Jurnal Farmasi dan Ilmu Kefarmasian Indonesia
Pharmacy and Pharmaceutical Sciences Journal
Published by Faculty of Pharmacy Universitas Airlangga
OPEN ACCESS
E-ISSN 2580-8303
P-ISSN 2406-9388

Jurnal Farmasi dan Ilmu Kefarmasian Indonesia Vol. 11 No. 3 December 2024, 345-355
DOI: 10.20473/jfiki.v11i32024.345-355
Available online at https://e-journal.unair.ac.id/JFIKI/

Phytochemical Screening and Anti-hyperglycemic Effect Test of Ethanol Extract of Waru Leaf (*Hibiscus tiliaceus*) on Glucose-loaded Mice

Vivi Sofia*, Tya Novita Firdaus, Muharni Saputri
Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy and Health, Tjut Nyak Dhien University, Medan, Indonesia

*Corresponding author: vivi@utnd.ac.id
Orcid ID: 0009-0005-3175-4731

Submitted: 17 November 2024
Revised: 19 December 2024
Accepted: 26 December 2024

Abstract

***Background**: **The** treatment of diabetes mellitus relies on synthetic drugs with various side effects. Therefore, it is necessary to explore alternative treatments with herbal therapies, such as Hibiscus tiliaceus leaves. **Objectives**: The aim of this study was to determine the anti-hyperglycemic effect of an ethanol extract from Hibiscus tiliaceus leaves in glucose-loaded mice. **Methods**: The initial stage of dried leaf characterization was to ensure the identity, quality, purity, and safety to be used, and then extracted using the maceration method with 70% ethanol solvent. The next step was phytochemical screening to identify secondary metabolite content. The anti-hyperglycemic effect was evaluated using the oral glucose tolerance test (OGTT) on 25 male mice divided into five treatment groups. The negative control group was given Na CMC 0.5% w/v Na CMC, and the positive control group was administered glibenclamide. The ethanol extract of Hibiscus tiliaceus leaves was administered at doses of 200, 400, and 800 mg/kg. Approximately 200 µL of blood was collected and analyzed for glucose levels. The data were statistically analyzed using one-way analysis of variance (ANOVA) with SPSS ver. 25 program. **Results**: Hibiscus tiliaceus leaves contain alkaloids, flavonoids, saponins, and tannins. The highest decrease in blood glucose levels was observed in the ethanol extract group at a dose of 400 mg/kg BW, with a decrease of 78.52%, followed by a dose of 200 mg/kg BW of 76.63%, a dose group of 800 mg/kg BW of 73.48%, and a positive control group (glibenclamide) of 34.68%, which was significantly different from the negative control group (Na CMC 0.5%) ($p < 0.05$). **Conclusion:** The ethanol extract of H. tiliaceus has anti-hyperglycemic effects.*

***Keywords**: anti-hyperglycemia, Hibiscus tiliaceus, mice, phytochemical screening*

How to cite this article:
Sofia, V., Firdaus, T. N. & Saputri, M. (2024). Phytochemical Screening and Anti-hyperglycemic Effect Test of Ethanol Extract of Waru Leaf (*Hibiscus tiliaceus*) on Glucose-loaded Mice. *Jurnal Farmasi dan Ilmu Kefarmasian Indonesia*, 11(3), 345-355. http://doi.org/10.20473/jfiki.v11i32024.345-355

P-ISSN: 2406-9388
E-ISSN: 2580-8303

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder characterized by sustained hyperglycemia. This disorder results from a dysfunction in insulin production, response to insulin, or a combination of both. Uncontrolled increases in blood sugar levels can lead to serious complications in the cardiovascular system, vision, kidney function, and the nervous system (Naenggolan et al., 2023). The incidence of diabetes mellitus continues to increase both globally and nationally in Indonesia. Therefore, there is an urgent need to develop effective and safe therapeutic strategies. Conventional therapies using synthetic drugs have proven effective in controlling blood glucose levels. However, the use of these drugs often leads to undesirable side effects. This encourages researchers to explore alternative treatments from natural sources that have minimal side effects (Zebua et al., 2024). Natural herbs and their bioactive compounds can be effectively used as alternative therapies through a variety of drug therapy target pathways. In recent years, the scientific community has paid special attention to the use of traditional medicinal plants for diabetes management because of their significant therapeutic potential (Zhou & Yu, 2022). Waru (Hibiscus tiliaceus) is one of the herbal plants widely used by the community. Waru is one of the local names for herbal plants in Indonesia.

Researchers have identified *Hibiscus tiliaceus* as a plant with potential for the development of anti-hyperglycemic agents. Recent studies have shown that *Hibiscus tiliaceus* leaf extract contains flavonoid compounds that play an important role in antidiabetic activity. Flavonoid test screening results showed that methanol extracts and ethanol extracts of tree bark had higher total flavonoid levels than ethanol solvents in *Hibiscus tiliceus* bark extracts positive for flavonoids. The 72-hour maceration method with methanol solvent produced the extract (Putri, 2024). Bioactive compounds in waru leaves interact in a complex manner and affect certain metabolic pathways, resulting in antihyperglycemic activity. *Hibiscus tiliaceus* leaves showed strong DPPH free radical-scavenging activity and activity against MCF-7 cells. The results showed that H. tiliaceus leaves have antioxidant potential (Andriani et al., 2020). This mechanism is important in the management of diabetes mellitus. Rahmawati and Handayani (2020) reinforced these findings using a comprehensive phytochemical study. They identified various bioactive compounds in *Hibiscus tiliaceus* leaves that have the potential to exert anti-diabetic effects (Rahmawati and Handayani, 2020). Various studies have demonstrated the promising anti-hyperglycemic potential of waru leaves. However, some aspects require further investigation. Naenggolan et al. (2023) emphasized the need for the development of standardized extraction methods and a thorough evaluation of the safety of the long-term use of waru leaf extracts. Researchers also need to deepen their understanding of the molecular mechanisms underlying anti-hyperglycemic effects. Further research is needed to determine the optimal dose and compare its effectiveness with that of standard antidiabetic drugs.

Based on the previous explanation, the researchers wanted to conduct further research using various dose variations of the ethanol extract of *Hibiscus tiliaceus* leaves to reduce blood glucose levels in male white rats using the oral glucose tolerance method.

MATERIALS AND METHODS

Materials

The materials used in this study were *Hibiscus tiliaceus* leaves from Tanjung Harapan Dusun 1, Air Putih District, Batubara Regency, North Sumatra, Na CMC (Sigma Aldrich®), Aquadest, 70% ethanol (Smart Lab®), and HCl. The materials used were concentrated (Smart Lab®), H_2SO_4 (Merck®), NaOH (Merck®), $FeCl_3$ (Merck®), glacial acetic acid (Merck®), and reagents (Mayer, Dragendorff, Liebermann-Bouchardart).

Tools

Analytical balance (Ohauss®), glucometer (EasyTouch®), Erlenmeyer flask (Pyrex®), measuring cylinder (Pyrex®), water bath (Memmert®), blender (Miyako®), and mobile camera (Samsung A52®), gas stove (Rinnai®), drying oven (Horja®), oven (Memmert®), rotary evaporator (Buchi®), animal scale (Kenmaster®), mortar, pestle, animal cage, parchment, syringe (Terumo®).

Method

Plant identification

Plant identification was performed at the University of North Sumatra's Herbarium Medanense (MEDA) with specimen number 1486/MEDA/2023. Identification begins by sending plant parts such as leaves, stems, and roots. The accompanying cover letter does not include the name of the region of origin of the test plants.

Collection and extraction of dried leaves

Hibiscus tiliaceus leaves obtained from Tanjung Harapan Village, Air Putih District, Batubara Regency, North Sumatra, were dried in an oven at 40°C for 48 h and then pulverized using a blender until powdered. The

P-ISSN: 2406-9388
E-ISSN: 2580-8303

extraction process was performed using a maceration method with 70% ethanol. Dried plant powder (500 g) was placed into the macerator, and the solvent was added until it was completely submerged. After that, the mixture was allowed to stand for five days while stirring frequently and repeatedly. After five days, the filtrate was filtered, and the filtered pulp was returned to a dark-colored bottle and rinsed again with 70% ethanol. The extraction process was repeated twice using the same type and amount of solvent. The liquid extract was then concentrated using a 50^0C rotary evaporator until a thick extract was obtained. The yield of the extract is then calculated by calculating the percentage between the weight of the extract and the weight of the dried leaves (Kemenkes RI, 2017).

Ethical clearance

This research was approved by the Ethics Commission of the Faculty of Medicine, Prima University, as the research institution with No.080/KEPK/UNPRI/11/2024.

Specific organoleptic test of the herbal extract

The specific organoleptic test of the dried plant preparation entails a description of the form, odor, color, and taste using the five senses (Kemenkes RI, 2017).

Non-specific parameters for testing herbal extract

Moisture content

A total of 200 ml of toluene and 2 ml of distilled water were placed in a round-bottom flask and distilled for 2 h. Then, the toluene was allowed to cool for 30 min, and the volume of water was read in the receiver tube with an accuracy of 0.05 ml; then, 5 g of dried leaf powder was placed into a flask that had been carefully weighed and then heated carefully for 15 min. After the toluene boiled, the drip speed was set at two drops per second until most of the water was distilled, and then the drip speed was increased to drops per second. After all water was distilled off, the interior of the cooler was rinsed with toluene. Distillation continued for 5 minutes; then, the receiving tube was allowed to cool at room temperature. After the water and toluene were completely separated, the volume of water was read with an accuracy of 0.05 ml; the difference between the two volumes of water read corresponds to the water content contained in the material being examined. Moisture content is calculated in percentages (Kemenkes RI, 2017).

$$\%\text{moisture content} = \frac{\text{initial volume} - \text{final volume}}{\text{dried leaves weight}} \text{ x } 100\%$$

Total ash content

Dried leaves weighing approximately 2-3 grams, were placed in a pre-weighed crucible. The crucible containing dried leaves was then transferred to a burner. The combustion process was carried out at 500-600°C for 4-6 hours or until the residue turned white ash or grayish ash. After the combustion was complete, the crucible was removed from the furnace using heat-resistant tongs and immediately placed in a desiccator. The crucible was left in a desiccator until it cooled to prevent the absorption of water vapor from air. After the crucible had cooled, the crucible was weighed along with the ash (Kemenkes RI, 2017).

$$\text{Total ash content} = \frac{\text{initial weight} - \text{final weight}}{\text{dried leaves weight}} \text{ x } 100\%$$

Acid insoluble ash content

The ash obtained in the determination of total ash content was boiled with 25 ml of dilute hydrochloric acid for 5 min. The part that did not dissolve in acid was filtered using ash-free filter paper, washed with hot water, and incinerated in a crucible until the weight remained at 600 ^{0}C. The insoluble ash content in acid was calculated based on the weight of the test in %w/w (Kemenkes RI, 2017).

The water-soluble essence content

Approximately 5 g of the powder, which was air-dried, was weighed with precision. The solution was transferred to a stoppered flask and 100 ml of chloroform-saturated water was added. The contents were shaken repeatedly for the first 6 h and left for 18 h. The solution was then evaporated to dryness in a flat-bottomed cup that had been heated and torn. The remaining solution was heated to 105°C, and the weight was recorded (Kemenkes RI, 2017).

$$\text{Water soluble essence content} = \frac{\text{extract weight}}{\text{dried leaves weight}} \text{ x } \frac{100}{20} \text{ x}100\%$$

Ethanol soluble essence content

Five grams of air-dried powder was weighed, placed in a flask with a lid, 100 ml of ethanol was added, shaken repeatedly for the first 6 h, allowed to stand for 18 h, and then filtered quickly to avoid evaporation of ethanol. Approximately 20 ml of the filtrate was evaporated to dryness in a heated and calibrated cup, and the rest was heated at 105 ^{0}C until the weight remained (Kemenkes RI, 2017).

P-ISSN: 2406-9388
E-ISSN: 2580-8303

$$\text{Ethanol soluble essence content} = \frac{\text{extract weight}}{\text{dried leaves weight}} \text{ x } \frac{100}{20} \text{ x100\%}$$

Phytochemical screening

Alkaloid

A total of 2 mL of the solution was evaporated in a porcelain cup until the residue was obtained. The residue was then dissolved in 5 mL of 2 N HCl. The obtained solution was divided into three test tubes. Two N HCl was added to the first tube is added with 2 N HCl, which served as a blank. Dragendorff reagent was added to the second tube as much as three drops, and Mayer reagent was added to the third tube as much as three drops. The formation of an orange precipitate in the second tube and a white-to-yellowish precipitate in the third tube indicates the presence of alkaloids (Lolok et al., 2020)

Flavonoids

One milliliter of the test extract solution was evaporated to dryness, the remainder was moistened with acetone P, and a small amount of boric acid fine powder P and oxalic acid fine powder P were added, carefully heated in a water bath, and excessive heating was avoided. The remainder obtained was mixed with 10 mL of ether P, observed under UV light at 366 nm, and fluoresced intensely yellow, indicating the presence of flavonoids (Lolok et al., 2020).

Tannin test

A total of 1 mL of the test solution was reacted with 10% iron (III) chloride solution; if a dark blue, blue-black, or greenish-black color occurs, it indicates the presence of polyphenol compounds and tannins. (Lolok et al., 2020).

Saponin test

The test extract was placed in a test tube and 10 mL of hot water was added, cooled, and shaken vigorously for 10 s. A steady froth for not less than 10 minutes as high as 1-10 cm. The foam did not disappear with the addition of 2 N HCl (Lolok et al., 2020).

Steroid/ triterpenoid test

The extract was dissolved in chloroform (0.5 mL) and added to anhydrous acetic acid (0.5 mL). Furthermore, this mixture was dripped with 2 mL concentrated sulfuric acid through the tube wall. The formation of a bluish-green color indicated the presence of sterols. A brownish or violet ring on the border of the two solvents indicated the presence of triterpenoids (Lolok et al., 2020).

Anti-hyperglycemic effect test

An experiment was conducted to test the anti-hyperglycemic effects of the substance in question. A study was conducted on the oral glucose tolerance method using 25 male mice aged 2-3 months and weighing 20-35 grams. Animals were randomly divided into five groups. The first group served as the negative control and was administered 0.5% sodium carboxymethyl cellulose. Na CMC is used as a suspending agent that can make the suspension homogeneous and evenly dispersed, making it easy to use. As a carrier, Na-CMC is neutral, inert, and does not have pharmacological effects. The second group served as the positive control and was administered with glibenclamide at a dose of 0.013 mg/kg BW. The remaining three groups were treated with test extracts, with each dose consisting of 200 mg/kg BW, 400 mg/kg BW, and 800 mg/kg BW. Prior to the commencement of the study, the mice were acclimated to their surroundings for seven days. The mice were first fed for 16 h, and their initial blood glucose levels were measured (T0). The test preparation was administered 30 min prior to administration of the glucose induction load to the test animals. Subsequently, each experimental animal was administered a glucose solution at a dose of 0.195 g/kg BW 30 min after administration of the test preparation. Subsequently, blood glucose levels were assessed at 30, 60, 90, 120, and 150 min after the administration of the glucose load. Blood glucose levels were monitored by collecting a drop of blood from the tail of each rat using a sterile lancet. The blood sample was then applied to a test strip that had been paired with an easy-to-touch strip on a glucometer. The resulting blood glucose level data were expressed in mg/dL, and the Area Under Curve (AUC) was calculated using the following formula:

$$AUC_{0\text{-}150} = \frac{t1-t0}{2}(C_0 + C_1) + \frac{t1-t0}{2}(C_1 + C_2) + \frac{t1-t0}{2}(C_n + C_{n-1})$$

t = time (in minutes)
C = blood glucose level (in mg/dL)
$AUC_{0\text{-}150}$ = the area under the curve from minute 0 to 150

Once the $AUC_{0\text{-}150}$ value is calculated, the percentage reduction in blood glucose levels (% RBG) can be determined using the following equation:

$$\% \text{ RBG} = \frac{AUC\,0-150\ (\text{negative control}) - AUC\,0-150\ (\text{treatment control})}{AUC\,0-150\ (\text{negative control})} \text{ x } 100\ \%$$

Statistical analysis

The data were analyzed for normality using the *Shapiro-Wilk* test and for homogeneity using the *Levene*

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E-ISSN: 2580-8303

test, with a 95% confidence level. Subsequently, an ANOVA one-way test was conducted using the SPSS.25 software, followed by the *Post Hoc Tukey* test.

RESULTS AND DISCUSSION

The results and subsequent discussion pertain to the yield of *Hibiscus tiliaceus* leaf extract. Extraction was conducted using the maceration method with 70% ethanol as the solvent. Table 1. shows the results of the fresh *Hibiscus tiliaceus* leaf extracts. From 1.8 kg of fresh leaves, a thick extract with a yield of 4.68 % was obtained. The results of the organoleptic examination of the *Hibiscus tiliaceus* leaves are shown in Table 2.

From the results of the examination of non-specific parameters of dried leaves for five parameters, all of them meet the requirements that have been set by the 2nd edition of the Herbal Pharmacopoeia 2017, which is shown in Table 3 (Kemenkes, 2017).

Phytochemical screening results

Phytochemical screening was conducted using qualitative analysis and the tube test method, which involves color reactions and precipitation. The results of these tests are listed in Table 4.

The results of phytochemical analysis showed that *Hibiscus tiliaceus* leaves contain alkaloids, flavonoids, tannins, and saponins (Table 4). This is reinforced by the findings of other researchers (Hidayah et al., 2021).

The anti-hyperglycemic efficacy of the *Hibiscus tiliaceus* leaf extract was evaluated. The effects of oral glucose administration on the blood glucose levels of mice are shown in Table 5.

Table 1. Extract yield results

Extraction time (hour)	Fresh leaf (g)	Dried leaves weight (g)	Extract weight (g)	% Yield
72	1800	500	84.24	4.68

Table 2. The macroscopic and organoleptic examination of the *Hibiscus tiliaceus* leaf

Number	Description	Information	
		Whole Leaf	Powder
1	Shape	cordatus	powder
2	Color	dark green	dark green
3	Smell	aromatic	aromatic
4	Taste	bitter	bitter
5	Leaf bones	pincer	-
6	Leaf tips	tapered	-
7	Leaf base	grooved	-
8	Leaf surface	Thin hairy	-
9	Leaf edge	crenate	-
10	Leaf size	long: 8 - 19 cm Wide: 7.5- 10 cm	-

Table 3. Results of examination of non-specific parameters of dried leaves

Number	Characteristic of dried leaves	Result	Farmakope Herbal Indonesia 2thEd (Kemenkes RI, 2017)	Criteria
1	Moisture content	7.32 %	≤ 10%	meet the requirements
2	Total ash content	9.98 %	≤ 10%	meet the requirements
3	Acid insoluble ash content	5.31 %	≤ 7%	meet the requirements
4	Water soluble essence content	11.50 %	≥ 10%	meet the requirements
5	Ethanol soluble essence content	12.79 %	≥12%	meet the requirements

Table 4. Results of the phytochemical screening test of *Hibiscus tiliaceus* leaf

Number	Screening	Reagent	Observation	Result
1	Alkaloids	Dragendorff	Brown precipitate	(+)
		Bouchardat	Brown	(+)
		Mayer	no white precipitate	(-)
2	Flavonoids	Zn + HCl(p)	red orange	(+)
		Mg + HCl(p)	yellow	(+)
3	Tannin	FeCl 5%	blackish green	(+)
4	Saponins	Whisked with hot water + HCl(p)	there is foam	(+)
5.	Steroids/Triterpenoids	Liebermann-Burchard	green	(-)

Remarks: (+) contains the compound examined, (-) does not contain the compound examined

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E-ISSN: 2580-8303

Table 5. Average results (mg/dL) and standard deviation in the oral glucose tolerance test method

Group	Average ± SD blood glucose level (mg/dL) at the minute...					
	0	**30**	**60**	**90**	**120**	**150**
Negative control (CMC Na 0,5% w/v)	76.83 ± 8.61	275.62 ± 10.48	255.81 ± 9.06	233.18 ± 11.3	213.62 ± 6.24	177.42 ± 10.34
Positive control (glibenclamide 0,013 mg/kg BW)	81.82 ± 15.06	257.25 ± 8.10	237.63 ± 5.85	177.84 ± 24.57	138,78 ± 19.11	98,23 ± 7.56
Ethanol extract of *H. tiliaceus* leaf (200 mg/kg BW)	130.62 ± 55.99	207.41 ± 45.69	180.29 ± 47.98	171.26 ± 36.26	141.81 ± 46.35	133.83 ± 53.11
Ethanol extract of *H. tiliaceus* leaf (400 mg/kg BW)	103.45 ± 24.23	176.28 ± 82.27	148,07 ± 62.02	124,45 ± 34.39	114.42 ± 27.13	118.76 ± 25.06
Ethanol extract of H. *tiliaceus* leaf (800 mg/kg BW)	90,01 ± 11.84	187,05 ± 5.58	149.23 ± 10.97	114.38 ± 16.93	104.83 ± 11.58	97.85 ±9.36

Table 6. Average change in glucose levels (mg/dL)

Group	Average change in glucose levels ± SD (mg/dL) at the minute...					
	0	**30**	**60**	**90**	**120**	**150**
Negative control (CMC Na 0,5% w/v)	0	198.79 ± 1.87	173.99 ± 4.50	156.35 ± 2.69	136.79 ± 2.37	100.59 ± 1.73
Positive control (glibenclamide 0,013 mg/kg BW)	0	175.43± 7.04	155.81 ± 6.96	96.02 ± 9.21	56.96 ± 4.05	16.41 ± 7.50
Ethanol extract of *H. tiliaceus* leaf (200 mg/kg BW)	0	76.79 ± 10.30	49.67 ± 8.01	40.64 ± 19.73	11.19 ± 9.64	3.21 ± 2.88
Ethanol extract of *H. tiliaceus* leaf (400 mg/kg BW)	0	72.83± 58.04	44.62 ± 37.79	21.00 ± 10.16	10.97 ± 2.90	15.21 ± 0.83
Ethanol extract of *H. tiliaceus* leaf (800 mg/kg BW)	0	97.04 ± 6.26	59.22 ± 0.87	24.37 ± 5.09	14.82 ± 0.26	7.84 2.48

Table 7. Decrease in blood glucose levels

Group	Average of AUC_{0-150} (mg. minute/dL) ± SD	Percent decrease in blood glucose levels (%)
Negative control (CMC Na 0,5% WV)	11497.65 ± 815.27	0
Positive control (glibenclamide 0,013 mg/kg BW	7509.95 ± 985.55*	34.68
Ethanol extract of *H. tiliaceus* leaf (200 mg/kg BW)	2722.50 ± 107.48*	76.63
Ethanol extract of *H. tiliaceus* leaf (400 mg/kg BW)	2469.47 ± 215.58*	78.52
Ethanol extract of *H. tiliaceus* leaf (800 mg/kg BW)	3049.31 ± 450.17*	73.48

* = significantly different from the negative control group (CMC Na 0.5% w/v)

P-ISSN: 2406-9388
E-ISSN: 2580-8303

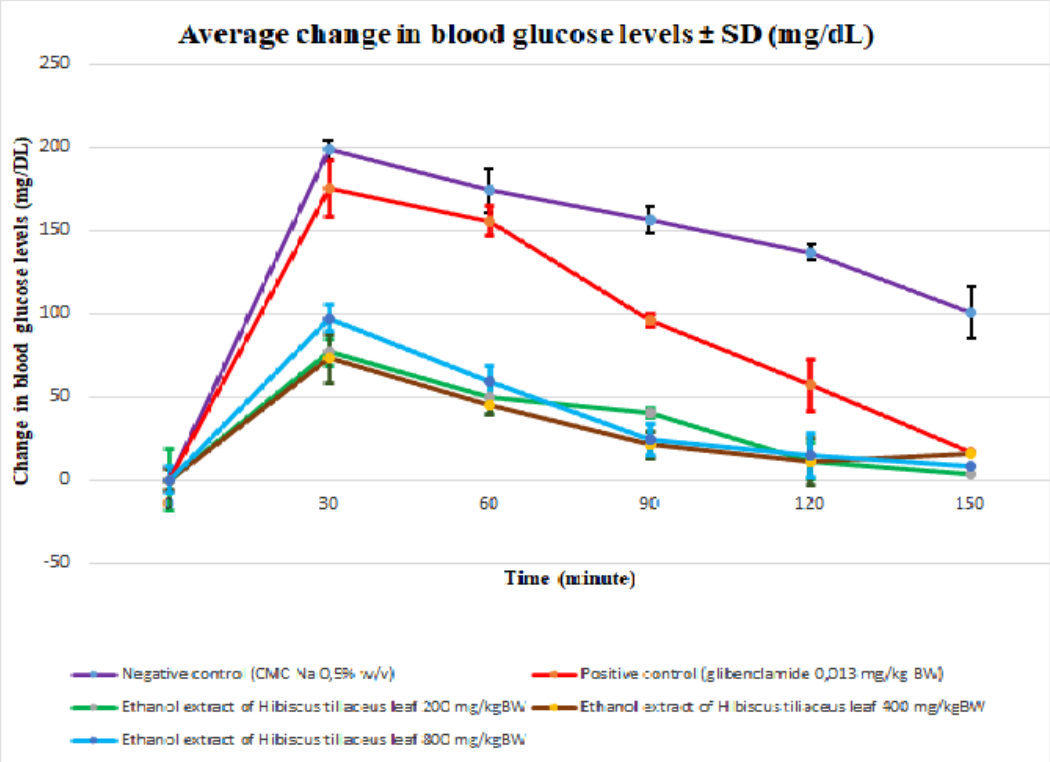

Figure 1. Average change in blood glucose levels

Blood was collected from the orbital sinus of the eye at minute (-90) before treatment to determine the normal blood glucose level of rats, where minute 0 was the time of glucose loading. Minute (-60) is the time of blood collection from test animals immediately after being given glibenclamide or suspension of ethanol extract of *Hibiscus tiliaceus* leaves before being given glucose at minute 0. After the test animals were administered glucose, blood was collected at the 30^{th}, 60^{th}, 90^{th}, $120^{th,}$ and 150^{th} min so that the blood of each test animal from the orbital sinus as a whole was taken at the 90^{th}, 60^{th}, 0, 30^{th}, 60^{th}, 90^{th}, 120^{th} and 150^{th} minutes. To compare the anti-hyperglycemic effect between the groups, the changes in blood glucose levels of each treatment group were calculated, and the area under the curve from the 0th minute to the 150^{th} minute (AUC_{0-150}) was calculated. The value AUC_{0-150} of each treatment group shows the amount of change in blood glucose levels for 150 min because of each treatment in each group. The AUC value was inversely proportional to the antihyperglycemic effect of the preparation. The smaller the AUC value, the greater the antihyperglycemic effect of the preparation. Data on the changes in blood glucose levels and AUC_{0-150} can are seen in Tables 6 and 7. From the AUC_{0-150} value, it can be said that the one that shows the most significant effect of reducing blood glucose levels is the treatment with ethanol extract of *Hibiscus tiliaceus* leaves at a dose of 400 mg/kg BW (body weight), followed by treatment with the ethanol extract of *Hibiscus tiliaceus* leaves (200 mg/kg BW), treatment with ethanol extract (800 mg/kg BW), and positive control (0.013 mg/kg BW). The AUC value provides an overall picture of the effect of a compound or drug on the change in the measured parameter over time. In antihyperglycemic studies, the AUC shows how much and how long the compound is effective in reducing blood glucose levels. The calculation of the AUC allows for a more accurate quantitative assessment than just looking at a specific time point, so that differences between doses or treatments are more clearly visible.

The glucose levels of all test groups showed a significant increase ($p<0.05$) after a glucose induction dose of 0.2 g/kg BW. This indicates that the induction of a glucose dose of 0.2 g/kg BW successfully caused hyperglycemia in the test animals, which reached a peak at the 30^{th} minute. All test groups of H. tiliaceus ethanol extract at doses of 200, 400, and 800 mg/kg BW showed a decrease in blood glucose levels that were significantly different when compared to the negative control group ($p<0.05$) from the 30^{th}-minute observation to the 150^{th} minute. This indicates that all test groups of the ethanol extract of waru leaves at doses of 200, 400, and 800

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E-ISSN: 2580-8303

mg/kg BW exhibited anti-hyperglycemic effects (Table 5 and Figure 1).

The highest decrease in blood glucose levels was observed in the *Hibiscus tiliaceus* leaf ethanol extract group at a dose of 400 mg/kg BW, with a reduction of 78.52%, followed by a dose of 200 mg/kg BW (76.63%), a dose group of 800 mg/kg BW (73.48%), and a positive control group (glibenclamide) of 34.68%. The results of Tukey's Post Hoc test conducted to further evaluate differences between groups showed that in the positive control group (glibenclamide), the *Hibiscus tiliaceus* leaf ethanol extract group at doses of 200, 400, and 800 mg/kg BW, there were significant differences compared to the negative control group (NaCMC 0.5%). This indicates that the ethanol extract of *Hibiscus tiliaceus* leaves administered at doses of 200, 400, and 800 mg/kg BW can effectively reduce blood glucose levels in rats. Between the dose groups of 200, 400, and 800 mg/kg BW of *Hibiscus tiliaceus* leaf ethanol extract, there was no statistically significant difference in their ability to lower blood glucose levels ($p<0.05$). In addition, the decrease in blood glucose levels observed in all test groups did not lead to hypoglycemia.

In this study, increasing the dose of *Hibiscus tiliaceus* extract did not show a linear increase in effect, indicating that the decrease in blood glucose levels did not depend on the increase in the dose administered. In drugs derived from natural ingredients, there are several component compounds that interact with each other to cause a response; with increasing doses and the increasing number of chemical compounds contained, there are interactions that cause a decrease in effect. The results of this study show that increasing the dose did not increase this effect. There is a phenomenon that can explain the inconsistency of increasing dose to pharmacological response, namely, the nonmonotonic dose-response relationship (NMDR). There are various mechanisms in NMDR, including cytotoxicity, cell- and tissue-specific receptors and cofactors, receptor selectivity, receptor down-regulation and desensitization, inter-receptor competition, and endocrine negative feedback (Lagarde et al., 2015). In this study, it was unclear which mechanism explains the relationship between dose and pharmacological response.

Based on the results of phytochemical screening, the ethanol extract of *Hibiscus tiliaceus* leaves contains alkaloids, flavonoids, saponins, and tannins. There has been no research to test the anti-hyperglycemic effect of *Hibiscus tiliaceus* leaf, but another study, which belongs to the same family as the plant, namely ethanol extract of hibiscus leaves (*Hibiscus rosa sinensis* L.), has shown an anti-diabetic effect on hyperglycemic mice at a dose of 300 mg/kg BW (Meilina et al., 2022). Previous research has demonstrated that *Hibiscus tiliaceus* exhibits antioxidant and cytotoxic properties. The *Hibiscus tiliaceus* leaf extract fraction has been shown to possess considerable antioxidant activity, largely because of its flavonoid content. Another study reported that *Hibiscus tiliaceus* leaf has antioxidant properties that can neutralize free radicals because *Hibiscus tiliaceus* leaf contains chemical compounds, including anthocyanins, amides, coumarins, phenols, organic acids and compounds from the flavonoid group identified in the leaves, stems, and bark of the waru plant using several types of solvents, namely methanol, water, chloroform, and ethanol. The part of the plant that has anti-oxidant activity is the leaves which have strong anti-oxidant activity (IC50 = 86.5 µg/mL) (Suarantika & Patricia, 2023).

According to Tang et al. (2017), alkaloids found in the seeds of *Nigella glandulifera* F plants can increase glycogen synthesis through the inhibition of Protein of Tyrosine Phosphatase 1 B (PTP1B) and can also increase the activation of insulin signaling pathways that contribute to anti-diabetic effects. Alkaloids can also stimulate the expression of insulin by activation of glucose transporter 4 (GLUT 4), AMP-activated protein kinase (AMPK) and glycogen synthase kinase-3 (GSK3). Another study revealed that the anti-hyperglycemic effect of alkaloids found in *Rhizoma Copditis* plants was obtained through the suppression of insulin resistance in the liver, muscle, and adipose tissue, increasing insulin levels, regulating intestinal hormones, and improving the composition of the intestinal microbiota. Berberine is the most dominant type of alkaloid in *Rhizoma Copditis* and aims to improve the condition of diabetic nephropathy by reducing fibrosis in the renal tissue through inhibition of epithelial to mesenchymal transition, reducing extracellular matrix accumulation, and extinguishing inflammatory reactions through inactivation of the nuclear factor kappa B (NF-kB) pathway and regulation of G proteins (Ma et al., 2019).

Flavonoids are a group of natural polyphenolic secondary metabolites that have been reported to have various pharmacological effects, such as antidiabetic and anti-inflammatory effects, through various molecular mechanisms of action. Flavonoids can inhibit the activity of glycogen phosphorylase, which catalyzes the breakdown of glycogen into glucose in the liver and has been shown to modulate blood glucose levels (Jaitak

P-ISSN: 2406-9388
E-ISSN: 2580-8303

et al., 2019). Yang et al., in 2021, identified 30 flavonoid compounds from *Potentilla anserina* L rhizomes that have potential as anti-hyperglycemia agents by inhibiting the alpha-glucosidase enzyme. Inhibition of this enzyme is very important in the management of type 2 diabetes mellitus because alpha-glucosidase catalyzes the hydrolysis of starch into simple sugars (Yang et al., 2021). Another study conducted by Li et al. (2024) on the anti-diabetic effect of five types of flavonoids of the *Tamarix* sp. plant with the mechanism of inhibition of the alpha-glucosidase enzyme reported that all flavonoid compounds in this plant showed a higher percentage inhibition than acarbose (Li et al., 2024). The results of a study by Dhanya R et al. (2017) on the molecular mechanism of action of quercetin (a type of flavonoid) found in *Citrus* sp. plants on cell culture of L6 myotubes (skeletal muscle cells) explained that the anti-hyperglycemic effect of quercetin occurs through the AMP-activated protein kinase (AMPK) pathway, with its downstream target being the inhibition of p38 MAPK (p38 mitogen-activated protein kinase). Type 2 diabetes is characterized by insulin resistance and chronic inflammation in body tissues, and p38 MAPK is involved in the inflammatory response that can worsen insulin resistance. Activation of p38 MAPK in fat and muscle tissue can increase the production of pro-inflammatory cytokines, such as TNF α (Tumor Necrosis Factor α (TNF-α), interleukin 6 and MCP-1 (Monocyte Chemoattractant Protein-1), which can interfere with insulin signaling and increase insulin resistance (Dhanya et al., 2017).

Hibiscus tiliaceus leaves contain saponin compounds, which are secondary metabolites with a bitter taste. Saponins have become the focus of researchers because of their many pharmacological effects, including immunomodulatory, antioxidant, anti-inflammatory, anti-obesity, antibacterial and anti-diabetic effects (Jeepipalli et al., 2020; Wang et al., 2020; He et al., 2019). Research conducted by Keller et al. in 2021 on obese C57BLK/6 mice who were administered the Saponin Rich Factor (SRF) fraction of the *Momordica charantia* L plant at a dose of 0.5 mg/g BW for 4 weeks reduced fasting blood glucose levels, improved glucose tolerance, and modulated insulin sensitivity (Keller et al., 2021). In 2017, Ma et al. reported that the administration of *Momordica charantia* L ethanol extract (containing 43% saponins) at doses of 100, 200, and 400 mg/kg BW for 8 weeks in diabetic mice induced with streptozotocin (25 mg/kg BW) reduced blood glucose levels through the regulation of Glucose Transporter GLUT-4, Suppressor of Cytokine Signaling 3 (SOCS-3), c-Jun N-terminal kinase (JNK), interleukin 6, and Tumor Necrosis Factor (TNF) α. GLUT-4 is responsible for insulin-regulated glucose uptake by fat and muscle cells, SOCS-3 and JNK. JNK suppression can increase the occurrence of insulin resistance and glucose intolerance by inhibiting phosphorylation of the insulin receptor, whereas SOCS-3 can cause insulin inactivation (Ma et al., 2019).

Another secondary metabolite that plays a role in reducing blood glucose levels is tannins. Tannins are polyphenols with various molecular weights, and in recent years, research on tannins has increased because of their benefits in treating chronic diseases, including diabetes mellitus (Ajebli et al., 2019). Research conducted by Sanvee et al. in 2020 reported that the administration of tannin fraction extract from *Bridelia ferruginea* (Benth) plants at 200 mg/kg BW for 28 days in fructose-induced mice can significantly reduce blood glucose levels (Sanvee et al., 2020). Tannins can inhibit the activity of alpha-glucosidase by changing the conformation and hydrophobicity of the enzyme. Tannins also have a strong inhibitory effect on glycation products such as fructosamine and dicarbonyl compounds, so tannins have the potential to be used as anti-diabetic drugs (Huang et al., 2019).

CONCLUSION

Hibiscus tilaceus leaves contain secondary metabolites including alkaloids, flavonoids, saponins, and tannins. The ethanol extract of waru leaves, administered at doses of 200, 400, and 800 mg/kg BW, has been observed to exert an anti-hyperglycemic effect in male white mice. Of the doses tested, 400 mg/kg BW demonstrated the most pronounced reduction in blood glucose levels.

ACKNOWLEDGMENT

The authors thank the pharmacology and phytochemistry laboratory staff of the Faculty of Pharmacy and Health, Tjut Nyak Dhien University.

AUTHOR CONTRIBUTIONS

Conceptualization: V.S., T.N.F., M.S.; Methodology, V.S., M.S.; Software, V.S., T.N.F.; Validation, V.S., T.N.F.; Formal Analysis, V.S., T.N.F., M.S.; Investigation: V.S., T.N.F., M.S.; Resources, T.N.F.; Data Curration; V.S., T.N.F.; Writing - Original Draft, V.S., T.N.F.; Writing - Review and Editing, V.S., T.N.F., M.S.; Visualization, V.S., T.N.F.; Supervision: V.S., T.N.F., M.S.; Project Administration, V.S., T.N.F., M.S.; Funding Acquisition, V.S., T.N.F.

P-ISSN: 2406-9388
E-ISSN: 2580-8303

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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P-ISSN: 2406-9388
E-ISSN: 2580-8303