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Production of α-Amylase Inhibitors of *Aspergillus* RD2 from Dewandaru (*Eugenia uniflora* L.) as Diabetes Drug

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Abstract. Diabetes mellitus is a metabolic disorder characterized by above-normal blood glucose levels. An α-amylase inhibitors have inhibitory activity against α-amylase enzymes and cause a decrease in glucose absorption. Dewandaru (*E. uniflora* L.) has the potential to produce compounds capable of controlling blood glucose levels. *Aspergillus* RD2 from twigs of the Dewandaru plant is expected to be able to produce antidiabetic compounds. This study aimed to determine the ability of the *Aspergillus* RD2 to produce α-amylase inhibitors by determining the production time, extracting α-amylase inhibitor compounds, testing the activity of the supernatant α-amylase inhibitor and ethyl acetate extract with various concentrations of 2%, 4%, 6%, 8%, 10%, and GC-MS analysis. The results showed that *Aspergillus* RD2 was able to produce α-amylase inhibitors with an inhibitory activity of 59.71%. The incubation time of the *Aspergillus* RD2 in producing the highest α-amylase inhibitor was on the 7th day. The highest α-amylase inhibitor activity was at a concentration of 6% extract with an inhibition percentage of 82.79%. 9-Octadecenoic acid, 9-Octadecenal, and n-Hexadecanoic acid were identified as having α-amylase inhibitor and antidiabetic activity. *Aspergillus* RD2 can produce α-amylase inhibitor compounds that have the potential to be used as antidiabetic drugs.

Keywords: α-amylase Inhibitor, *Aspergillus* RD2, Dewandaru plant, Antidiabetic, Endophytic Fungi

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INTRODUCTION

Diabetes is a chronic disease, expressed as hyperglycemia that occurs due to a decrease in the production of the hormone insulin in adipose tissue, peripheral tissue, and liver. Diabetes mellitus (DM) includes metabolic disorders caused by various factors such as insulin resistance, chronic hyperglycemia, or Langerhans cell abnormalities (Manaithiya *et al*, 2021). There are more people with type 2 diabetes than with type 1 diabetes. The number of people with type 2 diabetes continues to increase in every country, about 80% of which come from developing countries. The IDF estimates that as many as 439 million people will have type 2 diabetes by 2030 (IDF, 2011). Control of blood glucose levels is an important factor in the treatment of diabetes mellitus.

Diabetes drugs or oral antidiabetic drugs (OAD) are therapy to control blood glucose levels in patients with type 2 diabetes mellitus. The decrease in blood glucose levels by antidiabetic drugs is done by inhibiting the action of carbohydrate hydrolyzing enzymes, namely α-glucosidase and α-amylase enzymes which are naturally produced. in the human body (Michelle de Sales et al, 2012). Drug that is widely used commercially to reduce blood glucose levels is acarbose. Which works competitively by inhibiting the action of enzymes, able to delay the breakdown of carbohydrates thereby reducing the postprandial rise in plasma glucose. Inhibition by α-amylase and α-glucosidase inhibitors allows pancreatic cells more time to secrete insulin in response to increased plasma glucose levels. The use of this class of drugs is limited because it provides side effects including diarrhea, flatulence, and abdominal pain (Olokoba et al, 2012).

The use of α-amylase inhibitors as an antidiabetic drug is an alternative because of its ability to reduce postprandial blood glucose levels by interfering with the work of the amylase enzyme that converts starch into glucose (Kotowaro et al, 2006). Many medicinal plants are utilized as the main agent to reduce blood glucose levels. In Beutong District, they used leaves the part of a medical plant such as *Catharanthus roseus* (Tapak Dara Plant), fruit

Syzygium cumini by drinking as an antidiabetic agent (Putrimarlin et al, 2022), and Dewandaru leaves (Goncalves et al, 2010). Dewandaru fruit is widely used as an antidiabetic drug because it contains flavonoids and phenolics that play a role in lowering blood glucose levels through a hypoglycemic mechanism that inhibits glucose reabsorption from the kidneys and can increase the solubility of glucose levels, making it easier to excrete through urine (Santoso et al, 2018).

The limited land for planting use of an endophytic fungi to produce antidiabetic metabolites is an alternative that is suitable for current conditions. Endophytic fungi are microorganisms that live in plant tissues. Endophytic fungi are capable of producing secondary metabolites, similar or derivative compounds produced by the host plant (Malik et al, 2020). Endophytic fungi are capable of producing secondary bioactive compounds needed in the pharmaceutical, industrial and agricultural fields. Endophytic fungi are often used as a source of α-glucosidase and α-amylase inhibitor compounds to reduce blood glucose levels. *Aspergillus layori* obtained from *Acacia nilotica* leaves has inhibitory activity against α-glucosidase and α-amylase enzymes Singh and Kaur (2015).

The production of α-Amylase inhibitor compounds from the Endophytic fungi *Aspergillus* RD2 is the renewal of this study, most researchers use plant organs directly to obtain secondary metabolites. However, due to limited land due to the development of industrial estates, the use of Endophytic fungi as secondary metabolite production agents is a separate alternative. Therefore, this study aims to collect in-depth information regarding the production of α-amylase inhibitors from *Aspergillus* RD2 that can reduce blood glucose levels. The purpose of this study was to find out which chemical compounds can be utilized as oral antidiabetic drugs for patients with type 2 diabetes mellitus.

METHODS

Rejuvenation of *Aspergillus* RD2

Rejuvenation of *Aspergillus* RD2 aims to regenerate isolates when endophytes have been stored in Biotechnology Laboratory of the Biology Program, Faculty of of Mathematics and Natural Sciences, Diponegoro University. Rejuvenation was carried out by taking a few hyphae using a sharp loop, then placing them on PDA media in a petri dish. Mold isolates were grown for 5-7 days at room temperature.

Observation of the growth curve of *Aspergillus* RD2

Aspergillus RD2 that were 7 days old were added with 5 mL of distilled water, the spores were destroyed. As much as 1 mL of spore suspension was put in 20 mL PDB medium, then incubated for 11 days at room temperature, speed of 120 rpm. The mycelia of *Aspergillus* RD2 were dried at 50°C for 1x24 hours and the dry weight of the mycelia was calculated by calculating the difference between the weight of the empty filter paper and the filter paper containing mycelia.

Test the activity of the supernatant α-amylase inhibitor for initial screening of the of *Aspergillus* RD2

The supernatant from the culture was taken as much as 500 L and added with 500 L of α-amylase enzyme from Bacillus sp. (Sigma, A6380) with phosphate buffer pH 6.9. The mixed solution was incubated at 25°C for 10 minutes. After that, 1000 L of starch substrate was added to the mixed solution and incubated again at 25°C for 10 minutes. The mixed solution was added 2000 L of dinitrosalicylic acid (DNS) reagent and heated in boiling water for 15 minutes. The mixed solution has measured the absorption value of α-amylase inhibitor using a spectrophotometer at a wavelength of 540 nm. The solution used as a comparison is a 1% acarbose solution.

$$\% \text{ Inhibition: } \frac{Abs.\ control - Abs.sample}{Abs.\ control} \text{ x100\%}$$

Production curve of α-amylase inhibitor

Observation of the production curve of α-amylase inhibitor was carried out for 11 days. Sampling was carried out every 24 hours by taking 1 mL to observe the optimum production time of α-amylase inhibitors. Samples were centrifuged at 10,000 rpm for 15 minutes. The supernatant was used to test the activity of α-amylase inhibitor by spectrophotometric method at a wavelength of 540.

Production and extraction of α-amylase inhibitor from *Aspergillus* RD2

Aspergillus RD2 7 days old as much as 4 ml was put into 500 ml Potato Dextrose Broth (PDB) media and incubated at room temperature for 7 days with a shaker speed of 120 rpm. Ethyl acetate is used as a solvent for inhibitor compounds. The fermentation medium was

mixed with ethyl acetate at a ratio of 1:1. The ethyl acetate extract was homogenized using a magnetic stirrer for 30 minutes. The ethyl acetate fraction was evaporated until a brownish extract was obtained (Yunarto, 2015). The ethyl acetate extract was diluted using DMSO solvent with various concentrations of 2%, 4%, 6%, 8%, and 10% and tested for α-amylase inhibitor activity.

GC-MS analysis

GC-MS analysis was performed on the ethyl acetate extract to determine the content of compounds that have α-amylase inhibitor activity. The samples were sent to the UPT Laboratorium Terpadu UNDIP for detection of secondary metabolites using the GC-MS instrument.

RESULTS AND DISCUSSION

An α-amylase Inhibitor Activity Test Supernatant Sample

The sample used was the supernatant as a result of fermentation of the *Aspergillus* RD2 with an incubation time of 5 days which had entered the stationary phase, referring to Figure 1 of the observed growth curve data. The use of the supernatant as a sample was due to secondary metabolites produced by the *Aspergillus* RD2 during the stationary phase to the growth environment, namely PDB media. This is to the opinion of Pinu and Villas-Boas (2017), during the growth period, microorganisms secrete secondary metabolites into the growth medium. Separation of pellets and supernatants through centrifugation aims to obtain supernatants containing extracellular metabolites.

If the supernatant contains an α-amylase inhibitor, the inhibitory reaction is indicated by a bright yellow mixed solution and is indicated by a smaller absorbance value. Both of these indicated that only a small amount of reducing sugar (not orange color) was produced because the enzyme activity in catalyzing the substrate had been inhibited by α-amylase inhibitors. According to Barrett and Udani (2011), α-amylase inhibitors prevent the breakdown of starch into glucose by binding to the active site of the enzyme, so that the enzyme-substrate complex is not formed.

Based on Table 1, it is known that the supernatant has an inhibition percentage of 55.84%, while the percentage inhibition of acarbose as a comparison solution is only 47.77%. This shows that *Aspergillus* RD2 has the potential to produce α-amylase inhibitors because its activity is higher than acarbose as a comparison solution.

According to Meilia and Purwandarie's research (2017), acarbose was used as a comparison and used as a standard for enzyme inhibition. Acarbose is one of the commercial oral drugs with the principle of inhibiting the carbohydrate hydrolyzing enzyme which is

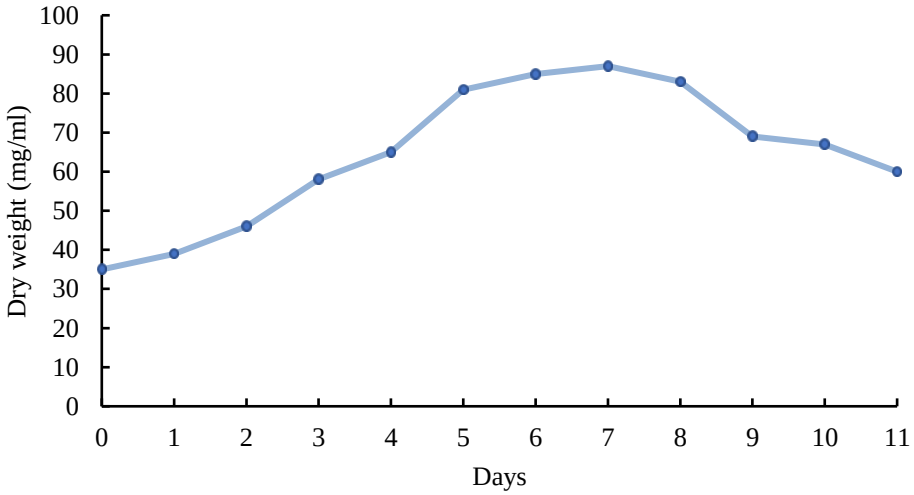

Figure 1. The growth curve of *Aspergillus* RD2

Table 1. The Activity of α-amylase Inhibitor Supernatant *Aspergillus* RD2

No.	Sample	Absorption (540 nm)		(%) Inhibition
		Control	Supernatant	
1	*Aspergillus* RD2	0.539	0.238	59.71
2	Acarbosa 1%	0.561	0.293	46.23

widely consumed by people with type 2 diabetes mellitus to reduce post-prandial blood glucose levels. This is following the opinion of Dinicolantonio (2015), acarbose plays an important role as an inhibitory agent of carbohydrate absorption during the breakdown of polymeric sugars. Acarbose drugs are used for the treatment or prevention of type 2 diabetes mellitus.

Production Curve of α-amylase Inhibitor

Based on the production curve of α-amylase inhibitor, incubation for 9 days (Figure 2) on day 0 has detected 16% of α-amylase inhibitor activity followed by a decrease on day 1 with a percentage inhibition of 11%. Entering the 2nd day of incubation, there was an increase in inhibitory activity until day 6 with the percentage values of inhibition being 17%, 20%, 31%, 47%, and 49%, respectively. On the 7th day, α-amylase inhibitory activity was recorded at 55%, followed by a decrease in the inhibition percentage on the eighth day by 50% until the 9th day by 37% so that the observation of incubation time was stopped.

Based on the explanation above, the 7th day is the incubation time that is capable of producing large amounts of α-amylase inhibitors, as indicated by the high activity of α-amylase inhibitors through the calculation of the percentage of inhibition. Referring to the growth curve, the *Aspergillus* RD2 entered the stationary growth phase on day 7, which means that the fungus can produce inhibitors as secondary metabolite products. This is supported by the research of Ferniah *et al*. (2018), the growth of the fungus *A. niger* entered the stationary phase on days 5 to 7, as seen from the fixed dry cell weight, the formation of horizontal lines on the growth curve and the production of secondary metabolites in the media. In the study of Rumidatul *et al*. (2021), the highest production of secondary metabolites from Endophytic fungi was found in the stationary period. The incubation time of 7 days was used as a reference for producing and extracting ethyl acetate extract from the Endophytic fungi *Aspergillus* RD2.

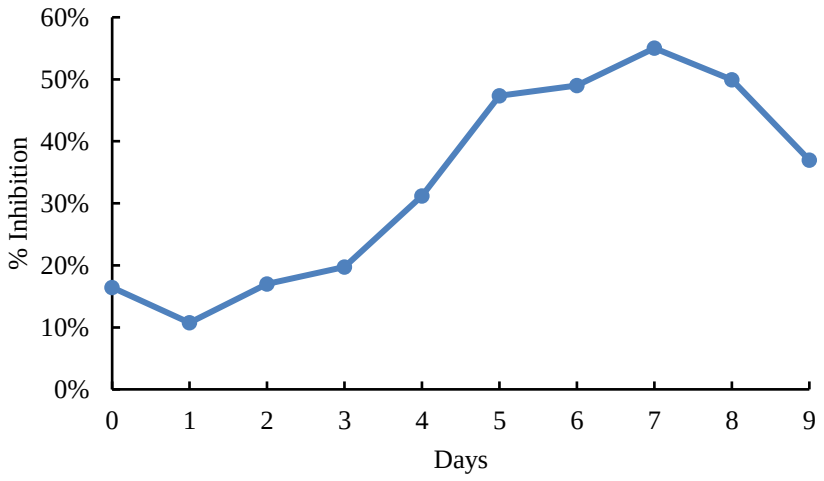

Figure 2. Production of α-Amylase Inhibitor from *Aspergillus* RD2 for 9 days

Table 2. Percentage inhibition of extract ethyl acetate

Concentration of Extract Ethyl Acetate (%)	Mean of Percentage Inhibition (%)
Control	41.43[a]
2	71.12[b]
4	76.10[c]
6	82.79[d]
8	83.02[d]
10	84.64[d]

The production of ethyl acetate extract from the *Aspergillus* RD2 aims to obtain extracts containing more bioactive compounds than the supernatant mixed with other solutions.

An α-amylase Extract Ethyl Acetate Inhibitor Activity Test

Fermentation of the *Aspergillus* RD2 using Potato dextrose broth (PDB) as a production medium which was incubated at room temperature, 120 rpm, for 7 days, because the seventh day was the best optimum time to produce large amounts of inhibitor compounds. *Aspergillus* RD2 can grow well because the PDB media contains potato (carbohydrate) as a carbon source. According to Octavia and Wantini (2017), PDA is a semi-synthetic medium containing potato, dextrose, and agar which are very important for the growth and reproduction of fungi, especially molds. Potato is a natural material that functions as a carbon source, dextrose as a source of sugar and energy, and agar as a PDA media compactor.

The inhibitor extract contained in the production medium was dissolved with ethyl acetate. Ethyl acetate is a semi-polar solvent that is often used as a solvent because it can attract many compounds, both polar and non-polar compounds. This is supported by the research of Kifle *et al*. (2021) that the ethyl acetate solvent used to extract the α-amylase inhibitor had an inhibition percentage of 61.57% (IC50, 18.73 g/ml), greater than chloroform solvent with a percentage inhibition value of 56.87% (IC50, 21.57 g /ml).

The concentration of ethyl acetate extract affected the inhibitory activity of the α-amylase enzyme, the highest increase in inhibitory activity was 84.63% at a concentration of 10% and the lowest inhibitory activity was 71.12% at a concentration of 2%, which means that each addition of extract concentration increased the activity of α-amylase inhibitors. Following the research of Pujiyanto *et al*. (2019), the increase in the dilution concentration of the extract was directly proportional to the activity of the α-amylase inhibitor, which means that the greater the concentration of the extract, the higher the percentage of inhibitor. The value of the scattered inhibitory activity was 95.06% at the extract concentration of 1000 g/mL, while the lowest α-amylase inhibitor activity was -31.32% at the extract concentration of 62.5 g/mL.

Table 3. The identified compounds by GC-MS and its bioactivity

Potential Compounds	Area	Synonyms (%)	Group	Bioactivity
4. 9-*Octadecenoic acid* (Z)	Δ9-cis-*Oleic acid*	21.04	Fatty acid	α-amylase and α-glucosidase Inhibitor (Chelladurai and Chinnachamy, 2018), Antihiperglycemic (Hashim *et al.* 2013), Antidiabetic (Palomer *et al.* 2017)
15. 9-*Octadecenal* (Z)	*Octadeceny l aldehyde*	12.40	Fatty acid	Antidiabetic (Iwara. 2022)
5. n- *Hexadecanoic acid*	*Palmitic acid*	9.10	Fat Aldehyde	α-amilase Inhibitor (Un *et al.* 2022); (Dai *et al.* 2019), stimulates glucose uptake (Jing *et al.* 2011), Antihiperglycemic (Rashid *et al.* 2020)

The concentration variation of 6% gave the most significant effect on the activity of α-amylase inhibitors with an average inhibition value of 82.79% compared to other concentrations of ethyl acetate extract (Table 2). the higher the concentration of the extract was related to the content of α-amylase inhibitor compounds in the ethyl acetate extract. The more α-amylase inhibitor compounds, the inhibition of the work of the α-amylase enzyme so that the process of breaking down polysaccharides into disaccharides is slowed down, resulting in suppressed glucose absorption.

Ethyl acetate extract has the potential to produce inhibitory compounds that can be used as antidiabetic agents because there is an inhibitory activity against the α-amylase enzyme. The compounds in the extract are identical to secondary metabolites in the Dewandaru plant as a host plant which is widely used as an antidiabetic. According to Fidelis *et al*. (2022), the Dewandaru plant has antihyperglycemic and antidiabetic activity and according to Borges *et al*. (2016) pitanga fruit has the inhibitory activity of α-amylase, α-glucosidase, and pancreatic lipase. In addition, in the research of Andrade et al. (2010) Dewandaru seeds contain palmitic acid which is a group of fatty acids that has the potential as an inhibitory agent for the α-amylase enzyme.

Identification of Antidiabetic Compounds Ethyl Acetate Extract

The results of the analysis in Table 3 show that there are 15 secondary metabolite compounds detected by the GC-MS instrument because there are 15 peaks that appear on the chromatogram with different retention times. The further to the right of the peak, the longer the retention time required for the compound to leave the column to the detector. This is influenced by the molecular weight and boiling point of each secondary metabolite compound.

Based on these 15 compounds, there are three compounds known to have α-amylase inhibitor activity, including 9-Octadecenoic acid (Z) which is the 2nd highest compound with an area value of 21.04%, 9-Octadecenal (Z) compound has an area of 21.04%. 12.40% as the 3rd highest compound and n-Hexadecanoic acid is ranked 5th with an area of 9.10%.

The fourth compound, 9-Octadecenoic acid or known as oleic acid, belongs to the group of unsaturated weak acids, having a double bond at C-9 in the form of the Z (cis) isomer. According to Hashim et al. (2013), oleic acid has an antihyperglycemic activity that can suppress the increase in blood glucose levels through the inhibition of enzymes in producing glucose. Further research by Chun-Han *et al*. (2013), oleic acid and linoleic acid showed enzyme inhibitory activity in controlling postprandial glucose levels. Then in the research of Chelladurai and Chinnachamy (2018), the stem extract of Salacia oblonga has a percentage of α-amylase inhibitor activity of 59.46±0.04% at a concentration of 100 mg/mL with an IC50 value of 73.56 mg/mL.

The 15th compound, 9-Octadecenal (Z) belongs to the aldehyde fatty acid group. According to FoodB (2022), 9-Octadecenal or octadecenyl aldehyde belongs to a class of organic compounds known as fatty aldehydes. The long chain of aldehydes consists of 12 carbon atoms. According to research by Iwara et al. (2022), Peritrophe bicalyculata extract and quercetin administered to a diabetic rat model showed antidiabetic activity. Based on GCMS analysis, the extract contained oleic acid (34.11%), hexadecanoic acid (16.68%), octadecanoic acid (9.97%), 9-octadecenal (8.43%).

The fifth compound, n-Hexadecanoic acid a synonym for palmitic acid, is a saturated fatty acid with 16 carbon backbone chains. In the research of Bhaskar *et al*. (2011), palmitic acid is the main compound contained in *Mucuna pruriens* extract with a concentration value of 48.21%. This extract was effective in lowering glucose levels from 242.4 ± 9.2 mg/dl to 91.0 ± 5.2 mg/dl in a diabetic rat model. In addition, palmitic acid in *Clausena indica* fruit extract was significantly able to inhibit the α-amylase enzyme with IC50 values of 0.07 and 1.52 mg/mL. This is supported by the research of Siregar *et al*. (2022) extract from the Endophytic fungi of the raru plant contains compounds of palmitic acid, oleic acid, isopthalic acid, and palmitin which can be used as α-amylase inhibitors.

To sum up, the use of Endophytic fungi, *Aspergillus* RD2, is an alternative method to produce secondary metabolites because it is able to produce compounds similar to as the Dewandaru plant which can be used as an oral antidiabetic drug. The chemical compound produced by *Aspergillus* RD2 has α-amylase inhibitory activity which is needed by T2DM patients as therapy in lowering blood glucose levels.

CONCLUSION

The *Aspergillus RD2* had α-amylase enzyme inhibitory activity which effective to lowering glucose concentration for patients with diabetes mellitus type 2. There were three chemical compounds 9-Octadecenoic acid (Z), 9-Octadecenal (Z) and n-Hexadecanoic acid identified as compounds in the ethyl acetate extract that have α-amylase inhibitor activity. We suggest for further research needs to be done, such as conduct molecular identification to determine the species of Endophytic fungi *Aspergillus* RD2. In addition, molecular docking simulations were carried out to determine the interaction of the above compounds with the α-amylase enzyme.

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