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Activity of α-Amylase Inhibition Against Active Compound from Raru Wood (*Cotylelobium melanoxylon*)

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Abstract Diabetes is a chronic disease identified by the elevation of blood glucose and disturbance of carbohydrate, fat, and protein metabolisms that occurs either when the pancreas does not produce enough insulin or when the body cannot effectively utilize the insulin it produces. In a previous study, an α-glucosidase inhibition test was conducted on the crude extract of raru bark (*C. melanoxylon*) which showed high inhibitory activity, which was 94.86%. In this study, further purification of the raru wood extract was carried out so that the activity test of an active compound was more specific. The main purpose of this study was to determine the α-amylase inhibitory activity of the active compound in raru wood. The extraction method used a maceration technique with methanol as a solvent, while the compound purification used a column chromatography and TLC preparative technique with several ratios of organic solvents. Sample analysis using UPLC-MS showed a retention time of 6.224 and 7.061 minutes, with m/z 680.2125 and 694.1919 respectively. FT-IR analysis showed absorption at 3388.93 cm^{-1} and 831.32 cm^{-1}, which indicated the presence of phenolic groups in the structure of the compound. Based on this analysis, the suggested compound is a stilbene namely vaticanol G/A/E or viniferol D and cotylelophenol. This study is the first to report the presence of cotylelophenol in the raru wood species (*C. melanoxylon*). The results of the α-amylase inhibition test showed that the purified fraction and acetone fraction was able to inhibit the activity of the α-amylase enzyme very well at a concentration of 2000 ppm, with the highest inhibition percentage of 49.79% and 53.97% respectively, when compared to acarbose (77.22%) with the same concentration.

Keywords raru wood, *Cotylelobium melanoxylon*, α-amylase, diabetes mellitus

1. Introduction

Changes in food consumption and human lifestyle have led to an increase in diseases, one of which is diabetes mellitus [1]. Diabetes mellitus (DM) is a chronic metabolic disorder characterized by increased blood glucose (hyperglycemia) due to impaired work and/or insulin secretion. DM is one of the most critical global health problems because it is the world's fifth leading cause of death [2]. Several pharmacological approaches have been

used to control blood sugar based on different modes of action, such as stimulation of insulin release, increased glucose transport activity, inhibition of gluconeogenesis, and reduced glucose absorption from the gut [3]. Unfortunately, many currently available antidiabetic agents cause serious side effects and are even fatal if taken in the long term [4]. So, research and development of hypoglycemic agents that are more effective and safer from natural sources continue to be challenging for researchers.

In this context, one particular group of natural products derived from wood, namely stilbenoid or stilbene, has become increasingly popular, due to their broad spectrum of biological activity and interesting molecular structures. [5]. Based on the biosynthesis of stilbene, these compounds are related to each other, because they have the same general framework, but differ in the types and positions of functional groups on the aromatic ring. Stilbene in Dipterocarpaceae undergoes regioselective biosynthesis through oxidative coordination of resveratrol to produce phenoxyl radicals [6]. Over the past few years, stilbene-based compounds have been studied extensively for their diverse biological roles in humans [7]. Resveratrol is the most popular and most studied stilbenoid to date. This is due to the amount of biological activity that resveratrol has; such as antioxidants [8], antitumoral [9], antiviral [10], and anti-inflammatory [11]. Some stilbenoid dimers, for example, isomers of the compound viniferin, exhibit better α-amylase inhibition than the reference drug acarbose. While at the same concentration, the racemic mixture of viniferin was a more effective inhibitor than the pure enantiomer alone [12].

People in North Sumatra use the leaves and bark of the raru plant (*C. melanoxylon*) as a traditional medicine to treat several diseases such as diabetes, malaria, and diarrhea [13]. This is supported by several studies which revealed that stilbene compounds (in the raru plant) increased insulin sensitivity in type 2 DM patients and diabetic rats [14]. Recent research tested the inhibition of α-glucosidase activity in the crude extract of raru bark from Central Tapanuli, North Sumatra [15]. The crude extract of the raru bark showed high inhibitory activity, which was 94.86%, compared to the positive control (glucobay), which inhibited 97.05%. Stilbene compounds were also found in *C. melanoxylon* plant wood obtained from Thailand, namely melanoxylin A, vaticanol G, (+)-ampelopsin F, (+)-isoampelopsin F, and (+)-ε-viniferin, then tested for antidiabetic activity [16]. The results showed that the MeOH extract from the plant *C. melanoxylon* was able to significantly inhibit the increase in glucose levels in the plasma of rats fed sucrose at a dose of 250 mg/kg, per os (p.o).

Previous research was carried out on the enzyme α-glucosidase or α-D-glucoside glucohydrolase, an enzyme that forms glucose in the small intestine. In principle, compounds that have the potential as antidiabetic agents can inhibit the α-glucosidase to break down the polysaccharides and complex carbohydrates into monosaccharides and delay glucose absorption in the blood. The α-amylase enzyme has almost the same role in breaking oligosaccharides and disaccharides into monosaccharides. α-amylase is an endo-hydrolase enzyme that acts on 1,4-glucosidic bonds in linear regions of suitable length in starch, glycogen, and in various oligosaccharides. The activity of this enzyme can release simple sugars which are then converted into glucose and easily absorbed into the human intestine. Inhibition of α-amylase enzymes in the body can reduce the bioavailability of oligosaccharides that can be absorbed by the body so that it affects the reduction of postprandial hyperglycemia [17][18]. Inhibition of the α-amylase can inhibit carbohydrates from being digested in the body by lowering the rate of glucose absorption and preventing an increase in postprandial plasma glucose levels [19]. This study aimed to conduct further purification of the raru plant extract and then the purified fraction carried out an inhibitory activity test against α-amylase.

Material and Methods

Plant Material and Chemicals

Raru woods (*C. melanoxylon*) were collected in Bona Lumban, North Sumatra, Indonesia. Silica gel 60 GF_{254} glass plate 20x20 cm for preparative thin layer chromatography purchased from Merck (Darmstadt, Germany). TLC Silica gel 60 RP-18 F_{254}s and GF_{254} for thin layer chromatography purchased from Merck (Darmstadt, Germany). The α-amylase enzyme was from Sigma Aldrich. Chloroform was purchased from Merck, and other reagents were an analytical grade and used with a further distillation treatment.

Extraction and Isolation

Raru wood powder (1.8 kg) was extracted by the maceration method (28ºC) for 3x24 hours in 6.5 L MeOH. The MeOH extract was concentrated with a rotary vacuum evaporator at 40ºC (p. 122 mbar). The crude extract was dissolved in methanol and put in a separating funnel for liquid-liquid extraction. To separate the non-polar fraction, hexane solvent was added to the funnel and shaken to mix the two phases, then allowed to stand. The MeOH extract (151.39 g, polar phase) was concentrated with a rotary vacuum evaporator, then dissolved in acetone. The result was a gel-like solid; only 108.9 g of the extract was soluble in acetone. The acetone-soluble fraction was then taken as much as 11.36 g for fractionation by vacuum liquid chromatography in a silica column [hexane-EtOAc 10% gradient], yielding five combined fractions {fr. B (49.9 mg), fr. C (162.1 mg), fr. D (1.27 g), fr. **E** (1.52 g), and fr. F (3.14 g). Then selected Fr. **E** (1.52 g) for further purification by silica gel column chromatography [$CHCl_3$-MeOH (4:1)→EtOAc-DCM (3:2), v/v] yielded five combined fractions {fr. E9 (1.3 mg), fr. **E10** (238.6 mg), fr. E14 (74.4 mg), fr. E15 (668.5 mg), and fr. E19 (19.3 mg). Fr. E10 (238.6 mg) was purified by preparative TLC [$CHCl_3$-MeOH (4:1), glass plate TLC: Silica gel 60 GF_{254} 20x20] to produce **E10a** fraction. Next, Fr. E10a (100.3 mg) was carried out by column chromatography with silica gel [$CHCl_3$-MeOH (4:1), [v/v] to produce a fraction of **E10a4**. Fr. E10a4 (51.1 mg) Fr. E10a4 (51.1 mg) was added to a silica gel chromatography column [acetone-DCM (7:13), v/v] to produce a fraction of **E10a4a** (45.7 mg). Fr. E10a4a was further analyzed by FT-IR and UPLC-MS to obtain spectroscopic data and possible chemical structure.

UPLC-MS Analysis Conditions

The liquid chromatography system used Waters™ ACQUITY Ultra Performance Liquid Chromatography (UPLC) H-Class System coupled with Waters™ Xevo G2-S QT mass spectroscopy (positive mode) to conduct the analysis. The analysis was performed with column: ACQUITY UPLC HSS C18 Column, 100Å, 1.8 µm, 3 mm X 100 mm, while the mobile phases were a mixture of solvent A (water + 5mM ammonium formic) and solvent B (acetonitrile + 0.05 formic acid). The injection volume was 5 µL (filter through 0,2 µm syringe), and the flow rate was 0,2ml/min. Compound identification by Masslynx Software, including ChemSpider library.

α -Amylase Inhibition Test

Samples that act as inhibitors are fraction E10a4a, acetone fraction (crude extract), and acarbose (commercial diabetes drug as standard) with various concentrations of 2000 ppm, 1500 ppm, 1000 ppm, and 500 ppm. α-Amylase inhibition test using a substrate of 1% starch (0.15 g starch in 15 mL H_2O) [20]. There were four conditions for this test, the A1 and A2 test tube were made for each sample concentration. the A1 test tube was added 250 µL of the α-amylase enzyme and 250 µL inhibitor. Then in the A2 test tube, 250 µL H_2O and 250 µL inhibitor were added without enzymes. In test tube A3, 250 µL of α-amylase and 250 µL H_2O were added without any inhibitor. 500 µL of H_2O was added to the A4 test tube without the addition of enzymes and inhibitors.

All test tubes were incubated for 10 minutes at 37ºC. After incubation, each test tube was added with 250 µL of starch solution and incubated again at 37ºC. After 30 minutes, the test tube was removed and 250 µL 1M HCl was added to stop the enzyme reaction. Then 250 µL of iodine indicator was added to each test tube to form a complex with starch (blue). Added 4 mL of H_2O, then the absorbance of the sample was measured with a UV-Vis spectrophotometer at a wavelength of 600 nm.

Results and Discussion

Isolation and Characterization

In the stationary phase, the acetone fraction (11.36 g) was purified by column chromatography with silica gel. In the final stage, preparative TLC was used to produce the E10a4a fraction (45.7 mg, yield 4.2%). To determine the

components in the E10a4a fraction, monitoring by thin layer chromatography was carried out with silica gel as the stationary phase and CH-Cl_3-MeOH (4:1, v/v) as the mobile phase. Based on the TLC chromatogram (Fig. 1), this fraction has an almost single stain, with an apparent stain at Rf= 0.30. It is estimated that the E10a4a fraction is close to pure.

a) b) c)

Figure 1: TLC chromatogram of E10a4a fraction with silica [CH-Cl_3-MeOH (4:1, v/v)] under a) UV light 254 nm; b) UV light 366 nm; c) cerium sulfate

E10a4a was analyzed using Fourier Transform Infrared Spectroscopy (FT-IR) to determine the functional groups of compounds contained in this fraction. The FT-IR spectrum (Fig.2) shows the characterization of functional groups with O-H stretching vibrations that extend at a wavenumber of 3388.9 cm^{-1} and C-O vibrations in the region of 1695.43 cm^{-1}. The spectrum of sample E10a4a also shows the absorption of aromatic C-H stretching vibrations (aromatic substitution) in the 831.32 cm^{-1} regions. Based on the FT-IR spectrum, the E10a4a fraction shows the characteristics of the phenolic group compounds.

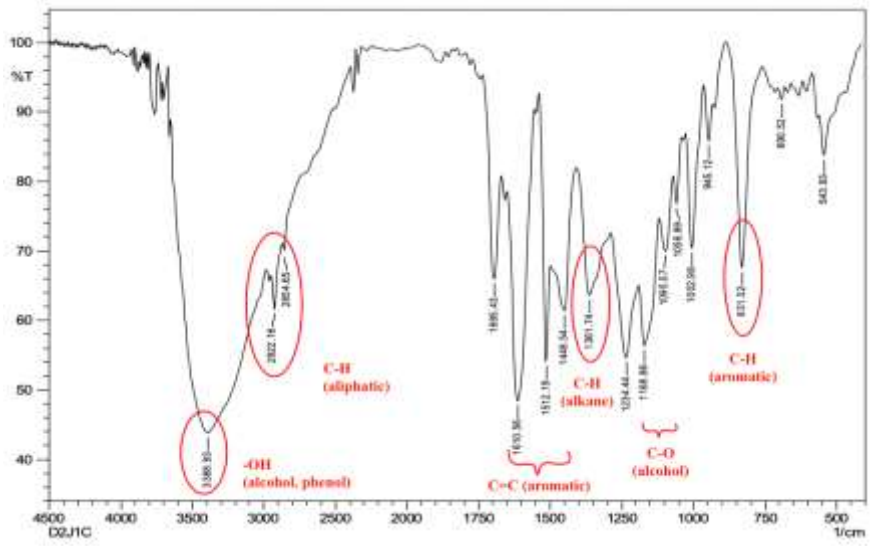


Figure 2: FT-IR Spectrum of E10a4a

E10a4a was then analyzed using UPLC-MS to determine the compounds contained in the fraction based on their molecular mass. Based on the UPLC chromatogram (Fig. 3), some compounds were identified from the E10a4a fraction of *C. canephora*. Based on the chromatogram showing several peaks that emerged from the retention time (RT) of 0-23 minutes, the E10a4a fraction is not a single compound. Furthermore, two peaks indicated the presence of stilbene compounds in this chromatogram, namely at RT= 6.224 minutes and RT= 7.061 minutes; the MS spectrum can be seen in Fig. 4.

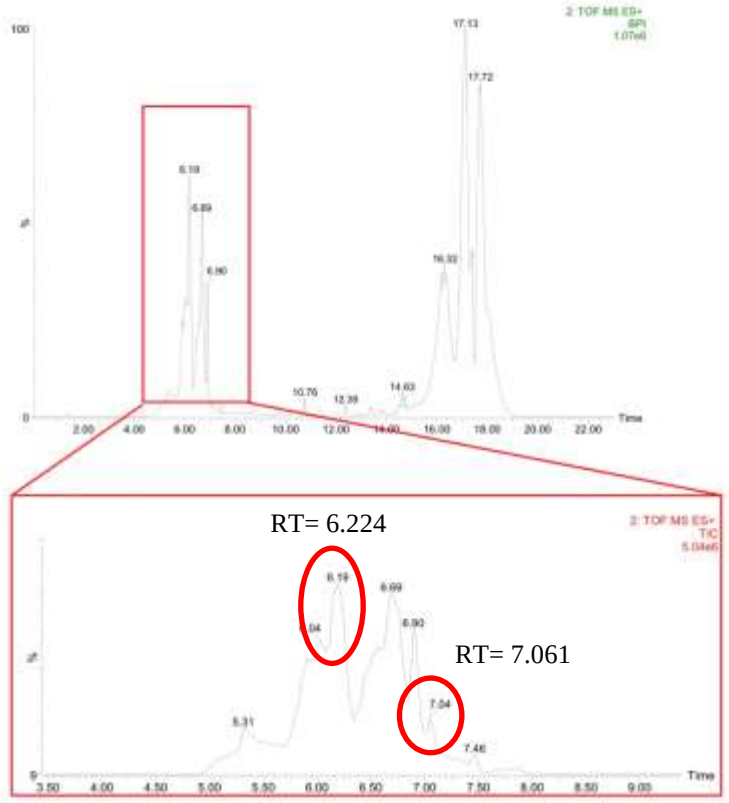


Figure 3: UPLC Chromatogram of E10a4a

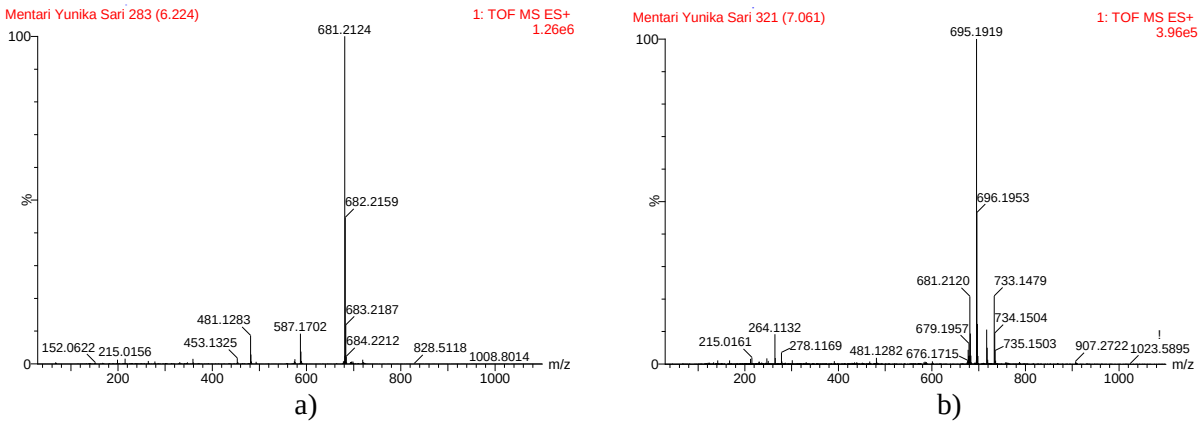
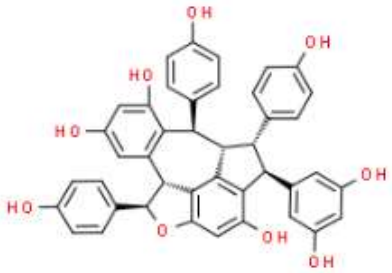


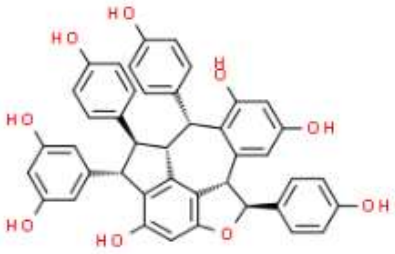
Figure 4: Mass Spectrum of E10a4a at a) RT= 6.224 and b) RT= 7.061

In spectrum **a**, a compound with m/z 680.2125 was detected, a compound with the molecular formula $C_{42}H_{32}O_9$. Based on the data obtained from chemspider.com, several possible compounds exist. However, the compound from

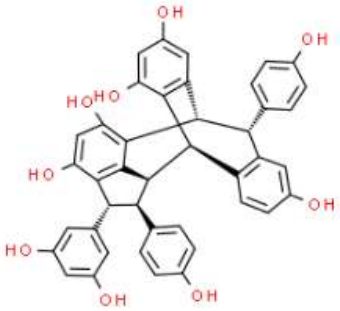
the stilbene group with that m/z value was vaticanol A/E/G or viniferol D, with a compound structure as shown in Fig. 5.



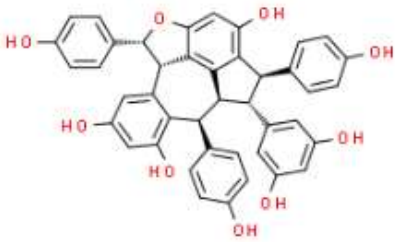
(-)-vaticanol A



vaticanol E



vaticanol G



viniferol D

Figure 5: Structures of Vaticanol A/E/G and Viniferol D (source: chemspider.com)

Previous studies have shown that the dimeric stilbene compound in the stem wood of *C. melanoxylon* is vaticanol G, the test showed that it increased blood glucose at a dose of 50 mg/kg, p.o. [16]. Thus, it is presumed that the E10a4a fraction contains vaticanol G compounds. However, it does not rule out that vaticanol A and E compounds are present in this fraction. Vaticanol G obtained from Hopea nigra has an antioxidant activity that is classified as active as a free radical scavenger by the Halliwel method, with an IC_{50} value of 683.96 g ml^{-1}. However, the anticancer test on HeLa cells did not show anticancer activity [21]. Meanwhile, until now, vaticanol A, E, and viniferol D have not been tested for their bioactivity.

Observations on spectrum **b** showed the presence of a molecular ion peak at m/z 694.1919, with the molecular formula $C_{42}H_{30}O_{10}$. Based on the data downloaded from chemspider.com, several possible compounds have the molecular formula $C_{42}H_{30}O_{10}$. However, the data shows that the stilbene group of compounds is cotylelophenol with the structural formula shown in Fig. 6.

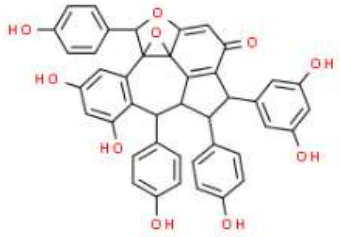


Figure 6: Structure of Cotylelofenol (source: chemspider.com)

Based on previous literature shows that previous studies have found two trimer stilbenes from the *C. lancelatum* (*Dipterocarpaceae*) plant, namely cotylelophenol A and B [22]. Cotylelophenol A is the first resveratrol trimer to undergo rearrangement in the hydroxyphenyl group. Currently, no research proves the presence of cotylelophenol found in raru plant species from North Sumatra. Therefore, this study is the first to report the presence of cotylelophenol in the raru wood species (*C. melanoxylon*).

α -Amylase Inhibition Test

Samples and enzymes in the test tubes were incubated for 10 minutes at 37ºC. After incubation, each tube was given additional substrate in the form of starch solution and incubated again at 37ºC. After 30 minutes, the test tube was removed, 1M HCl was added to stop the enzyme reaction, and starch was added [9]. Then the absorbance of the sample was measured with a UV-Vis spectrophotometer at a wavelength of 600 nm. The absorbance in test tubes A3 and A4 were not varied, 0.0223 and 1.7643 respectively. Then, % inhibition was calculated by the equation:

$$\% \text{ inhibition} = [\ 1 - \left(\frac{A2-A1}{A4-A3}\right)] \text{ x } 100 \quad (1)$$

Table 1: The results of the calculation of the sample inhibition of the α-amylase enzyme

Sample	Concentration (ppm)	% Inhibition
E10a4a	2000	**49.785 ± 0.004**
	1500	48.774 ± 0.580
	1000	48.829 ± 0.812
	500	48.441 ± 0.353
Acetone Fraction	2000	**53.967 ± 1.900**
	1500	48.266 ± 0.162
	1000	47.836 ± 0.942
	500	43.777 ± 0.690
Acarbose	2000	**77.216 ± 0.471**
	1500	74.653 ± 1.652
	1000	70.505 ± 3.694
	500	63.350 ± 0.694

*Values were shown as mean ± SD

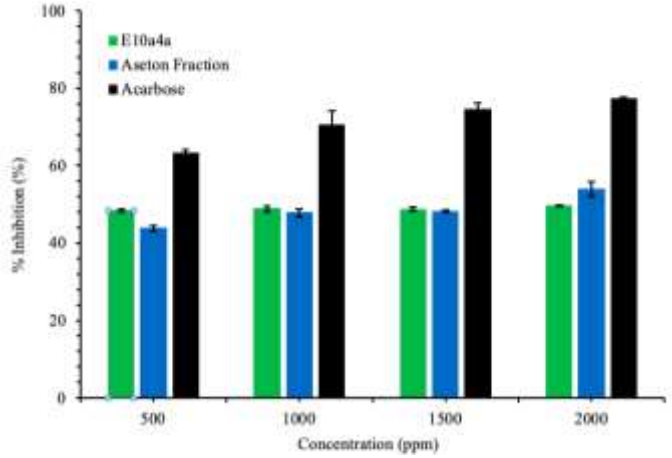


Figure 7: Graph of the α-amylase inhibition values with varying concentrations

Based on the data above, the highest % inhibition was at a sample concentration of 2000 ppm. The inhibition values for the E10a4a fraction and the acetone fraction at these concentrations were 49.788% and 53.967%, respectively. As observed from the graph above, the change in concentration in each sample showed a constant increase in % inhibition, although the increase was insignificant. Thus, the variation in sample concentration needs to be increased for further testing. Further purification is also needed to give an absolute structure of a compound from this fraction.

Conclusions

Based on UPLC-MS analysis, in the E10a4a fraction was found a stilbene compound; they were vaticanol A/E/G or viniferol D with formula $C_{42}H_{32}O_9$ (m/z 680.2125) and cotylelophenol with formula $C_{42}H_{30}O_{10}$ (m/z 694.1919). Currently, no research proves the possibility of cotylelophenol found in the raru plant, so this study is the first to prove the presence of cotylelophenol in the wood from *C. melanoxylon*. Based on the α-amylase inhibition test results, the highest inhibition of E10a4a and acetone fraction is at concentration 2000ppm (49.8% and 54.0%). This result is relatively high compared to the standard acarbose (77.21%). Stilbenoid oligomerization products have high structural complexity and three-dimensional architecture, thus leading to a higher possibility for selective binding to specific macromolecular targets (either catalytic or regulatory proteins, receptors, or transporters), so further research on the mechanism of action is needed.

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