β-Sitosterol Compound from Dichloromethane Extracts of Kalanchoe tomentosa (Crassulaceae) Leaves and Inhibition of α-amilase Activity

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Abstract

Kalanchoe tomentosa is one of the plants of the Crassulaceae tribe that can lower blood sugar and contains compounds of terpenoids, flavonoids, alkaloids, saponins, tannins, fatty acids, steroids, and triterpenoids. This study aims to isolate and identify chemical compounds from dichloromethane extract of Kalanchoe tomentosa leaves, as well as to test the inhibitory activity of the α-amilase enzyme. Extraction was carried out by maceration using dichloromethane as a solvent, then dichloromethane extract was purified using column chromatography, the pure isolate was obtained in the form of white powder, and an inhibition test was carried out against the α-amilase enzyme. The thin layer chromatography data of pure isolates compared to pure β-sitosterol are similar. Based on the research data, it can be concluded that the chemical structure of the pure isolate is β-sitosterol, the methylene chloride extract of K. tomentosa leaves has an inhibitory activity against the α-amilase enzyme with an inhibition value of 65%. This value is greater than the positive control of acarbose which only has 37% inhibition and β-sitosterol compound by 6.7%. This value is smaller when compared to the control of acarbose at the same concentration which obtained 5% inhibition.

Keywords: α-amilase, β-sitosterol, Crassulaceae, enzyme, Kalanchoe tomentosa.

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1. INTRODUCTION

Diabetes mellitus (DM) is a metabolic disease that has become a serious problem in the world. According to the International Diabetes Federation (IDF, 2013), Indonesia is included in the top 10 countries with the highest diabetes cases, to be precise at number 7 with 10 million diabetes cases. Up until now, diabetes has still become one of the biggest causes of death in Indonesia. This was proved by the Data Sample Registration Survey in 2014 where diabetes is the 3rd cause of death in Indonesia after stroke and coronary heart disease (Kemenkes, 2014). Diabetes mellitus cannot be cured, but diabetes mellitus can be controlled. Treatment given to DM patients aims to normalize blood sugar levels and prevent complications. The patient's blood sugar level will be kept in the range of 80-130 mg / dL before meals, and below 180 mg / dL two hours after eating (Widodo, 2014). Controlling postprandial glucose levels is an important strategy in preventing type 2 diabetes, so that a therapeutic approach can be taken by delaying glucose absorption by inhibiting carbohydrate hydrolysis enzymes, such as α-glucosidase in the digestive organs (Kim et al., 2005). Some of the inhibitors that have been used clinically are acarbose and miglitol which inhibit glycosidases such as α-glucosidase and α-amilase. However, many hypoglycemic agents have limitations since they cause side effects and increase diabetes complications. The main side effects of α-glucosidase inhibitors on the gastrointestinal tract including bloating, nausea, diarrhea, and fluctuation. Natural α-glucosidase inhibitors derived from natural ingredients can be used as a therapeutic approach to treat postprandial hyperglycemia because they have low side effects and are more affordable than synthetic anti-hyperglycemic drugs (Li et al., 2005).

Based on this information, researchers are encouraged to develop natural herbal
medicines to treat diabetes mellitus. More than 400 types of plants have been shown to have hypoglycemic activity because they contain compounds that have anti-diabetic properties such as polysaccharides, proteins, flavonoids, alkaloids, terpenoids and steroids (Kim et al., 2006).

One of the plants that has anti-diabetic properties is the Kalancheoe plant. Kalancheoe is known as a medicinal plant in traditional medicine. The Kalancheoe genus is reported to have contained bufadienolides, triterpenoids, flavonoids, triterpenoids and steroids (Saleh et al., 2014).

An amount of research has been conducted upon the Kalancheoe genus, but not much research on Kalancheoe tomentosa was conducted. Aisyah et al., (2017) have reported a flavonoid that has cytotoxic activity against P-388 murine leukemia cells from ethyl extracts of K. tomentosa. Saleh et al, (2014) have reported antioxidant activity of various K. tomentosa extracts, where the extracts were methylene chloride extract, ethyl acetate extract, and n-butanol extract with the IC$_{50}$ value of 71.3 µg / mL, 35.4 µg / mL, 99.3 µg / mL, respectively.

Based on the report, it is necessary to do further research on K. tomentosa plants considering that these plants have never been tested for hypoglycemia activity. The objective of this study was to determine the inhibitory activity of the α-amylase enzyme from β-sitosterol compounds found in K. tomentosa.

2. MATERIALS AND METHODS

Instruments

Laboratory glassware, a set of maceration tools, Blender (Philips HR 2102), chamber, capillary tube, vials, analytical balance (Mettler AE 260 DeltaRage), tweezers, ultraviolet lamp (Vilber Lourmat VL-8.LC), rotary evaporator (Heidolph Laborota 4000), a set of chromatography tools with vacuum liquid, infrared spectrophotometer (Shimadzu Prestige-21), 1H-NMR spectrometer (JEOL JNM-ECZ500R/S1 500 MHz).

Materials

Kalanchoe tomentosa leaves, distilled water, acetone (CH$_3$COCH$_3$), ethyl acetate (CH$_3$COOC$_2$H$_5$), dichloromethane (CH$_2$Cl$_2$), methanol (CH$_3$OH), n-hexane (C$_6$H$_{14}$), chloroform (CHCl$_3$) pa, F$_{254}$ Thin Layer Chromatography plate (Merck), 60 (0.2- 0.5 mm) silica gel (Merck), 60 G silica gel (Merck), amylase enzyme, phosphate buffer (pH 7)

3. RESULT AND DISCUSSION

White color needle crystal isolate was obtained from the isolation process. The isolate dissolved in polar solvents and A comparison of TLC analysis of compound 1 with β-sitosterol from the plant Kalancheoe blossfeldiana showed that Rf value and spots
obtained were very similar. Therefore, isolate was identified as β-sitosterol (Fig 1). Isolated crystal needles were observed to melt at 145-148 °C.

Based on the results of TLC, IR and 1H-NMR, isolate 1 is similar with the pure β-sitosterol compound from Kalanchoe blossfeldiana plant (figure 1). FTIR Analysis: The IR spectrum (Fig. 2) showed absorption peaks at 3415.93 cm⁻¹ (O‐H stretching); 2945.30 cm⁻¹ and 2866.22 cm⁻¹ (aliphatic C‐H stretching); 1649.14 cm⁻¹ (C=C absorption peak). Other absorption peaks include 1454.33 cm⁻¹ (CH₂); 1371.39 cm⁻¹ (OH def), 1047.35 cm⁻¹ (cycloalkane).

**Figure 1.** Comparison of standard β sitosterol with precipitated compound 1 (a) and Chemical Structure of β-sitosterol compound (b)

![FTIR spectra of compound 1](image)

**Figure 2.** FTIR spectra of compound 1

![Chemical shift of the proton compound 1](image)

**Figure 3.** Chemical shift of the proton compound 1
Table 1. Proton chemical shift of the compound 1 and β-sitosterol

| C  | δH (ΣH, m, J Hz) Compound 1** | δH (ΣH, m, J Hz) β-sitosterol *) Yun, 2015 |
|----|--------------------------------|---------------------------------------------|
| 1  | 1.05 : 1.02 (2H, dd, 10.5 : 5.5)| 1.07 : 1.02 (2H, dd, 10.5 : 5.5)            |
| 2  | 1.44 : 1.47 (2H, td, 9.5 : 6.0) | 1.44 : 1.48 (2H, td, 9.5 : 6.0)             |
| 3  | 3.49 (1H, m)                   | 3.51 (1H, m)                                |
| 4  | 2.23 : 2.31 (2H, m)            | 2.22 : 2.29 (2H, m)                         |
| 5  | -                              | -                                           |
| 6  | 5.35 (1H, t, 2.5)              | 5.35 (1H, br)                              |
| 7  | 1.85 : 2.01 (2H, dt, 5.6 : 8.5)| 1.85 : 2.01 (2H, dt, 5.6 : 8.5)             |
| 8  | 1.60 (1H, m)                   | 1.57 (1H, m)                                |
| 9  | 0.93 (1H, m)                   | 0.93 (1H, m)                                |
| 10 | -                              | -                                           |
| 11 | 1.42 : 1.47 (2H, m)            | 1.42 : 1.49 (2H, m)                         |
| 12 | 1.15 : 1.95 (2H, d, 5.6)       | 1.15 : 1.98 (2H, d, 5.6)                    |
| 13 | -                              | -                                           |
| 14 | 1.01 (1H, m)                   | 1.00 (1H, m)                                |
| 15 | 1.63 (2H, m)                   | 1.57 (2H, m)                                |
| 16 | 1.95 (2H, m)                   | 1.84 (2H, m)                                |
| 17 | 1.09 (1H, dt, 5.2 : 8.5)       | 1.09 (1H, dt, 5.2 : 8.5)                    |
| 18 | 0.68 (3H, s)                   | 0.68 (3H, s)                                |
| 19 | 1.01 (3H, s)                   | 1.01 (3H, s)                                |
| 20 | 1.31 (1H, m)                   | 1.36 (1H, m)                                |
| 21 | 0.69 (3H, d : 6.1)             | 0.92 (3H, d, 6.1)                           |
| 22 | 1.31 (2H, m)                   | 1.38 (2H, m)                                |
| 23 | 1.50 (2H, m)                   | 1.54 (2H, m)                                |
| 24 | 0.94 (1H, m)                   | 0.93 (1H, m)                                |
| 25 | 1.66 (1H, m)                   | 1.66 (1H, m)                                |
| 26 | 0.84 (3H, d, 6.2)              | 0.84 (3H, d, 6.2)                           |
| 27 | 0.92 (3H, d, 6.7)              | 0.92 (3H, d, 6.7)                           |
| 28 | 1.25 (2H, m)                   | 1.26 (2H, m)                                |
| 29 | 0.82 (3H, s)                   | 0.83 (3H, s)                                |

**1H-NMR CDCl₃, 500 MHz
*1H-NMR aseton-d₆, 500 MHz

Figure 4. Graph of the absorbance value of the sample
Figure 4 showed that the absorbance without the presence of enzymes is higher than the absorbance with the presence of enzymes. This occurred after the addition of the enzyme. The extract able to inhibit the hydrolysis of starch when enzymes are added. Starch or amylase will be easier to hydrolyze into simple molecules. The nature of the enzyme is the catalyst which can speed up the reaction without reacting. The activity of the α-amylase enzyme can be determined based on the absorbance value obtained. The α-amylase enzyme activity test was carried out to determine the ability of the α-amylase enzyme to hydrolyze starch into simple sugars. According to Saleh et al. (2014), the final product of α-amylase is oligosaccharide of varying length and α-configuration and α-limit dextrin which consists of a mixture of maltose, maltotriose, and oligosaccharide branches (6-8 glucose units) which contain α-1-4 and α-1-6.

The activity of the α-amylase enzyme was observed as a decrease in the intensity of the blue color in the iodine-starch complex due to reduced starch substrate due to hydrolysis carried out by the α-amylase enzyme. The blue color of the iodine-starch complex can be observed by spectrophotometer. The darker blue color shows the excess amount of starch. This occurred due to the formation of iodine and starch complexes where iodine is trapped in the helical structure of starch (amylose).

Figure 5 showed the specific activity of the enzyme in determining the inhibition percentage of each extract. An enzyme activity unit is the amount of enzyme that causes a change of 1 µmol (10-6) of the substrate per minute at 25 °C under optimum conditions. The specific activity is the number of enzyme activity units per milligram of protein (Saleh et al., 2014). In the control test for inhibition of the α-amylase enzyme, acarbose was used since acarbose is an antidiabetic drug used to inhibit the action of the α-amylase enzyme, inhibits carbohydrate processes in the digestive system, and reduces glucose absorption thereby preventing postprandial plasma glucose rise (Saleh et al., 2014). This medicine helps lower blood sugar levels after eating. According to Andayani et al., (2009), therapeutic combination of sulfonylurea, metformin, and acarbose in type 2 diabetes mellitus patients whose blood glucose control is poor, can lead to the risk of developing complications and drug side effects.

Testing the sample with the addition of methylene chloride extract with a concentration of 5% gave an inhibition value of the amylase enzyme of 65%, while the sample added with the pure compound β-sitosterol with the same concentration gave an inhibition value of 6.7%. When compared with the control drug, acarbose, at the same concentration with 37% inhibition of methylene chloride extract had a greater inhibitory value, while the pure compound β-sitosterol had a smaller inhibition value. According to Saifudin, (2014), crude extracts...
show an active or more active effect with positive control, often semi-pure ingredients have a loss of pharmacological effects. This is due to the synergistic effect, in which the presence of one or several compounds causing the strengthening of the active effect, where a single pure compound has no effect or is very weak. Separation causes weak activity or no activity. The percentage of inhibition of pure α-sitosterol at a concentration of 5% was not too high, which was only 6.7%. It was caused by the structure of the β-sitosterol compound where there are not many –OH groups present. The –OH group is able to act as a compound that can neutralize free radicals, thus preventing damage to pancreatic beta cells that produced insulin.

4. CONCLUSION

Based on the results of the research, it can be concluded that the isolate was obtained as a white powder with the form of a needle. It is assumed that isolate 1 is a β-sitosterol compound. The methylene chloride extract of K. tomentosa leaves has inhibitory activity against the α-amylase enzyme with an inhibition value of 65%. This value is greater than the positive control of acarbose which only has 37% inhibition and β-sitosterol compounds of 6.7%. This value is smaller compared with the acarbose at the same concentration of 5%.

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