Trad. Med. J., September-December 2023
Vol. 28(3), p 221-229
ISSN-p : 1410-5918 ISSN-e : 2406-9086

Submitted : 15-06-2023
Revised : 17-10-2023
Accepted : 16-11-2023

Identification of Compounds and Antidiabetic Activities of the Ethyl Acetate Fraction of Yacon (*Smallanthus sonchifolius*) Leaves Using In Silico and In Vitro Approaches

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ABSTRACT

Yacon (*Smallanthus sonchifolius*) is a plant that is vastly cultivated in Indonesia and has anti-diabetic activity. The purpose of this study was to isolate the compounds from the semipolar fraction of yacon leaves, as well as test them in silico and in vitro approaches. In this study, we use maceration for extraction, fractionation with n-hexane and ethyl acetate as solvent, and separation by preparative TLC method. The compounds tested were in silico by molecular docking using plant applications and in vitro by alpha-glucosidase inhibition assay. The difference between the molecular docking scores of an obtained molecule was calculated with Pair T-test methods. The results of the separation of the ethyl acetate fraction yielded a fraction, namely the H fraction. GC-MS analysis and IR spectroscopy showed that the H fraction contained the dominant compound called phthalic acid, di(2-propyl pentyl) ester. The docking score of the phthalic acid di(2-propyl pentyl) ester molecule and the alpha-glucosidase receptor, it is showed lower potency than 1-deoxynojirimycin as an alpha-glucosidase inhibitor. The in vitro test results showed that isolate H (IC50 = 130.479 ppm) from yacon leaves was no better than acarbose as an alpha-glucosidase enzyme inhibitor because the IC50 value was > 100 ppm.

Keywords: Yakon (*Smallanthus sonchifolius*); alpha-glucosidase inhibitor; molecular docking

INTRODUCTION

Diabetes mellitus is a disease with high prevalence in the world, which was estimated at 7,5% in 2019, or 373,9 million people (Saeedi et al., 2019). Meanwhile, Indonesia is one of the countries with a high number prevalence of diabetes mellitus, which is about 10.7 million patients in 2019 (Kementrian Kesehatan Republik Indonesia, 2020). Diabetes mellitus is caused by a lack of insulin levels or decreased insulin sensitivity. It can increase blood sugar levels and can become uncontrolled. Patients with diabetes mellitus especially type 2 will receive regular medication to keep their blood sugar levels under control (Banday et al., 2020). Research to identify potential medicinal plants with antihyperglycemic activity was done to find alternative therapy for patients with diabetes mellitus. The use of herbal medicine can reduce the risk of side effects from the synthetic drugs drug. One of the plants that has the potential to an antihyperglycemic is the yacon plant (Baroni et al., 2016).

Yacon (*Smallanthus sonchifolius*) is widely grown in South America. This plant belongs to the Asteraceae family. Yacon is widely cultivated in America, Europe, and Asia. Indonesia is one of the countries that has started cultivating this plant because the leaves of the yacon plant can be used for alternative therapy in patients suffering from diabetes mellitus type 2 (Baroni et al., 2016). Ethanol extract from the yacon plant can reduce blood sugar levels in animals that were induced with streptozotocin (Baroni et al., 2016). The mechanism of reducing blood sugar levels by yacon is still being studied to date. Research by (Z. Aziz et al., 2021) et all explained that based on in silico studies, yacon's leaves extract has a mechanism for inhibiting the alpha-glucosidase enzyme (Zulhemi. , Aziz et al., 2019). In vitro testing of the ethanol extract of the yacon leaves showed good inhibition for alpha-glucosidase enzyme when compared to acarbose (Serra-Barcellona et al., 2017).

In this research, the identification of compounds from the yacon plant will be carried out. Identification of antidiabetic activity will be observed through an in silico approach by molecular docking and in vitro tests on inhibition of the enzyme alpha-glucosidase.

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METHODOLOGY

Materials

Yacon's leaves powder (*Smallanthus sonchifolius*) were obtained from the Wonosobo region, water, distilled water, 70% ethanol (technical), n-hexane p.a. (MERCK), ethyl acetate p.a (MERCK), toluene p.a. (MERCK), TLC plates (MERCK), Silica gel 60 (MERCK), vanillin- sulfuric acid.

Tools

Analytical balance (OHAUS), pan, water bath, volumetric flask, Erlenmeyer glass, test tube, test tube rack, tray, filter paper, porcelain dish, UV254 and UV366 lamps, UV-Vis spectrophotometer (Thermo Scientific), infrared spectrophotometer, gas chromatography (Thermo Scientific) with mass spectrophotometer detector (Thermo Scientific), computational programs (Plants, YASARA, Passonline, Swiss Target).

Method

Plant Determination

The determination of yacon leaves (*Smallanthus sonchifolius*) was carried out at the Phytochemical Laboratory, Department of Pharmaceutical Biology, Faculty of Pharmacy, UGM. The determination aims to ensure the correctness of the plants used for research.

Extraction and Fractionation

Yacon leaves powder (1 kg) was extracted by maceration method with 10 liters of 70% ethanol for 24 hours assisted by a stirring process. The residue was remacerated with 5 liters of 70% ethanol for 24 hours. The whole obtained extract was mixed and then evaporated at room temperature to obtain a thick extract. The yield of the extract was calculated. Fractionation was carried out by solid-liquid extraction method with 2 different solvents, n-hexane p.a, and ethyl acetate p.a, The obtained extract was weighed as much as 30 grams was macerated with 500 mL of n-hexane p.a solvent. The insoluble residue of n-hexane then was macerated with 500 mL of ethyl acetate p.a. All obtained fraction was evaporated at room temperature until became a thick extract and then was identified by thin layer chromatography (TLC).

Mobile phase optimization

The mobile phase composition used was toluene: ethyl acetate formic acid with three types of comparisons:

1. toluene: ethyl acetate: formic acid (3: 1.5: 0.5);
2. toluene: ethyl acetate: formic acid (3.5: 1: 0.5);
3. toluene: ethyl acetate: formic acid (0.5: 1.5: 0.5)

One composition will be chosen that gives good separation results as the mobile phase in the preparative TLC isolation process.

Isolation and identification of compounds

Isolation of the ethyl acetate fraction used the preparative thin layer chromatography (TLC preparative) method. The mobile phase chosen was toluene: ethyl acetate: formic acid (3:1.5:0.5) in the amount of 20 mL. The size of the TLC plate used was 20 X 20 cm with an elution distance of 17 cm. The elution results before spraying vanillin-sulfuric acid reagent and after spraying vanillin-sulfuric acid were observed in UV254 and UV366 lamps. Bands that showed good separation were scraped off and then analyzed by infrared spectrophotometer, and gas chromatography tandem mass spectrometer.

In Silico Studies

The results of isolation from yakon leaves will be continued for activity exploration studies with an in silico approach with the docking method. The step is based on the bioinformatics of target proteins with anti-diabetic activity through the RCSB website. Selected target proteins were validated with the YASARA program (RMSD value ≤ 2 amstrong) (Ambarsari & Sumaryada, n.d.). After the validation process, the protein will be docked with a molecule identified by GC-MS (as a ligand) to determine the docking score. The docking scores will be a statistical analysis of the Independent SampleT–test (Unpaired T-Test) between the isolated molecular docking scores and the native ligands from PDB to find out significant differences.

In Vitro Test

In vitro testing refers to the method (Seo et al., 2009). The test solution was dissolved with DMSO until a final concentration of 500 ppm was reached. Variations in the concentration used for testing were 20, 40, 80, 120, and 180 ppm for the test sample and 0.1; 0.5; 1; 5; and 10 ppm for acarbose. For the assay, add 50 µL of 0.1 M phosphate buffer (pH 7.0), 25 µL of 4-nitrophenyl α-D-glucopyranoside 0.5 mM, 10 µL of the test sample (each variation of concentration) and 25 µL of α-glucosidase solution into microplate well. The mixed solution was incubated for 30 minutes at 37^0 C. The reaction was terminated by adding 100 µL of 0.2 M sodium carbonate solution. The hydrolysis reaction was analyzed with a microplate reader at a wavelength of 410 to see the amount of p-nitrophenol.

RESULT AND DISCUSSION

The results of plant determination carried out at the Phytochemical Laboratory, Department of Pharmaceutical Biology, Gadjah Mada University, showed that the yacon leaf sample used for this study was indeed *Smallanthus sonchifolius*, from the Asteraceae family.

The extraction process uses the maceration method with 70% ethanol solvent. The advantage of the maceration method is that it decreases the risk of damaging or losing compounds during the extraction process (Baroni et al., 2016). The

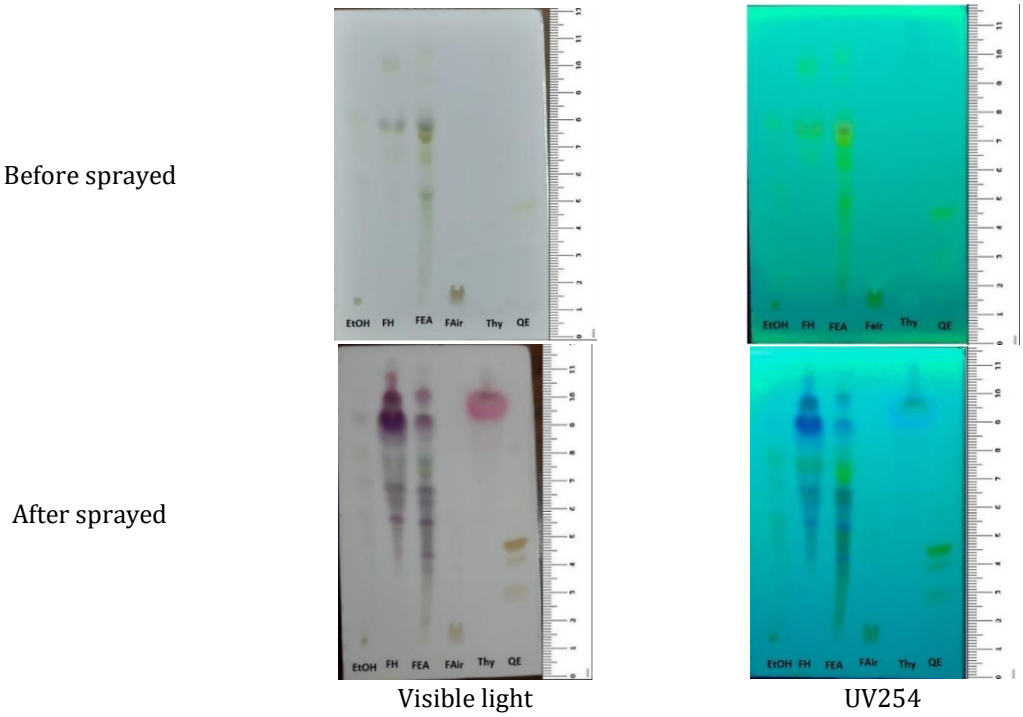

Figure 1. Thim layer chromatography for each obtained fraction from yacon leaves fractionation with thymol and quercetin reference, before and after sprayed with vanillin-sulphuric acid reagent. Silica plate as stationary phase and toluene: ethyl acetate:formic acid as mobile phase. The ethyl acetate fraction showed good separation compared to the other. The ethyl acetate fraction shown has the same spot as thymol reference.

extraction results obtained were 121.7 grams of thick extract (12.17%). It is about 30 grams thick extract was fractionated with 3 different solvents n-hexane, ethyl acetate, and water. The yields obtained from the water fraction, ethyl acetate fraction, and n-hexane fraction were 23.77 grams; 2.061 grams; and 0.781 grams. The highest yield value obtained was in the water fraction (76%), then the ethyl acetate fraction (6.6%), and the n-hexane fraction (2.5%). The fractions obtained were then observed for separation using the TLC method with selected mobile phase as toluene: ethyl acetate: formic acid (3.5: 1.5: 0.5) to see the pattern of separation in the three fractions. The results of the elution by thin layer chromatography method of the extracts for each solvent with thymol and quercetin as comparators showed that in the ethyl acetate fraction, a stain appeared which had an RF value close to the thymol. Thymol belongs to terpenoids, so it can be used as a marker for the identification of terpenoid compounds (Nagoor et al., 2017). The elution results were sprayed with vanillin-sulfuric acid reagent, showing a change in color to purplish in the thymol as well as in the n-hexane and ethyl acetate fractions. The vanillin-sulfuric acid reagent was chosen because it can identify terpenoid compounds (Ambarwati et al., 2015). Previous research explained that one of the compounds isolated from yacon leaves is an enhydrin compound that belongs to the terpenoid group(Lin et al., 2003). Enhydrin belongs to the sesquiterpene lactone group, which is a derivative of the sesquiterpene group. Enhydrin has quite a lot of activities, one of which is as an anti-diabetic(Salazar-Gómez et al., 2020). Therefore, the ethyl acetate fraction was chosen to proceed to the isolation stage with TLC preparative because it was suspected that it contained terpenoid compounds and the results of observations with TLC showed better separation when compared to the n-hexane fraction (Figure 1).

The ethyl acetate fraction obtained was separated by preparative TLC method with selected mobile phases, toluene: ethyl acetate: formic acid (3: 1.5: 0.5). The advantages of the preparative TLC method compared to other methods are the time needed is faster, easier to apply, the costs required are relatively cheaper, and the separation of compounds is quite good

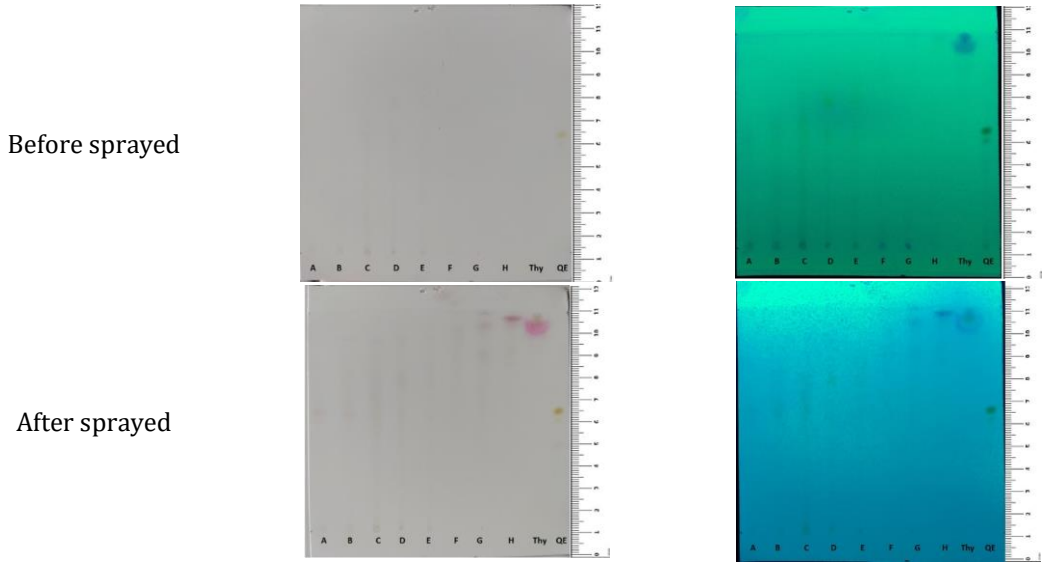

Figure 2. Preparative TLC resulted in 8 spots that appeared. The H spot was chosen for further analysis and identification for the IC50 score and molecular docking.

(Rabel & Sherma, 2017). Separation in the preparative TLC method will be influenced by the composition of the mobile phase used, so it is necessary to optimize the mobile phase to obtain the best separation (Rabel & Sherma, 2017). The results of the separation obtained as many as 8 bands on the silica plate (bands A, B, C, D, E, F, G, H) (Figure 2). All bands obtained were scraped off and then observed using the TLC method. The elution was carried out with the same mobile phase as the comparison of thymol and quercetin and sprayed using vanillin sulfuric acid reagent. Observation results in visible light show faint bands that appear in the sample. Observation under UV 254 light showed a clear attenuation of the spotting points of the thymol and quercetin compounds, and a slight attenuation in the H band. Observations under UV 366 light in the G and H bands, and the thymol compound glowed at almost the same elution distance. Observations were more clearly seen in samples that had been sprayed with vanillin sulfuric acid reagent. There was a color change from faint to purple in the G, H, and thymol bands.

The isolate obtained from the isolation process was 28.5 mg from 500 mg of the ethyl acetate fraction. The H fraction was chosen for the analysis and identification stage of the compound, because it has the most similar elution distance to standard thymol, and changed color after being sprayed with vanillin reagent sulfuric acid.

Functional group analysis was performed using infrared spectrophotometry. The results of the infrared spectra of the H fraction are as follows (Figure 3). The resulting spectra show a response at a frequency of 2920.98 cm-1 (-CH stretch). Another response at a frequency of 1463.90 cm-1 shows that there are aromatic ring groups. The hydroxy group (-OH) can be identified because of the response that appears at a frequency of 3455.77 cm-1 with a wide spectrum shape. The ester group was also identified in the H fraction of the response that appeared at a frequency of 1703.83 cm-1. The H fraction was then analyzed by gas chromatography-mass spectrometry (GC-MS).

The results of the analysis of the H fraction using the gas chromatography-mass spectrometry method, obtained the chromatogram as follows (Figure 4). The results of the GC-MS chromatogram analysis showed that in the H fraction, 1 peak appeared which was the most dominant, namely compound number 65. The compound was identified as bis(2-propyl pentyl) benzene-1,2-dicarboxylate as IUPAC name or can be called phthalic acid, di(2-propyl pentyl) ester ($C_{24}H_{38}O_4$, BM: 390) with a structure as shown in (Figure 5). Phthalic acid and di(2-propyl pentyl) esters are included in the ester derivatives of phthalic acid compounds. Ester phthalic acid is often used as a plasticizer which is often added to materials or polymers to increase the elasticity of a material. In addition, phthalic acid also has several other activities as an antimicrobial and insecticide (Huang et al., 2021). There are no studies that report the antidiabetic activity of phthalic acid,

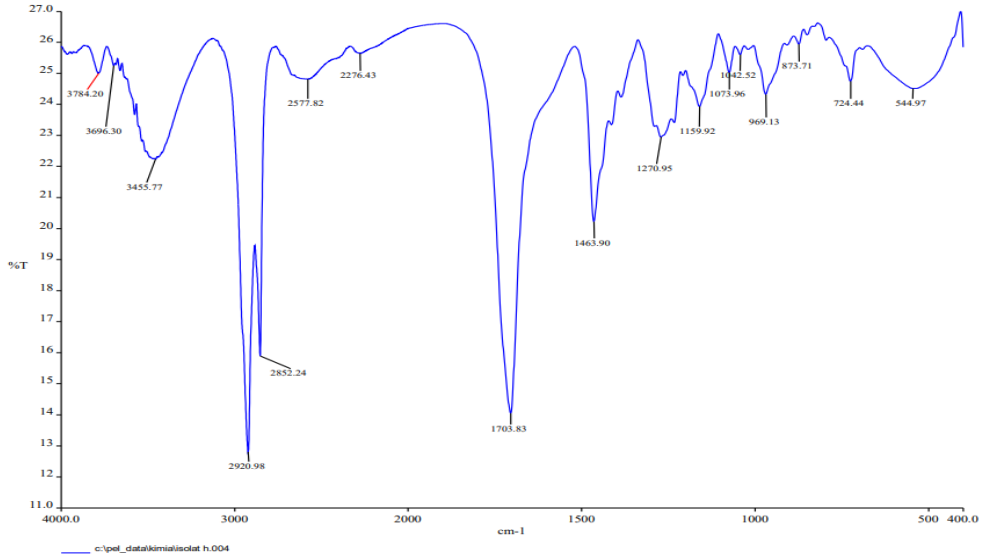

Figure 3. Infrared spectra of isolate H obtained from analysis with spectroscopy infrared with KBr pellets. Wave number used between 500 – 4000 nm

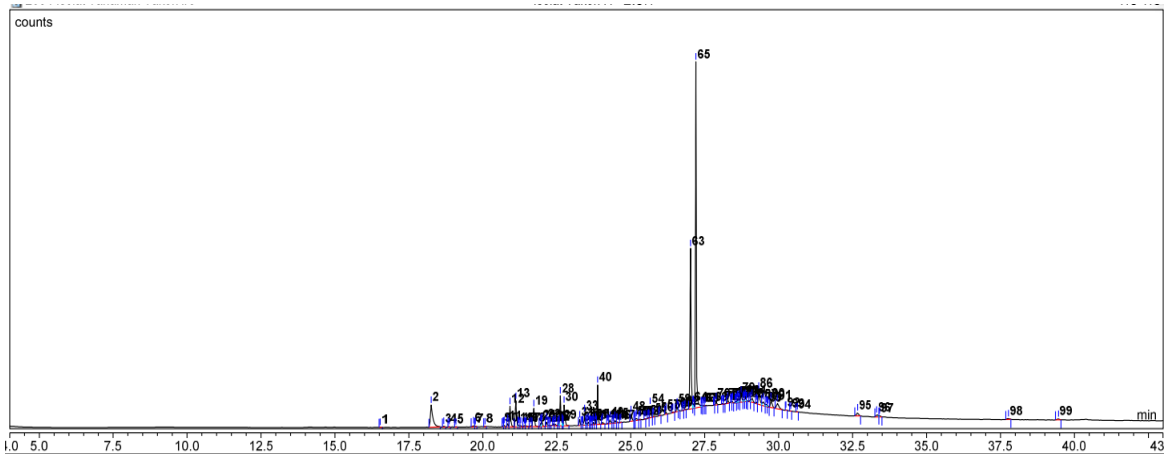

Figure 4. GC-MS chromatograms of isolate H showed that the highest peak was number 65. The dominant compound of H isolate was number 65 peak and it was Phthalic acid, di(2-propylpentyl) ester molecule (bis(2-propylpentyl) benzene-1,2-dicarboxylate).

di(2-propyl pentyl) ester compounds. Because Phthalic acid, di(2-propyl pentyl) ester has a known molecular structure, and this compound is suspected to be the most dominant in the H fraction, this compound will be docked with an enzyme that plays a role in blood glucose metabolism to see its potential antidiabetic activity.

Molecular docking is a computational chemical method that can generally be used to predict the activity of a chemical molecule. Molecular docking is used to see the interaction of the molecular structure and the target ligand (Palermo & De Vivo, 2015). The docking process uses several web-based instruments. Receptor target search via http://www.way2drug.com/passonline/predict.php. The search results for predicting the activity of the phthalic acid, di(2-propylpentyl) ester as an antidiabetic have not been found on the Passonline website. Therefore, in this study, an experiment was carried out using alpha-glucosidase receptors as protein targets for docking.

The protein target that has been selected is searched for its protein data bank (PDB) through the website https://www.rcsb.org. Proteins with PDB code 2JKE were obtained as alpha-glucosidase enzymes. The protein obtained was validated with

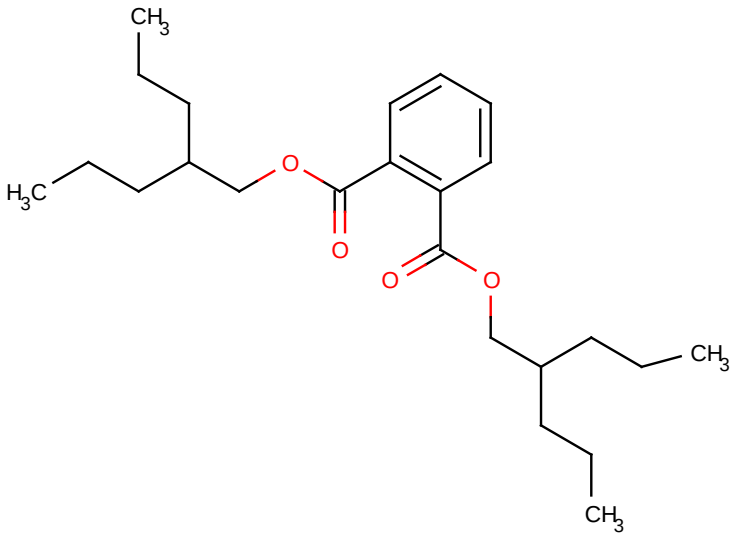

Figure 5. Bis(2-propyl pentyl) benzene-1,2-dicarboxylate structure or can be called phthalic acid, di(2-propyl pentyl) ester molecule

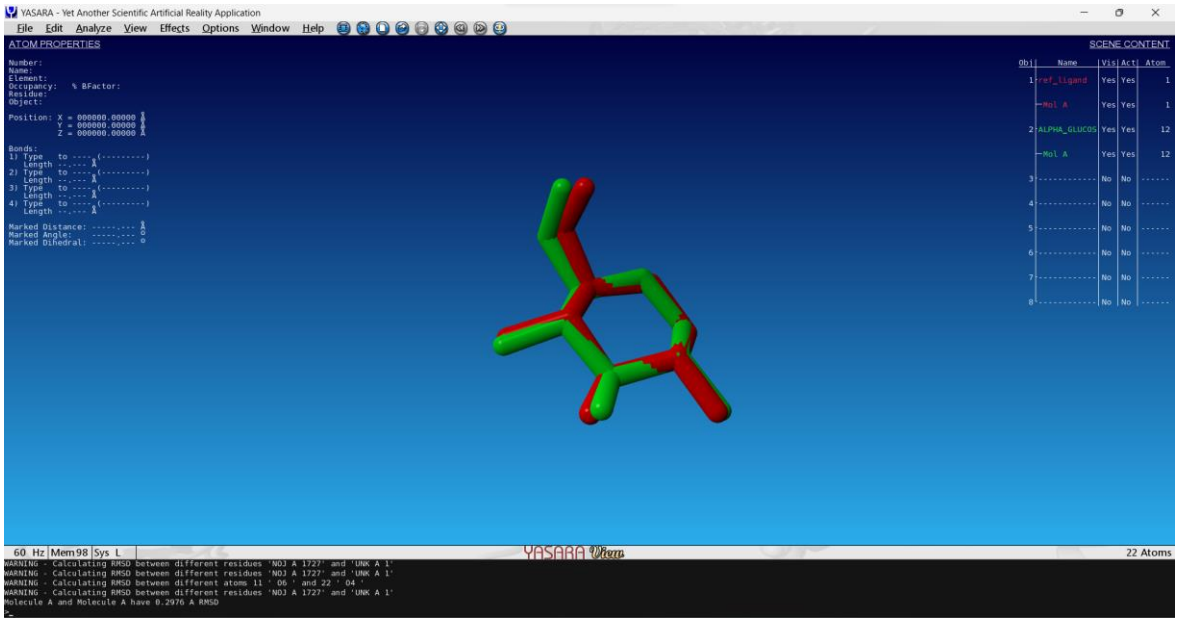

Figure 6. Validation result of protein 2JKE with programs YASARA. It showed that the RMSD score was 0,2976 amstrong (< 2 amstrong), so the protein used was valid.

the YASARA application, the purpose of carrying out the protein validation process was to simulate the docking process to find out how well the binding ability of the ligand and protein was. Good validation results are shown from the RMSD (Root Mean Standard Deviation) value, the expected RMSD value is < 2 Armstrong (Nursanti et al., 2022). The validation results obtained from the 2JKE protein are 0.276 Amstrong (Figure 6). These results indicate that the 2JKE protein is valid so that docking parameters can be applied for docking with isolated compound molecules (Nursanti et al., 2022.). The PDB code 2JKE is the code for the alpha-glucosidase protein which has a role in glucose metabolism. This protein will be docked with phthalic acid, di(2-propyl pentyl) ester molecule as a ligand. The docking process uses the PLANTS application to see the affinity and interaction between ligand receptors. The lower docking score obtained indicates that the bond between the ligand-receptor is more stable, so the interaction is better (Sari et al., 2020). The docking

Table I. Docking score of phthalic acid, di(2-propyl pentyl) ester molecule with alpha-glucosidase enzyme compared with 1-deoxynojirimycin

1-deoxynojirimycin	Phtalic Acid, di(2-propylpentyl) ester	X1 Mean	X2 Mean	Delta X Mean	Tcount	α=0.005 Ttable	α=0.05 Ttable	α=0.025 Ttable	α=0.01 Ttable
-76.961	-58.076	-77.124	-61.606	-15.518	14.725	2.879	1.734	2.101	2.552
-77.291	-53.396								
-76.914	-68.779								
-77.056	-60.327								
-77.250	-62.262								
-77.280	-56.104								
-77.020	-67.730								
-77.198	-56.994								
-77.078	-65.567								
-77.196	-66.824								

score of the phthalic acid molecule, di(2-propylpentyl) ester will be compared with the native PDB ligand, namely the 1-deoxynojirimycin molecule which is an inhibitor molecule of the alpha-glucosidase enzyme. Docking scores can be seen in Table I.

The docking results obtained showed that there were significant differences between the molecules of the compound isolated from phthalic acid, di(2-propylpentyl) ester compared to the native ligand 1-deoxynojirimycin. The difference in these results shows that the phthalic acid compound, di(2-propylpentyl) ester tends to be less potent than 1-deoxynojirimycin as an alpha-glucosidase enzyme inhibitor, so the potential of the compounds as anti-diabetics is lower than that of 1-deoxynojirimycin (Sari et al., 2020). The docking results of phthalic acid, di(2-propylpentyl) ester (-61.606) molecules were not good, because the average docking value was greater than that of 1-deoxynojirimycin (-77.124) (Ambarsari & Sumaryada, 2014). The smaller the docking value obtained, the interaction between the ligand-receptor will be more stable, so the inhibitory activity will be better (Nursanti et al., 2022).

In previous studies, it was stated that the yacon plant has inhibitory activity against the alpha-glucosidase enzyme. This is because the isolated compound obtained is nystose (Zulhemi. , Aziz et al., 2019), so the mechanism obtained is different from the current research.

Alpha-glucosidase is an enzyme that plays a role in the process of glucose metabolism in the body. One of the mechanisms for reducing blood sugar levels is by inhibiting the alpha-glucosidase enzyme (Malunga et al., 2016). Drugs that have an inhibitory mechanism against this enzyme are acarbose (Hanefeld, 2018). Alpha-glucosidase enzyme inhibition testing is often used in the drug development process to see the activity of a compound extracted or synthesized (Ikrom et al., 2014). The mechanism of inhibition of the alpha-glucosidase test in vitro was the observation of the intensity of the yellow color resulting from the reaction of alpha-glucosidase and p-nitrophenyl-alpha-D-glucopyranoside. The hydrolysis reaction of p-nitrophenyl-alpha-D-glucopyranoside by alpha-glucosidase will produce glucose and p-nitrophenol which are yellow. Inhibition of the alpha-glucosidase enzyme will affect the intensity of the yellow color produced. The stronger the alpha-glucosidase inhibition, the lower the intensity of the yellow color and the less glucose produced (Kusumawati & Indrayudha, 2021) In this study, the ethanol extract, ethyl acetate fraction, and H isolate of yakon leaves were tested in vitro using the alpha-glucosidase enzyme to see their inhibitory activity. The test results are listed in Table II.

The results of the alpha-glucosidase inhibition test, the IC50 value of the ethanol extract of yacon leaves (3.627x1013 ppm) and the ethyl acetate fraction of yacon leaves (0 ppm) did not show inhibitory activity against the alpha-glucosidase enzyme. Meanwhile, isolate H (IC50 = 130.479 ppm) had alpha-glucosidase inhibitory activity although not as good as acarbose (IC50 = 0.2474 ppm). A sample or compound is declared to have high/good inhibitory activity if the IC50 value is <50 ppm, whereas an IC50 value between 50-

Table II. IC50 value of ethanol extract, ethyl acetate fraction, and isolate H of yacon leaves from alpha-glucosidase inhibition assay

Sample	Concentration	%inhibition	SD	IC50 (ppm)
Ethanol extract	180	4.3790	0.3460	$3.627x10^{13}$
	120	2.5670	0.0654	
	80	1.5478	0.2357	
	40	0.4908	0.1308	
	20	0.2643	0.2850	
Ethyl acetate fraction	180	-0.2265	0.5190	0.00
	120	0.1888	0.5352	
	80	-0.3775	0.3640	
	40	-0.1510	0.5700	
	20	0.0000	0.6795	
H Isolate	180	56.6629	0.3460	130.4799
	120	48.0181	0.7426	
	80	40.2039	0.5190	
	40	27.1423	0.5352	
	20	13.4390	0.6440	
Acarbose	10	95.7627	0.4237	0.2474
	5	84.8164	0.1223	
	1	68.7147	0.4893	
	0.5	56.0028	0.2446	
	0.1	39.9718	1.7641	

100 ppm is stated to be active as an enzyme inhibitor (Widowati et al., 2021). Research (Widowati et al., 2021) reported that the ethanol extract of yakon leaves had alpha-glucosidase inhibitory activity with an IC50 value in the range of 50-100 ppm. One of the effects of the difference in results with previous research is possible because the samples were taken from different areas and there is a possibility of influence from the post-harvest process of yakon leaves, so different results are obtained.

CONCLUSION

Yacon leaf ethyl acetate fraction (*Smallanthus sonchifolius*) has low potential as an antidiabetic. The results of identification using the GC-MS method showed that one of the most dominant compounds contained in the ethyl acetate fraction of yacon leaves was phthalic acid, di(2-propyl pentyl) ester which belongs to the phthalic acid group. The results of the in silico isolate of yacon leaves (docking score -61.606) showed lower inhibitory activity against alpha-glucosidase enzyme than the 1-deoxynojirimycin (docking score -77.124) because the docking score obtained was greater, resulting in lower affinity for the receptor. This is supported by the IC50 value obtained by yacon leaf isolate (IC50 = 130.479 ppm) of more than 100 ppm, so its activity in inhibiting the alpha-glucosidase enzyme is lower than acarbose (IC50 = 0.2474 ppm).

ACKNOWLEDGEMENT

Thanks to the thesis supervisor, Faculty of Pharmacy UGM, and RTA UGM Batch II program in 2022 (according on letter number 2920/UN1/DITLIT/Dit-Lit/PT.01.05.2022) for the support of knowledge, funds, and motivation.

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