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Phytochemical Screening and In Vitro Antidiabetic Test of Ethyl Acetate Fraction of Tiger's Betel (*Piper porphyrophyllum)*

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Abstract
Piper porphyrophyllum or known as tiger's betel, is a medicinal plant of the Piper genus that is quite widespread in Indonesia, especially on Java, Sumatra, and Kalimantan island. Tiger's betel is widely used traditionally to treat headaches, bone pain, chest tightness, skin diseases, relieve inflammation, and diabetes mellitus. This study aims to determine the type of secondary metabolite compounds contained in the ethyl acetate fraction of tiger's betel and determine its antidiabetic activity. Determination of the type of compound is conducted by phytochemical screening by a qualitative test using various reagents to identify flavonoids, phenolics, saponins, triterpenoids, steroids, alkaloids, and coumarins. The antidiabetic assay was carried out by inhibiting α-glucosidase activity and the results were reported as IC_{50} values. The ethyl acetate fraction of tiger's betel positively contains alkaloids, flavonoids, and phenolics. The antidiabetic activity is classified as very strong, with an IC_{50} value of 14.06 µg/mL.

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INTRODUCTION

Diabetes Mellitus is one of the chronic diseases that cause the highest mortality in Indonesia. According to data from the Institute for Health Metrics and Evaluation, diabetes is the 3rd highest cause of death in Indonesia in 2019, which is around 57.42 deaths per 100,000 population. Diabetes Mellitus (D.M.) is a metabolic disease characterized by chronic hyperglycemia and carbohydrate, protein, and fat metabolism abnormalities. This disease is characterized by increased blood glucose levels, commonly called hyperglycemia. Various efforts have been made to deal with the incidence of diabetes mellitus, one of which is by inhibiting the work of α-glucosidase, a crucial enzyme in carbohydrate digestion that catalyzes the final stages of disaccharides and starch. The inhibiting α-glucosidase is considered effective in delaying the breakdown of carbohydrates in the small intestine and can reduce blood glucose levels in diabetics (Kazeem et al., 2013).

Diabetes Mellitus management, especially type 2 D.M., which has been commonly known to the public is the use of oral hypoglycemia drugs. Available oral hypoglycemia drugs such as sulfonylurea (glibenclamide), biguanides (metformin), meglitinides, thiazolidinediones, and α-glucosidase inhibitors can be used either as monotherapy or combination therapy, which is more effective in controlling blood glucose levels (Chukwunonso Obi et al., 2016; Jia et al., 2015). However, the use of these drugs has side effects. The most common side effects of the use of metformin as monotherapy are gastrointestinal disorders such as diarrhea, nausea, vomiting, and abdominal pain (Zhai et al., 2016). Therapy using glibenclamide causes side effects in the form of weight loss and hypoglycemia (Lamos et al., 2012). Glibenclamide is

reported to cause more hypoglycemia effects than other sulfonylurea-class drugs (Abdulkadir & Thanoon, 2012). Therefore, the use of medicinal plants is starting to be looked at in D.M. therapy which is expected to be safer and have fewer side effects (Utomo et al., 2022).

Medicinal plants have been used in traditional medicine for generations by various ethnic groups in Indonesia, including a plant with the genus Piper from the Piperaceae family. Some of the plants in this genus are used for stomach pain and gonorrhea and have antibacterial activity against *Bacillus subtilis*, *Micrococcus luteus*, and *Escherichia coli* (Alqadeeri et al., 2019). Tiger's betel (*Piper porphyrophyllum*) is traditionally used to treat headaches, bone pain, chest tightness, leprosy, stomach pain, inflammation, skin diseases, and diabetes (Ahmad et al., 2014). Many studies have proven that phytochemical compounds have the ability as antidiabetic agents to inhibit the work of α-glucosidase, such as compounds from the triterpene group, for example, oleanolic acid with an IC_{50} value of 5.0 µM (Javed et al., 2022), coumarin, for example, psoralidin with an IC_{50} value of 3.5 µM (Jiang et al., 2018), tannins (e.g., 1,2,3,6-tetra-O-galloyl-4-O-cinnamoyl-β-D-glucose with IC_{50} value of 2.9 µM) (Young et al., 2017), and flavonoids as the largest group that has α-glucosidase inhibitory activity, (e.g., flavones, morusin with IC_{50} value of 3.19 µM) (Dirir et al., 2022).

Therefore, this study aims to determine the content of secondary metabolite compounds in the ethyl acetate fraction of tiger's betel by color test method and determine the antidiabetic ability of the ethyl acetate fraction by α-glucosidase enzyme inhibition method. The novelty of this study is that there has been no research on the phytochemical content of the ethyl acetate fraction of tiger's betel and the antidiabetic test of this fraction. In the previous study, the sample tested was ethyl acetate extract of tiger's betel that extracted for 24 hours (Kuspradini et al., 2023). Meanwhile, this study uses the ethyl acetate fraction derived from the methanol extract of tiger's betel.

METHOD

Sample Preparation

Samples of tiger's betel plants were taken from the Andalas University Biology Education and Research Forest in Bukit Kamulau, Limau Manis, Padang, West Sumatra (0°54'S, 100°28'E).

The dried sample of tiger's betel was extracted by maceration method using methanol solvent for 14 repetitions. The methanol extract was multistage fractionated with hexane and ethyl acetate solvents, resulting in hexane fraction, ethyl acetate fraction, and methanol fraction (Efdi et al., 2022). The ethyl acetate fraction was selected for this study.

Phytochemical Screening

Phytochemical screening was carried out with modifications based on procedures by (Soamole et al., 2018). The classes of compounds identified in this study are alkaloids, flavonoids, phenolics, saponins, coumarins, triterpenoids, and steroids. The ethyl acetate fraction of tiger's betel was put into a test tube, and then chloroform and distilled water (1:1) were added. Then, separate the water and chloroform layers. The water layer was used to test the flavonoids, phenolics, and saponins content. At the same time, the chloroform layer was used to test the content of triterpenoids and steroids.

Flavonoid Test

A total of ± 2 mL of water was put into a test tube, two drops of concentrated HCl and a little magnesium powder were added, and the solution was then observed for color changes. If an orange-to-pink color is formed, it indicates that the sample is positive for flavonoids (Soamole et al., 2018).

Phenolic Test

A total of ± 2 mL of water layer was put into a test tube, and a 1% $FeCl_3$ solution was added as much as two drops. The solution's color change was observed. If the solution is blue-black, the sample is positive for phenolic content (Soamole et al., 2018).

Saponin Test

A total of ± 2 mL of water layer was put into a test tube, shaken, and a few drops of concentrated HCl were added. If a stable foam is formed that does not disappear for ± 30 seconds, the sample is positive for saponins (Soamole et al., 2018).

Triterpenoid and Steroid Test

The chloroform layer was put as many as three drops in the hole of the drip plate, then the solvent was evaporated, and two drops of concentrated sulfuric acid and two drops of acetic anhydride were added. If a red or purple color is formed, indicating the sample positively contains triterpenoids, and if a green or blue-green ring is formed, indicating the sample contains steroids (Soamole et al., 2018).

Alkaloid Test

The ethyl acetate fraction of tiger's betel was added with 5 mL chloroform and 5 mL ammonia-chloroform 0.05 N and then filtered. The mixture was put into a test tube, and 2 mL of 2 N sulfuric acid was added, then stirred and allowed to stand until two layers were formed, namely the acid layer and the chloroform layer. In the acid layer, add a few drops of Dragendorff reagent. If an orange precipitate is formed, it indicates that the sample contains alkaloids (Soamole et al., 2018).

Coumarin Test

The ethyl acetate fraction of tiger's betel was dissolved with ethyl acetate, then spotted on the Thin Layer Chromatography (TLC) plate and eluted with the suitable eluent. The eluted TLC plate was observed under UV light at 366 nm. If there is blue fluorescence and after spraying with 1% NaOH, the color is brighter, indicating that the sample is positive for coumarin (Soamole et al., 2018).

Antidiabetic Activity Test with α-Glucosidase Inhibition Method

The method used is a modification of the procedure carried out by (Etsassala et al., 2020). Standard acarbose and ethyl acetate fraction were made into test solutions with various concentrations, i.e., 1000, 500, 125, 62.5, and 15.63 µg/mL.

A total of 50 µL of 0.1 M phosphate saline buffer (pH 6.9) was put into the plate, then 10 µL of 1 µg/mL α-glucosidase enzyme was added and 20 µL of test solution was added. The well plate was covered with aluminum foil and incubated for 15 minutes at 37ºC. Then, 20 µL of 5 mM p-nitrophenyl-α-D-glucopyranoside (p-NPG) solution was added to initiate the reaction. Incubation was carried out for 20 minutes at 37ºC. The reaction was stopped by adding 50 µL of 0.1 M Na_2CO_3. The yellow color formed from p-nitrophenol released from p-NPG was measured for absorbance at a wavelength of 405 nm.

The percentage of inhibition of the sample was calculated based on the following formula:

$$\% \text{ inhibition} = \frac{C - S}{C} \text{ x } 100\%$$

Description:
C = absorbance of control
S = absorbance of sample

RESULTS AND DISCUSSION

Phytochemicals

Table 1. Phytochemical screening results of ethyl acetate fraction of tiger's betel

No.	Secondary metabolites	Reagents	Observation	Conclusion
1	Flavonoids	Concentrated HCl + Mg	Yellow solution	(+)
2	Phenolics	$FeCl_3$	Blue-black solution	(+)
3	Saponins	Distilled water + HCl	No stable foam formed	(-)
4	Triterpenoids	Liebermann-Burchard	No color change	(-)
5	Steroids	Liebermann-Burchard	No color change	(-)
6	Alkaloids	Dragendorff	Orange precipitate formed	(+)
7	Coumarins	NaOH 1%	No visible bright blue fluorescence	(-)

Based on Table 1, the ethyl acetate fraction of tiger's betel positively contains alkaloids, flavonoids, and phenolics. Ethyl acetate is a semi-polar solvent, so it can extract compounds with a wide polarity range from polar to non-polar (Santoso et al., 2019).

The phytochemical results we obtained in this study are different from the previous study. In the previous study, ethyl acetate extract of *P. porphyrophyllum* obtained by a multistage maceration method for 24 hours showed the presence of alkaloids, flavonoids, and steroids compounds (Kuspradini et al., 2023). However, our study found no steroid compounds and there were additional compounds, namely phenolics. Differences in the extraction methods used can cause this. Steroid compounds tend to be nonpolar (Ergina et al., 2014), so it is possible if steroids have been extracted with nonpolar solvents such as hexanes.

Flavonoid Test

Flavonoids were tested using concentrated HCl and Mg. Flavonoid compounds will be reduced with Mg and HCl to produce complex compounds that are red, yellow or orange in color (Harborne, 1987 in Hasairin et al., 2021). Tiger's betel is rich in flavonoids. Previous research has successfully isolated seven flavonoids from hexane extract and ethyl acetate extract of tiger's betel (Rajudin et al., 2010). Flavonoids have great biological activities, including cytotoxicity, anti-inflammatory, anti-Alzheimer, anti-microbial, estrogenic, vasorelaxant, enzyme inhibition (Shi et al., 2021), antioxidant, antidiabetic (Sarian et al., 2017), and many more.

Phenolic Test

The identification of phenolic compounds was carried out using $FeCl_3$ solution. Fe^{3+} ions will be reduced to Fe^{2+} by phenolic groups in the sample to form green, blue, or black colors, as a clue to the presence of phenolic compounds (Mukhriani et al., 2019). Phenolic compounds have various bioactivities, such as antibacterial, anticancer, anti-inflammatory, antiproliferation, antioxidants (Xia et al., 2010), and also antidiabetic (Praparatana et al., 2022).

Saponin Test

A positive test for saponins is characterized by its foam formation ability. The foam that arises is due to saponins containing compounds that are partially soluble in water (hydrophilic) and soluble in nonpolar solvents (hydrophobic) as surfactants that can reduce surface tension (Harborne, 1987). When shaken, the hydrophilic group will bind to water, while the

hydrophobic group will bind to air to form the foam. In this study, no saponins were found, similar results were also obtained in previous studies that no saponins were found in the ethyl acetate extract and ethanol extract of *P. porphyrophyllum* (Kuspradini et al., 2023). Saponins have good antioxidant and antidiabetic activity (Hussain & Ikram, 2020).

Triterpenoid and Steroid Test

No triterpenoids and steroids were found in this study. Triterpenoids and steroids tend to be nonpolar, so they may have dissolved in hexane solvent. So, only a few triterpenoids and steroids were extracted in ethyl acetate solvent, and when tested with Liebermann-Burchard reagent, there was no visible change in the color of the solution. Color changes in positive tests for triterpenoids and steroids are due to oxidation reactions by forming conjugated double bonds (Cannell, 1998). Triterpenoids and steroids have bioactivities such as anti-inflammatory, cytotoxic (Manivannan & Johnson, 2020), antioxidant (Garg et al., 2020), antidiabetic (Verma et al., 2021), and others.

Alkaloid Test

Alkaloid test with Dragendorff reagent showed a positive result with the formation of an orange precipitate. The precipitate is potassium alkaloid. The nitrogen atoms in alkaloids with dragendorff reagent will form a coordinate covalent bond with K^+ ions (Khafid et al., 2023). Alkaloids have many bioactivities such as antimicrobial, antioxidant, anti-inflammatory, anticholinesterase, antineurodegenerative, anticancer, antidiabetic, antivenom, larvicidal, antihypertensive, wound healing, analgesic, and many other activities (Naidoo et al., 2021).

Coumarin Test

Coumarin was not found in the ethyl acetate fraction of tiger's betel in this study. Coumarin test using 1% NaOH can increase fluorescence because the reaction between coumarin and NaOH causes changes in the chemical structure of coumarin. NaOH is a base that can divert protons from the hydroxyl group on coumarin, resulting in a more stable and conjugated form. This increases the fluorescence efficiency, resulting in brighter light being emitted. Coumarins have bioactivities as antioxidants, antitumor (Khalil & Mustafa, 2020), anticancer (Al-Warhi et al., 2020), antidiabetic (Ranđelović & Bipat, 2021), and many more.

Antidiabetic Activity Test with α-Glucosidase Inhibition

Table 2. Results of α-glucosidase activity inhibition test of acarbose and ethyl acetate fraction

Sample	IC_{50} (µg/mL)
Acarbose (positive control)	4.64×10^{-3}
Ethyl acetate fraction of tiger's betel	14.06

This method is used because α-glucosidase is an enzyme that breaks down carbohydrates into glucose. Inhibition of this enzyme decreases glucose uptake by the small intestine, which will lower blood glucose levels (Kumar et al., 2011).

In this study, the ethyl acetate fraction of tiger's betel was used to be an inhibitor of the work of the α-glucosidase enzyme. Acarbose is used as a comparison, where acarbose has become an antidiabetic drug that is widely known in the community and readily available. Acarbose is an oligosaccharide that inhibits the α-glucosidase enzyme located in the wall of the small intestine. The substrate p-nitrophenyl-α-D-glucopyranoside (p-NPG) is also used as a model that represents carbohydrates in the body, where enzymes will break down the substrate into glucose and p-nitrophenol (Figure 1). Following the principle of this test is the measurement of enzyme activity based on the absorbance of p-nitrophenol, which is the result of hydrolysis of the p-NPG substrate. The higher the ability of the sample to inhibit the α-glucosidase

enzyme, the smaller the p-nitrophenol product formed, which is indicated by a change in the color of the substrate, a faded yellow color (Maryam et al., 2020).

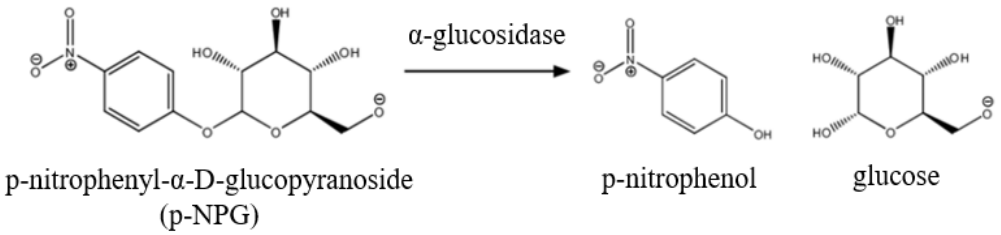

Figure 1. Enzymatic reaction of α-glucosidase and p-NPG
(Fatin et al., 2018)

Inhibition activity is represented by the IC_{50} value. The IC_{50} value represents the concentration of a substance required to inhibit a process by 50% of the population. The sample is declared to have very strong inhibitory activity if the IC_{50} value is <50 ppm, strong if the IC_{50} value is 50-100 ppm, moderate if the IC_{50} value is 100-150 ppm, weak if the IC_{50} value is 151-200 ppm, and declared inactive if it has an IC_{50} value >200 ppm (Nicoli et al., 1999 in Antarti & Lisnasari, 2018).

Thus, acarbose as a comparison and ethyl acetate fraction of tiger's betel has a very strong α-glucosidase inhibitory activity with IC_{50} values of 4.64×10^{-3} µg/mL and 14.06 µg/mL, respectively. The ethyl acetate fraction of tiger's betel has high antidiabetic activity because it has been shown to contain various secondary metabolite compounds that have antidiabetic abilities, such as alkaloids, phenolics, and flavonoids as the largest group that has α-glucosidase inhibitory activity (Dirir et al., 2022).

CONCLUSION

Phytochemical screening of the ethyl acetate fraction of tiger's betel has been successfully carried out and showed the presence of alkaloids, phenolics, and flavonoids compounds. This study can complete information about bioactive compounds from tiger's betel and the ability of antidiabetic activity of ethyl acetate fraction, which has never been reported before. The ethyl acetate fraction of tiger's betel is declared to have a very strong ability to inhibit α-glucosidase activity. Therefore, the use of tiger's betel plants can be considered an alternative in the treatment of diabetes, but other medical reviews are needed.

RECOMMENDATIONS

Based on the results of this study, recommendations for further research include conducting phytochemical screening on various fractions of tiger's betel, such as the hexane and methanol fractions, and comparing the strength of their antidiabetic activity. The research can also be completed by quantitatively calculating the content of secondary metabolite compounds.

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