Potency of Kitolod (*Isotoma longiflora* L presl.) fruit ethyl acetate extract as antioxidant, α amylase and α-glucosidase enzyme inhibitor for anti-diabetic prevention

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Abstract. This research was carried out to determine the chemical composition, antioxidant, and anti-diabetic potential of ethyl acetate extract of kitolod fruit (*Isotoma longiflora* (L) Presl). The design of this research was carried out descriptively. To obtain bioactive compound components, the Kitolod fruits were dried at a temperature of 50°C and then ground into powder until they passed through an 80 mesh sieve. Extraction was carried out using the maceration method at 30°C for 48 hours with an ethyl acetate solvent. Ethyl acetate extracts had been fractioned with n-Hexan and identified through LC-MS examination. The dominant chemical composition of ethyl acetate extract was sesquiterpene (66.74%), followed by flavonoids (18.11%). Antioxidant potential was evaluated through DPPH free radical scavenging activity and inhibition of α-amylase and α-glucosidase enzyme activity. Ethyl acetate extract showed moderate levels of antioxidant activity. The inhibitory activity of α-amylase enzyme is lower than quercetin as a positive standard, but the inhibitory activity of α-glucosidase enzyme is extreme with an IC_{50} value of 47.33 ±1.40 μgmL^{-1}. These results suggest that ethyl acetate extracts from the fruit have the potential for antidiabetic-based natural products and antioxidant agents.

1 Introduction

Plants are a source of bioactive compounds used as antioxidants to maintain health caused by free radicals [1]. Natural antioxidants function as singlet oxygen binders, pro-oxidant metal complexes, reducing agents, and free radical scavengers, thereby protecting against cellular damage caused by free radicals [2]. One of the secondary metabolites of plants is

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flavonoids, which have a role in plant growth physiology[3]. As an antioxidant, flavonoids prevent degenerative diseases[4] and avoid the negative effects of free radicals [5].

Kitolod (*Isotoma longiflora* (L) Presl.) as a plant that can be found in Indonesia, has been assessed, especially regarding its ability of antioxidant, anticancer, and antibacterial [6]. Hapsari Kitolod also has been screened for secondary metabolites such as flavonoids, phenolics, chlorophylls, carotenoids, and ascorbic acid from the root, stem, flower, leaves, and fruit [7]. Kitolod leaves were extracted by ethanol and tested for their activity against the inhibition of Hella cells [8] and tuberculosis bacteria[10]. Extract ethanol of Kitolod also possessed antimicrobial activity [11]. Moreover, Kitolod extracted by methanol inhibited the growth of *Streptococcus mutans* [6]. However, the extraction of Kitolod leaves with ethyl acetate has never been done. Although many other leaf extraction studies used ethyl acetate to evaluate its antioxidant activity [[12].

Based on research, the types of active compounds that have antioxidant properties have yet to be disclosed. This research will extract Kitolod fruit with ethyl acetate. This study aimed to investigate the bioactive compounds in the ethyl acetate fraction of Kitolod fruit and determine the antioxidant activity, inhibition of alpha-amylase activity, and alpha-glucosidase activity.

2 Materials and Methods

2.1 Plant Extraction

Natural populations of kitolod fruits were gathered at Merjosari, Lowokwaru, Malang, East Java, Indonesia. After being collected, the fruits were rinsed under running water and allowed to drip for three hours at 27°C. The fruit must then be dried for 36 hours at 50°C in a cabinet dryer (GETRA, FJ-15C, China). Before being used, dried fruit is pounded into a powder, sieved through a 60 mesh sieve pit, sealed in a beaker, and kept in the dark at -20°C (LG, GN 304SL, South Korea). For 48 hours at room temperature (27°C), 100 g of Kitolod fruit powder was macerated with 1 L of ethyl acetate (99.9%, Merck, USA). Using a Buchner funnel lined with Whatman No. 1 filter paper, the resultant mixture was filtered.

2.2 Liquid chromatography (LC)-mass spectrometry (MS)

Bioactive chemicals are identified using the Shimadzu LCMS-8040 LC/MS, Shimadzu Column, and FC-ODS Shim Pack (2 mm × 150 mm, 3 μm). The column temperature is kept at 35°C while the injection volume is fixed at 1 μL. At 0/0 or 0 minutes, 15/85 at 5 minutes, 20/80 at 20 minutes, and 90/10 at 24 minutes, eluents are pumped in gradients. 90% methanol in water at a flow rate of 0.5 mL min-1 dissolves at 350 °C. Fragmentation is induced by low-energy CID[13]. The study's chemicals and other reagents were acquired from Sigma Aldrich Chemical Co. and were categorised as analytical grade.

2.3 Antioxidant activity

2.3.1 2, 2-diphenyl-1-picrylhydrazil (DPPH) radical scavenging assay

Antioxidant activity was determined by the IC_{50} value based on the method of [14]. The IC_{50} value showed the level of extracts that can inhibit half of the free radicals added. The ethyl acetate extract and its fraction were prepared in various concentrations (0, 20, 40, 60, 80, 100, 120,140,160 µg / mL) into a 10 ml test tube, then added a DPPH solution of 2.10^{-4} mmol L^{-1}, and homogenized. The mixture was kept for 30 minutes at room temperature in the dark and tightly closed. The formed purple-to-yellow color was absorbed at a wavelength (λ) of 517 nm. Antioxidant activity was calculated based on the equation (1):

$$\text{antioxidan activity (\%)} = \frac{\text{Absorbance (control)- Absorbance (sample)}}{\text{Absorbance (control)}} \times 100$$

(1)

The absorbance of the negative control reaction is known as the absorbance control, and the absorbance in the presence of plant extracts is known as the absorbance sample. IC50, or the concentration (µg mL–1) of the ethyl acetate extract that scavenges 50% of the DPPH• radical, was used to communicate the results.

2.3.2. Inhibition of α-glucosidase assay

Dimethyl Sulfoxide was used to dissolve ethyl acetate extracts that were generated in a range of concentrations (2 to 200 µg mL^{-1}). After transferring a 30 µL sample into a microplate, 17 µL of 4 mM p-nitrophenyl-α-D-glucopyranose (p-NPG) substrate (Sigma-Aldrich, Switzerland) and 36 µL of quasi-phosphate buffer at pH 6.9 were added. For five minutes, the mixture was incubated at 39°C. To aid in substrate reduction, 25 µL of an α-glucosidase enzyme solution (0.8 U mL^{-1}, Saccharomyces cerevisiae, Sigma-Aldrich, Germany) was then added, and the mixture was incubated for 15 minutes at 39°C. 100 µL of a 200 mM Na_2CO_3 solution (Merck, USA) was added to stop the reaction. A microplate was used to measure the amount of nitrophenol released from p-NPG at 400 nm in order to assess the level of α-glucosidase inhibition.

Inhibition (%) = [(blank absorption – sample absorption) / blank absorption] × 100 (2)

Results were expressed as IC_{50}, the concentration (µg mL^{-1}) of ethyl acetate extract required to inhibit 50% of α-glucosidase activity.

2.3.3 Inhibition of α-amylase assay

Testing of alpha-amylase inhibition follows the standard method. Inhibition activity of α-amylase from ethyl acetate extract of kitolod fruit [16]. A 0.5% (w/v) starch solution, prepared by boiling potato starch in distilled water for 15 minutes, served as the substrate. For the enzyme solution, 1 mg of alpha-amylase from porcine pancreas was dissolved in a 20 mM phosphate buffer (pH 6.9, 100 mL). Ethyl acetate extract samples were prepared in various concentrations (10 to 100 mg mL^{-1}) using dimethyl sulfoxide (Merck, USA). A DNS solution (Merck, USA) was used as a dye, prepared with 20 mL of 96 mM 3,5-

dinitrosalicylic acid, 12 g of sodium potassium tartrate, 8 mL of 2 M NaOH, and 12 mL of deionized water.

For the inhibition analysis, each test was repeated three times, with blanks and controls. The enzyme solution (1 mL) was added to each test mixture in test tubes and incubated at 25°C for 30 minutes. A 1 mL sample from each mixture was then combined with a 1 mL starch solution and incubated again at 25°C for 3 minutes. Afterwards, 1 mL of DNS solution was added, and the tubes were sealed and heated in a water bath at 85°C for 15 minutes. Once cooled, the reaction mixtures were diluted with 9 mL of distilled water, and absorbance was measured at 540 nm. The blanks, where DNS was added before the starch solution, and the control, where 1 mL of dimethyl sulfoxide was used instead of the extract, are crucial for establishing a baseline for the experiment and ensuring that any observed effects are due to the extract and not other factors. Acarbose (Glyco AC 50, Mumbai, India), an established antidiabetic agent, was used as a positive control. The percentage inhibition was calculated using Equation (3).

$$\text{Inhibition (\%)} = [(AC - AS) / AC] \times 100 \quad (3)$$

In this assay, AC represents the control absorbance, while AS is the absorbance measured in the presence of the plant extract. The result is expressed as IC_{50}, which is defined as the hexane extract concentration (μg mL^{-1}) required to inhibit 50% of α-amylase activity.

3 Statistics analysis

The research data were collected and presented as the mean ± standard deviation from three independent trials. The correlation coefficient and IC_{50} values were calculated using linear regression analysis in Microsoft Excel, and GraphPad Prism 7 (GraphPad Software, La Jolla, California, USA) was used for statistical analysis. A significance level of $P < 0.05$ was considered statistically significant.[17]

4 Results and discussion

4.1 Chemical Compounds

We have investigated ethyl acetate extract from kitolod fruit flour obtained by maceration process for 48 hours. Two hundred grams of Kitolod fruit powder macerated with ethyl acetate for 48 hours. Furthermore, ethyl acetate extract was fractionated with hexane so that an ethyl acetate fraction of 7.60 g and a hexane fraction of 37.12 g were obtained. Identification of chemical components with LC-MS shows that ethyl acetate extract obtained 55 types, as shown in Fig. 1. The results of the compound spectrum analysis of each peak are shown in Table 1. The chemical composition of ethyl acetate extract can be grouped into 3, namely sesquiterpenes (63.89%), flavonoids (18.81%), and organic acids (15.16%). As for sesquiterpenes, there were 10 types whose number is between 2.13 – 3.87%, namely: Chrysanthenone, Scopoletin, Chrysandiol, Santamarine, Chrysartemin A, Angeloylcumambrin B, Chrysanin, Scopolin, Sesamin. While the order of flavonoid content was Apigenin OH > Quercetagetin > Centaureidin > Luteolin > Quercetagetin 3' methyl ether.

Santamaria was also classified as a sesquiterpene with antioxidant activity [18]. Sesquiterpenes in essential oils from various plant extracts have a role in antioxidant and

antimicrobial activity [19]; besides that, according to [20] said that terpenoid compounds contained in plant extracts have synergism in capturing free radicals

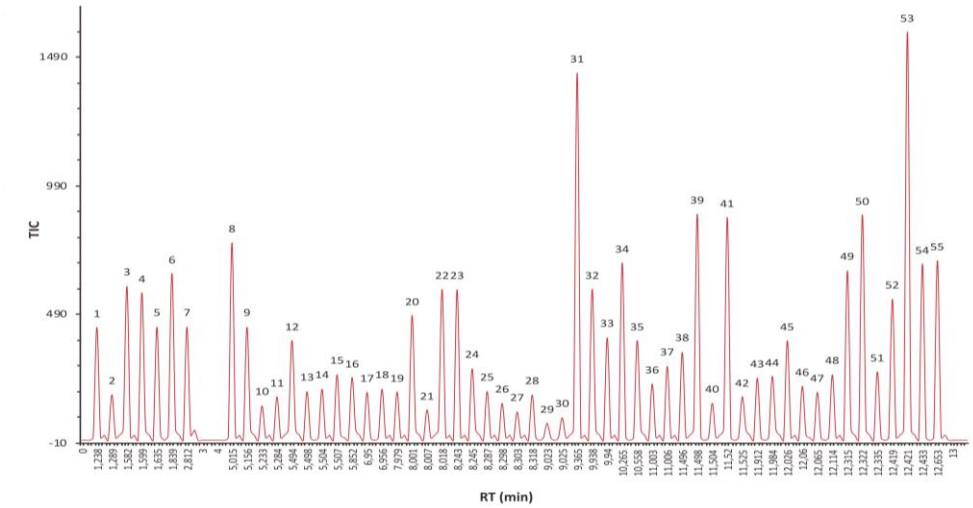

Fig. 1. Chromatogram graph of LC-MS analysis of ethyl acetate extract of fruits Kitolod

Table 1. The chemical compound contained in ethyl acetate extract from Kitolod fruits based on the chromatogram in Figure 1.

Peak No	RT (min)	Compound name	% Area	Chemical Formula	Exact Mass (g mol^{-1})	Molecular Weight (g mol^{-1})
1	1.238	Fumaric acid	1,92872	$C_4H_4O_4$	116,0110	116,0720
2	1.289	Benzoic acid	0,77717	$C_7H_6O_2$	122,0368	122,1230
3	1.582	Cinnamic acid	2,63117	$C_9H_8O_2$	148,0524	148,1610
4	1.599	Chrysanthenone	2,52187	$C_{10}H_{14}O$	150,1045	150,2210
5	1.635	Borneol	1,93268	$C_{10}H_{18}O$	154,1358	154,2530
6	1.839	p Coumaric acid	2,85097	$C_9H_8O_3$	164,0473	164,1600
7	2.812	Capillin O	1,93267	$C_{12}H_8O$	168,0575	168,1950
8	5.015	Scopoletin	3,37668	$C_{10}H_8O_4$	192,0423	192,1700
9	5.156	Bornyl acetate	1,93272	$C_{12}H_{20}O_2$	196,1463	196,2900
10	5.233	Calacorene	0,58822	$C_{15}H_{20}$	200,1565	200,3250
11	5.284	Calamene	0,74160	$C_{15}H_{22}$	202,1722	202,3410
12	5.494	β Caryophyllene	1,70370	$C_{15}H_{24}$	204,1878	204,3570
13	5.498	β Farnesene	0,82940	$C_{15}H_{24}$	204,1878	204,3570
14	5.504	β Elemene	0,87341	$C_{15}H_{24}$	204,1878	204,3570
15	5.507	Germacrene D	1,11942	$C_{15}H_{24}$	204,1878	204,3570
16	5.852	cis Spiroketalenolether polyyne	1,07163	$C_{14}H_{14}O_2$	214,0994	214,2640
17	6.95	Nerolidol	0,81959	$C_{15}H_{26}O$	222,1984	222,3720
18	6.956	t Muurolol	0,87387	$C_{15}H_{26}O$	222,1984	222,3720
19	7.979	Kikkanol B	0,82496	$C_{15}H_{24}O_2$	236,1776	236,3550
20	8.001	Chrysandiol	2,13389	$C_{15}H_{24}O_3$	252,1725	252,3540
21	8.007	Clovane 2 β,9 α diol	0,52192	$C_{15}H_{26}O_2$	238,1933	238,3710

22	8.018	Santamarine	2,57910	$C_{15}H_{20}O_3$	248,1412	248,3220
23	8.243	Chrysetunone	1,22137	$C_{15}H_{24}O_3$	252,1725	252,3540
24	8.245	Kikkanol C	0,83435	$C_{15}H_{24}O_3$	252,1725	252,3540
25	8287	Kikkanol E	0,63219	$C_{15}H_{24}O_3$	252,1725	252,3540
26	8.298	Kikkanol A	0,48326	$C_{15}H_{26}O_3$	254,1882	254,3700
27	8.303	Kikkanol D	0,77521	$C_{15}H_{26}O_3$	254,1882	254,3700
28	8.318	Kikkanol F	0,28954	$C_{15}H_{26}O_3$	254,1882	254,3700
29	9.023	Tatridin A	0,38467	$C_{15}H_{20}O_4$	264,1362	264,3210
30	9.025	Tatridin B	0,38467	$C_{15}H_{20}O_4$	264,1362	264,3210
31	9.365	Apigenin OH	6,27835	$C_{15}H_{10}O_5$	270,0528	270,2400
32	9.938	Chrysartemin A	2,58341	$C_{15}H_{18}O_5$	278,1154	278,3040
33	9.94	Chrysartemin B	1,75254	$C_{15}H_{18}O_5$	278,1154	278,3040
34	10.265	Luteolin	3,03151	$C_{15}H_{10}O_6$	286,0477	286,2390
35	10,.58	Pyrethrosine	1,70465	$C_{16}H_{22}O_5$	294,1467	294,3470
36	11.003	Kikkanol D monoacetate	0,96171	$C_{17}H_{28}O_4$	296,1988	296,4070
37	11.006	Kikkanol F monoacetate	1,26386	$C_{17}H_{28}O_4$	296,1988	296,4070
38	11.496	Arteglasin A	1,50347	$C_{17}H_{20}O_5$	304,1311	304,3420
39	11.498	Chrysanolide	3,86581	$C_{17}H_{20}O_5$	304,3420	304,3420
40	11.504	Cumambrin A	0,63263	$C_{17}H_{22}O_5$	306,1467	306,3580
41	11.52	Quercetagetin	3,80837	$C_{15}H_{10}O_8$	318,0376	318,2370
42	11.525	β Cyclopyrethrosin	0,74208	$C_{17}H_{22}O_5$	306,1467	306,3580
43	11.912	Dihydrocumambrin A	1,06284	$C_{17}H_{24}O_5$	308,1624	308,3740
44	11.984	Chlorochrymorin	1,09356	$C_{15}H_{19}ClO_5$	314,0921	314,7620
45	12.026	Quercetagetin 3' methyl ether	1,70321	$C_{16}H_{12}O_8$	332,0532	332,2640
46	12.06	Eupatilin	0,92220	$C_{18}H1_6O_7$	344,0896	344,3190
47	12.065	Lobelin!	0,82060	$C_{22}H_{27}NO_2$	337,2042	337,4630
48	12.114	Lobelanidine	1,11887	$C_{22}H_{29}NO_2$	339,2198	339,4790
49	12.315	Angeloylcumambrin B	2,89536	$C_{20}H_{26}O_5$	346,1780	346,4230
50	12.322	Chrysanin	3,85113	$C_{20}H_{26}O_5$	346,1780	346,4230
51	12.335	Nevadensin	1,17263	$C_{18}H_{16}O_7$	344,0896	344,3190
52	12.419	Scopolin	2,41202	$C_{16}H_{18}O_9$	354,0951	354,3110
53	12.421	Chlorogenic acid	6,97555	$C_{16}H_{18}O_9$	354,0951	354,3110
54	12.433	Sesamin	3,01393	$C_20H_{18}O_6$	354,1103	354,3580
55	12.653	Centaureidin	3,07061	$C_{18}H_{16}O_8$	360,0845	360,3180

4.2 Antioxidant Activity in IC_{50} Values

This study revealed that the level of DPPH radical scavenging derived from ethyl acetate extract of Kitolod fruit was moderate with IC_{50} values of 92.30 ± 0.95 μgmL^{-1} and when compared with quercetin standards as a positive control, as shown in Table 2. IC_{50} is a value that indicates the concentration of a substance which can reduce DPPH radicals by up to 50%. The lower the IC_{50} value, the higher the antioxidant activity [21]. When compared with ethyl acetate extract from *H. colorant* and cinnamon leaves which have IC_{50} values of 108 and 289.8 ± 0.32 μgmL-1 [22], ethyl acetate extract of child fruit has more vigorous activity.

The reduction in antioxidant power is an essential indicator of potential antioxidant activity. Ethyl acetate extract from Kitolod leaves showed strong antioxidant activity; this was also consistent with ethyl acetate extract from fruits [23]. Antioxidants are needed to prevent the harmful effects of free radicals in food and biological systems [24]. The

electron transfer reaction influences the increase in antioxidant capacity [25]. The hydroxyl functional group or modification of the hydroxyl group in the flavonoid structure or the free hydroxyl group at position C-4 'determines the anti-radical activity [26]. This diversity of components and chemical structures may be the basis for the high activity of antioxidants and radical scavengers [27].

4.3. Enzyme inhibitory

4.3.1 Inhibition of α-amylase

Afterr being put in the mout, foodh will be chewe, and enzymest help break down starch into simple sugars. The enzyme that plays a role is alpha-amylase found in the mouth. The inhibition of the enzyme alpha amylase is carried out to inhibit the breakdown so that it remains in the form of complex carbohydrates. Diabetics use acarbose to inhibit the enzyme. Terpenoids and flavonoids have a role in inhibiting the α-amylase enzyme in breaking down oligosaccharides and disaccharides into monosaccharides [28]. This mechanism effectively helps reduce postprandial blood sugar levels [29].

Referring to Table 2, shows that ethyl acetate extract has lower IC_{50} values (138.27 ± 0.89) μg mL^{-1} compared to IC50 values of quercetin and akarbose (125.19 ±1.07) μg mL^{-1} and (133.647 ± 0.59) μg mL^{-1}) . Although its activity is lower than quercetin and acarbose, the difference is not too high. The potential of ethyl acetate extract in inhibiting α-amylase is more likely due to the presence of flavonoids [30]. The fact that α-amylase inhibition plays a role in the process of starch and glycogen digestion is the basic idea for treating impaired carbohydrate absorption in diabetics [31][.

4.3.2. α-glucosidase inhibitory activity

Blood glucose levels rise when oligosaccharides are hydrolysed into monosaccharides by the enzyme α-glucosidase, which is then absorbed by the small intestine [32]. The activity of the α-glucosidase enzyme can be suppressed in in vitro using Kitolod fruit ethyl acetate extract, using quercetin and acarbose as favourable controls. Terpenes, found in a variety of therapeutic plants, are also involved in preventing the action of α-glucosidase. [33].

Ethyl acetate extract has a more vital IC50 value (47.33 ±1.40) μg mL-1 than quercetin, but it is lower than that of acarbose (86.051 ± 0.43) μg mL-1 and (14.891 ± 0.11) μg mL-1, according to Table 2. However, the flavonoids in ethyl acetate extract are more likely to be the cause of its efficacy in blocking α-glucosidase [30]. The fundamental concept for treating reduced carbohydrate absorption in diabetics is based on the notion that inhibition of α-amylase plays a substantial role in the digestion of starch and glycogen [31]. By blocking some enzymes, particularly the alpha-glucosidase enzyme, and altering intestinal glucose absorption, certain flavonoids can influence glucose metabolism [34].

Table 2. Radical scavenging activity, α-amylase activity, and α-glucosidase activity inhibition of hexane extracts of kitolod leaves and fruits.

Materials	Radical scavenging activity IC_{50} (µg mL^{-1})	Inhibition of α-amylase activity IC_{50} (mg mL^{-1})	Inhibition of α-glucosidase activity IC_{50} (µg mL^{-1})
Fruit	92,30 ± 0,95 [b]	138,27 ± 0,89 [b]	47,33 ±1,40 [b]
Quercetin	29,93 ± 0,34 [a]	125,19 ±1,07[a]	86.051 ± 0.43[c]
Acarbose	-	133.647 ± 0.59 [b]	14.891 ± 0.11[a]

Note: The above value is mean ± sd. Subcritp shows a meaningful difference with $P < 0.05$

5 Conclusion

Fifty-five different compounds were found in ethyl acetate extract by LC-MS analysis. Sesquiterpene compounds and flavonoids make ethyl acetate extract effective in the inhibitory potential of alpha-amylase enzyme activity, alpha-glucosidase, and antioxidants. Our study revealed that ethyl acetate extract obtained from kitolod fruit powder might be a promising source of effective antioxidants and inhibitory of alpha-amylase and glucosidase enzymes and may play an essential role in supporting the prevention of diabetes Mellitus.

Conflict of Interests

The authors declare that there are no conflicts of interest related to the publication of this

Acknowledgments

This work was supported by a research grant from the Univesity of Muhammadiyah Malang, Indonesia

Author's Contribution

Sukardi, prepared the extracts and carried out all the experiment processes.
Warkoyo, providing materials for research
Damat, Chemical Component Analysis
Lili Zalizar, Proofreading articles
Noor Harini, Statistical Analysis

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