Total flavonoid contents and in silico study of flavonoid compounds from Meniran (*Phyllanthus niruri L.*) towards alpha-amylase and alpha-glucosidase enzyme

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ABSTRACT

Meniran (*Phyllanthus niruri* L.) is a plant that is reported to contain flavonoids and shows the activity as a type 2 anti-diabetic by a mechanism of inhibiting the absorption of glucose. Flavonoid compounds such as rutin, quercetin and hesperidin shows anti-diabetic activity. This research aims to determine the levels of total flavonoids and docking studies of flavonoids from *P. niruri* against α-amylase and α-glucosidase. Analysis of total flavonoid content utilizes colorimetric method using $AlCl_3$ and rutin standard, the docking study uses *Autodock Vina* program with the assistance of *AutoDockTools* (ADT) and visualization of docking results using *Discovery Study*. The results of the determination of total flavonoids level was 12.32±0.53 mgRE/g extract whereas in ethyl acetate and water fraction each were 19.33±0.68 and 20.07±1.23 mgRE/g, respectively. Results of all flavonoids docking study showed the potential to bind to both receptors, rutin has better binding energy towards α-amylase whereas against α-glucosidase is demonstrated by 3-*O* -*β*-D-glucopyranosyl-(2-1)-*O*-*β*-D-xylopyranoside quercetin.
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Keywords: *Phyllanthus niruri* L., flavonoid, docking studies, α-amylase, α-glucosidase

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INTRODUCTION

Traditionally, many plants can be used as an anti-diabetic medication. One of the medicinal plant often used by the local community as an Original Indonesian Medicine (OIM) or by the international community is *Phyllanthus niruri* L. which is from *Phyllanthaceae* family. In vivo study of *P. niruri* leaves and fruits water extract at 150-600 mg/kgbw/day is effective in controlling insulin resistance in mice inducted with 10% of sucrose (Adeneye, 2012). The methanol extract of this herb is also capable of lowering blood sugar and body weight of rats which is induced using alloxan and could inhibit α-amylase, and α-glucosidase enzyme with IC_{50} of 2.15±0.1 and 0.2±0.02 mg/mL (Okoli *et al*., 2011). Secondary metabolites in *P. niruri* were alkaloid, flavonoid, hydrolyzed tannin (ellagitanin), lignin, polyphenol, sterol and essential oil (Patel *et al*., 2011). Quercetin and rutin flavonoids in a dose of 100 mg/kgbw each significantly (p<0.05) lower blood sugar level in diabetic rats. Rutin and quercetin shows best antidiabetic activity each at the 2nd and 4th hour of administration. Rutin lowers blood sugar more compared to the positive control, glibenclamide (Jadhav and Puchchakayala, 2012). Quercetin and rutin is a flavonoid contained in *P. niruri*. Other than those two flavonoids, *P. niruri* also contains gallocathechin, nirurin, niruriflavone, quercetin, quercitrin, astragalin and 3-*O*-*β*-D-glucopyranosyl-(2-1)-*O*-*β*-D-xylopyranoside quercetin (Bagalkotkar *et al*., 2006).

Potential medicine from natural materials need to be developed, as most of conventional medicines are derived from plants. Medicinal compounds derived from natural materials can bind with one or more receptor (Siswandono, 2000). Interaction between the compound and receptor can be known using docking study. Docking studies has been proven to found the effectiveness of a compounds with receptors based on energy and the bonding type (Yanuar, 2012). Some of the compounds from plants are known to have the potency as type 2 anti-diabetic medication with the mechanism of inhibiting alpha amylase and glucosidase enzyme. Docking study results stated that compounds like curcumin, 16-hydroxychloride-3,13 dina-16,15-olide (16H), docosanol, tetracosanol, anto quinolol, berberine, catechin, quercetin, actinodafnine and rutin has the ability to bind to both alpha amylase and alpha glucosidase receptors (Jhong *et al*., 2015). Based on these research, flavonoids compound is potential to be an anti-diabetic agent. Research regarding the docking study of flavonoids and total flavonoid contents from *P. niruri* has never been done. This research aims to determine the total flavonoid content from extract and fraction of *P. niruri* and its flavonoid affinity against α-amylase and α-glucosidase receptor through docking studies.

MATERIALS AND METHOD
Equipment and Materials

Laptop (Asus X455L with Intel Core i3™), *ChemDraw* program (version 15.0) to create 2-dimensional (2D) structure, *ChemOffice* 3D (version 15.0) to create a 3-dimensional structure (3D), a database of protein from the protein data bank and NCBI, *Autodock Vina* (Version 1.1.2) with the assistance of *Auto Dock Tools* (ADT) and visualization results of Docking by using Discovery Studio, UV-Vis Spectrophotometer (UV Thermo Scientific Genesys 10 UV Scanning), rotary evaporator (Rotavapor BUCHI II), analytical scale, UV 254 and 366 nm lamp, *P. niruri* powder, Quercetin (sigma Aldrich), $AlCl_3$ (Merck), methanol p.a (Merck), n-hexane (Merck), ethyl acetate p.a. (Merck), working ethanol, $FeCl_3$, Mg tape, HCl p.a. (Merck), H_2SO_4 p.a (Merck), *Mayer* reagent, *Dragendorff* reagent, *Wagner* reagent, glacial acetic acid (Merck), gelatin 1%, NaCl 10%, TLC (silica gel GF_{254}), butanol p. a. (Merck), toluene p.a. (Merck), Sodium acetate (Merck) and aquadest.

Methods

This research starts from the extraction of oil, and subsequently phytochemical screening and determination of total flavonoids from the extracts and fractions of *P. niruri* docking studies of the flavonoid compounds which structure known from literature study.

Sample preparation, extraction and fractionation

Dried *P. niruri* herb were sourced from the area of Yogyakarta, Indonesia through supplier and distributor of herbs/herbal (*Herbal Anugrah Alam*). Dried herbs were powdered and microscopically tested to ensure the species of the samples. Subsequently, maceration was done using 70% ethanol. Liquid extract obtained were collected into one and condensed using a rotary evaporator at a temperature of $50^{\circ}C$ to obtain thick extract. Obtained thick extract were partitioned by liquid-liquid partition method using a separatory funnel. Thick extract was dissolved into warm aquadest, then n-hexane was added and shaken inside a separatory funnel. After shaking finishes, two layer of immiscible liquid will form, the water layer was separated from the n-hexane. This process is repeated until the n-hexane solvent became clear. The water layer was added with ethyl acetate and was fractionated repeatedly until the ethyl acetate layer became clear. n-Hexane, ethyl acetate and aqueous fraction was then condensed with vacuum oven up to obtain condensed fraction (Ahmad *et al*., 2018).

Determination of total flavonoid content

Analysis of total flavonoid content uses aluminum chloride ($AlCl_3$) colorimetric method. An aliquot of extract and fraction are inserted into a 25 mL measuring flask (concentration of 500-1000 µg/mL) was diluted in methanol. About 2 mL of the sample was then added with 0.15 mL of $NaNO_2$ and incubated for 6 minutes, 10% $AlCl_3$ was added into the sample and incubated for another 6 minutes, and 2 mL of 4% NaOH were added after the incubation. After 15 minutes of incubation, the result of the reaction was ready to be measured in a UV-VIS Spectrophotometer at a wavelength of 511.5 nm (Surana *et al*., 2016).

Docking study

Docking study begins with the preparation of ligands i.e. creating a 2D structure of rutin, quercetin, gallocathechin, niruriflavone, nirurin, quercetol, quercitrin, astragalin, and 3-*O* -*β*-D-glucopyranosyl-(2-1)-*O*-*β*-D-xylopyranoside quercetin using ChemDraw Program (version 15.0). Each of the structure above were then made into 3D structure using ChemOffice 3D (version 15.0) which can observe the stereochemistry 3D form and set up the most stable form of the compounds by minimalizing the energy using MM2 Minimize energy tools in the program, to obtain the most compatible form in binding the stereochemistry receptor (Liu-Qiong Hou *et al*., 2015). Detection of active site, and analysis of bind amino acid receptor was done using *Autodock Vina* (version 1.1.2) with the assistance of *AutoDockTools* (ADT) was done to all detected potential active site (cavity) within alpha-amylase and alpha-glucosidase receptor. Energy is rated based from binding energy (kcal/mol) and RMSD (Root Mean Square Deviation). In addition, the possibility of hydrogen bonds between donor and acceptor (receptor ligands) were also observed. Parameters which were commonly used to observe the drug-receptor bond was binding energy, while visualization of docking results was done using *Discovery Studio*, and then the data was analyzed (Jhong *et al*., 2015).

RESULTS AND DISCUSSION
Extraction and fractionation

Extraction of 1,128.5 g of powdered sample resulted in 284.14 g of thick extract with yield of 25.17%. The extraction was done by maceration, by soaking the powdered sample using solvents inside a maceration vessel. Maceration was chosen because it is safe for compounds that

are not resistant to high temperatures, furthermore the method is simple and uses basic instruments. The use of powdered sample aims to expand the surface on which the sample will be extracted, allowing for the extraction of more chemical components from the sample. Ethanol 70% is used as a solvent due to its nature as a polar solvent, which would optimize the extraction by extracting a number of chemical components that are either polar or nonpolar. In accordance with the laws of solubility: "*like dissolves like*", which means that solubility will occurs when both solvent and solute has the same polarity, which can dissolve metabolite-secondary metabolite in plants (Harborne, 1987).

Fractionation or partition, also called liquid-liquid extraction is a process of separating a substance in 2 immiscible liquids or in other words, a comparison of the concentration of a substance dissolved in organic and water solvent. The result of fractionation in each solvent produces different amount of fraction, this is possible because of the nature of the substance which can be dissolved into water and there are substances that can be dissolved in an organic solvent (Rydberg, 2004), there is also a difference in the level of solubility of each solvent used. In this research, fractionation is done gradually from non-polar solvent to polar solvent, in order to separate a substance based on its solubility (Simon Gibbons and Alexander, 2006). n-Hexane, ethyl acetate and aqueous fraction which is obtained from 168.94 g of ethanol extracts were 5.63; 10.47 and 51.40 g, with yield of 3.33; 6.20 and 30.43%, respectively. Then the chemical content extracts and fractions were identified through phytochemical screening.

Phytochemical screening of extract and fraction

The extract which is obtained in the form of ethanol extracts with dark green color and has specific odor. Phytochemical screening of ethanol extracts and fractions of *P. niruri* showed the ethanol extracts, ethyl acetate fractions and aqueous fractions contain flavonoid but n-hexan fractions not yet. Phytochemical screening of the extracts and fractions of *P. niruri* as listed in Table I.

Table I. Phytochemical screening results

Plant Sample of *P. niruri* L.	Class of Compound					
	Phenol	Flavonoid	Tannin	Steroid	Alkaloid	Saponins
Ethanol Extract	+	+	+	+	+	+
n-hexane Fraction	+	-	+	+	-	-
Ethyl Acetate Fraction	+	+	+	+	+	-
Aqueous Fraction	+	+	+	-	-	-

The result of total flavonoid content analysis

Based on the results of phytochemical screening, flavonoids were identified in the sample ethanol extracts, ethyl acetate fractions and aqueous fractions. The determination of total flavonoid content in the sample ethanol extracts, ethyl acetate fractions and aqueous fraction are then conducted. Determination of total flavonoid content with $AlCl_3$ colorimetric method uses rutin as the base standard. The determination of maximum wavelength resulted in 511.5 nm. A standard curve is made to calculate total flavonoid content in the extracts and fractions of *P. niruri*. The result of total flavonoid content determination is 12.32 ± 0.53 mgRE/g extracts while the result for ethyl acetate and aqueous fractions were 19.33±0.68 and 20.07±1.23 mgRE/g

respectively. Flavonoids have the ability to ward off free radicals and binds to metal, this gives relationship hypotheses between diabetes and the inflammation, as well as the potency of flavonoids to protect the body from free radicals and other pro oxidative compounds. The consumption of flavonoid-rich foods or flavonoids can reduce the risk of diabetes (Vinayagam & Xu, 2015). Some flavonoids from various plants have been tested in-vitro as well as in-vivo with various methods, which indicates anti-diabetic activity like quercetin, hesperidin, caempherol, apigenin, luteolin, naringenin, isorhamnetin and rutin (Vinayagam & Xu, 2015).

Docking Study Result

Docking method used in this study is *AutoDock Vina* with the assistance of *AutoDockTool* (ADT). Study of *docking* is done by comparing the drug compounds which activity is biologically proven to the target receptors of the drug. This research uses Acarbose as the comparison substance. Medicinal compound, hereinafter referred to as ligand is analyzed for its physicochemical properties using *ChemOffice*. The structure of quercetin, rutin, gallocathechin, niruriflavone, nirurin, quercetol, quercitrin, astragalin, and 3-*O*-β-D-glucopyranosyl-(2-1)-*O*-β-D-xylopyranoside quercetin are drawn in two dimensions (2D).

Table II. The result of ligands physicochemical properties determination

Compound Name	Log P	MW
Rutin	-2.28	610.15
Quercetin	0.35	302.04
Gallocathechin	1.11	306.07
Niruriflavone	1.33	364.03
Nirurin	0.96	516.16
Quercetol	2.74	354.15
Quercitrin	-0.54	448.10
Astragalin	-1.2	432.11
3-*O*-β-D-glucopyranosyl-(2-1)-*O*-β-D-xylopyranoside quercetin	997.13	626.15
Acarbose (Positive Control)	-6.8	645.60

The results of the physicochemical analysis based on the structure, molecular weight, log P values as listed in Table II. It can be seen that most of the molecular weight is < 500, but rutin, nirurin and 3-*O*-β-D-glucopyranosyl-(2-1)-*O* -β-D- xylopyranoside quercetin were > 500. The average log P values is < 5, but it was > 5 in 3-*O*-β-D-glucopyranosyl-(2-1)-*O* -β-D-xylopyranoside quercetin. The number of H donor (OH and NH cluster) is > 5. Based on the Lipinski's rule of five, < 5 means it has excellent permeability. Compounds with MW < 500 g/mol can easily penetrate biological membranes. The value of log P describes the ability of a compound to dissolve in biological membrane liquid. The optimum value for log P based on Lipinski's rule of five is < 5. Based on the data analysis, flavonoids are easily absorbed and have excellent permeability, except for 3-*O*-β-D-glucopyranosyl-(2-1)-*O*-β-D-xylopyranoside quercetin (Block, 2011). The 2D structure compounds are drawn in 3D form using Chem Office 3D. Compounds that bind to the receptor should be drawn in 3D as pdb format. This is due to the compound that will be docked can only be visualized in the form of 3D formats such as pdb by *Autodock Vina*. Pdb format files have the required data for the docking purposes, such as atom positions, coordinates of molecules and chains of amino acids of a macromolecule (Yanuar, 2012).

Table III. The result of molecular docking towards α-amylase and α-glucosidase receptor

Compound Name	Docking Score towards α-Amylase	Docking Score towards α-Glucosidase
Rutin	-9.6	-6.8
Quercetin	-8.5	-6.4
Gallocathechin	-8.5	-6.0
Niruriflavone	-8.3	-6.1
Nirurin	-9.4	-6.7
Quercetol	-8.9	-6.7
Quercitrin	-8.8	-6.8
Astragalin	-9.1	-6.7
3-*O*-*β*-D-glucopyranosyl-(2-1)-*O*-*β*-D-xylopyranoside quercetin	-8.9	-7.5
Acarbose (Positive Control)	-8.1	-7.2

The data in Table III shows that the binding energy of *P. niruri* flavonoid compounds towards receptor α-amylase is lower than acarbose. It can illustrate that flavonoids from *P. niruri* are potentially forming a bond with α-amylase. The binding energy of *P. niruri* flavonoid compounds towards α-receptor glucosidase is greater than acarbose but the binding energy of 3-*O*-*β*-D-glucopyranosyl-(2-1)-*O*-*β*-D-xylopyranoside quercetin is lower. Overall, these compounds have the potential to bind to α-amylase or α-glucosidase receptor. This is in accordance with the theory of energy enthalpy, where the lower binding energy is (exothermic) between a compound with a receptor (product) compared to compound itself, then it is easier to form a bond between compounds with receptor (Chan, 2010; Trott & Olson, 2010). Based on the data above, the next selected compounds with the highest docking score (binding energy) be visualized as a result of docking. In this research, rutin is further visualized towards α-amylase while 3-*O*-*β*-D-glucopyranosyl-(2-1)-*O*-*β*-D-xylopyranoside quercetin is visualized towards α-glucosidase using *Autodock Vina* version 1.1.2 with the assistance of *AutoDockTools* and visualization of the docking result using *Discovery Studio*.

The molecular stuctures of rutin by high docking scores on α-amylase as shown in Figure 1 and the 3-*O*-*β*-D-glucopyranosyl-(2-1)-*O*-*β*-D-xylopyranoside quercetin on α-glucosidase inhibitors detected in virtual screening were shown in Figure 3. Rutin ligan (in rutin-α-amylase complex) perform hydrogen bonding with amino acid residues Gln A:63 and Glu A:233 and has π-π interaction with amino acid residues Trp A:59 and Thr A:163 while acabose as positive control only perform hydrogen bonding with amino acid residues Asp A:197, Glu A:233, Asn A:105, Gln A:63 and Thr A:163 as shown in Figure 2. The 3-*O*-*β*-D-glucopyranosyl-(2-1)-*O*-*β*-D-xylopyranoside quercetin-α-glucosidase complex shown the amino acid residues that participated in hydrogen bonding were Arg A:655, Glu A:765 and Phe A:727. This compound also has π-π interaction with amino acid residues Thr A:648. Hydrogen binding also performed between acarbose with α-glucosidase inhibitors. The amino acid residues are Gln A:655, Tyr A:729, Leu A:726 and Glu A:765 as shown in Figure 4. Some of the research in-vitro shown the flavonoids have shown promising inhibition of α-amylase and α-glucosidase (Kim *et al*., 2010; Pereira *et al*., 2011). Flavonoid it was possible to observe that the flavone with a -C_1 ion at 3-position of C-ring, an –OH group at 3'- and 4'- positions of B-ring and at 5- and 7-positions of A-ring and the $C_2=C_3$ double bond, was the most active tested flavonoid, through competitive inhibition (Proenca, *et al*., 2019).
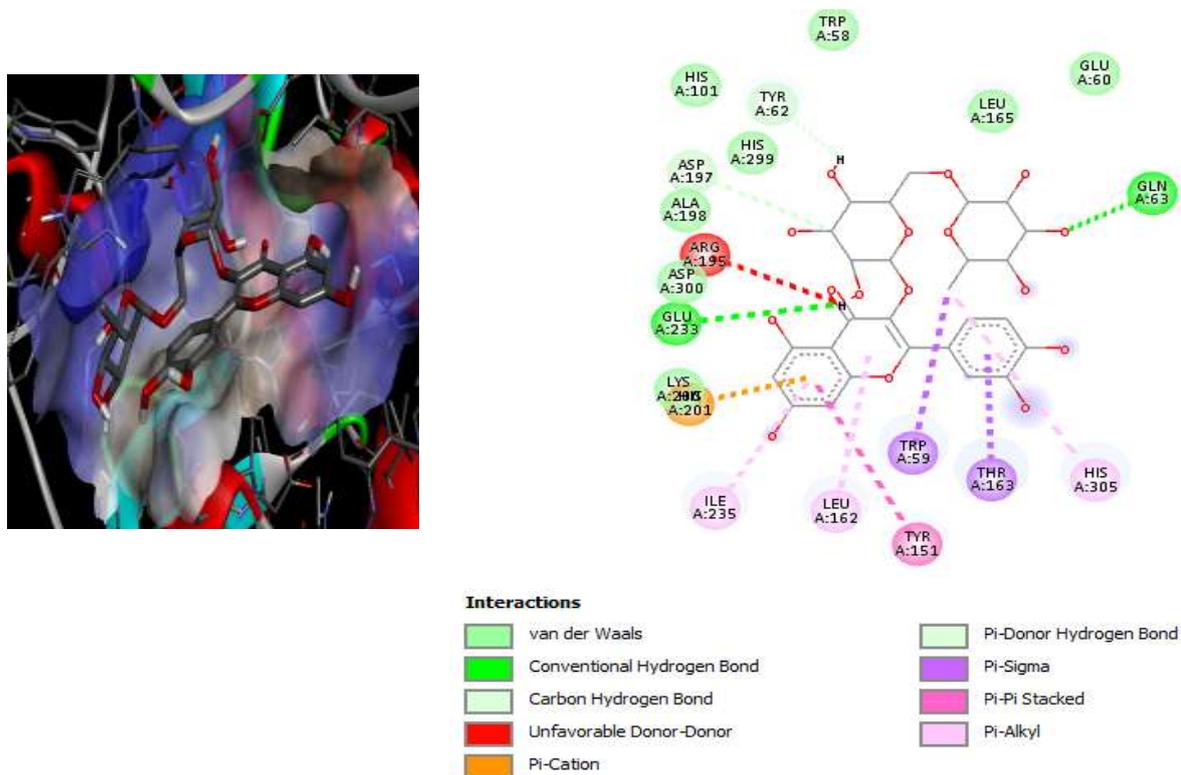
Figure 1. Interaction hydrophobic visualization and interaction 2D rutin with α-amylase receptors
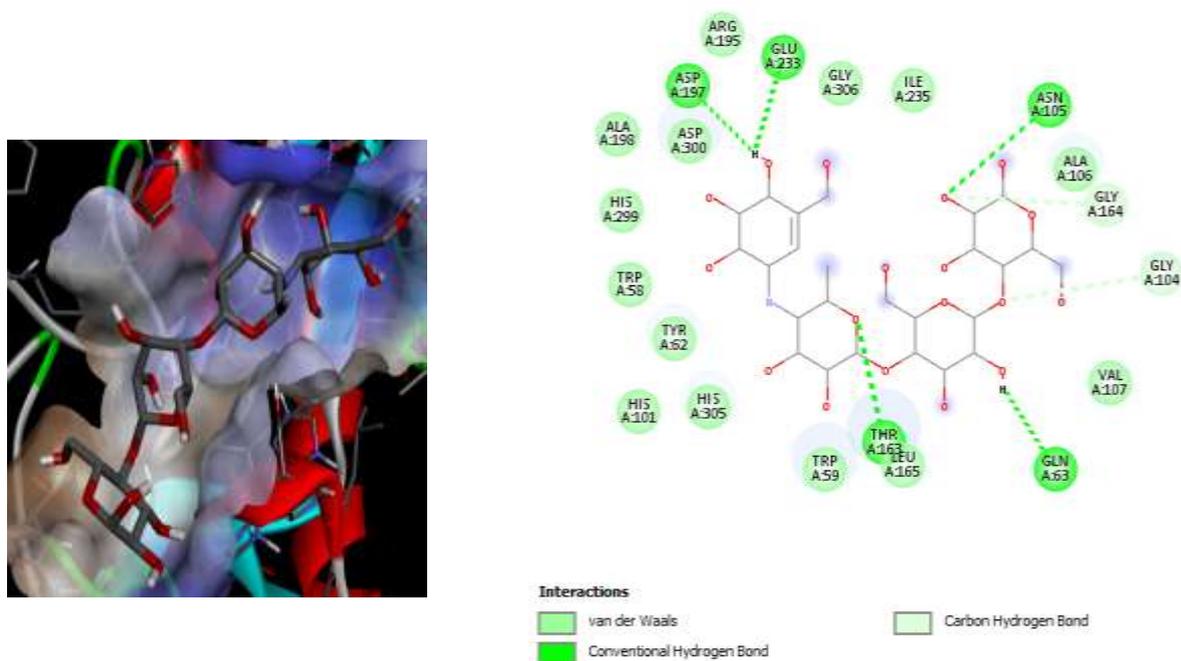

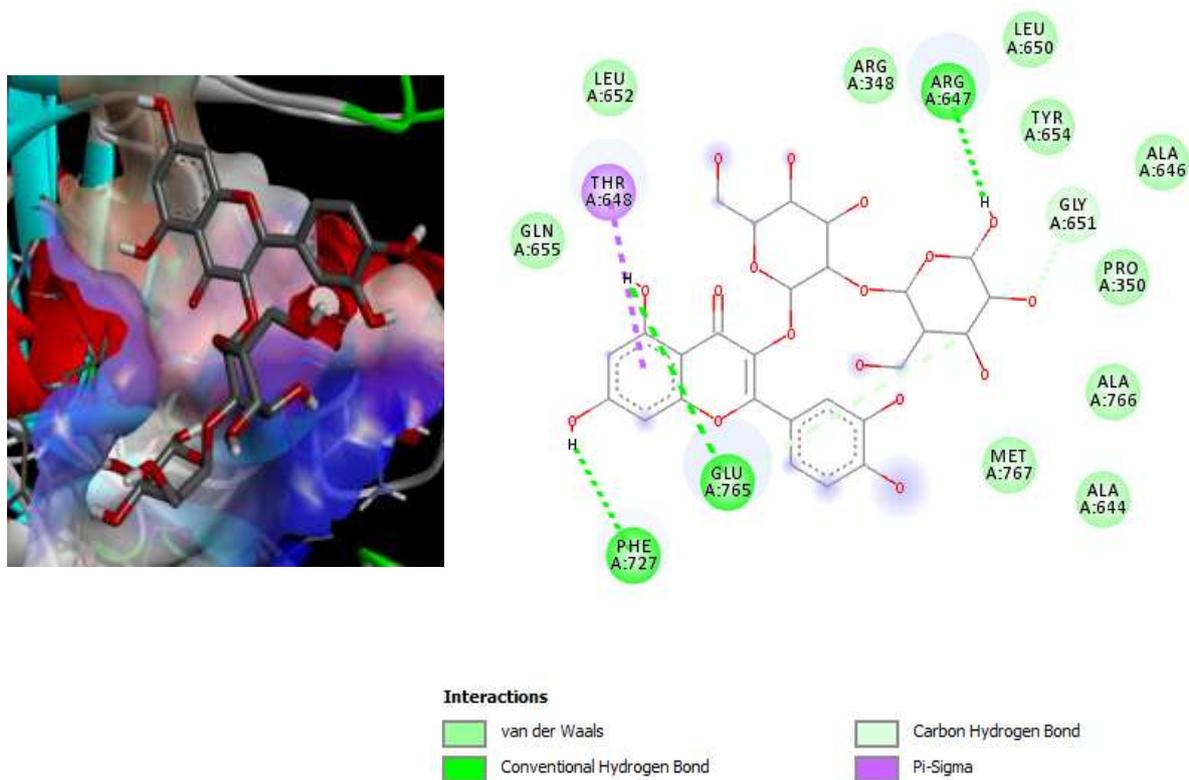**Figure 2. Interaction hydrophobic visualization and interaction 2D acarbose with α-amylase receptors**

Figure 3. Interaction hydrophobic visualization and interaction 2D quercetin 3-O-β-D-glucopyranosyl-(2-1)-O-β-D-xylopyranoside with α-glucosidase receptors
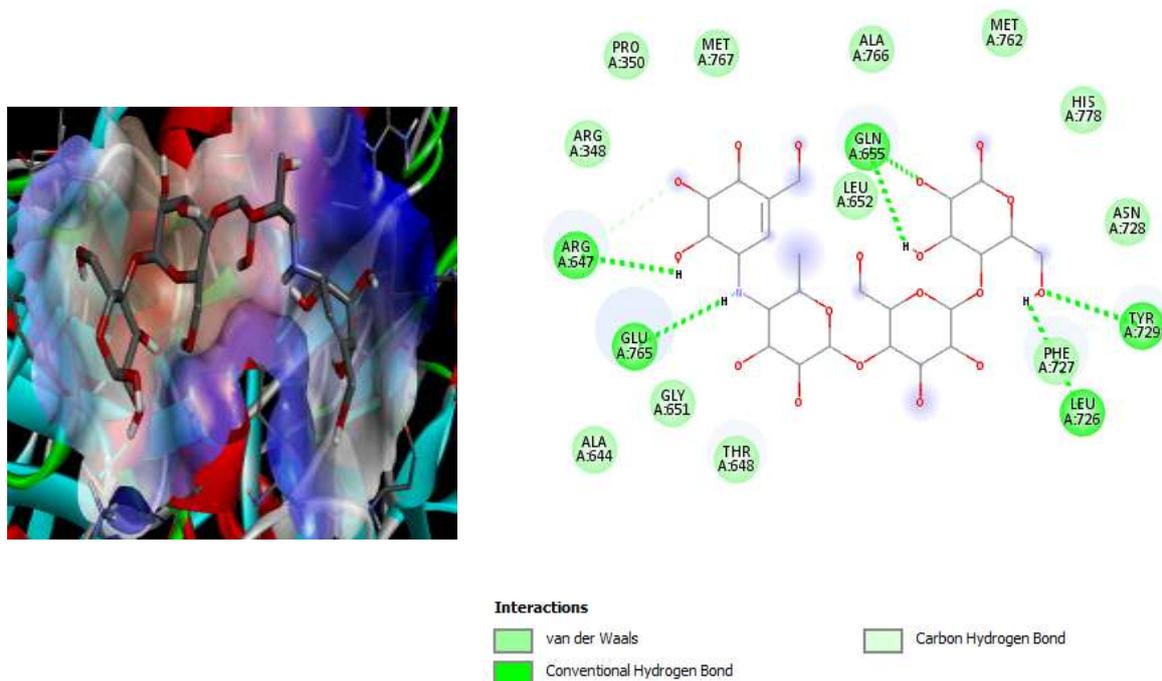

Figure 4. Interaction hydrophobic visualization and interaction 2D acarbose with α-glucosidase receptors

CONCLUSION

Total flavonoid content is higher in fraction than in extract. Flavonoid compounds of *P. niruri* potentially inhibit α-amylase and α-glucosidase, rutin showed better activity than acarbose towards α-amylase and 3-*O*-*β*-D-glucopyranosyl-(2-1)-*O*-*β*-D-xylopyranoside quercetin is better than acarbose towards the α-glucosidase. Results of all flavonoids docking study showed the potential to bind to both receptors, rutin has better binding energy towards α-amylase whereas against α-glucosidase is demonstrated by 3-*O* -*β*-D-glucopyranosyl-(2-1)-*O*-*β*-D-xylopyranoside quercetin.

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