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Original article

Inhibitory activity of endophytic fungi against alpha-amylase isolated from raru (*Cotylelobium melanoxylon)*

Pebrianiafrahul I Siregar[1], Sri Pujiyanto[2*], Arina T Lunggani[2]

[1]Master Program, Department of Biology, Faculty of Mathematics and Natural Sciences, University of Diponegoro, Semarang, Indonesia
[2]Department of Biology, Faculty of Mathematics and Natural Sciences, University of Diponegoro, Semarang, Indonesia

Abstract

Diabetes mellitus is a metabolic disorder characterized by elevated blood glucose levels. Alpha-amylase inhibitors are chemicals that prevent amylase from degrading carbohydrates into monosaccharides. For instance, the Raru plant (*Cotylelobium melanoxylon*), which was widely used by the local people, particularly in Sumatra, was used to lower blood glucose levels. The goal of this study was to isolate an endophytic fungus from the Raru plant and test its alpha-amylase activity by optimizing treatment time, substrate concentration, and alpha-amylase inhibitor compound extraction. The activity of alpha-amylase was measured using spectrophotometry, and starch was used as the substrate. Four isolates of the endophytic fungus were isolated from Raru bark, including TR1, TR2, TR3, and TR4. Of these, the isolate TR3 had the highest alpha-amylase activity, reaching 88.71%. Alpha-amylase was optimally inhibited for 7 days with a percentage of 90.13%. At a substrate concentration of 2%, the inhibition of alpha-amylase was observed at 96.34%. While the highest extraction of alpha-amylase inhibitory compound was observed at 0.13 ppm with a percentage of 74.71% inhibition. Based on GC-MS (gas chromatography mass spectrometry) analysis, compound 24, identified as1,3-benzenedicarboxylic acid, bis (2-ethylhexyl) ester, isophthalic acid group, was produced by endophytic fungi, exerting alpha-amylase inhibitory activity. This research would provide a new source of chemical agents to combat diabetes.

Keywords: alpha-amylase, inhibitory activity, endophytic fungi, raru plant, diabetes mellitus.

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Introduction

Diabetes has been known as an age-old disease since 3000 years ago and is popular as a metabolic disorder characterized by blood sugar concentrations exceeding normal limits. This disease is not only responsible for causing premature death worldwide, but is also one of the leading causes of diseases, including blindness, stroke, heart disease, kidney disease, impotence, and psychological problems, as well as complications (Ministry of Health of the Republic of Indonesia, 2020). The International Diabetes Federation (IDF) reported that Indonesia is ranked seventh out of ten countries in Southeast Asia with 10.7 million patients suffering from diabetes. The prevalence of diabetes cases in Indonesia is also estimated to be high (Ministry of Health of the Republic of Indonesia, 2020). The use of plants and spices to treat diabetes has existed since the past, such as aloe vera (*Aloe vera* L.), Japanese red pine (*Pinus densiflora*) (Sharma et al., 2021) and the Raru plant (*Cotylelobium melanoxylon*) (Dompeipen & Simanjuntak 2015). Thus, exploring the potential of chemicals derived from plants could be potential strategy for treating this disease.

The utilization of raru plants by the local community can traditionally treat diabetes, such as the Karo people in North Sumatra, Indonesia. The plant contains several beneficial compounds, including alkaloids, flavonoids, phenolic and terpenoid compounds, that can reduce blood sugar levels (Situmorang et al., 2015). Previous study reported that inhibitory activity of glucubay was observed in bark extract of raru, reaching a 97% inhibitory effect (Pasaribu, 2011), while methanol extract from raru bark inhibits plasma glucose expansion from sucrose (Matsuda et al., 2009). As the demand for medicinal raw materials grows in both quantity and variety, the potential of natural resources, particularly endophytic fungi of raru plants, which are endemic plants, should be explored and developed.

Endophytic fungi yield valuable compounds, having the identical properties as the host plant's metabolites. Endophytic fungi such as *Chaetomella raphigera* isolated from the medicinal plant *Terminalia arjuna* produce Taxol (anti-cancer) (Chhipa & Deshmukh 2019). While endophytic fungi, *Diaporthe phaseolorum* from *Pinus densiflora* (Saravanakumar et al., 2021) and *Penicillium canescens* from *Juniperus polycarpos* (Malik et al., 2020), exert antidiabetic activity. Endophytic fungi derived from plant tissues are genetically exchanged, potentially producing the same metabolite compounds without harming the host plant (e.g., alpha-amylase inhibitors). Alph- amylase inhibitors are compounds inhibiting the activity of the alpha-amylase enzyme in degrading carbohydrates into simple sugars (e.g., glucose) (Khirzin, 2020).

Previous study found that extracts from endophytic fungi isolated from jambolan plants (*Syzygium cumini* L.) exerts inhibitory effect against alpha-amylase enzyme

*Corresponding Author:
Name: Sri Pujiyanto
Department of Biology, Faculty of Mathematics and Natural Sciences, University of Diponegoro, Semarang, Indonesia
Phone: 081325495389
E-mail: spbio92@gmai.com

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(Khan et al., 2019). Briefly, complex carbohydrates will be digested by digestive enzymes in the small intestine, namely the alpha-amylase enzyme. This enzyme catalyzes the release of alpha glucose polysaccharide starch into short-chain amylose, which is then absorbed in the intestinal lumen and will enter the blood circulation, causing hyperglycemia. Thus, inhibiting alpha-amylase could be delayed and prolong the digestion time of carbohydrates, thus decomposing alpha glucose. In this scenario, exploring chemicals exerting inhibitory effect against alpha-amylase enzyme could be potential as antidiabetics agent. This study was aimed to isolate the endophytic fungi derived from the raru plant and characterize their inhibitory activity against alpha-amylase. This study will provide a new source of chemical agents to fight diabetes disease.

Methods

Isolation of endophytic bacteria from raru plant

The bark of the raru plant was collected from Bonalumban Natural Forest, Tukka District, Central Tapanuli Regency, North Sumatra, Indonesia. The bark of the raru plant was taken based on several parameters, including a length of 30 cm, a width of 5 cm, a diameter ranging from 30-50 cm at an altitude of 100 m above sea level (Pasaribu, 2011). The bark of the raru plant was sliced crosswise into 1x1 cm and placed in a Petri dish containing *Potato dextrose agar* (PDA) media, and incubated at room temperature for 5-7 days. The isolated endophytic fungi from the sample showing the fungal morphology were transferred to new PDA containing chloramphenicol (PDA) media (Prahesti et al., 2018).

Purification of endophytic bacteria

Each isolate of endophytic fungi was transferred into PDA media. This purification step aims to separate endophytic colonies with unique morphologies into separate isolates. Morphological observations were conducted after 5-7 days of incubation. If macroscopically different colony growth was observed, the isolate would be re-transferred until pure isolates were obtained. Endophytic fungi are incubated for 3-5 days according to their growth. The colony stocks were grown in the test tubes containing PDA media and incubated at room temperature for 5-7 days until sporulation. Then, the colony stock was stored at 2-4°C until further analysis (Ratnawati & Kasman 2021). Four endophytic fungi were finally isolated and purified from the raru plant, namely TR1, TR2, TR3 and TR4.

Production of alpha-amylase inhibitors

Each endophytic fungus, isolate TR1, TR2, TR3 and TR4, was taken and tranferred into 5 mL sterile distilled water and centrifuged for 10 min at room temperature. About 1 mL culture was transferred into a 100 mL Erlenmeyer containing 30 ml *Potato dextrose broth* (PDB), and shaken at 120 rpm for 5 days. Next, 1 ml culture was collected and centrifuged at 4000 rpm for 20 min. The supernatant was collected for testing the inhibitory activity against alpha-amylase and the absorbance value was measured using spectrophotometer. The endophytic fungi exerting the highest activity against alpha-amylase were selected for further analysis (Pujiyanto & Ferniah, 2010; Prahesti et al., 2018).

Inhibitory test of endophytic fungi against alpha-amylase

The collected supernatant was used to determine the inhibitory activity against alpha-amylase. Briefly, 500 µL of supernatant was mixed with 500 µL of alpha-amylase enzyme (0.5 U/mL), which was dissolved in phosphate buffer pH 7.0. The mixture was then incubated at 25°C for 10 min. The mixture was then added with 1000 µL of amylum substrate (500 mg amylum and 100 mL distilled water), incubated at 25°C for 10 min, and then added with 2000 µL of DNS reagent (Dinitrosaliclic Acid). The mixture was heated in boiling water for 15 min to stop the reaction, then the absorbance value was measured using an UV spectrophotometer vis at 540 nm. Akarbosa solution was used as a comparison solution (Pujiyanto et al., 2016). The test was carried out 2 times (Pujiyanto et al., 2016).

Time-based optimization assay

The selected endophytic fungal isolates were grown on PDA medium for 5 days at room temperature. The isolate was transferred into 5 mL of sterile distilled water and homogenized. 1 mL suspension was added to 150 mL PDB media, incubated at room temperature, and shaken at 120 rpm. Every 24 h for 11 days, 1 mL of culture is collected, centrifuged at 4000 rpm for 15 min, and measured for its inhibitor activity in the supernatant (Prahesti et al., 2018).

Substrate-based optimization assay

The selected endophytic fungal isolates were grown on PDA media for 5 days at room temperature. The isolate was transferred into 5 mL of sterile distilled water and homogenized. About 1 mL suspension was added to 150 mL PDB media, incubated at room temperature, and shaken at 120 rpm for 7 days. The cultures were centrifuged at 4000 rpm for 15 min and the inhibitory activity of the supernatant against alpha-amylase was measured at different substrate concentrations of 0.5%, 1%, 1.5%, 2.0%, and 2.5% (Pujiyanto et al., 2019).

Extraction of alpha-amylase inhibitor

Ethyl acetate solvent was used to extract alpha-amylase inhibitor compounds. Briefly, the supernatant was mixed with ethyl acetate in a ratio of 1:1. The extraction was then homogenized using a magnetic stirrer for 2 h until a water fraction and a solvent fraction were formed. The solvent fraction was carried out using a rotary evaporator until a concentrated fraction was obtained, then the weight was calculated until further analysis (Pujiyanto et al., 2016). Alpha-amylase inhibitors were tested with different concentrations, including 0.13 ppm, 0.065 ppm, 0.0325 ppm, and 0.01625 ppm. Data analysis was performed by calculating the percentage of alpha-amylase inhibition. Data obtained from absorbance measurements was calculated as % inhibition using the following formula:

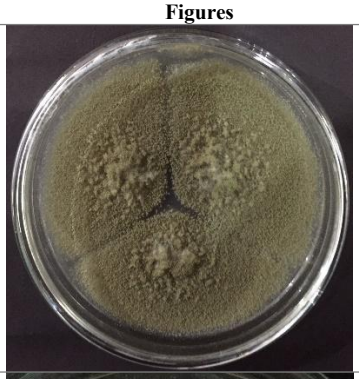
$$\% \text{ inhibition: } \frac{\text{abs 540 (control)} - \text{abs 540 (sample)}}{\text{abs 540 (control)}} \times 100\%$$

Results

Endophytic bacteria isolated from raru plant

Four isolates of endophytic fungi, namely isolates TR1, TR2, TR3 and TR4, were isolated and purified from the stem bark of raru plants (Table 1). Based on the analysis morphological of the Food Technology and Agricultural Products test laboratory, Faculty of Gadjah Mada, the four endophytic bacteria isolates are identified as *Aspergillus sp* (TR1 and TR4) and *Penicillium sp.* (TR2 and TR4) (Table 2)

Table 1. Four endophytic bacteria isolated from raru plant

No.	Isolates	Macroscopic Characteristics	Figures
1.	TR1	White colonies, white mycelial hyphae and green spores.	
2.	TR2	White edge color, ash colonies and ash spores.	
3.	TR3	White colonies, orange mycelial hyphae and white spores	
4.	TR4	White colony edges, firm colonies, ash-colored hyphae and mycelium	

Discussion

An alpha-amylase inhibition test is used to determine which endophytic fungal isolate has the highest inhibitory activity against alpha-amylase. According to the results of the test, isolate TR3 has the highest inhibitory rate of 88.71% with descriptive statistical data X ± sd 83.98 ± 5.95 (Fig. 1). The bioactivity to inhibit alpha-amylase enzyme activity was attributed to secondary metabolites produced by isolate TR3. The reaction between reducing sugar and DNS reagent (3,5-Dinitrosalicylic acid) produces a lower absorbance with a bright yellow color, and this value is decreasing due to the alpha-amylase enzyme's inhibition of amylum hydrolysis to produce products. In this condition, the reduced product produced by the substrate and enzyme is indicated by a decrease in color intensity; thus, the lower the color intensity, the less product is formed (Prahesti et al., 2018).

Table 2. Identification isolate (Genus)

No.	Code/sample	Identified isolate Genus
1.	TR1	*Aspergillus sp.*
2.	TR2	*Penicillium sp.*
3.	TR3	*Penicillium sp.*
4.	TR4	*Aspergillus sp.*

The DNS reagent can be used to measure reducing sugar concentration and determine small enzyme activity. The results of four endophytic fungal isolates tested revealed that TR3 had the best alpha-amylase inhibition activity. This activity is similar because it is caused by the same endophytic fungal isolate, whereas in the previous study, different isolates caused different bioactivities in roots, stems, leaves, and petioles (Ginting et al., 2020). Isolate TR3 is then selected to optimize alpha-amylase inhibitors production, such as time-based optimization, substrate-based optimization, and alpha-amylase inhibitor extraction.

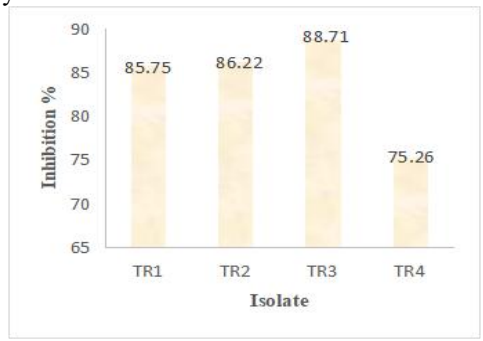


Figure 1. Inhibition activity of endophytic fungi against alpha-amylase isolated from bark of raru plants

Time-based optimization of alpha-amylase inhibitors

To determine the optimal time for production of alpha-amylase inhibitors, the production time for alpha-amylase inhibitors was optimized. The optimum time curve of alpha-amylase inhibitor production activity reveals TR3 endophytic fungal isolates (Fig. 2)**.** The optimal time was obtained on the seventh day with a percentage of 90.13%. The previous study reported that the optimum time of alpha-amylase inhibitor production activity of DT isolate was observed on the eighth day with a percentage of 71.98% (Prahesti et al., 2018). On day seven, the fermentation of endophytic fungal isolates was halted due to a decrease. On day seven, isolate TR3 has entered the stationary phase. On the 6th day there was a decline due to a technical tool error. Because the number of cells that grow equals the number of cells that die, the stationary phase has a fixed cell population. Many secondary metabolite compounds can be harvested during this phase (Indrawati et al., 2018).

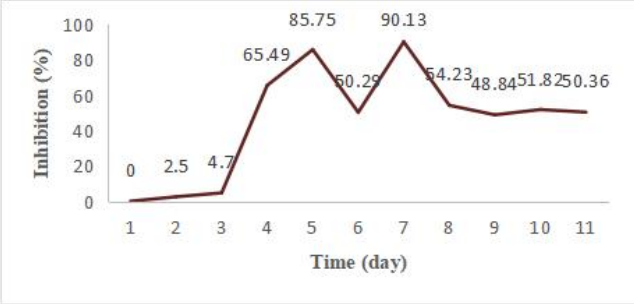


Figure 2. Inhibition activity of isolate TR3 against alpha-amylase during 11 days.

Effect of substrate concentration on alpha-amylase inhibitor activity

Substrate is the primary source of nutrients for microorganisms such as fungi. In this study, amylum substrate was used to determine the ability of various concentrations to produce alpha-amylase inhibitors with the highest production. According to the inhibitory test analysis (Fig. 3). the 2% concentration has the best inhibitory activity against alpha-amylase enzyme, with a percentage of 96.34%. According to previous research, 47.77% of amylum inhibitor compounds are produced (Pujiyanto & Ferniah 2010). Based on statistical analysis, substrate concentration treatment affects alpha-amylase inhibition with a decision of 0.007 > 0.005, implying that the data variants obtained are homogeneous. This process demonstrates the existence of glycoside bonds that play a role in the biosynthesis of alpha-amylase inhibitors, thereby accelerating the alpha-amylase inhibitor biosynthesis reaction. The results of the inhibitory test indicate that the fungus can naturally produce the alpha-amylase enzyme that converts amylum to glucose (Indrawati et al., 2018).

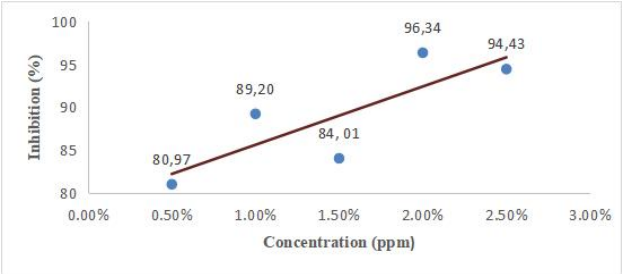


Figure 3. The test results for the substrate concentration of alpha amylase inhibitor isolate TR3

Extraction of alpha-amylase inhibitor compounds

To extract inhibitor compounds from the production media, extraction of compounds from isolate TR3 can be achieved by applying a suitable solvent. According to a previous study, from preliminary experiments of various solvents, including chloroform, ethyl acetate, and methanol, ethyl acetate gave the best results as a solvent (Pujiyanto et al. 2016). Thus, ethyl acetate solvent was used in this study. Accordingly, TR3 isolate extract was tested to observe the inhibitory activity against the alpha-amylase enzyme.

At a concentration of 0.13 ppm, the highest rate of inhibition was observed (60.89% inhibition), followed by 0.065 ppm (44.44% inhibition), 0.0325 ppm (31.40% inhibition), and 0.01625 ppm (11.34% inhibition) with descriptive statistical data X ± sd 51.76 ± 22.24 (Fig. 4). In this condition, the higher the concentration of the extract, the higher the inhibition activity. The previous study showed that the JP-3 culture inhibitor activity of ethyl acetate extract gave 78.07% inhibition (Pujiyanto et al., 2016). The lower the concentration of the extract, the lower the inhibition of enzyme activity.

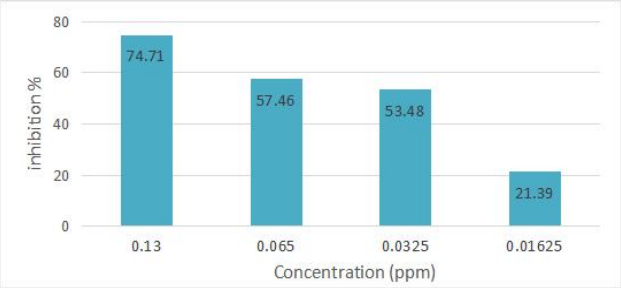


Figure 4. Bioactivity of metabolite extract TR3 isolate against alpha-amylase

Identification of the compounds by GC-MS analysis

The identification of compounds contained in the isolate can be expressed in the form of a chromatogram. A chromatogram is a graph between the retention time and peak height of the compound. Peak height indicates the number of compounds contained in the extracted isolate (Pravst, 2014). The results showed the presence of several compound peaks, indicating that endophytic fungal isolates have secondary metabolite chemical compounds (Table 3).

Table 3. Identification of eluean ethyl acetate GC-MS which has inhibitory activity

Peak	Retention	Peak area	Area %	Compound name
1	3.079	22833896	3.60	Acetic acid, butyl ester (CAS)
2	3.710	3241840	0.51	Ethylbenzene
3	3.821	3509385	0.55	Benzene, 1,2-dimethyl-(CAS)
4	4.412	49345991	7.79	Ethanol, 2-butoxy
5	7.351	2149070	0.34	2-Butoxyethyl acetate
6	9.638	16458867	2.60	Ethanol, 2-(2-butoxyethoxy)- (CAS)
7	16.455	368025	0.58	Ethyl trans-3-(2-oxo-5-methylcyclohexyl) propionate
8	16.605	1123955	0.18	4-Undecene, 4-Methyl-, (Z)-
9	16.780	5596958	0.88	10-Undecenyl Chloride
10	20.084	2848529	0.45	Propanoic acid, 2-methyl-, 1-(1,1-dimethylethyl)-2-methyl-1,3-propanediyl
11	21.022	1275797	0.20	1,3-Butanedione, 1-(1,3,5-Trimethyl-1h-Pyrazol-4-Yl)-
12	26.522	8318579	1.31	1-Tetradecanol (CAS)
13	28.625	29016558	4.58	n-Hexadecanoic acid
14	29.096	1560219	0.25	Ethyl 2-Cyano-3-(2-Furyl)-3-(2-Methoxyphenyl) Propanoate
15	30.597	2390595	0.38	1-Hexadecanol (CAS)
16	32.048	45972042	7.25	Oleic Acid
17	32.370	6350969	1.00	Octadecanoic acid
18	32.659	8986512	1.42	Hexadecanamide (CAS)
19	33.712	974785	0.15	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester (CAS)
20	33.910	3386879	0.53	9-Octadecenal, (Z)- (CAS)
21	34.031	3878295	0.61	9-Octadecenoic acid (Z)-, 9-octadecenyl ester, (Z)- (CAS)
22	34.160	3791599	0.60	9,12,15-Octadecatrienoic acid, 2-(acetyloxy)-1-(acetyloxy) methyl ethyl
23	34.508	9581376	1.51	Hexadecanoic acid, 2-hydroxy-1,3-propanediyl ester (CAS)
24	35.948	305848940	48.26	1,3-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester
25	36.215	4326626	0.68	Tetradecanamide
26	36.538	2040376	0.32	1,3-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester
27	36.765	1632530	0.26	9-Tetradecenal, (Z)-
28	37.543	14537252	2.29	9-Octadecenal, (Z)- (CAS)
29	37.921	8159335	1.29	Hexadecanoic acid, 2-hydroxy-1,3-propanediyl ester (CAS)
30	38.298	60942692	9.62	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester (CAS)

As shown in Table 4, several active substances have been identified by producing a peak. However, only the four highest substances, including 13- n-hexadecanoic acid, 16-oleic acid, 24-1,3-benzenedicarboxylic acid, bis (2-ethylhexyl) ester, and 30 hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester (CAS), were observed based on their inhibitor activity. Compound 13, n-hexadecanoic acid, is a class of palmitic acid, an unsaturated fatty acid that can prevent beta cell apoptosis (Yang et al., 2011). This group's function reduces weight gain, improves the development of hyperglycemia and hypertriglyceridemia, and improves insulin sensitivity

(Orhan et al., 2015), indicating that it has anti-diabetic potential.

Compound 16, oleic acid, is a weak unsaturated acid that is more effective than palmitic acid in the prevention and postponement of diabetes (Palomer et al., 2017). Furthermore, *Capparis decidua*, one of the traditional medicines used for treatment in Pakistan, contains a variety of compounds, one of which is oleic acid. The previous study discovered significant antioxidant and antihemolytic activity (Zia-ul-haq et al., 2011). Another study found that polypeptides and oils isolated from *Momordica charantia* seeds were 79.18% effective as alpha-amylase inhibitors and alpha-glucosidase inhibitors (Ahmad et al., 2012).

Compound 24, 1,3-benzenedicarboxylic acid, bis (2-ethylhexyl) ester, has the highest peak in the sample. This isophthalic acid compound group is a saturated acid compound that is also found in the ethanol extract of *Amaranthus tristis* Linn leaves, showing alpha-amylase and alpha glucosidase enzyme inhibitor activity (Rajan, 2017), as well as the compounds 9 (-octadecenal, (Z) (CAS)), and compound 30 (hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester (CAS)). Oleic acid and palmitic acid groups exert antidiabetic activity. Palmitic acid compounds also function as inhibitors of diabetes-causing enzymes. As a result, ethyl acetate extracts from endophytic fungal cultures of *Aspergillus* sp. can be used as alpha-amylase inhibitors.

Table 4. The identified compounds by GC-MS and its bioactivity

Identified compounds	Group	Bioactivity
13. n-hexadecanoic acid	Palmitic acid	Obesity disorder (Saraswathi et al., 2020), reduction in hyperglycemia (Yang et al., 2011), antidiabetic (Orhan et al., 2015)
16. oleic acid	Oleic acid	antidiabetic (Palomer et al., 2017); (Carullo et al., 2017); (Muhoya et al., 2017), antidiabetic and antioxidant (Zia-ul-haq et al., 2011), antidiabetics and alpha glucosidase inhibitors (Wheni et al., 2018), diabetic retinopathy (Alcubierre et al., 2016), cytotoxic antidiabetics and antioxidants (Silva et al., 2019), inhibition of alpha-amylase and alpha glucosidase enzymes (Ahmad et al., 2012)
24.1,3-benzenedicarboxylic acid, bis (2-ethylhexyl) ester	Isophthalic acid	Antidiabetic (Patel et al., 2016); (Acta, 2021); (Choudhary et al., 2011), alpha-amylase and alpha glucosidase inhibitors (Rajan, 2017), antidiabetic and antioxidant (Abusufyan et al., 2018)
28. 9-octadecenal, (Z)-(CAS)	Oleic acid	(Iwara et al., 2022)
30. hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester (CAS)	Palmitin	Antidiabetic (Bharti et al., 2018); (Savych et al., 2021), alpha-amylase inhibitors and antioxidant (Ahmed & Ashiq 2014)

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