ATLANTIS PRESS

Advances in Engineering Research, volume 190

International Conference of CELSciTech 2019 - Science and Technology track (ICCELST-ST 2019)

Isolation, Characterization, in Vitro and in Silico Antidiabetic Activity of Bioactive Compound of Katemas Leaves (Euphorbia Heterophylla, L.)

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***Abstract*—The purpose of this study is to isolate, purify and characterize active compounds from *E. heterophylla* L. plants and to know the antidiabetic activity of pure Katemas (*E.heterophylla*, L.) leaf compounds in vitro and in silico (molecular docking) through inhibition of alpha glucosidase enzymes. In this study sample extraction was carried out using multilevel maceration, starting with n-hexane, then with ethyl acetate. Isolation was done by VLC. Antidiabetic activity was tested for pure compounds through inhibition of the α-glucosidase enzyme in vitro using a microplate reader and in silico (molecular docking) using Discovery Studio 4.1 software. VLC results from the ethyl acetate extract of katemas leaves obtained 21 fractions and taken f2 because of the potential for more and stable crystals. F2 is predicted to be a DS-01 diterpenoid compound according to the results of UV, IR, H^1-NMR, C^{13}-NMR and MS spectroscopy. The results of antidiabetic activity test in vitro obtained IC_{50} values of 138.63 µg/mL for ethyl acetate extract and IC_{50} values of 191.82 µg/mL for diterpenoid DS-01 compounds. The results of molecular docking showed that the active compounds contained in the extract and also DS-01 diterpenoids were able to form hydrogen bonds between the ligand with the receptor, but less when compared with the acarbose.**

Keywords*—*E.heterophylla* L, *alpha glucosidase, in vitro, in vivo

I. INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder characterized by hyperglycemia or an increase in blood glucose levels as well as interference or changes in the metabolism of carbohydrates, proteins and fats as a result of abnormal secretion or insulin activity [1]. According to data from the International Diabetes Federation (IDF) in 2013, it was reported that ± 382 million people worldwide suffer from diabetes, and is estimated to reach 592 million people in 2035, and its prevalence in Indonesia ± 4.8% [2].

The mechanism of action of antidiabetic drugs is to enhance the action of the alpha glucosidase enzyme which is a key enzyme that converts disaccharides into simple monosaccharides which will be absorbed in the gastrointestinal tract [3]. Alpha glucosidase inhibition is a major therapeutic strategy for controlling postprandial hyperglycemia in DM type 2 [4]. Clinically available alpha glucosidase inhibitors such as acarbose are currently given orally as monotherapy or in combination with other oral antidiabetic drugs [5]. However, the price is relatively expensive and is known to have side effects on gastrointestinal, and long-term use also has an effect on the heart [6].

Finding natural medicine sources for alpha glucosidase inhibitory activity is very possible because it has a risk of fewer side effects and a higher level of safety [7]. Traditional medicine usually utilizes medicinal plants which are an alternative step to overcome DM [8]. One of the plants used as traditional medicines is katemas leaf (*Euphorbia heterophylla* L.).

According to the literature [9], *E. heterophylla* L. leaf extract contains bioactive alkaloids, phenolic compounds, steroids, tannins and terpenoids. Ethyl acetate extract (semi-polar in nature) was shown to have a high antioxidant activity (IC_{50}= 129,79 ppm), due to its alkaloid and phenolic content. Ethanol extract is also active against *P. vulgaris* and *S. Aureus* bacteria [10], so it can be potential as an antidiabetic.

The purpose of this research is to isolate, purify, characterize active compounds from *E. heterophylla* L. leaves and test the inhibitory activity of α-glucosidase in vitro and in silico (molecular docking) to confirm the hydrogen bonds and the binding energy of pure compounds with amino acids from the alpha glucosidase enzyme protein.

II. RESEARCH METHOD

A. Tools and Materials Used

The tools used are: oven, analytical balance, rotary evaporator (Buchi®), distillation apparatus, aluminum foil, pH meter, 96 wells microplate reader, Cromatography, FTIR (Shimadzu Prestige-21), Fisher John Melting Point Apparatus (SMP 11-Stuart®), UV-Vis spectrophotometer (Genesys 10S UV-VIS v4.002 2L9N175013), NMR spectrometers (JEOL ECA 500 1H-RMI, 500 MHz), mass spectrometers (MS water LCT premiere XE positive mode),

incubators, centrifuges, micro pipettes 10-100 µl, eppendrorft tube and glass equipment commonly used in laboratories. The hardware in the form of an internet-connected computer with computer specifications used in this study is, Intel® Core ™ i5 computer, 4 core server, 500GB hard drive, 4GB random access memory (RAM), and 2GB display card (VGA). Completeness of the computer namely the monitor, mouse and keyboard. The software used is Discovery studio 4.1, UCSF CHIMERA.

The materials used in this study were katemas *(E.heterophylla* L.) leaves, n-hexane solvent, ethyl acetate, dichloromethane (DCM), methanol, absolute ethanol, HCl, diethyl ether, and aquadest. While the antidiabetic ingredients are, dimethylsulfoxide (DMSO), phosphate buffer solution pH 7 and Na_2CO_3 solution. The 3-dimensional protein structure is taken from the protein bank website (www.pdb.org) with a 3W37 access code, alpha glucosidase protein with acarbose standard ligand.

B. Research Procedures

Sample Preparation

Katemas leaves were taken on Kartama Street, Pekanbaru Riau for ± 6000 grams. Katemas leaves were cleaned, and then air dried until constant weight. After dry, katemas leaves were cut into small pieces, chopped and mashed to obtain a dry powder called simplicia as much as 3000 grams.

Extraction and Isolation

Extraction was carried out using a multilevel maceration method for n-hexane, ethyl acetate and methanol solvent. A total of 3000 grams of dried katemas leaf powder was soaked with n-hexane solvent in a dark container until it was completely submerged. Simplicia was macerated for 3 days and repeated until the filtrate was clear. The residue in the form of pulp from the first maceration was re-macerated with ethyl acetate solvent for 3 days with 3 times repetition. Then the ethyl acetate maceration was filtered and ethyl acetate filtrate and residues were obtained. Furthermore, the maceration was concentrated using a rotary evaporator (50 ^oC) until a thick extract of ethyl acetate was obtained as much as 20.03 gr.

A total of 10 grams of ethyl acetate extract was separated using vacuum liquid chromatography. The column used is 5 cm in diameter and 20 cm in height filled with silica gel 60 GF_{254} to a height of approximately 10 cm. Column filling was carried out under vacuum, in order to obtain the maximum density of the stationary phase. The extract to be VLC was pre-absorbed and then put into a column.

The tracing was carried out with various eluents which have increased polarity using n-hexane (100%), n-hexane: ethyl acetate, ethyl acetate (100%), ethylacetate: methanol and methanol (100%). The results of the separation were stored in an erlenmeyer that has been numbered according to their fractions and produced 21 fractions. Furthermore, the solvents were evaporated in each fraction using a rotary evaporator and thin layer chromatography (TLC) was performed on each fraction. Recrystallization or purification was carried out at fraction 2 (6.01 gr) because it provided a fairly good chromatogram and had many and stable crystals. After recrystallization, it was obtained in the form of DS-01 isolate in the form of yellowish white crystals (weight ± 36 mg) and melting point of 86-88 ^oC.

Characterization

Elucidation of the structure of the isolated compound was carried out by UV, FTIR, ^{1}H-NMR, ^{13}C-NMR, and MS spectroscopy.

Alpha Glucosidase Inhibitory Activity Test

The alpha glucosidase inhibitory activity was determined according to the method modification [11] using a microplate (ELISA reader). The reagent mixture used in this test contained 50 µL phosphate buffer 0.1 M (pH 7.0), 25 µL p-nitrophenyl-alpha-D-glucopyranoside 0.55 mM, 10 µL test sample and 25 µL enzyme solution glucosidase (1 mg / mL). The reaction mixture was then incubated at 37 ^0C for 30 minutes. The reaction was stopped by the addition of 100 µL Na_2CO_3 0.2 M. The enzymatic hydrolysis of the substrate was monitored by the amount of *p*-nitropenol released in the reaction mixture at 410 nm. Positive control used is acarbose. Blanks are used to correct absorbance. Blank is a test solution consisting of enzyme preparations without extracts [12]. The test was carried out three times. Inhibitory activity (%) is calculated using the following equation:

$$\% \text{ Inhibition} = \frac{Ac - As}{Ac} \times 100\ \%$$

where; Ac = Absorbance control,
As = Absorbance sample.

The percent inhibition value calculated from each concentration is then used for the IC_{50} calculation. IC_{50} or 50% inhibitor concentration is the value of the concentration of a material to inhibit the activity of the α-glucosidase enzyme by 50%. The concentration value of the diluted solution from the sample and the percent inhibition were plotted on the x and y axes, then the IC_{50} value was calculated by linear regression , $y = a \ln(x) + b$

Note: y = % Inhibition, X = Sample concentration,
a = Interception, b= Slope

Molecular Docking

Discovery studio ® 4.1 and UCSF CHIMERA were used to perform molecular docking of compounds isolated according to the method [12]. The 3-dimensional protein structure was taken from the protein bank website (www.pdb.org) with a 3W37 access code, α-glucosidase protein with acarbose ligand. Protein molecules were prepared by removing H_2O groups (if any), the separation of natural found in protein molecules and adding hydrogen atoms and determining the active side of protein. The proposed compound that would be docking is called a ligand. Ligands must also be prepared before docking is carried out to obtain the most stable ligand conformation, adding charge to each ligand making atom, adding hydrogen atoms to the ligand, and minimizing energy [12].

The docking process was first performed with the standard ligand and must get a small Root Mean Square Deviation (RMSD) value of 2 Å. This serves as a validation of the docking method to be used. If these requirements are met, then the docking process of the proposed compound with the target protein can be carried out. The docking process results in the form of energy and binding sites between ligands and amino acid residues. The interactions that arise can be in the form of hydrogen bonds and phi bonds, with each bond distinguished from the color of the bond formed. The smaller the binding energy between the ligand and the protein produced, the more stable the bond that occurs between the ligand and protein.

III. RESULTS AND DISCUSSION

A. Isolation and Elucidation of Structure of Diterpenoid DS-01 Compound

Diterpenoid DS-01 compound obtained yellowish white crystals, with the molecular formula $C_{20}H_{30}O_4$, with a molecular weight (m/z) of 332,327 $[M-H]^+$. Tests using a UV spectrophotometer show the presence of conjugated bonds through the maximum wavelength (λ) and absorbance of 230 nm (2.504) indicating the existence of double bonds C=O, 341 nm (0.046) and 396 nm (0.011) indicate double bonds C=C .

The results of the IR spectrophotometer analysis showed that the Diterpenoid DS-01 compound, namely the wave number 2916.49, and 2848.98 cm^{-1} was the absorption of the aliphatic C-H group (stretch), the absorption band at the wave number 1736.97 was the absorption of carbonyl (C=O) and the absorption band at wave number 1463.07 was the absorption of methylene (CH_2)

B. Spectrum analysis of ^{1}H-NMR DS-01 compound

Analysis of the 1H-NMR spectrum of the DS-01 compound showed that the number of protons from the compound obtained was in accordance with the results of characterization by mass spectroscopy. Based on Table 4.2 interpretation of 1H-NMR data, it is seen that at a chemical shift δ 5.36 ppm *(t,* 2H, *J* = 6.3 Hz) indicated is the proton from methyl (CH_3), at a chemical shift δ 5.11 ppm (*s*, 1H) is a proton bound to C that has no H atom, at a chemical shift δ 4.58 ppm (*d*, 1H, *J* = 7.4 Hz), δ 4.12 ppm (*d*, 1H, *J* = 7.1 Hz) is the H proton of methyl (CH_3), the chemical shift δ 4.05 ppm (*t*, 1H, *J* = 6.75 Hz) is the H proton of methylene. Chemical shift at δ 3.66 ppm indicates H proton bound to O (OH) with the singlet peak, chemical shift δ 2.80 ppm (*s*, 1H) which is also the peak of the singlet is indicated as H proton from methyl (CH_3), at a shift of δ 28 2.28 ppm (*t*, 2H, *J* = 7.5 Hz) indicated the proton H of methylene (CH_2). Furthermore, at the chemical shift δ 2.04 ppm, δ 1.63 ppm, δ 1.30 ppm and, δ 0.89 ppm also indicated is the chemical shift of the proton H from methylene (CH_2) has a multiplet splitting with a number of consecutive integrations 4, 6, and 9. The results of the ^{1}H-NMR spectrum analysis of the DS-01 compound showed that the number of protons from the compounds obtained was in accordance with the results of characterization by mass spectroscopy (MS) which obtained the number of H protons was 38. This corresponds with the formula that has been given to the authors. From the results of the ^{1}H-NMR spectrum, it can be seen that the isolated DS-01 compound can be identified as a diterpenoid group.

C. Spectrum analysis of ^{13}C-NMR DS-01 compound

Analysis of the ^{13}C-NMR spectrum of the DS-01 compound showed the presence of a peak that was typical for the DS-01 compound. At a chemical shift δ 64.56 ppm indicates the presence of carbon quarterners. Then in the chemical shift δ 37.45 ppm, δ 34.59 ppm, δ 32.09 ppm, δ 29.86 ppm, δ 29.82 ppm, δ 29.75 ppm, δ 29.69 ppm, δ 29.64 ppm, δ 29.52 ppm, δ 29.44 ppm, δ 29.42 ppm and δ 29.33 ppm respectively are methylene groups (CH_2). Furthermore, at the chemical shift δ 28.82 ppm, δ 26.10 ppm, δ 25.20 ppm, δ 22.85 ppm and, 14.27 ppm respectively are signals from the methyl group (CH_3). Analysis of the ^{1}H-NMR and ^{13}C-NMR spectrum in the DS-01 compound provided information that in the DS-01 compound there were hydroxyl groups, methyl groups and methylene groups, the same that researchers had found [13] from the *Euphorbia fischeriana* plant. The following is a comparison of the spectrum analysis of the DS-01 compound with the literature compound.

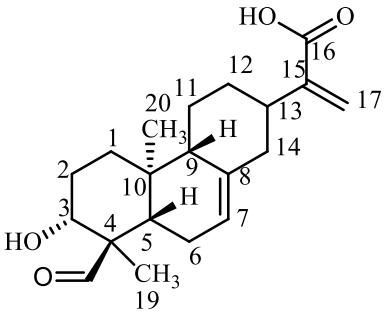


Figure 1. Structure of DS-01 (Fischerianoid A) Compound

Table 1. Data Interpretation of ^{1}H-NMR and ^{13}C-NMR ($CDCl_3$) of Diterpenoid DS-01 Compound

No of C	DS-01 compound		Literature Compound [13]	
	δ_H (ppm)	**δc (ppm)**	**δ_H (ppm)**	**δc (ppm)**
1		37,45	1,24 m; 1,82 m	38,4 t
2		28,82	1,94 m	28,2 t
3	4,12 (d, 1H, *J* = 7,1 Hz)		4,27 m	73,7 d
4			-	43,4 s
5			1,93 m	43,3 d
6		22,85	2,03 m	23,7 t
7			5,42 s	121,3 d
8			-	137,4 s
9	1,63 (m, 9H)		1,75 m	52,8 d
10		34,59	-	35,6 s
11		26,10	1,23 m; 1,76 m	26,1 t
12	1,30 (m, 6H); 2,04 (m, 4H)	32,09	1,28 m; 2,04 m	32,5 t
13	2,80 (s, 1H)		2,80 m	40,0 d
14			2,02 m; 2,66 m	41,9 t
15			-	147,8 s
16			-	170,1 s

17	5,36 (t, 2H, *J* = 6,3 Hz)		5,64 s; 6,54 s	122,1 t
18	3,66 (s, 1H); 4,05 (t, 1H, *J* = 6,75 Hz)	64,56	3,68 d (10,6) ; 4,14 d (10,6)	67,7 t
19	1,63 (m, 9H)		1,16 s	13,4 q
20	0,89 (m, 9H)	14,27	0,93 s	16,2 q

D. Antidiabetic Activity Test.

Based on the α-glucosidase enzyme inhibition test, Katemas leaves ethyl acetate extract and Diterpenoid DS-01 showed that IC_{50} values were not too much different. IC_{50} value of ethyl acetate extract was 138.6295 µg/mL and DS-01 compound was 191.82 µg/mL. This could be due to the extract sample containing several active compounds that allow the reaction of protagonists between compounds in the sample [17], but it is still lower than the acarbose (standard) with an IC_{50} value of 0.6799 µg/mL.

Table 2. Percentage of acarbose inhibition and sample

Sample	IC 50 (µg/mL)
Ethyl acetate extract of katemas leaf	138,63
DS-01 compound	191,82
Acarbose	0,68

From the % data of the extract inhibition and sample, it can be seen that extract activity was better than diterpenoid DS-01 compound, as shown in the graph in figure 2.

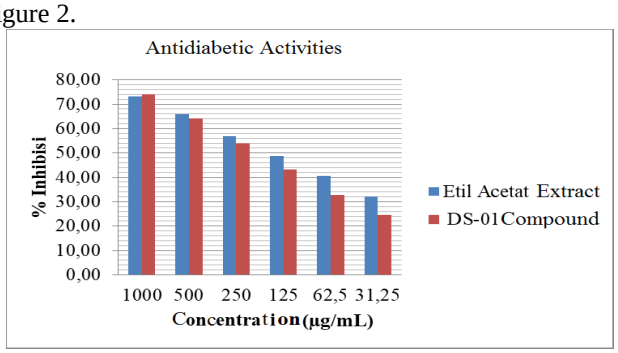
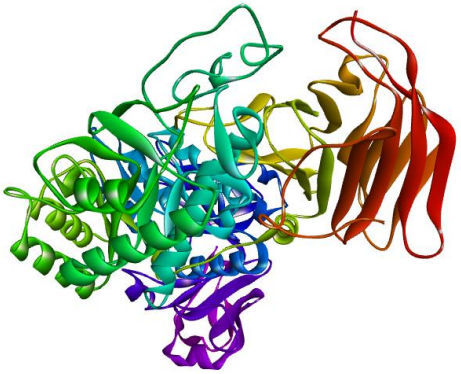


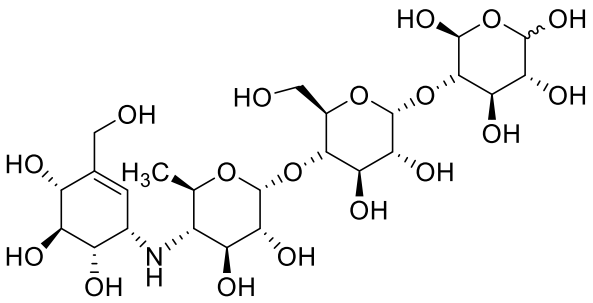
Figure 2. Graph of antidiabetic activity test

E. Molecular Docking

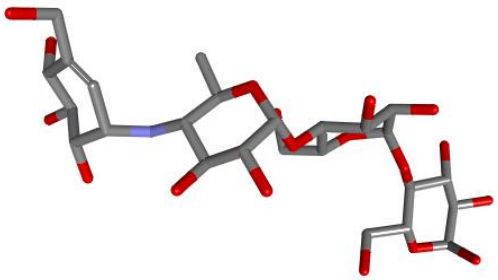
DS-01 compound docking is carried out on the α-glucosidase protein which plays a role in converting carbohydrates into glucose which results in higher blood glucose levels in diabetics. Therefore, the work of this enzyme in the intestine must be inhibited. This protein is taken from a protein bank data with access code 3W37 in the form of a complex crystal with acarbose standard ligand which can be seen in Figure 3.



(a)



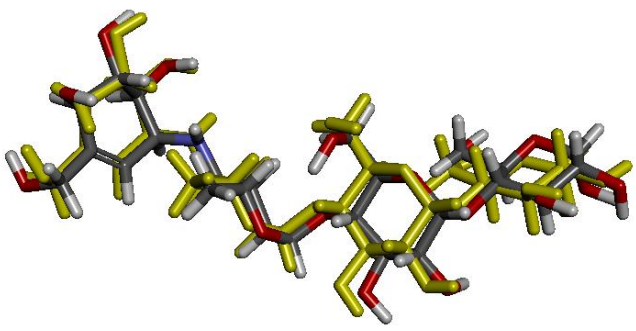
(b)



(c)

Figure 3. (a) 3D cristal structure of 3W37 protein and (b),(c) acarbose ligand standar.

The RMSD value generated through ACARBOSE re-docking to 3W37 protein is 1.6 Å, so it can be said that the molecular docking method is valid because the RMSD value <2 Å and has a re-docking ligand pose that is very similar to the original pose which can be seen in Figure 4. Then the docking process of the DS-01 compound can be carried out with 3W37 protein.



(a)

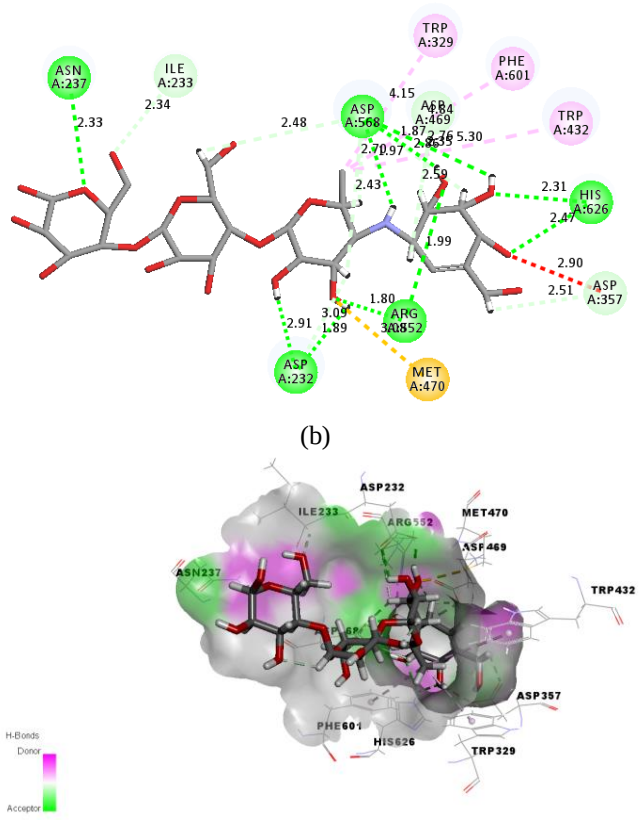


Figure 4. (a) Initial ligand pose (color matches the atom) and pose after re-docking (yellow); (b) and (c) standard ligand interaction (acarbose) with 3W37 protein

Molecular docking aims to see the interaction between ligands and proteins that produce hydrogen bonds to amino acid residues and -CDOCKER-ENERGY values. Important amino acid residues that act as active centers of antidiabetic formed by the rootbose ligands are ASP232, ARG552, ASP357, ASP568 and HIS626. The interactions formed between ligands and proteins can be seen in Table 4. In general, DS-01 compounds form interactions with amino acid residues LYS506. The DS-01 compound does not form the same hydrogen bond as the hydrogen bond formed by the acarbose standard ligand.

Table 4. Protein docking result with standard ligand and proposed ligand

No	Molecular Name	-CDOCKER-ENERGY	Hydrogen Bonding
1.	Acarbose	-25,5384	ASP232, ARG552, ASP357, ASP568, HIS626
2.	Diterpenoid DS-01	-27,2025	LYS506

The smaller the energy cDOCKER-ENERGY produced, the more stable the ligand bonds with the protein formed so that the more active the ligand used. The easier and more stable the ligand-protein bonds are formed, the lower the binding energy produced.

The results of molecular docking in Figure 5, show that the DS-01 compound is able to form hydrogen bonds between the ligand with protein, but less when compared to the acarbose and it can be concluded that the DS-01 compound is predicted to have antidiabetic activity.

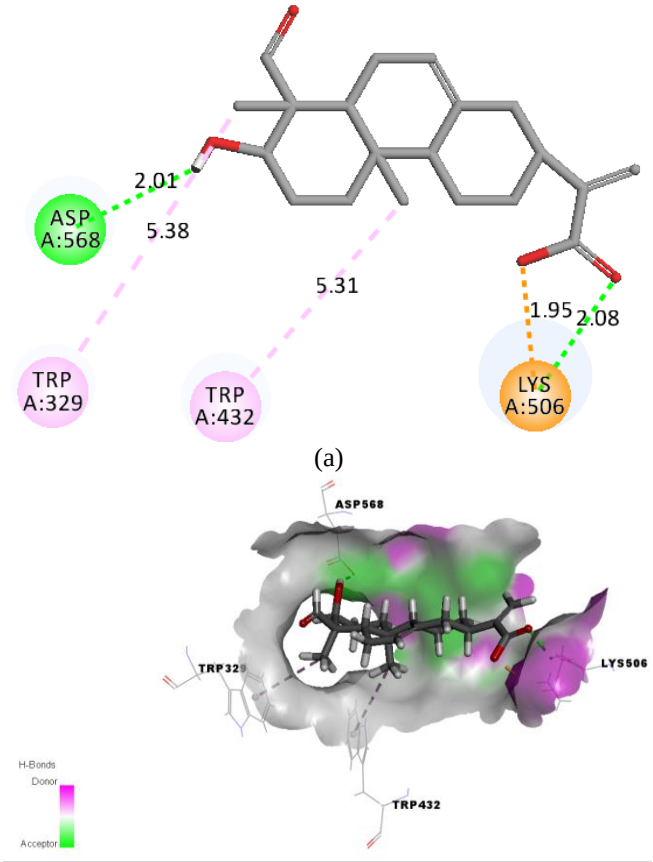


Figure 5. (a) and (b) Ligand interaction of DS-01 compound with protein

IV. CONCLUSION AND SUGGESTIONS

The results of research on the isolation of bioactive compounds from Katemas *(E. heterophylla* L) leaves of ethyl acetate extracts showed that pure bioactive compounds contained in extracts were diterpenoid groups based on characterization by UV, IR, ^{1}H-NMR, ^{13}C-NMR, and MS spectroscopy. In vitro antidiabetic activity test results, it obtained IC_{50} values of 138.63 µg/mL for ethyl acetate extract and IC_{50} values of 191.82 µg/mL for the Diterpenoid DS-01 compound. The results of molecular docking showed that the active compounds contained in the DS-01 Diterpenoid compound were able to form hydrogen bonds between the ligand with the receptor, but were less and not the same as the acarbose.

ACKNOWLEDGMENT

The authors would like to thank Universitas Muhammadiyah Riau for funding this research with the 2019 Academic Lecturer Research Scheme (PDM) in 2019 with contract No.017/KONTRAK-PENELITIAN/K10/KM/ 2019

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