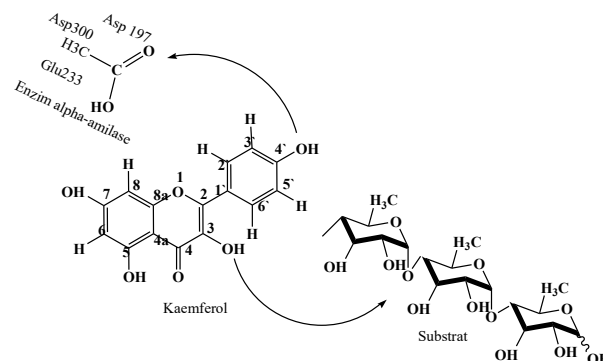


Figure 6. Binding of compounds to substrate and enzymes

#### 4. Conclusions

Based on the results of the study, it can be concluded that the isolation of the compound from the crude extract of *K. tomentosa* ethyl acetate leaves produced a secondary metabolite compound identified as a flavonoid compound and has a structural similarity to the synthetic antidiabetic compound, kaempferol. The  $\alpha$ -amylase enzyme inhibitory activity test showed that the IC<sub>50</sub> values of crude ethyl acetate and isolate 1 were 346  $\mu$ U/mL and 379.8  $\mu$ U/mL, respectively. The crude extract of ethyl acetate and the kaempferol compound showed a higher IC<sub>50</sub> value compared to the acarbose control, which had a value of 39.3  $\mu$ U/mL.

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