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Full Length Article

Alpha-Glucosidase Inhibitor Activity Test of Fractions and Isolate from Leaves of *Tristaniopsis obovata*

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Abstract

Diabetes mellitus is a metabolic disease characterized by an uncontrolled increase of blood glucose levels. Diabetes drugs such as metformin, acarbose, and others have the side effects that can harm to the human body such as liver, kidneys and other organs. *Tristaniopsis obovata* Benn. leaves have efficacy as an *α*-glucosidase inhibitors (AGIs), but the active compounds contained in *T. obovata* leaves have not been disclosed. This study aimed to determine the active compound in 70% ethanolic extract of *T. obovata* leaves which has the potential as AGIs. *T. obovata* leaves were macerated using 70% ethanol for 3×24 h gave ethanol extract-*T. obovata* leaves in 22.7% yield. Analysis of total phenolic and flavonoid contents of ethanol extract-*T. obovata* leaves showed that its total phenolic content was 31.1 mg GAE g^{-1} and total flavonoid content was 10.9 mg QE g^{-1}. Solvents partition of ethanol extract gradually gave *n*-hexane, ethyl acetate, methanol, and water fractions in 10.0, 32.3, 41.0, and 8.9% yields, respectively. *In vitro* bioassay of extract and its fractions for *α*-glucosidase enzyme showed that the IC_{50} values were 3.2, 3.9, 5.1 and 7.5 μg mL^{-1} for *n*-hexane fraction, ethyl acetate fraction, methanol fraction and water fraction, respectively, of ethanol extract. Separation of methanol fraction by column chromatograph using silica gel and *n*-hexane/ethyl acetate/methanol (4/1/0 – 0/1/1) as a mobile phase produced 17 subfractions (F-1 to F-17). *In vitro* bioassay of F-11, F-15, and F-16 for *α*-glucosidase enzyme gave the IC_{50} values of 40.0, 5.0, 3.6 μg mL^{-1}, respectively. Analysis by FT-NMR spectroscopy, it was predicted that F-16 was myricetin 3-*O*-*α*-rhamnoside, which was the first time found in the *T. obovata*.

Keywords: *α*-Glucosidase; Inhibitory activity; Myricetin 3-*O*-*α*-rhamnoside; *Tristaniopsis obovata*

Introduction

Diabetes mellitus is a metabolic disorder characterized by an uncontrolled increase of blood glucose levels (hyperglycemia). This is associated with abnormalities in the metabolism of carbohydrates, fats, and proteins due to decreased in insulin secretion or insulin action, or both. The chronic diabetes mellitus could cause a long-term damage, dysfunction, and failure of several organs, such as the blood vessels, kidneys, eyes, and heart (American Diabetes Association 2010). Based on Indonesia Basic Health Research (Ministry of Health of the Republic of Indonesia 2018), it was estimated that the number of diabetes mellitus sufferers in Indonesia aged > 15 years was 14 million people i.e., 8.5% of Indonesia's population in 2013, which increased to 11.1% in 2018. This situation encourages diabetes mellitus sufferers to seek the alternative treatments that are cheap, efficacious, and have no side effects (Saputra 2021).

Currently, public awareness of going back to nature is increasing, as seen from the use of medicinal plants for the treatment and prevention of various diseases is increasing (Toktonalieva and Toktonaliev 2020; Ferdosi *et al.* 2021; Javaid *et al.* 2022). In Indonesia, herbal medicine known as "jamu", has also been widely used by the Indonesian people for centuries for maintaining their health and treating disease. One of these plants is *T. obovata*, the endemic plant that grows in the Bangka Belitung Province and several areas of Sumatra Island. The *T. obovata* plant is trusted by the people of Bangka Belitung as a medicinal plant that can be used to reduce fever and high blood pressure, anti-cholesterol, anti-inflammatory (Enggiwanto *et al.* 2018), antidiabetic, and treating a cough (Akbarini 2016). The *T. obovata* plant has also been used as a new medicinal plant for the treatment of stroke, but it has not been examined scientifically. It was also reported that the 96% ethanolic extract of *T. obovata* leaves

has the inhibitory activity against *α*-glucosidase enzyme with an IC_{50} of 74 *μ*g mL^{-1} (Budiana *et al.* 2020). The active compounds might be come from flavonoids, steroids, triterpenoids, tannins, and saponins contained in the *T. obovata* leaves (Kadri *et al.* 2019).

Until now, the active compounds contained in *T. obovata* leaves which have the inhibitory activity against *α*-glucosidase enzyme have not been disclosed. The aim of this study was to isolate the active substance contained in the 70% ethanol extract of *T. obovata* leaves (Ethanol Extract-*T. obovata* leaves) which have *in vitro* inhibitory activity against *α*-glucosidase enzyme and to determine it by FT-NMR spectroscopy.

Materials and Methods

Materials and equipments

Leaves of *T. obovata* were collected from the Pelawan Forest of Namang, Central Bangka Regency, Bangka Belitung Province, Indonesia and the sample was observed by BRIN "Herbarium Bogoriense" Cibinong, number: B-942/V/D1.05.07/12/2021. The chemicals used were 70% ethanol, *n*-hexane, ethyl acetate, methanol, water, *p*-nitrophenyl-*β*-D-glucopyranose (p-NPG) substrate (Sigma Aldrich N 1337-1G), *α*-glucosidase enzyme (Sigma Aldrich), DMSO, CD_3OD, phosphate buffer pH 7.0, Na_2CO_3, quercetin (Merck, Germany), TLC plates (Sigma Aldrich), silica gel GF_{254} (Sigma Aldrich), celite 545, *α*-glucosidase enzyme target protein from Saccharomyces cerevisiae (PDB:3A4A). The equipments used were analytical balance (Precisa 240A), blender, vacuumized rotary evaporator (Stuart), spectrophotometer UV-visible (Simadzu 1240), waterbath (Memmert), incubator (Firlabo), ELISA reader (Spectra Max), and NMR spectrometer (Jeol-ECZ, 500R, 500 MHz).

Sample preparation

An amount of 10 kg of *T. obovata* leaves was sorted, washed, and dried at room temperature to get the water content < 10%, then the dried *T. obovata* leaves were powdered by blender and sieved by 4/18 mesh size.

Extraction and fractionation

T. obovata leaves sample (749.5 g) was macerated with 70% ethanol (10 L) for 3x24 h at room temperature then filtered. The obtained filtrates were then evaporated using a vacuumized rotary evaporator (Atmajani *et al.* 2018) to give ethanol extract. Subsequently, 20 g of ethanol leaf extract was gradually partitioned with *n*-hexane, followed by ethyl acetate, methanol, and the remained residue was dissolved with water. The *n*-hexane, ethyl acetate, and methanol soluble portions were concentrated with a vacuumized rotary evaporator while the water fraction was concentrated with a water bath gave fractions: *n*-hexane, ethyl acetate, methanol and water.

Determination of total phenolic content

The total phenolic content of *T. obovata* leaves was determined using Folin-Ciocalteu spectrophotometric method (Singleton *et al.* 1999; Winarno *et al.* 2023). The absorbances of the sample solutions were measured using ultraviolet–visible spectrophotometer at 765 nm wavelength. Each treatment was done in two repetitions. The total phenolic content value was calculated as gallic acid equivalent per ethanol extract of *T. obovata* leaves using the linear calibration curve of standard gallic acid.

Determination of total flavonoid content

The total flavonoid contents of Ethanol Extract-*T. obovata* leaves was determined by using aluminium chloride colorimetric method (Chang *et al.* 2002; Winarno *et al.* 2023). The absorbances of the sample solutions were measured using ultraviolet–visible spectrophotometer at 510 nm wavelength. Each treatment was done in two repetitions. The total flavonoid content value was calculated as quercetin equivalent/Ethanol Extract-*T. obovata* leaves using the linear calibration curve of standard quercetin.

Bioassay against *α*-glucosidase enzyme and IC_{50} value

Bioassay of the samples for *α*-glucosidase enzyme based on *in vitro* enzymatic reaction was done as described by Dewi *et al.* (2014). The absorbances of the samples were measured by spectrophotometer ultraviolet-visible using a microplate reader at 400 nm wavelength. Each treatment was done in two repetitions. The inhibitory activity percentage against *α*-glucosidase enzyme was calculated by equation 1, where *C* was the absorbance of blank and *S* was the absorbance of sample.

$$\text{Inhibitory activity (\%)} = \frac{(C-S)}{C} \times 100\ \% \quad (1)$$

The half-maximum inhibition concentration (IC_{50}) which is the concentration of sample that has the ability for inhibiting the 50% of *α*-glucosidase enzyme was obtained from the equation of linear regression Y = aX + b and was calculated by equations (2) and (3), where, Y was inhibitory activity percentage and X was sample concentration for subfraction F-11, F-15 and F-16, and logarithm of sample concentration for extract (Ethanol Extract-*T. obovata* leaves) and fractions (*n*-hexane, ethyl acetate, methanol and water fractions).

$$IC_{50} = \frac{50-a}{b} \quad (2)$$

$$IC_{50} = \text{antilogarithm of } \frac{50-a}{b} \quad (3)$$

Separation and isolation of compounds by column chromatography

An amount of 10 g of the active fraction (methanol fraction) was added celite 545 (8.0 g), then crushed and dried.

The sample was subjected into a chromatography column containing silica gel 60 (300 g) then eluted with *n*-hexane/ethyl acetate/methanol (4:1:0 ~ 0:1:1). The results of the fractionation were analyzed by silica gel 60 F_{254} Thin Layer Chromatography (TLC) aluminium plate with the mobile phase of *n*-hexane/ethyl acetate (4/1). Visualization of the spots was done by UV lamp (254 and 366 nm wavelength), followed by sprayed with H_2SO_4 and heated on a hot plate. The fractions that gave the same chromatogram pattern were combined (Tetti 2014).

Identification of compounds

The isolate showing a potent inhibitory activity against *α*-glucosidase enzyme was analyzed its ^{1}H- and ^{13}C-spectra by NMR spectrometer (Jeol-ECZ500R, 500 MHz, CD_3OD).

Results

Extraction and fractionation

Extraction of dried powder of *T. obovata* leaves (794.5 g) by 70% of ethanol gave 179.96 g (22.7%) of Ethanol Extract-*T. obovata* leaves. Further─fractionation of the ethanol extract (20 g) by using *n*-hexane, followed by ethyl acetate, methanol and water gave *n*-hexane, ethyl acetate, methanol, and water fractions as shown in Table 1. The methanol fraction (41.0%) showed the highest yield, followed by ethyl acetate fraction (32.3%), *n*-hexane fraction (10.0%) and water fraction (8.9%).

Total phenolic content and total flavonoid content

Determination of total phenolic content as gallic acid equivalent (GAE) showed that the total phenolic content in *T. obovata* leaves was 31.1 mg GAE g^{-1}. Then, determination of total flavonoid content as quercetin equivalent (QE) showed that the total flavonoid content in *T. obovata* leaves was 10.9 mg QE g^{-1}.

Bioassay for *α*-glucosidase inhibitor and its IC_{50} value

The inhibitory activity test of extract and fractions and there IC_{50} values against *α*-glucosidase enzyme were shown in Table 2. Furthermore, through the linear regression curve of inhibitory activity percentage versus logarithm of samples concentration (Fig. 1), the IC_{50} value of ethanol extract against α-glucosidase enzyme was 3.2 μg mL^{-1}, whereas the IC_{50} values of *n*-hexane, ethyl acetate, methanol and water fractions were 3.9, 5.1 and 7.5 μg mL^{-1}, respectively (Table 2).

Isolation of compounds by column chromatography

Since, all fractions were categorized as active fraction as α-glucosidase inhibitor, then the methanol fraction which has the highest yield was chosen for column fractionation on silica gel with the mobile phase of n-hexane/ethyl acetate/methanol (4:1:0 ~ 0:1:1) obtained 17 subfractions (F-1 to F-17). Among them, F-11, F-15 and F-16 were selected to test the inhibitory activity against *α*-glucosidase enzyme; this was due to the large yield compared to the other subfractions. The IC_{50} value of F-11, F-15 and F-16 was presented in Fig. 2 and Table 3.

Table 1: Yields of fractions from 20 g of ethanol extract-*T. obovata* leaves

Extract/Fraction	Yield (g) / (%)
Ethanol extract	179.96 (22.7)*
n-Hexane	0.20 (10.0)
Ethyl acetate	6.46 (32.3)
Methanol	8.19 (41.0)
Water	1.77 (8.9)

*Calculated from 794.5 g of dried *T. obovata* leaves sample

Table 2: Inhibitory activity percentage of extract and fraction against *α*-glucosidase enzyme

Sample	Linear regression equation; R	IC_{50} (μg mL^{-1})
EE-TOL	Y = 60.672 X + 19.73; R = 0.9799	3.2
EAF	Y = 43.204 X + 24.372; R = 0.9919	3.9
MEF	Y = 37.824 X + 23.278; R = 0.9881	5.1
WTF	Y = 35.417 X + 19.076; R = 0.9934	7.5

*EE-TOL : Ethanol Extract-*T. obovata* Leaves
EAF : Ethyl acetate Fraction
MEF : Methanol Fraction
WTF : Water Fraction

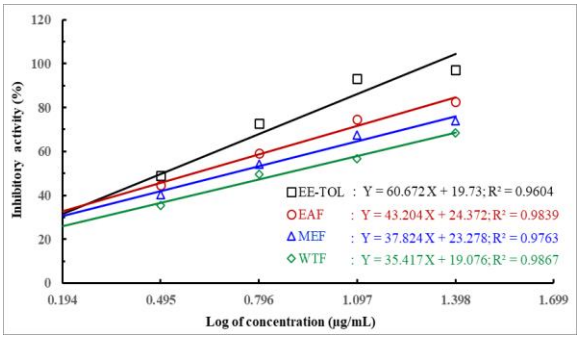

Fig. 1: Correlation between inhibitory activity percentage and logarithmic of concentrations of extract and fraction
Caption of figure:
EE-TOL : Ethanol Extract- *T. obovata* leaves
EAF : Ethyl Acetate Fraction
MEF : Methanol Fraction
WTF : Water Fraction

^{1}H- and ^{13}C-NMR spectrum measurement

For structure identification of isolate F-16, it was identified based on ^{1}H- and ^{13}C-NMR spectra data as shown in Table 4.

Discussion

Phenolic compounds, one of the important secondary metabolites act to defend against pathogens, and as

Table 3: Yield and IC_{50} value of F-11, F-15, and F-15 from 10 g of Methanol Fraction

Sample	Yield, mg (%)	Linear regression equation; R	IC_{50} ($\mu g\ mL^{-1}$)
F-11	40 (0.4 %)	Y = 0.899 X + 14.041; R = 0.9399	40.0
F-15	450 (4.5 %)	Y = 2.6043 X + 37.026; R = 0.9312	5.0
F-16	1.130 (11.3 %)	Y = 2.1397 X + 42.27; R= 0.9991	3.6

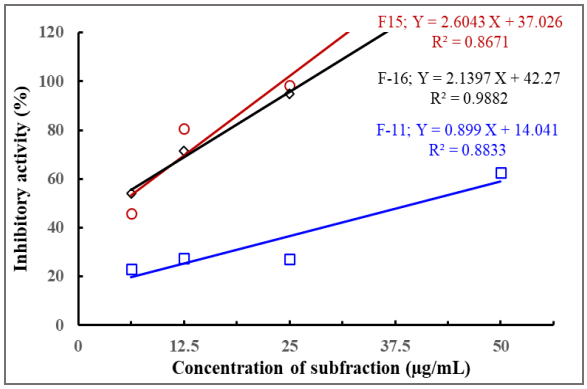

Fig. 2: Correlation between inhibitory activity percentage and concentration of subfractions (F-11, F-15, and F-16)

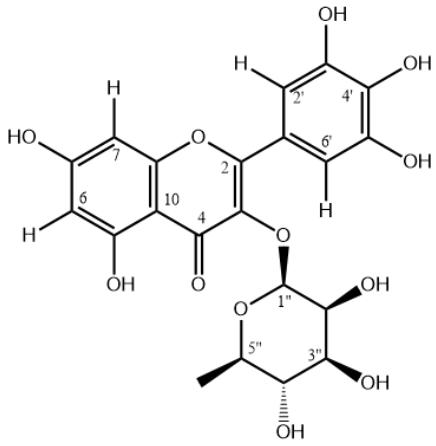

Fig. 3: The structure of myricetin-3-*O*-*α*-rhamnoside

antioxidants to scavenge the free radicals as well as to inhibit the oxidative mechanisms (Naveed *et al.* 2018; Shoaib *et al.* 2022; Winarno *et al.* 2023). The result in this study (31.1 mg GAE g^{-1}) was higher than total phenolic content in Ethanol Extract-*T. obovata* leaves (13.4 mg GAE g^{-1}) and in ethyl acetate extract of *T. obovata* stem (10.3 mg GAE g^{-1}) reported by Budiana *et al.* (2020). In contrast, the total phenolic content was lower compared to its content in *T. merguensis* ethyl acetate fraction (86.7 mg GAE g^{-1}) as reported by Mahardika *et al.* (2020).

Flavonoids are suggested to have a protective effect through several mechanisms, such as free radicals scavenging, enzymes inhibiting, and metal ions chelating, which depend on their structures, saturations, and substitution degrees. Furthermore, flavonoids exhibit biological activities, such as antioxidants, antiviral,

Table 4: Comparison of NMR data (500 MHz, CD_3OD) of isolate F-16 with myricetin 3-O-α-rhamnoside (Motlhatlego *et al.* 2020)

Position	Isolate F-16		myricetin-3-*O*-*α*-rhamnoside (Motlhatlego *et al.* 2020)	
	δ_H (ppm); mult, J_H in Hz	δ_C (ppm)	δ_H (ppm), mult, J_H in Hz	δ_C (ppm)
2		161.10		156.4
3		137.96		137.0
4		179.67		181.4
5		163.10		165.3
6	6.19 (d, 2.5)	99.92	6.21 (d, 2.0)	99.5
7		165.88		165.7
8	6.36 (d,= 2.5)	94.82	6.38 (d, 2.0)	94.3
9		158.51		158.5
10		104.50		105.7
1'		121.91		123.1
2', 6'	6.95 (2H, s)	109.68	6.96 (2H, s)	109.6
3', 5'		146.84		145.7
4'		136.31		136.4
1"	5.19 (d, J_H = 1.5 Hz)	103.64	5.32 (d, 1.5)	102.0
2"	3.71 (m)	73.33	4.23 (dd, 1.6; 3.3)	71.9
3"	3.49 (m)	71.90	3.79 (dd, 3.6; 9.6)	71.9
4"	3.40 (m)	72.91	3.37 (d, 9.6)	73.1
5"	3.85 (m)	72.11	3.53 (dd), 6.3; 9.6)	71.9
6"	0.96 (d, 7)	17.72	0.96 (d, 6.3)	17.1

s (single), d (doublet), dd (double doublet), m (multiplet)

antiinflammatory, antimicrobial, and antitumor (Kanwal *et al.* 2009, 2011; David *et al.* 2016; Winarno *et al.* 2023). Accordingly, it was important to determine the total flavonoid content in ethanol extract. Fortunately, the total flavonoid content in this study (10.9 mg QE g^{-1}) was higher than previous study reported by Budiana *et al.* (2020) that the total flavonoid content in ethanol extract and ethyl acetate extract of *T. obovata* leaves were 2.5 mg QE g^{-1} and 1.5 mg QE g^{-1}, respectively.

In this study, it was demonstrated that the Ethanol Extract-*T. obovata* leaves has a potent inhibitory activity against *α*-glucosidase enzyme with the IC_{50} value of 3.2 $\mu g\ mL^{-1}$. This result was higher than previous study conducted by Kissinger *et al.* (2012) that the IC_{50} value of ethanol extract was 4.4 $\mu g\ mL^{-1}$. In addition, all fractions also showed a potent inhibitory activity against *α*-glucosidase enzyme with the IC_{50} values of 3.9, 5.1 and 7.5 $\mu g\ mL^{-1}$ for ethyl acetate, methanol, and water fractions respectively. Nevertheless, the inhibitory activity of the extract (Ethanol Extract-*T. obovata* leaves) significantly higher than its fractions, it was probably caused by synergistic effect of some components present in extract rather than in the fractions (Abu-Lafi *et al.* 2018; Caesar and Cech 2019).

Analysis by ^1H and ^{13}C-NMR spectroscopy, the isolate contained 21 carbon atoms, namely one CH_3, nine CH, 10 C, and one C=O. Among them, 15 carbon atoms with 4 x CH, 10 x C, and C=O, namely at δ_C = 94.82 ppm (CH), 99.92 ppm (CH), 104.5 ppm (C), 13 p7.96 ppm (C), 158.51 ppm (C), 161.1 ppm (C), 163.1 ppm (C), and 165.88 ppm (C) was indicated the ring A and C of flavonoid group, whereas the six carbon atoms at δ_C = 121.91 ppm (1 C); δ_C = 109.68 ppm, δ_H = 6.95 ppm (s) (2 x

CH); δ_C = 146.84 ppm (2 x C), and δ_C = 1336.31 ppm (1 C) indicated tetra-substituted aromatic ring (ring B) of the flavonoid group (Silverstein *et al.* 2005). Subsequently, the presence of six carbon atoms at δ_C = 71.90, 72.11, 72.91, 73.33, 103.64, and 103.64 ppm, and an anomeric proton signal at δ_H = 5.31 ppm (d, 1.5 Hz), an HC-OH signal at δ_H = 3.5 ppm, and a methyl signal at δ_H = 0.96 ppm (d, 7 Hz) was characteristic of the rhamnoside. Finally, it was clarified that ^{1}H and ^{13}C-NMR spectra was similar to those myricetin-3-*O*-*α*-rhamnoside found in *Newtonia buchananii* leaves as reported by Motlhatlego *et al.* (2020) and also found in *Albizia amara* leaves reported by Kassem *et al.* (2016). In this study, the presence of isolate contained in *pelawan* (*Tristaniopsis obovata* Benn.) leaves was reported for the first time. Liu *et al.* (2021) reported that myricetin-3-*O*-*α*-rhamnoside (Fig. 3) isolated from *Rhus chinensis* Mill. fruits shoed the *α*-glucosidase inhibitor with the IC_{50} value of 93 μg mL^{-1}. The comparison of NMR data (500 MHz, CD_3OD) of isolate F-16 with myricetin-3-*O*-*α*-rhamnoside (Motlhatlego *et al*. 2020) is summarized in Table 4.

Conclusion

The 70% ethanolic extract of *T. obovata* leaves showed a potent inhibitory activity against *α*-glucosidase enzyme with the IC_{50} value of 3.9 μg mL^{-1}. Solvent partition of extract followed by silica gel column chromatography resulted isolation of myricetin 3-O-*α*-rhamnoside that showed a potent inhibitory activity against *α*-glucosidase enzyme with the IC_{50} value of 3.6 μg mL^{-1}. The presence of the compound in *T. obovata* leaves was reported for the first time.

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Author Contributions

AFD planned the experiments, MH and HW interpreted the results. AFD, MH, and HW made the write up and statistically analyzed the data and made illustrations.

Conflict of Interest

All authors declare no conflict of interest in this study.

Data Availability

Data presented in this study will be available on a fair request to the corresponding author.

Ethics Approval

Not applicable to this study.

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