LC-MS Profiling and α-Glucosidase Inhibitory Activity of the Ethyl Acetate Extract of *Hippobroma longiflora* Leaves

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Kitolod *(Hippobroma longiflora)* is traditionally used as a medicinal plant for treating various diseases. In a previous study, the ethyl acetate extract of kitolod leaves demonstrated the highest antioxidant activity in comparison to the n-hexane and methanol extracts. In this study kitolod leaves were extracted using ethyl acetate, after which the chemical composition and the α-glucosidase inhibitory potential of this extract were determined. Extraction was performed using the maceration method. The chemical composition was analysed using LC-MS. The in vitro α-glucosidase activity test used the pNPG method. The kitolod leaf extract was found to contain four major compounds: hexachlorophene, octaverine, difenoxin, and myristyl myristate. The extract also exhibited moderate inhibitory activity, with an IC_{50} value of 79.652 ± 0.410 µg/mL. The positive control, acarbose, showed a lower IC_{50} value of 55.433 ± 0.233 µg/mL indicating strong activity.

Keywords: Kitolod, Hippobroma longiflora, α-Glucosidase, LC-MS

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The leaves of the kitolod plant *(Hippobroma longiflora)* possess considerable potential for further exploration [1]. The kitolod plant is widely distributed in the Cilegon area of Banten Province [2]. This species is typically found in moist environments with high water content. A significant focus of research in the field of kitolod botany has been the study of the plant's leaves. A multitude of studies have documented the presence of flavonoid, terpenoid, and phenolic compounds in kitolod leaf extracts [3]. It has been documented that kitolod leaves demonstrate biological activities, including antihyperglycemic, antibacterial, and antioxidant properties [4, 5].

A previous study reported that the ethyl acetate extract of kitolod leaves exhibited higher total phenolic and total flavonoid contents compared to the ethanol extract. The antioxidant activity of the ethyl acetate extract was also reported to have the higher IC_{50} value, which was 150.542 ± 3.917 ppm [6]. Phenolic and flavonoid compounds have been reported to possess potential as inhibitors of the α-glucosidase enzyme [7, 8]. This is due to the interactions between their hydroxyl groups and aromatic rings with the active site of the enzyme [9], which have been demonstrated to inhibit the process of carbohydrate breakdown into glucose, thereby contributing to the regulation of blood glucose levels.

The relationship between antioxidant activity and α-glucosidase inhibitory activity is closely associated with diabetes management. In diabetic conditions, hyperglycaemia can increase the production of reactive oxygen species (ROS), leading to oxidative stress and cellular damage. Antioxidant compounds are able to neutralize free radicals and reduce oxidative stress, thereby helping to prevent diabetes-related complications.

α-glucosidase is commonly selected as a target enzyme in antidiabetic activity studies because it plays an important role in carbohydrate digestion and postprandial blood glucose regulation [10]. This enzyme is located in the small intestine and functions to hydrolyse complex carbohydrates into simple glucose molecules that can be absorbed into the bloodstream. Inhibition of α-glucosidase can delay carbohydrate digestion and glucose absorption, thereby reducing postprandial hyperglycemia. For this reason, α-glucosidase inhibitors are considered an effective therapeutic approach for the management of type 2 diabetes mellitus [7]. In addition, the α-glucosidase inhibition assay is widely used because the method is simple, rapid, sensitive, and relatively inexpensive [11]. The assay can be performed in vitro using spectrophotometric analysis, making it suitable for preliminary screening of natural products and plant extracts with potential antidiabetic properties. Many secondary metabolites, such as flavonoids, phenolic compounds, alkaloids, and tannins, have been reported to exhibit α-glucosidase inhibitory activity. Furthermore, standard antidiabetic

drugs such as acarbose may be used as positive controls to provide a reliable comparison for evaluating the effectiveness of tested samples.

This investigative study was conducted with the objective of analysing the chemical compounds present in the ethyl acetate extract of kitolod leaves and evaluating the potential of the extract in inhibiting α-glucosidase enzyme activity. The extraction process utilised the maceration technique. The analysis of chemical compounds was performed using a liquid chromatography–mass spectrometry (LC–MS) method. The α-glucosidase inhibitory activity was evaluated using the pNPG method.

EXPERIMENTAL

Materials

The leaves of kitolod (*Hippobroma longiflora*), collected from Cilegon, Banten, Indonesia, were utilized as the sample for this study. The chemicals utilized in this research included DMSO (MerckK GaA, Germany), buffer phosphate pH 6.9, NaOH (analytical grade), ethyl acetate (Merck KGaA, Germany), 3,5-dinitrosalicylic acid (DNS) (Merck KGaA, Germany), α-glucosidase from *Saccharomyces* purchased from Himedia Indonesia, and acarbose (analytical grade).

Sample Preparation

A total of 1 kg of fresh leaves were utilized in this study. The leaves were meticulously divided into diminutive fragments and subsequently desiccated at ambient temperature for a period of 14 days. Following the drying process, the sample was subjected to grinding using a blender until a simplicia powder was obtained. The leaves, stems, roots, and flowers were prepared as herbarium specimens for identification purposes. Subsequently, identification and authentication of the plant samples were conducted at the Herbarium Laboratory, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta. The identification results showed that the kitolod plant belonged to the genus *Hippobroma* and the species *Hippobroma longiflora*, with determination number 00686/S.Tb./VII/2024 by Prof. Dr. Ratna Susandarini, M.Sc.

Extraction of Kitolod Leaves

A total of 250 g of the powdered kitolod leaves were weighed and placed in a 2 L Erlenmeyer flask. The leaves were then macerated with 1500 mL of ethyl acetate until the sample was completely immersed. The sample was stirred at 6-hour intervals. The maceration process was conducted for a duration of 3×24 hours. Following this, the extract was filtered using filter paper. The resulting filtrate was subjected to concentration using a rotary evaporator at 40 °C. The concentrated extract was then placed in a desiccator at room temperature until all residual ethyl acetate solvent had evaporated. The final dried extract was weighed and stored in a sealed glass container prior to further analysis.

LC-MS Analysis of Ethyl Acetate Fraction of Kitolod Leaves Fraction

The sample's phytochemical composition was analysed using an Advion LC-MS instrument equipped with a C18 column (2.1 × 100 mm, 1.7 µm; Waters, USA) and operated under Electrospray Ionization (ESI) in positive and negative ionization modes. This technique facilitated the detection of secondary metabolite compounds present in the ethyl acetate extract. The ethyl acetate extract was accurately weighed (approximately 1–5 mg) and dissolved in HPLC-grade methanol to obtain a final concentration of 1 mg/mL. The solution was sonicated for 10 minutes to ensure complete dissolution and subsequently filtered through a 0.22 µm membrane filter prior to analysis. The filtered sample was transferred into an autosampler vial for injection. Chromatographic separation was carried out using a reversed-phase C18 column. The mobile phase consisted of water containing 0.1 % formic acid (solvent A) and acetonitrile containing 0.1 % formic acid (solvent B). A gradient elution program was applied as follows: 10 % B (0–2 min), increased to 100 % B (2–15 min), maintained at 100 % B (15–18 min), and returned to initial conditions for re-equilibration. The flow rate was set at 0.3 mL/min, with an injection volume of 5 µL and column temperature maintained at 30 °C.

Alpha Glucosidase Inhibitor Activity

The α-glucosidase inhibitory assay was performed according to the method described by Tinrat and Jiraprasertwong (2021), with several modifications [12]. The concentrations used were 20, 40, 60, 80 and 100 µg/mL. A total of 10 µL of the sample solution (EAK) was mixed with 120 µL of 0.1 M phosphate buffer (pH 6.8) and 20 µL of α-glucosidase enzyme solution in a 96-well microplate. The mixture was then subjected to an incubation period of 15 minutes at a temperature of 37 °C. This was followed by the addition of 20 µL of a 10 mM solution of p-nitrophenyl-α-D-glucopyranoside (pNPG), which served as the substrate. Subsequently, the reaction was subjected to incubation and terminated by the addition of 80 µL of a 0.2 M sodium carbonate solution. The pNPG substrate was utilized to monitor the enzymatic reaction. The absorption of the resulting yellow colour solution was subsequently measured at a wavelength of 415 nm by means of a microplate reader. Acarbose was utilized as the positive control [13]. The experiment was conducted in triplicate.

Table 1. LC-MS analysis of EAK.

Compound	Molecular formula	Molecular weight
Hexachlorophene	$C_{13}H_6Cl_6O_2$	403.8498
Octaverine	$C_{23}H_{27}NO_5$	397.1889
difenoxin	$C_{28}H_{28}N_2O_2$	424.2150
Myristyl myristate	$C_{28}H_{56}O_2$	424.7420

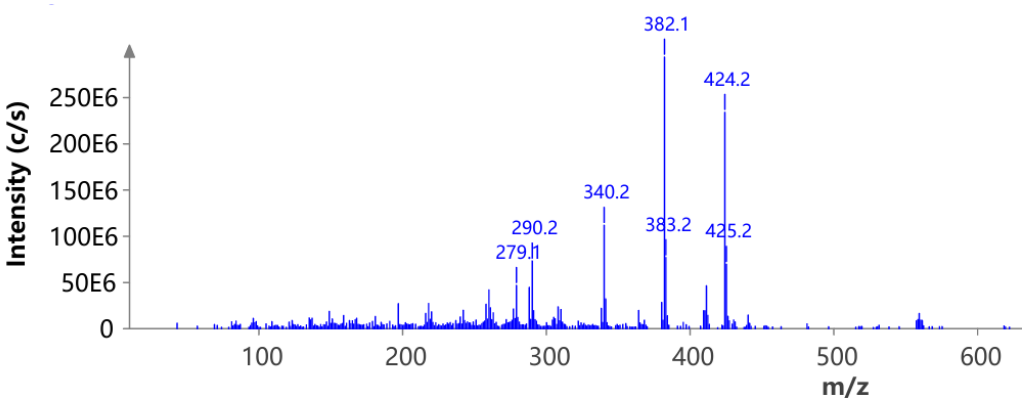

Figure 1. LC-MS Chromatogram of EAK.

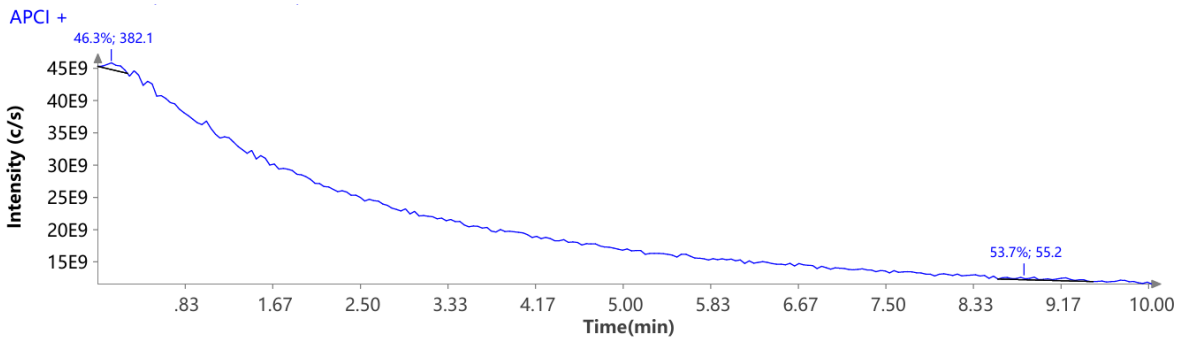

Figure 2. Total ion chromatogram (TIC) of EAK.

RESULTS AND DISCUSSION

Extraction of Kitolod Leaves

In this experiment, the ethyl acetate extract of kitolod was designated as EAK. The mass of EAK extract was 25.7 g, a yield of 9.7 %. The efficacy of ethyl acetate in extracting the kitolod leaf sample was determined by the yield value obtained, which approached 10 %. Ethyl acetate, a semi-polar solvent, has the capacity to extract both non-polar and semi-polar compounds [14]. It is well established that compounds belonging to the phenolic, flavonoid, alkaloid, and terpenoid groups are soluble in semi-polar solvents [15]. A study by Faradin et al. (2025) reported that phytochemical screening showed that the ethyl acetate extract of kitolod leaves contained flavonoids, phenolics, terpenoids, and alkaloids [6]. These compounds have been reported to possess antihyperglycaemic and antidiabetic activities [15, 16]. Compounds belonging to the flavonoid and phenolic groups, which are commonly found in plant extracts, have been shown to inhibit α-glucosidase activity through interactions with the enzyme's active site as well as through the formation of hydrogen bonds with amino acid residues of the enzyme [17]. Consequently, the ethyl acetate fraction of kitolod leaves has potential for further development as a natural source of compounds for controlling hyperglycaemia through the inhibition of α-glucosidase activity.

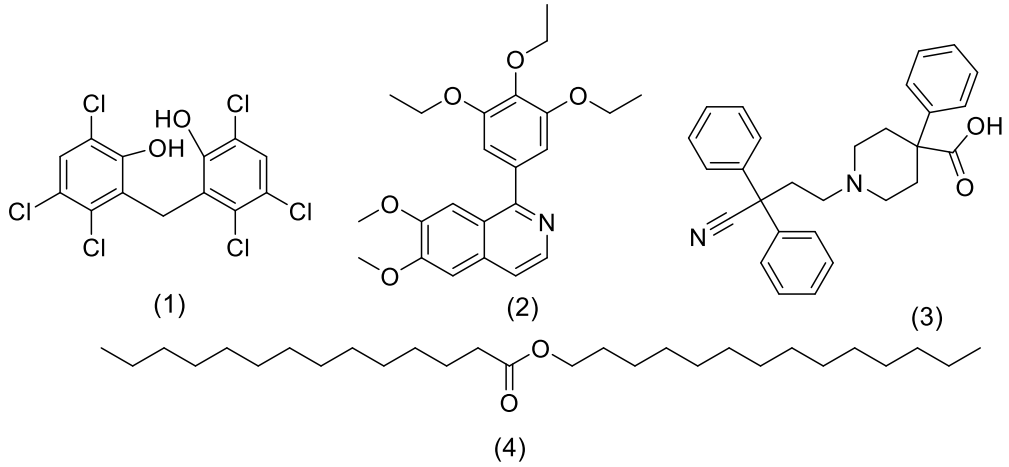

Figure 3. The chemical structures of compounds present in EAK: hexachlorophene (1), octaverine (2), difenoxin (3), and myrystil myristate (4).

LC-MS Analysis of EAK

The analysis of the chemical constituents of EAK showed the presence of four major compounds. The results are presented in Table 1. These four compounds consist of hexachlorophene, octaverine, difenoxin, and 6,6'-methylenebis(2,3,5-trichlorophenol). The chromatogram of EAK is shown in Figure 1, the total ion chromatogram (TIC) in Figure 2 and the chemical structures of its compounds in Figure 3.

The presence of these compounds in EAK indicates that ethyl acetate is capable of extracting compounds with semi-polar to non-polar characteristics, consistent with its nature as a solvent. Compounds such as hexachlorophene (Figure 3) and its derivatives are classified as chlorinated phenolics, which possess aromatic hydroxyl groups and are recognized for their biological activities, particularly as antimicrobial agents [18]. The phenolic groups in these structures may also be potentially involved in antioxidant activity through mechanisms of proton or electron donation. The detected hexachlorophene (1) compound is likely associated with environmental contamination or tentative database matching rather than a genuine plant secondary metabolite. Octaverine (2) was tentatively annotated based on LC-HRMS database matching; however, its occurrence as a natural plant metabolite remains uncertain. Difenoxin (3) was tentatively assigned through database matching; however, as this compound is primarily recognized as a synthetic pharmaceutical agent, its natural occurrence in plants is also doubtful. Myristyl myristate (4) is a class of lipid esters or wax esters composed of myristic acid and myristyl alcohol. This compound is classified as a lipophilic substance that can naturally be found in plant wax layers, leaf cuticles, and various tissues of living organisms. The presence of myristyl myristate in the kitolod extract indicates the existence of nonpolar lipid components that may play a role in protecting plant tissue surfaces against water loss, microbial exposure, and environmental stress [19].

When associated with α-glucosidase inhibitory activity, the identified compounds exhibit characteristics that support such activity. Phenolic compounds and their derivatives have been shown to inhibit α-glucosidase by binding to the enzyme's active site or allosteric site [20]. This binding results in the disruption of the hydrolysis of carbohydrates into glucose. Conjugated aromatic groups have been demonstrated to enhance binding affinity toward the target enzyme.

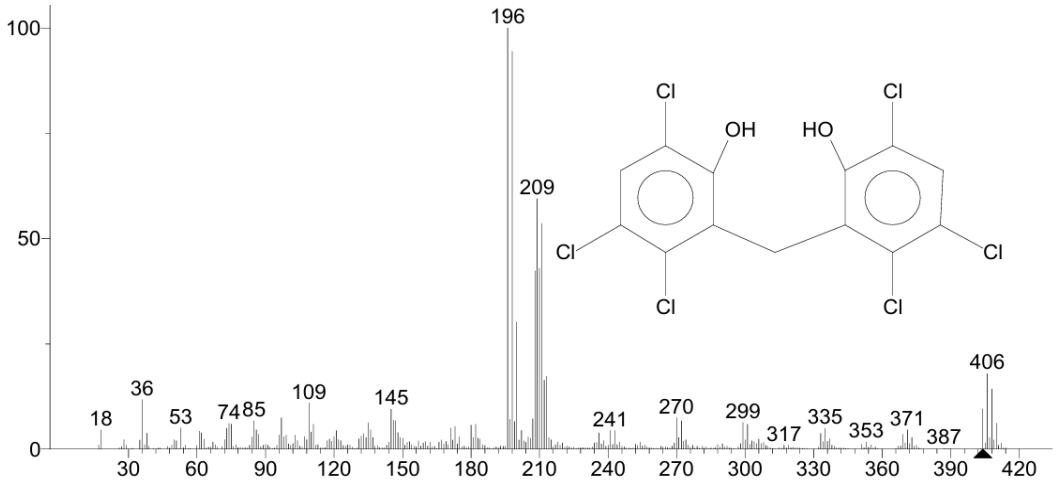

Figure 4. Chromatogram of hexachlorophene (1).

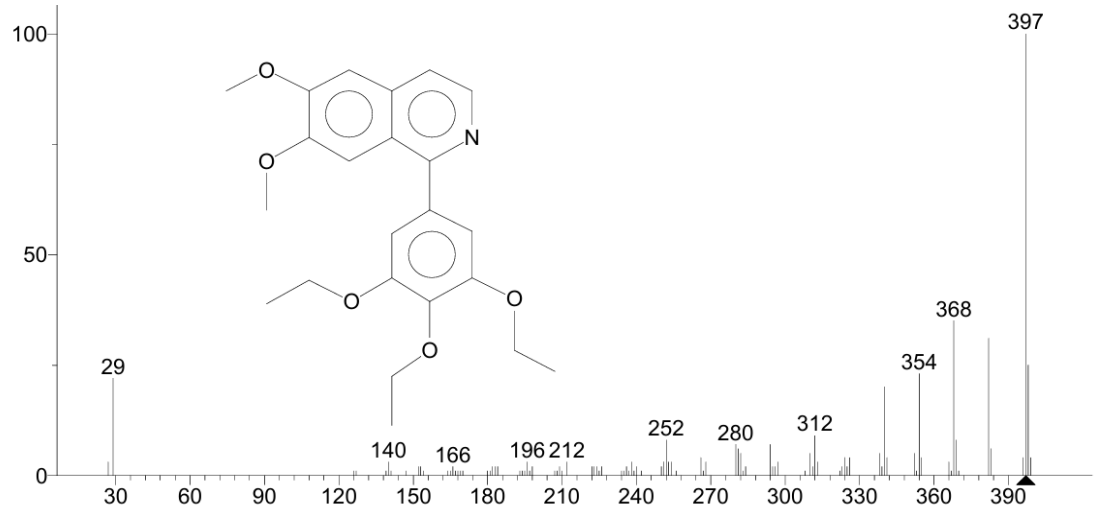

Figure 5. Chromatogram of octaverine (2).

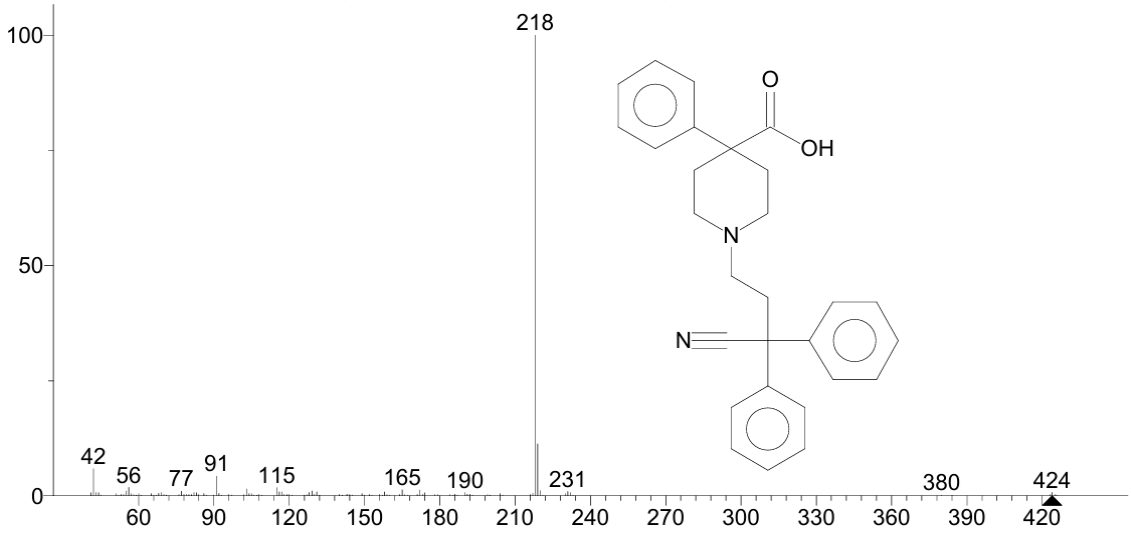

Figure 6. Chromatogram of difenoxin (3).

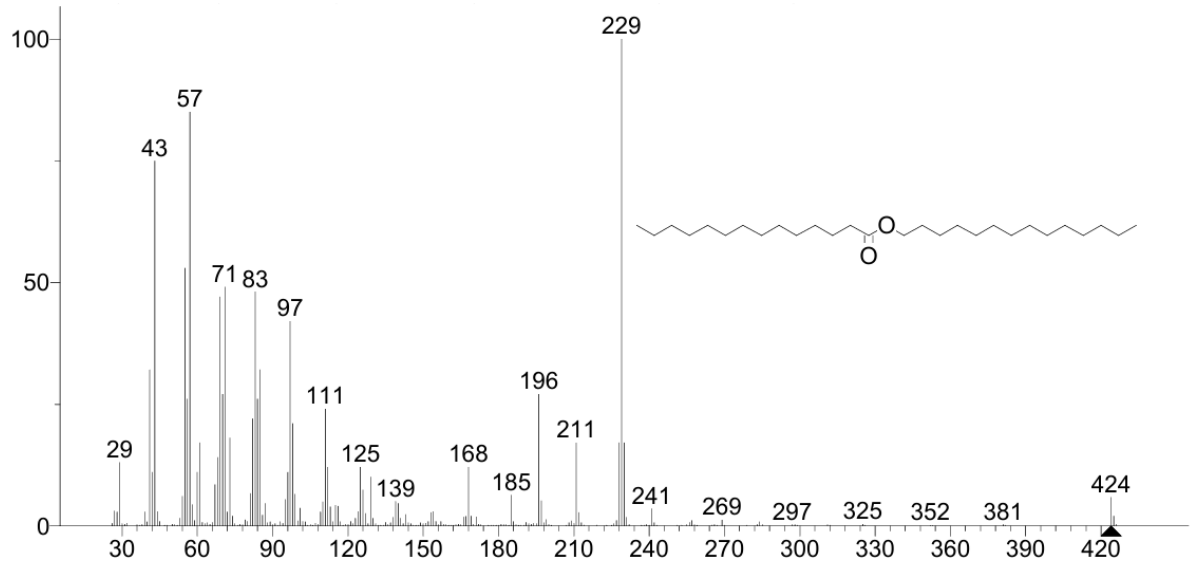

Figure 7. Chromatogram myristyl myristate (4).

Table 2. The concentration of EAK and its inhibition (%) against α-glucosidase.

Sample	Concentration (ppm)	Inhibition (%)		
		1	2	3
EAK	20	14.8477	13.2822	14.8477
	40	26.6497	27.0753	28.5532
	60	36.6751	37.2924	37.4365
	80	49.1116	47.2541	48.4771
	100	63.5786	64.1123	63.9593
Acarbose	20	31.0198	30.9557	29.6875
	40	44.9008	45.0784	45.7386
	60	53.5410	53.0670	53.5511
	80	60.6232	60.7703	61.5056
	100	70.9631	70.3281	71.3068

α-Glucosidase Activity of EAK

The α-glucosidase inhibitory activity assay was carried out using the pNPG method. The colour change that occurred was measured using a UV–Visible spectrophotometer at a wavelength of 417 nm. The results of the percentage inhibition calculation at various concentrations of EAK are presented in Table 2. The concentrations and linear regression curves of inhibition (%) are shown in Figure 8.

Based on the linear regression curve (Figure 8), the IC_{50} value of the ethyl acetate extract of kitolod leaves (EAK) was 79.652 ± 0.410 µg/mL, while the positive control, acarbose, showed a lower IC_{50} value of 55.433 ± 0.233 µg/mL. The IC_{50} value represents the concentration of a sample required to inhibit 50 % of α-glucosidase enzyme activity; therefore, a lower IC_{50} indicates stronger inhibitory activity.

The results of the study indicate that EAK exhibited moderate inhibitory activity against α-glucosidase, although its potency was lower compared to acarbose. This discrepancy was anticipated, as acarbose is a well-established synthetic inhibitor that has been specifically engineered to target α-glucosidase with high affinity. In contrast, EAK is a complex mixture of various phytochemical constituents, which may act synergistically but individually possess lower inhibitory strength [9].

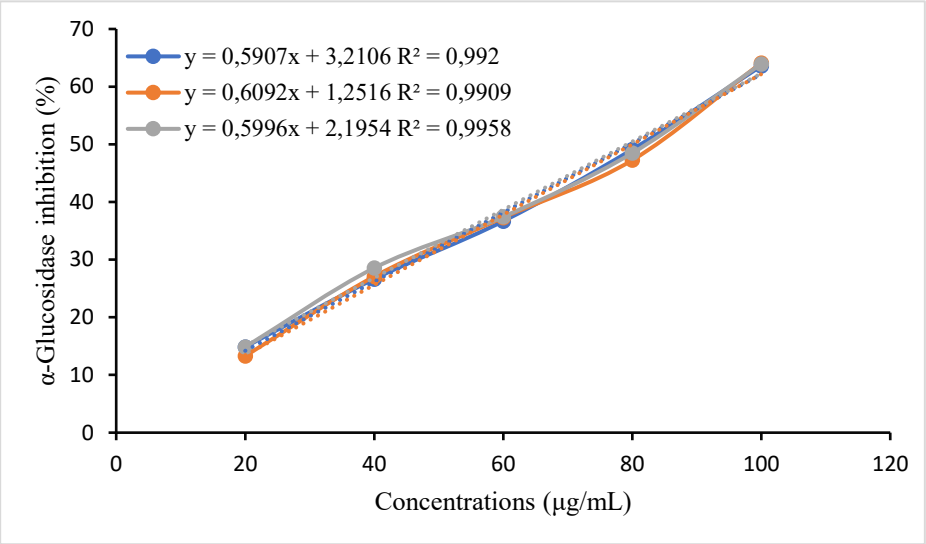

Figure 8. The linear regression curves for various concentrations of EAK against α-glucosidase inhibition.

The observed inhibitory activity of EAK may be attributed to the presence of bioactive compounds such as phenolics and alkaloids, as identified in the LC–MS analyses. These compounds have been shown to inhibit α-glucosidase through multiple mechanisms, including binding to the active site of the enzyme, altering enzyme conformation, or interacting with amino acid residues via hydrogen bonding and hydrophobic interactions.

In addition, the relatively close IC_{50} values of EAK and acarbose suggest that the extract has promising potential as a natural α-glucosidase inhibitor. Despite its reduced activity compared to the standard drug, EAK may offer certain advantages, including a reduced likelihood of adverse effects and the presence of multiple bioactive compounds that could provide additional therapeutic benefits, including antioxidant activity.

CONCLUSION

LC-MS analysis of the chemical constituents in the ethyl acetate extract of kitolod leaves identified four major compounds: hexachlorophene, octaverine, difenoxin, and myristyl myristate. The α-glucosidase assay indicated that EAK had an IC_{50} value of 79.652 ± 0.410 µg/mL. The positive control, acarbose, showed a lower IC_{50} value of 55.433 ± 0.233 µg/mL, indicating strong activity. Based on this study, EAK displayed moderate activity as a natural α-glucosidase inhibitor.

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