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Research Article

Antidiabetic Activity of Extract Combination of *Orthosiphon aristatus* and *Oryza sativa* L. var *glutinosa*

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Abstract

Traditionally and scientifically, research has shown that *Orthosiphon aristatus* and *Oryza sativa* L. var. *glutinosa* have antidiabetic activity. The combination of two medicinal plants can increase their biological activity. This study aimed to determine the antidiabetic activity of *O. aristatus* and *O. sativa* L. var. *glutinosa* on single and combined extracts. Phytochemical screening of the single extract was done qualitatively. The α-glucosidase inhibitory method was used as an antidiabetic activity. The results showed that every extract contained alkaloids, steroids/triterpenoids, flavonoids, tannins, quinones, and coumarins. A single extract of *O. sativa* L. var *glutinosa*, *O. aristatus*, and their combinations (1:1, 1:2, and 2:1) had an α-glucosidase enzyme inhibitory activity with an IC_{50} value of 67.82, 80.93, 73.81, 88.72, and 61.51 µg/ml, respectively. The combination shows that the ratio of 1:1 was nearly additive, 1:2 was slight to moderate antagonism, and 2:1 was moderate to slight synergism. The combination of 96% ethanol extract of *O. sativa* L. var. *glutinosa* and *O. aristatus* in a ratio of 2:1 was the most effective in increasing its inhibitory activity.

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INTRODUCTION

Diabetes mellitus (DM) is a condition in which an increase in blood sugar levels (hyperglycemia) that caused by a decrease in insulin secretion and activity[1]. It is because of the damage in the metabolic system, especially carbohydrates, fats, and proteins. Diabetes mellitus is one of five diseases with a high number of cases and death rates worldwide. The recent report estimates that diabetes mellitus attacked more than 463 million people worldwide and around 4.2 million of them died in 2019[2]. According to the Indonesian Health Ministry report, about 16.5 million Indonesians aged over 15 years were diagnosed with diabetes in 2018. In diabetic patients, the production of free radicals will be higher due to the auto-oxidation process of glucose[3].

Although diabetes mellitus is a chronic disease that does not cause immediate death, it can be fatal if the curing management is not proper. Management of diabetes mellitus requires drug therapy and non-drug therapy. Acarbose, a class of α-glucosidase enzyme inhibitors, is one of the drug therapies used in diabetic patients[4]. The α-glucosidase enzyme is an enzyme that plays a role in the breakdown of carbohydrates into glucose in the digestive tract to control glucose absorption[5]. Treatment of diabetes takes a lifetime at a relatively high cost. In

addition to treatment with synthetic drugs, natural drugs as antidiabetics are increasingly in demand. Although the effects of plant-derived compounds are not as effective as synthetic drugs, the risk of the side effects seems to be very rare[6]. Some natural medicines have been used for generations, but research ensures their efficacy and safety[7].

Research on black glutinous rice (*Oryza sativa* L. var. *glutinosa*) has been carried out previously in the in vivo study. The water extract of *O. sativa* L. var. *glutinosa* can reduce blood glucose levels, with the highest dose at 500 mg/kg BW[7]. In addition to *O. sativa* L. var. *glutinosa*, the Cat's whiskers tea or Java tea (*Orthosiphon aristatus*) is also used as traditional antidiabetic medicine. The 50% ethanol extract of *O. aristatus* can inhibit the α-glucosidase enzyme with an IC_{50} value of 4.63 mg/mL[8]. Meanwhile, Juliani *et al*.[9] reported that methanol *extract of O. aristatus* inhibited the α-glucosidase enzyme with an IC_{50} value of 465.83 µg/mL.

In addition to a single extract, several studies have also used a combination of two different types of plants for α-glucosidase enzyme inhibitory activity. To optimize the utilization of medicinal plant extracts as antidiabetic, combining them could be the best alternative for diabetic treatment. A previous study showed that the combination of *Eurycoma longifolia* and *Punica granatum* extracts could increase the inhibitory activity of the α-glucosidase enzyme compared to their respective single form[10]. Every extract of *O. sativa* L. var. *glutinosa* and *O. aristatus* has antidiabetic activity by inhibiting the α-glucosidase enzyme. Moreover, the combination of extracts can increase the inhibitory activity of the α-glucosidase enzyme. Therefore, this study aimed to determine the antidiabetic activity of *O. sativa* L. var. *glutinosa* and *O. aristatus* on single and combined extracts by inhibiting the α-glucosidase enzyme.

MATERIALS AND METHODS

Materials

ρ-nitrophenyl-α-D-glucopyranose (PNPG) (Sigma), α-glucosidase enzyme from *Saccharomyces cerevisiae* (Sigma), acarbose (Sigma), 96% ethanol (Brataco), n-hexane (Brataco), ethyl acetate (Brataco), methanol (Brataco), distilled water (Brataco), hydrochloric acid (Merck), ammonia (Merck), chloroform (Merck), potassium iodide (Merck), ether (Merck), anhydrous acetic acid (Merck), sulfuric acid (Merck), magnesium powder (Merck), amyl alcohol (Merck), sodium hydroxide (Merck), iron (III) chloride (Merck), dimethyl sulfoxide (DMSO) (Merck), potassium dihydrogen phosphate (Merck), dipotassium phosphate (Merck), sodium carbonate (Merck). The main instruments used in this study were analytical balance (Precisa 340A), incubator (Memmert), rotary vacuum evaporator (Janke & Kunkel RV 05-ST), UV-Vis Spectrophotometer (Hitachi U-3900H).

Methods

Plant extraction

Oryza sativa L. var. *glutinosa* (BKH) and *O. aristatus* (KK) were obtained from Sri Rahayu Grocery Store and Indonesian Spice and Medicinal Crops Research Institute (Balittro), Bogor, West Java, respectively. The samples were identified in the Herbarium Bogoriense, Research Center for Biology, Indonesian Institute of Sciences, Bogor, with report number 2339/IPH.1.01/If.07/X/2018. All samples were washed with tap water and then cut into small pieces, specifically for KK. The samples were dried under the sunlight. One hundred grams of BKH and KK were put into separate glass jars. They were extracted six times with 96% ethanol for 24 hours, filtered, and stored. All the filtrates obtained were collected and evaporated on a rotary evaporator. The combination of extracts used in this research were

1:1, 1:2, and 2:1 (w/w)[11]. All the extracts were weighed using analytical balance to determine the yield.

Phytochemical screening

Qualitative phytochemical screening was performed based on the standard procedures[12].

Antidiabetic activity assay

A total of 1 mg of an α-glucosidase enzyme from *S. cerevisiae* (9.8 units/mg) was dissolved in 1 mL of 0.01 M phosphate buffer (pH 7) as an enzyme stock solution (9.8 units/mL). Approximately 0.02 mL of the enzyme stock solution was dissolved to 5 mL in 0.01 M phosphate buffer (pH 7) to obtain a working solution (0.04 units/mL). The single and combined extract solutions in DMSO concentrations were 6.25, 12.5, 25, 50, and 100 µg/mL. Acarbose (in water) at the concentration series of 3, 6, 9, 12, and 15 µg/mL were made as a positive control. A total of 475 µL of 0.1 M phosphate buffer (pH 7), 250 µL of 0.2 M ρ-nitrophenyl-α-D-glucopyranoside (ρNPG), and 25 µL of each extract were put into a test tube. Then, they were incubated at 37°C for 5 minutes, followed by adding 250 µL of the enzyme solution and re-incubated at 37°C for 30 minutes. The enzyme reaction was stopped by added 1000 µL of 0.2 M sodium carbonate solution.

The inhibitory activity of α-glucosidase was calculated based on the absorption values at the wavelength of 400 nm using a UV-Vis spectrophotometer[13]. The IC_{50} value, whereas the extract concentration can inhibit 50% of the α-glucosidase enzyme activity, was calculated based on the linear regression equation[14].

Extract combination analysis

The inhibitory activity of the α-glucosidase enzyme from the combined extract was calculated using the combination index[15] between BKH and KK through the following **Formula 1**.

$$Combination\ index = \frac{D1}{(Dx)1} + \frac{D2}{(Dx)2} \dots [1]$$

Dx1 and Dx2 were the concentrations of one single extract needed to give effect (IC_{50} on the α-glucosidase enzyme activity), while D1 and D2 were the concentration of the two extracts (combination) to give the same effect. The results of Combination Index (CI) were interpreted as follows: <0.1: very strong synergism; 0.1-0.3: strong synergism; 0.3-0.7: synergism; 0.7-0.9: moderate to slight synergism; 0.9-1.1: nearly additive; 1,1-1.45: slight to moderate antagonism; 1.45-3.3: antagonism; and >3.3: strong to very strong antagonism[16].

RESULTS AND DISCUSSION

Plant extraction

The percentage of yields was obtained from the ratio of the extract and the number of herbal substances. In this study, the yield of KK extract was higher than BKH extract. The solvent used for extraction is more efficient in attracting the compound in the KK than BKH extract. A high yield value can indicate the number of bioactive compounds contained. Likewise, the higher the yield percentage, the more bioactive compounds contained in[17]. Therefore, we can assume that more bioactive components in KK compare to BKH extract. **Table I** showed that the 96% ethanol extract weight of BKH and KK were 7.29 and 21.56 g, and the yields were 7.27% and 21.52%, respectively.

Table I. Yield of 96% ethanol extract

Extracts	Weight of simplicia (g)	Weight of extracts (g)	Yields (%)
BKH	100.3	7.29	7.27
KK	100.2	21.56	21.52

Phytochemical screening

The phytochemical screening results of BKH and KK extracts were presented in **Table II**. It shows that the 96% ethanol extract of the KK did not contain triterpenoids and saponins. In contrast, the 96% ethanol extract of BKH did not contain steroid and saponin compounds.

The bioactive components in a medicinal plant extract are related to their biological activity. The 96% ethanol extract of KK and BKH contains various chemical compounds, roleplaying a vital role in inhibiting the activity of the α-glucosidase enzyme. Several studies reported that all compounds detected in these phytochemical screening tests have antidiabetic activity. The flavonoids were reported to have several types of inhibitory activity for the α-glucosidase enzyme, including in competitive, non-competitive, and mixed manner by receiving or donating protons to form hydrogen bonds with the active site the enzyme[18]. The presence of hydrogen bonds between flavonoids and enzymes will allow flavonoids to regulate glucose absorption to achieve stability of glucose levels through the disaccharide pathway, inhibitory effect on maltase enzyme activity, and decreases glycemia before glucose abundance[19].

Table II. Phytochemical screening of 96% ethanol extract of BKH and KK

Compounds	Results	
	BKH	KK
Alkaloids	A thick red precipitate was formed with Dragendorff's*	A thick red precipitate was formed with Dragendorff's*
Steroids	No blue or green color formed**	A green color was formed*
Triterpenoids	A red color was formed*	No red color formed**
Flavonoids	A red solution was formed in organic layer*	A red solution was formed in organic layer*
Saponins	A stable foam not formed after shaking**	A stable foam not formed after shaking**
Tannins	A greenish black solution was formed*	A greenish black solution was formed*
Quinons	A red solution was formed*	A red solution was formed*
Qoumarins	A fluorescence solution was formed under UV light*	A fluorescence solution was formed under UV light*

*: Positive result. **: Negative result.

Alkaloids have been reported can inhibit the α-glucosidase enzyme competitively[20] or non-competitively[21]. Inhibition of alkaloids against the α-glucosidase enzyme has several mechanisms, including the formation of hydrogen bonds, hydrophobic interactions, and cations[22]. Steroids also have the activity of inhibiting the activity of the α-glucosidase enzyme through the hydrophobic interaction pathway with enzyme attachment site as a target[23]. These compounds configure the hydrogen bonds with the enzyme's active site[24]. The terpenoid compounds were reported to have non-competitive α-glucosidase enzyme inhibitory activity via the formation of hydrogen and hydrophobic bonds with the enzyme's active site[25].

Tannin groups were reported as antidiabetic by non-competitive inhibition of the α-glucosidase enzyme[26]. Furthermore, tannin configures the hydrogen bonds with the enzyme's active site[27]. Coumarin has been reported as antidiabetic by inhibiting the α-glucosidase enzyme. This compound has non-competitive inhibition via hydrogen bonds with the enzyme's active site[28]. Emodin, one of the compounds in the quinone groups, was reported to have an inhibitory activity to the α-glucosidase enzyme through increasing glucose absorption[29].

Antidiabetic activity assay

The α-glucosidase enzyme inhibitory activity from the single extract of BKH and KK and their combination (1:1, 1:2, and 2:1) were presented in **Tables III** to **V**. In this study, KK's 96% ethanol extract had an inhibitory activity of the α-glucosidase enzyme with an IC_{50} value of 80.93 µg/mL. This value was better than in the research conducted by Juliani *et al.*[9], which reported that the IC_{50} value of *O. aristatus* butanol extract was 154.07 µg/mL. In 96% ethanol extract, BKH had α-glucosidase enzyme inhibitory activity with IC_{50} of 67.82 µg/mL. The activity in this study was slightly lower than the inhibitory activity of the α-glucosidase enzyme from methanol extract of *O. sativa* L. var. *glutinosa* RF6 that had an IC_{50} value of 54.93 µg/mL[30]. These gaps occur due to different places where they grow and the type of solvent that

affects the content of bioactive compounds in extracts of medicinal plants[31,32].

Table III. Analysis of the inhibitory activity of the α-glucosidase enzyme from the 96% ethanol extract of KK and BKH

Concentration (µg/mL)	Enzyme inhibition activity (%)	
	KK	BKH
6.25	14.84±1.12	12.00±1.00
12.5	25.59±0.99	26.33±1.15
25	32.69±0.99	27.67±1.53
50	44.30±0.37	38.00±1.73
100	53.98±4.30	68.00±2.65

Table IV. Analysis of the inhibitory activity of the α-glucosidase enzyme combination from the 96% ethanol extract of KK and BKH

Concentration (µg/mL)	Enzyme inhibition activity (%)		
	BKH-KK (1:1)	BKH-KK (1:2)	BKH-KK (2:1)
6.25	5.52±0.38	8.33±0.58	11.26±0.66
12.5	21.63±0.38	13.00±1.00	23.40±0.76
25	33.33±0.38	26.67±0.58	42.16±1.38
50	45.25±1.53	34.00±1.00	48.79±0.38
100	58.06±0.38	53.33±1.53	65.56±0.66

Table V. The IC_{50} value of the α-glucosidase enzyme inhibition extract of KK and BKH

Materials test	IC_{50} (µg/mL)
KK	80.93±7.58
BKH	67.82±3.06
BKH-KK (1:1)	73.81±1.02
BKH-KK (1:2)	88.72±2.63
BKH-KK (2:1)	61.51±1.15
Acarbose (positive control)	14.68±0.03

In carbohydrate metabolism, carbohydrates entering the digestive tract would be digested into simpler sugars and then absorbed by the small intestine. The α-glucosidase is an enzyme commonly used for *in vitro* antidiabetic activity assay. It is commonly found in the small intestine and converts disaccharides into monosaccharide carbohydrates[33]. Therefore, inhibition of the α-glucosidase enzyme activity will reduce the breakdown of disaccharides into glucose which eventually reduces the blood glucose levels[34]. In the *in vitro* study, the α-glucosidase enzyme hydrolyzes the substrate ρ-nitrophenyl-α-D-glucopyranoside to become yellow ρ-nitrophenyl and glucose[35].

Extract combination analysis

The effectiveness of the combination 96% ethanol extract BKH and KK on α-glucosidase enzyme inhibition, with the Combination Index (CI) value as the analysis parameter, were presented in **Table VI**. The use of a combination of extracts with a low ratio is a simple initial step to determine the effect of each extract on efficacy and toxicity. This is essential as initial data to determine which extract influences the efficacy and toxicity when the extract is combined so that the ratio of the combination of extracts to be used can be estimated[36,37].

A single extract of KK and BKH has a good antidiabetic activity via inhibition of the α-glucosidase enzyme. The antidiabetic activity of the plant extracts can be improved by combining them. The combination of *E. longifolia* and *P. granatum* extracts can increase the inhibitory activity of the α-glucosidase enzyme compared to their respective single extract[10]. In this study, the combination of the 96% ethanol extract of BKH and KK (2:1) had a lower IC_{50} value than the single one. This result indicated that the combination shows a synergism effect. It is in line with previous studies which reported that the plant extracts combination can increase the inhibition activity of the α-glucosidase enzyme[38]. The synergism effect is a positive interaction of two or more substances that show a higher mechanism than the sum of the single substance[39].

Table VI. The index value of the combination of 96% ethanol extract of KK and BKH on inhibition of the α-glucosidase enzyme

Ratio of BKH-KK	CI values	Categories
1:1	1.0003	nearly additives
1:2	1.1669	slight to moderate antagonism
2:1	0.8580	moderate to slight synergism

In addition to the synergism effect, the combination of extracts can also have additive and antagonism effects. In this study, the combination of 96% ethanol extract with a ratio of 1:1 produced a nearly additive effect. The combination of 96% ethanol extract of BKH and KK with

a combination of 1:2 has a slight to moderate antagonism effect. An additive effect occurs when the combination only has a biological enhancement effect from its single extracts. An antagonist effect occurs when the combination shows lower activity than every extract[38]. The additive and antagonism effects also occur in previous studies. Marianne *et al*.[11] reported that the combination of the ethanol extract of *Curcuma heyneana* rhizome and *Curanga fel-terrae* leaf with a 1:1 and 2:1 ratio had CI values at 1.09 and 1.21, respectively.

CONCLUSION

Both single and the combination of the 96% ethanol extract of *O. aristatus* and *O. sativa* L. var. *glutinosa* have an inhibitory activity of the α-glucosidase enzyme. The 96% ethanol extract of *O. sativa* L. var. *glutinosa* has better inhibitory activity than *O. aristatus*. The combination of 96% ethanol extract of *O. sativa* L. var. *glutinosa* and *O. aristatus* in a ratio of 2:1 is the most effective to increase the inhibitory activity.

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AUTHORS' CONTRIBUTION

Eris Septiana: supervision, conceptualization, antidiabetic activity, extract combination analysis, data entry and analysis. **Nurul Maulida Rizka**: antidiabetic activity, phytochemical screening, data analysis. **Yadi**: extraction. **Partomuan Simanjuntak**: supervision, conceptualization, phytochemical screening.

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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