JPSCR: Journal of Pharmaceutical Science and Clinical Research
Volume 9, Issue 1, Pages 105-117, March 2024
https://doi.org/10.20961/jpscr.v9i1.74955

ISSN 2503-331X (Online)

Antidiabetic Potential and Metabolite Profile of Leaf and Stem Extract of *Castanopsis tungurrut* (Blume) A.DC.

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Received: 13 June 2023; **Accepted**: 24 March 2024; **Published**: 04 April 2024

Abstract
Diabetes mellitus is a metabolic disorder caused by high blood sugar levels. One species in *the Castanopsis* genus is proven with a hypoglycemic effect. Therefore, the study aimed to discover the potential of *Castanopsis tungurrut* as an antidiabetic. Sample extraction, α-amylase inhibition, glucose diffusion analysis, GC-MS analysis, and molecular docking were applied in this study. Maceration of the leaf ethanol extract showed the highest yield value of 21.08%, while the stem extract was 14.04%. Leaf ethanol extract and stem ethyl acetate extract showed the highest inhibiting α-amylase activity with an inhibition value of 33.74%±1.54 and 34.45%±1.08 at 1 mg/mL concentration. The glucose entrapment assay showed that these two extracts could inhibit the diffusion of glucose in the dialysis bag. The final result was glucose concentration in dialysate for the two extracts of 0.114±0.001 mg/mL and 0.116±0.001 mg/mL which was lower than acarbose in 0.120±0.004 mg/mL. GC-MS analysis showed 6 metabolites in leaf ethanol extract and 22 metabolites in stems ethyl acetate extract from an alkane, salicylic, cinnamic, terpene, steroid, and fatty acid. Molecular docking resulting between the compounds with α-amylase enzymes complex showed γ-sitosterol and ß-bisabolene from *C. tungurrut* extract have the potential to be developed as an antidiabetic drug due to its good inhibitory activity with binding affinity values of -9.1 and -6.9 that considered better and quite close to acarbose as control of -7.7.

Keywords: Antidiabetic; *Castanopsis tungurrut*; Diabetes mellitus; Enzyme inhibition; Gass Chromatography-Mass Spectrometry (GC-MS)

1. INTRODUCTION

Diabetes mellitus is when blood sugar levels exceed normal limits, commonly called hyperglycemia (Deshpande et al., 2008). In 2021, The International Diabetes Federation (IDF) states that there are 537 million people with diabetes. Several factors, such as unhealthy lifestyles, limited access to healthy food, high rates of obesity, and genetic factors, lead to an increase in the number of people with diabetes (Betteng et al., 2014). There are two types of diabetes mellitus, namely type I diabetes mellitus, which is caused by damage to pancreatic β cells, and type II diabetes, which is caused by insulin resistance (Samocha-Bonet et al., 2021). Pharmacological treatments are now widely used to treat diabetes and have side effects in long-

term use. For example, the use of acarbose, which is an oral drug for diabetes with digestive enzyme inhibitory activity to prevent an increase in blood sugar, has side effects such as flatulence, diarrhea, abdominal distention, and borborygmus if used in the long term (Hilma et al., 2020). Alternative treatments, such as herbal plants, are now widely known to have hypoglycemic effects with minimal side effects, such as mild digestive discomfort. The hypoglycemic activity of this herbal plant is caused by phytochemicals such as alkaloids, terpenes, flavonoids, and saponins contained therein (Suvarna et al., 2021). Flavonoids isolated from *Tamarix gallica* (Hmidene et al., 2017) and kaempferol derivatives isolated from Sedum dendroidum leaf extract (Da Silva et al., 2014)have been reported to have in vitro and in vivo antidiabetic activity.

Plants from the *Castanopsis* genus, namely *Castanopsis costata*, have been shown to have antidiabetic activity in a study by Alkandahri et al (2016) on diabetes-induced rats. The diverse phytochemical content of the *Castanopsis* genus, such as terpenoids, galloyl acid, shikimic, glycosides, saponins, steroids, flavonoids, alkaloids, and a distinctive compound in the form of Castanopsinins is suspected to support the activity of this plant as an antidiabetic (Alkandahri et al., 2016; Kim et al., 2020; Prakash et al., 2020). *Castanopsis tungurrut* is a species of the genus *Castanopsis*, which is widely found in Indonesia. This plant is included in the endangered group according to the IUCN Red List, and research related to its use as a food and pharmacological ingredient has yet to be carried out. However, *C. tungurrut* has received limited attention regarding its bioactivity potential. Research on *C. tungurrut* has primarily focused on conservation initiatives, with less documentation on its ethnobotanical aspects. The recent publication by *C. tungurrut* demonstrated its potential as an antibacterial agent (Ilyas et al., 2023).

The study of the bioactive properties of a plant species now facing the threat of extinction is of utmost importance in facilitating ex-situ conservation initiatives that actively engage the public in conservation activities. The dissemination of information regarding the advantageous properties of *C. tungurrut* is anticipated to generate public attention toward cultivating the plant outside the confines of the national park. Therefore, this study aimed to explore the potential of *C. tungurrut* as an antidiabetic agent. This study included inhibition of α-amylase enzymes, glucose entrapment, and molecular docking to assess the antidiabetic activity of extracts from the leaves and stems of *C. tungurrut* in different solvents. Furthermore, a GC-MS analysis was conducted to determine the composition and relative quantities of secondary metabolites within the leaf and stem extracts of *C. tungurrut.*

2. MATERIALS AND METHODS

2.1. Materials

Simplicia from leaves and stems of *C. tungurrut* was provided by the Research Center for Plant Conservation, Botanic Garden and Forestry, National Research and Innovation

Agency (BRIN). For extraction, sterile distilled water, ethanol (Merck), and ethyl acetate (Merck). Acarbose (Sigma Aldrich, Merck), α-amylase enzymes (TCI), starch (Merck), DNS reagents consisting of 3,5-dinitrosalicylate (Sigma Aldrich, Merck), NaOH 2N (Merck), Na-K Tartrate (Merck), phosphate buffer pH 7 consisting of the compounds $NaH_2PO_4.2H_2O$ and $Na_2HPO_4.2H_2O$ (Merck), and DMSO for inhibition of the α-amylase enzyme. Dialysis bags (Sigma Aldrich, Merck) and D (+)-Glucose (Merck) for the glucose entrapment test.

2.2. Methods

2.2.1. Extraction

Extraction was done using sterile aquadest, 70% ethanol, and ethyl acetate for each leaf and stem simplicia. The simplicia was maceration (1:10) for 3×24 hours in ethanol and ethyl acetate solvents. The filtrate obtained was concentrated using a rotary evaporator with a temperature of 40°C. For the aqua dest solvent, maceration was carried out for 24 hours in the refrigerator for samples previously heated in a water bath at 50°C for 45 minutes. The filtrate obtained was then concentrated using a rotary evaporator at 50°C, followed by a water bath at 75°C until a paste-shaped extract was obtained. The pasta extract was transferred into dark vials and subsequently stored at 10°C to set up for the following study.

2.2.2. α-Amylase Enzyme Inhibition

The amylase inhibition test (Table 1) was carried out according to the protocol of Bahtiarsyah et al. (2023). The inhibition value was then calculated using the formula 1.

$$\% \text{ Inhibition} = \frac{C-S}{C} \times 100\%$$

Formula 1. Calculation of α-Amylase Enzyme inhibition value. Description: Absorbance Control (C) and S1-S0 (Apparent absorbance) (S).

Table 1. Protocol for α-Amylase inhibition test.

Volume (mL)	Blank	Control	Sample with enzyme	Sample without enzyme
	B	C	S1	S0
Extract	-	-	0.25	0.25
Incubation (5 min, 37°C)				
Phosphate Buffer pH 7	1	0.5	0.25	0.5
α-Amylase enzyme	-	0.25	0.25	-
Incubation (5 min, 37°C)				
Starch 1%	-	0.25	0.25	0.25
Incubation (5 min, 37°C)				
DNS	0.5	0.5	0.5	0.5
Heated in waterbath (5 min, 100°C), then cooled to room temperature				
Aquadest	5	5	5	5
Vortex, and measured the absorbance in a spectrophotometer (540 nm)				

2.2.3. Glucose entrapment

This experiment aims to assess the extract's capacity to effectively bind sugar, thereby simulating the conditions within the digestive system when glucose levels increase following a meal. Dialysis bags with a diameter of 10 cm were immersed for 5 minutes in distilled water. A combined volume of 6.25 mL of 0.02M glucose and 0.125 mL of extract or sample were placed in a dialysis bag with one end tied. Following the tying of the opposite end of the bag, the dialysis bag was inserted into an Erlenmeyer containing 50 mL of distilled water. The Erlenmeyer was subsequently shaken at 150 rpm for 120 minutes. At the end of incubation, 1 mL of glucose sample was collected and measured for the glucose diffused outside of the dialysis bag by adding 1 mL of DNS followed by 80°C to 100°C heating in a water bath for 5 minutes. Subsequently, 8 mL of distilled water was added to the mixture and mixed thoroughly. Next, the absorbance of the mixture was determined at 540 nanometers using a UV-Vis spectrometer. The negative control and positive control used distilled water and acarbose at a 0.1 mg/mL concentration, respectively. The glucose concentrations were determined by plotting the absorbance to the standard glucose curve in the series of 0.2, 0.4, 0.6, 0.8, and 1 mg/mL. This protocol refers to a study by Bahtiarsyah et al. (2023).

2.2.4. Gass Chromatography-Mass Spectrometry (GC-MS)

The metabolite profiles of *C. tungurrut* ethanol and ethyl acetate extracts were examined using GC-MS (Shimadzu QP2010SE). The machine's conditions were set as follows: the GC column used was the Rtx 5 MS with 30 m length, 0.25 mm diameter, and 0.25 µm thickness. Helium gas was the carrier gas with a 28 mL/min flow rate. An average of 3 µL of the sample was injected in the spitless mode. The temperature was set to 70–300 °C with 5 °C increments every 1 minute, with a total running time of 60 min. The mass spectrometry condition used was a 70-electron volt ionization electron detector (EI 70 Ev). The GC-MS condition followed the protocol of Calvaryni and Nuringtyas (2022).

2.2.5. Molecular docking

The protein receptor is an α-amylase pancreatic enzyme with PDB ID: 1B2Y obtained from the RCSB PDB website (rcsb.org). Seventeen compounds with the highest %area of the GC-MS profiles was chosen and were collected for their 3D structures on the Pubchem website (https://pubchem.ncbi.nlm.nih.gov). 3D structures not available in Pubchem were constructed manually using Novopro (https://www.novoprolabs.com/tools/smiles2pdb). Protein preparation was carried out using UCSF Chimera software and AutodockTools 1.5.7. Ligan preparation and running of molecular docking was carried out in PyRx 0.8 software. Docking results were visualized using Pymol and DS Visualizer software.

2.3. Data analysis

Data analysis was performed for data on % inhibition and glucose concentration in IBM SPSS Statistics 20 software for the Nested test, One-way ANOVA, and Duncan's MRT post hoc.

3. Results and Discussion

Extraction using three different solvents separately was intended to determine the most effective extract in its activity as an antidiabetic. Ethanol and aquadest are polar solvents, so compounds with the same polarity can be separated in the extraction process. Ethyl acetate, with its semipolar properties, can attract compounds with polar and non-polar properties. Extraction results of 25 g simplicial of *C. tungurrut* leaves and stems in 250 mL solvent (Table 2). The highest yield value was found in the ethanol extract of *C. tungurrut* leaves, indicating that ethanol extract could separate compounds better than other solvents. The findings indicate that the compounds present in the leaves and stems of *C. tungurrut* possess a polarity similar to that of ethanol. It aligns with the solvent's tendency to separate compounds according to their polarities (Savitri et al., 2017; Verdiana et al., 2018).

Table 2. Extraction result of *Castanopsis tungurrut* leaves and stems using aquadest, ethanol, and ethyl acetate solvents.

Sample	Solvent	Extract Mass (g)	Yield (%)
Leaves	Aquadest	3.63	14.52
	Ethanol 70%	5.27	21.08
	Ethyl Acetate	0.06	2.4
Stems	Aquadest	2.25	9
	Ethanol 70%	3.51	14.04
	Ethyl Acetate	0.02	0.8

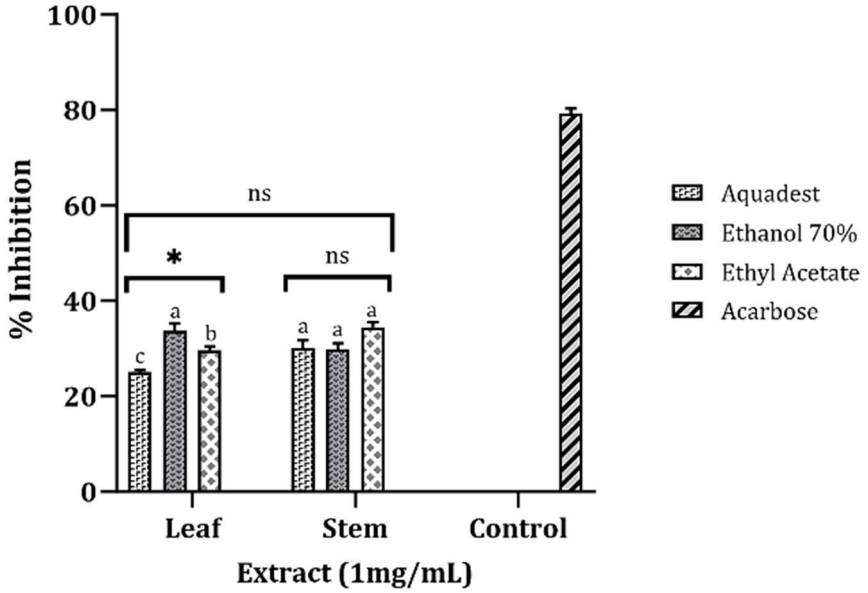


Figure 1. The α-Amylase inhibition activity of leaf and stem extract. The difference in letters is significant based on Duncan MRT (Multiple Range Test) at $p<0.05$.

In inhibiting the α-amylase enzyme, the percent inhibition in the leaves and stem samples of *C. tungurrut* was still lower than that of acarbose as the positive control. However, the best value was shown by the ethanol extract of leaves and ethyl acetate extract of *C. tungurrut* stems of 33.74 ± 1.54% and 34.45 ± 1.08%. Statistical test results using Nested ANOVA showed no significant difference in the % inhibition of the leaf and stem extract groups (p=0.536). However, the one-way ANOVA test for the three groups of solvents used had significantly different % inhibition values (p=0.003). The solvent groups that were significantly different were aquadest, ethanol, and ethyl acetate in the leaf extract, as indicated by the different subsets in Duncan's post hoc test (Figure 1). These results indicate that the inhibitory activity of α-amylase in both leaf and stem organs is not much different. However, the difference in the solvent used affects the inhibitory activity of the extract.

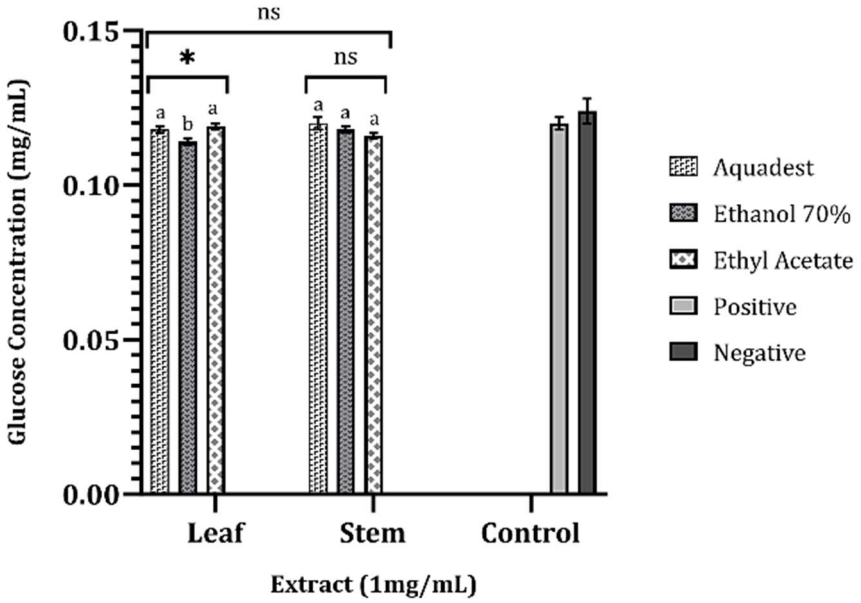


Figure 2. Inhibition of glucose diffusion from the dialysis bag in 120 min (mg/mL). The difference in letters is significant based on Duncan MRT (Multiple Range Test) at p<0.05.

The plant extract's limited inhibitory efficacy is attributed to several active chemicals involved in competitive binding with the enzyme. Furthermore, the higher potency of acarbose can be attributed to its competitive inhibition as a pure molecule, which has been demonstrated to possess significant inhibitory activity (Hilma et al., 2020; Wulandari et al., 2021). Hence, all plant extracts used in this study need to be concentrated 2-3 times to achieve a balance with acarbose's inhibitory effect. Fractionation may enhance the activity by separating the active molecules from the non-active ones through the division into multiple fractions. The inhibitory activity by plant extracts is due to the bonds that form between the hydroxy groups in the compound and amino acids on the active site of the enzyme, as a result of which enzymatic reactions can be blocked (Anugrahini and Wahyuni, 2021). In the α-amylase enzyme, inhibitor activity slows the digestion time of carbohydrates and reduces the rate of glucose absorption in the digestive tract. The hydrolysis reaction on the blocked enzyme causes disaccharides from

enzymatic reactions such as maltose, sucrose, and lactose not to be produced so postprandial blood glucose can be lowered (Torres-Naranjo et al., 2016; Wickramaratne et al., 2016).

The glucose entrapment test supported the results of α-amylase inhibition, which showed the best results in ethanol extract and ethyl acetate extract of *C. tungurrut* stems. Glucose entrapment aims to represent the process of glucose metabolism in the small intestine. The extract was tested for its ability to inhibit the transfer of glucose from the small intestine to the blood vessels, as represented by dialysis bags and dialysate (Qujeq and Babazadeh, 2013). The results obtained in this test are glucose concentration in the dialysate, indicating the possible glucose level in the blood (Figure 2).

The ethanol extract of the leaves showed the lowest glucose concentration of 0.114 ± 0.001 mg/mL, followed by the ethyl acetate extract of the stems of 0.116 ± 0.001 mg/mL. This result was lower than that of acarbose, the positive control, and aquadest, the negative control. The Nested ANOVA statistic test showed no significant difference in glucose concentration values between the leaf and stem groups (p=0,715). One-way ANOVA test showed that in the leaf group, there was a significant difference between the solvents used (p=0,008). Significant differences were shown in aquadest, significantly different from the other two solvents. The inhibitory activity by plant extracts in this glucose entrapment test was due to the concentration and molecular mass of soluble fiber in plants inhibiting the diffusion of glucose and delaying the absorption of carbohydrates. Plant fiber increases viscosity and performs binding activity, so glucose does not diffuse and glucose levels in the blood do not increase (Younas and Hussain, 2014).

Table 3. The secondary metabolite identified in the *Castanopsis tungurrut* leaf ethanol extract was analyzed using GC-MS.

No	R Time	Molecular ions (m/z)	Compounds	Area (%)	Group
1.	15.648	152,15	Methyl Salicylate	2.36	Salicylates
2.	17.927	152,24	Geranial	1.26	Monoterpenoid
3.	23.674	176,21	Ethyl Cinnamate	2.14	Cinnamates
4.	24.042	202	Curcumene	27.35	Sesquiterpenoid
5.	24.748	204,35	Beta-Bisabolene	12.52	Sesquiterpenoid
6.	38.027	296	Methyl petroselinate	1.88	Fatty acid

The GC-MS results showed that the ethanol extract of *C. tungurrut* leaves contained six types of secondary metabolites consisting of salicylic, terpenoid, cinnamic, and fatty acid groups (Table 3). The abundance of compounds indicated by % area showed that curcumene had the highest abundance of 27.35%, followed by beta-bisabolene with % area of 12.52%. Both of these compounds belong to the class of sesquiterpenoids. In the ethyl acetate extract of *C. tungurrut* stems, 22 types of compounds were found, dominated by alkane groups (Table 4). The highest abundance was shown by gamma-sitosterol, with a % an area of 16%, followed by palmitic acid, with 9.58%. Antidiabetic activity was found in ethyl cinnamate, curcumene, and

beta-bisabolene compounds in the ethanol extract of leaves (Dosoky and Setzer, 2018; Zhang et al., 2015). Meanwhile, in the ethyl acetate extract of the stems, neophyte diene, palmitic acid, oleic acid, linoleic acid, phytol, tetracosane, eicosane, 3-methyleicosane, and vitamin E compounds were reported to have antidiabetic activity in previous studies (Table 4). This activity includes inhibitory activity of related digestive enzymes and hypoglycemic activity by other assay methods (Rautela et al., 2018; Smorowska et al., 2021).

Compounds with antidiabetic activity on GC-MS identification and compounds with high abundance were subsequently subjected to molecular docking simulations to determine their inhibitory activity against α-amylase enzyme in docking simulation. This analysis was conducted to further explore the antidiabetic potential of the compound contained in the plant extract due to their weak inhibitory activity against the enzyme in previous tests. This may guide how the extract will be fractionated to obtain the active compounds.

Table 4. Secondary metabolite of *C. tungurrut* stems ethyl acetate extract analyzed using Gass Chromatogrpahy-Mass Spectrometry (GC-MS).

No	R Time	Molecular ions (m/z)	Compound	Area (%)	Group
1.	32.558	278	Neophytadiene	1.66	Sesquiterpenoids
2.	33.492	296,49	Phytol	0.67	Terpene
3.	35.283	256,42	Palmitic acid	9.58	Fatty acid
4.	38.692	280,45	Linoleic acid	5.42	Fatty acid
5.	39.208	282,47	cis-Oleic acid	1.37	Fatty acid
6.	39.942	338,69	Tetracosane	0.76	Alkane
7.	41.775	282,55	Eicosane	1.58	Alkane
8.	44.592	507	Hexatriacontane	0.79	Alkane
9.	45.217	338	Tetratetracontane	3.66	Alkane
10.	46.400	310,58	3-Methylheneicosane	0.61	Alkane
11.	47.625	296	Heneicosane	1.21	Alkane
12.	47.825	380	Pentatriacontane	1.10	Alkane
13.	48.408	394,83	Octacosane	4.61	Alkane
14.	48.867	605	Tritetracontane	4.37	Fatty acid
15.	49.350	380	Heptacosane	1.90	Alkane
16.	49.508	310	3-Methyleicosane	0.91	Alkane
17.	49.917	408	Nonacosane	4.00	Alkane
18.	50.367	801	1-Iodooctatetracontane	3.05	Alkane
19.	50.833	324	Tricosane	1.33	Alkane
20.	51.867	592	1-Hentetracontanol	1.50	Fatty acid
21.	55.433	430,71	Vitamin E	2.38	Terpenoid
22.	60.208	414,70	Gamma-Sitosterol	16.00	Sitosterols/steroid

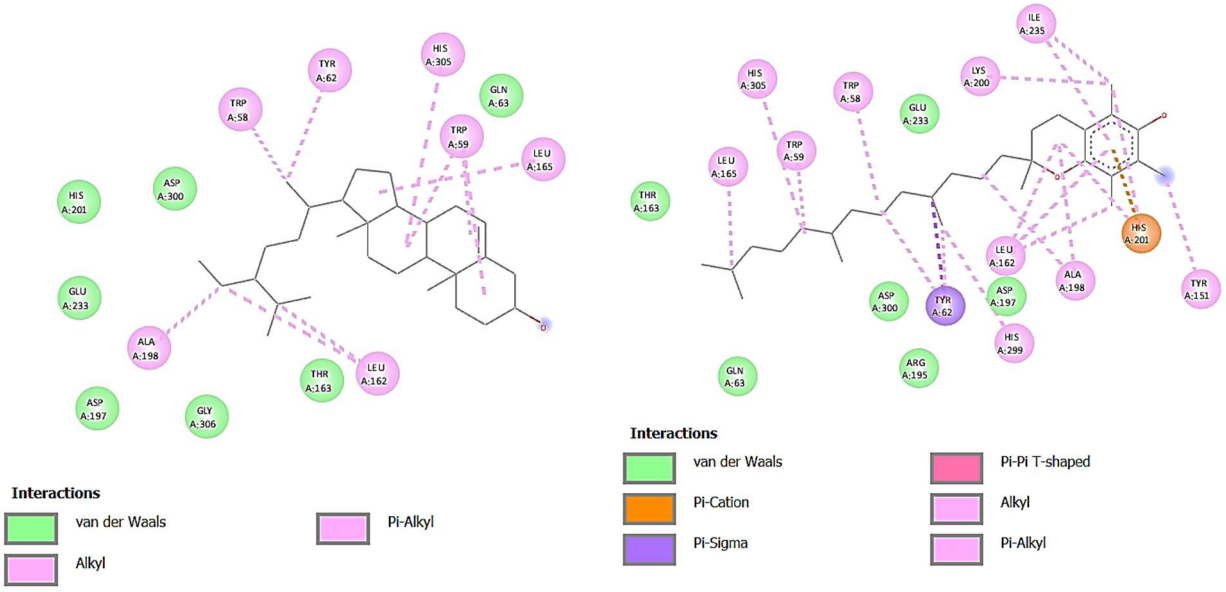
The results of molecular docking of the selected compounds with receptors in the form of α-amylase enzymes (PDB ID: 1B2Y) (Table 5). The gamma-sitosterol compound showed the best results, with the lowest binding affinity value of -9.1. This result was followed by a binding affinity value for vitamin E of -8.2 and beta-bisabolene of -6.9, close to the binding affinity of acarbose of -7.7. The lower the binding affinity value, the more likely the compounds

will bind to the protein target. The good results of these two compounds are supported by the many bonds formed with the amino acids on the receptors (Figure 3.). The gamma-sitosterol compound forms as many as 10 hydrophobic bonds in the form of Pi-Alkyl with the amino acid α-amylase enzyme.

Meanwhile, vitamin E forms as many as 17 hydrophobic bonds, and beta-bisabolene forms 9 bonds with amino acids in α-amylase consisting of Pi-Alkyl and Pi-Sigma bonds. These results indicate that these compounds have the potential to be diabetes drugs that can inhibit α-amylase enzyme activity. The activity of gamma sitosterol and vitamin E as enzyme inhibitors can be associated with their activity as antioxidants so that damage to the β cells responsible for producing insulin can be prevented and cell regeneration can be more effective. As a result, the secretion and sensitivity of insulin in controlling blood sugar levels can increase (Aly and Mantawy, 2012; Gaspersz et al., 2022). Another alternative compared to vitamin E that is commonly found is the beta-bisabolene compound, which also has an excellent binding energy during this docking. Previously, the beta-bisabolene compound had been reported to have antidiabetic activity, which was tested on the DPP-4 receptor in silico. The relatively good binding energy results indicate that this compound has potential as a type 2 diabetes mellitus drug that can handle DPP-4 protein. The results of this previous study support the activity of beta-bisabolene as an antidiabetic, which was tested against the α-amylase enzyme in this study (Sharma et al., 2022).

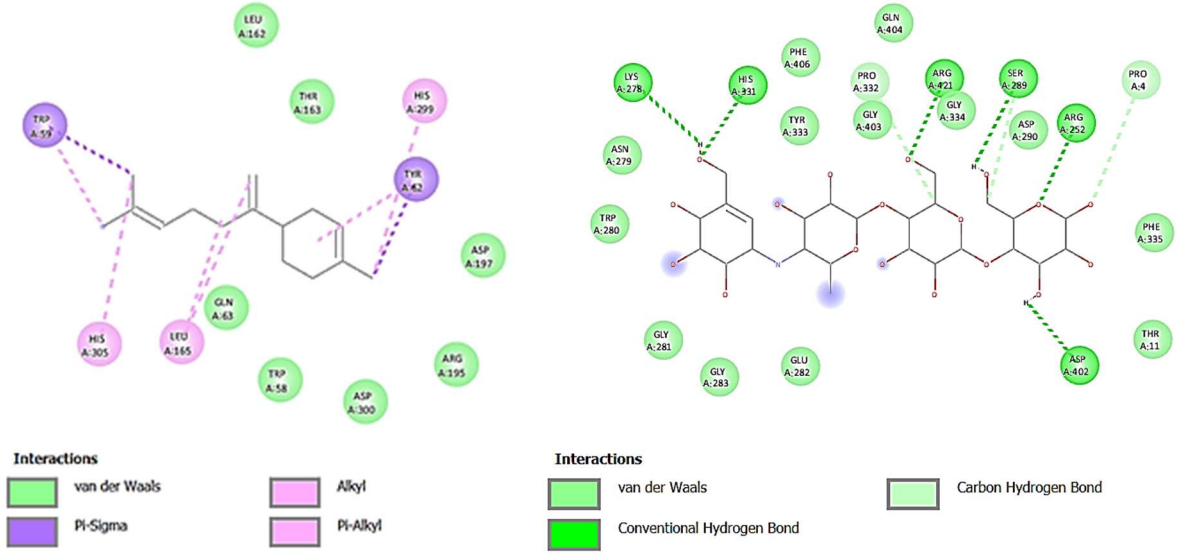
Table 5. Binding affinity of *C. tungurrut* secondary metabolite for 1B2Y Protein.

No	Ligan	Binding affinity (g/mol)
1.	Acarbose	-7,1
2.	Ethyl cinnamate	-6,1
3.	Curcumene	-6,5
4.	Beta-bisabolone	-6,9
5.	Neophytadiene	-5,5
6.	Phytol	-6,0
7.	Palmitic Acid	-5,6
8.	Linoleic Acid	-5,5
9.	Oleic Acid	-5,1
10.	Tetracosane	-5,1
11.	Eicosane	-5,0
12.	3-Methyleicosane	-4,9
13.	Nonacosane	-5,1
14.	Vitamin E	-8,2
15.	Octacosane	-4,8
16.	Heptacosane	-4,9
17.	Gamma Sitosterol	-9,1



(a) (b)

Figure 3. 2D Visualization of 1B2Y receptor docking results with gamma sitosterol (a), vitamin E (b), beta-bisabolene (c), and acarbose (d).



(c) (d)

Figure 4. 2D Visualization of 1B2Y receptor docking results with gamma sitosterol (a), vitamin E (b), beta-bisabolene (c), and acarbose (d) (*Continued*).

4. CONCLUSION

This study concludes that ethanol extract from leaves and ethyl acetate extract of *C. tungurrut* has good inhibitory activity for the α-amylase enzyme. However, a 2-3 times higher concentration is needed for maximum inhibitory activity. The glucose entrapment test showed that the activity of inhibiting glucose diffusion in the ethanol extract of leaves and ethyl acetate extract of *C. tungurrut* stems was better than acarbose. The in-silico antidiabetic potential of *C.*

tungurrut leaf and stem extracts also showed promising results, especially for the compounds gamma-sitosterol and beta-bisabolene, which are natural compounds in *C. tungurrut* plants. Gass Chromatography-Mass Spectrometry (GC-MS) analysis showed that the ethanol extract of leaves and ethyl acetate extract of *C. tungurrut* stems had secondary metabolites dominated by terpenoids, steroids, fatty acids, and alkanes.

ACKNOWLEDGMENT

This study was supported by the Research Center for Plant Conservation, Botanic Garden and Forestry, and the National Research and Innovation Agency (BRIN). This article is part of Bilqis Zahra Nabila's writings thesis entitled In Vitro Antidiabetic Activity and Secondary Metabolite Distribution of Leaves and Stems of *Castanopsis tungurrut* (Blume) A.DC.

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