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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 activity1. 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 20192. 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 glucose3. 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 a-glucosidase enzyme inhibitors, is one of the drug therapies used in diabetic patients4.

The a-glucosidase enzyme is an enzyme that plays a role in the breakdown of carbohydrates into glucose in the digestive tract to control glucose absorption5. 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 rare6. Some natural medicines have been used for generations, but research ensures their efficacy and safety7. 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 BW7. 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 a-glucosidase enzyme with an IC50 value of 4.63 mg/mL8. Meanwhile, Juliani et al.9 reported that methanol extract of O. aristatus inhibited the a-glucosidase enzyme with an IC50 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 a-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 a-glucosidase enzyme compared to their respective single form10. Every extract of O. sativa L. var. glutinosa and O. aristatus has antidiabetic activity by inhibiting the a-glucosidase enzyme. Moreover, the combination of extracts

can increase the inhibitory activity of the a-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 a-glucosidase enzyme. MATERIALS AND METHODS Materials ?-nitrophenyl-a-D-glucopyranose (PNPG) (Sigma), a-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 procedures12.

Antidiabetic activity assay A total of 1 mg of an a-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 ?-nitrophenyl-a-D-glucopyranoside (?NPG), 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 a-glucosidase was calculated based on the absorption values at the wavelength of 400 nm using a UV-Vis spectrophotometer13. The IC50 value, whereas the extract concentration can inhibit 50% of the a-glucosidase enzyme activity, was calculated based on the linear regression equation14.

Extract combination analysis The inhibitory activity of the a-glucosidase enzyme from the combined extract was calculated using the combination index15 between BKH and KK through the following Formula 1. ?????????????????????? ??????????= ??1 ???? 1 + ??2 ???? 2 … [1] Dx1 and Dx2 were the concentrations of one single extract needed to give effect (IC50 on the a-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 antagonism16. 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 in17. 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 a-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 a-glucosidase enzyme, including in competitive, non-competitive, and mixed manner by receiving or donating protons to form hydrogen bonds with the active site the enzyme18. 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 abundance19. 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 a-glucosidase enzyme competitively20 or non-competitively21.

Inhibition of alkaloids against the a-glucosidase enzyme has several mechanisms, including the formation of hydrogen bonds, hydrophobic interactions, and cations22. Steroids also have the activity of inhibiting the activity of the a-glucosidase enzyme through the hydrophobic interaction pathway with enzyme attachment site as a target23. These compounds configure the hydrogen bonds with the enzyme's active site24. The terpenoid compounds were reported to have non-competitive a-glucosidase enzyme inhibitory activity via the formation of hydrogen and hydrophobic bonds with the enzyme's active site25.

Tannin groups were reported as antidiabetic by non-competitive inhibition of the a-glucosidase enzyme26. Furthermore, tannin configures the hydrogen bonds with the enzyme's active site27. Coumarin has been reported as antidiabetic by inhibiting the a-glucosidase enzyme. This compound has non-competitive inhibition via hydrogen bonds with the enzyme's active site28. Emodin, one of the compounds in the quinone groups, was reported to have an inhibitory activity to the a-glucosidase enzyme through increasing glucose absorption29.

Antidiabetic activity assay The a-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 a-glucosidase enzyme with an IC50 value of 80.93 µg/mL. This value was better than in the research conducted by Juliani et al.9, which reported that the IC50 value of O. aristatus butanol extract was 154.07 µg/mL. In 96% ethanol extract, BKH had a-glucosidase enzyme inhibitory activity with IC50 of 67.82 µg/mL.

The activity in this study was slightly lower than the inhibitory activity of the a-glucosidase enzyme from methanol extract of O. sativa L. var. glutinosa RF6 that had an IC50 value of 54.93 µg/mL30. 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 plants31,32. Table III. Analysis of the inhibitory activity of the a-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 a-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 IC50 value of the a-glucosidase enzyme inhibition extract of KK and BKH Materials test _IC50 (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 a-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 carbohydrates33. Therefore, inhibition of the a-glucosidase enzyme activity will reduce the breakdown of disaccharides into glucose which eventually reduces the blood glucose levels34. In the in vitro study, the a-glucosidase enzyme hydrolyzes the substrate ?-nitrophenyl-a-D-glucopyranoside to become yellow ?-nitrophenyl and glucose35.

Extract combination analysis The effectiveness of the combination 96% ethanol extract

BKH and KK on a-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 estimated36,37.

A single extract of KK and BKH has a good antidiabetic activity via inhibition of the a-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 a-glucosidase enzyme compared to their respective single extract10. In this study, the combination of the 96% ethanol extract of BKH and KK (2:1) had a lower IC50 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 a-glucosidase enzyme38.

The synergism effect is a positive interaction of two or more substances that show a higher mechanism than the sum of the single substance39. Table VI. The index value of the combination of 96% ethanol extract of KK and BKH on inhibition of the a-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 extract38. 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 a-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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