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Review Article

In vitro antioxidant activity and α-amylase inhibitory activity of ethanol extract and fraction of *Ganoderma lucidum*

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Abstract: The objective of this study is to determine the antioxidant and antidiabetic potential of ethanol extracts and fractions of Ganoderma lucidum. G. lucidum has been traditionally used for its medicinal properties, and recent studies suggest it may possess significant bioactive compounds with therapeutic potential. To investigate these properties, the study utilized two distinct methodologies: (1) antioxidant tests using the ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) assay, which evaluates the capacity of a substance to scavenge free radicals, and (2) antidiabetic tests involving alpha-amylase enzyme inhibition to assess the extract's ability to modulate blood sugar levels. The results indicated that the ethanol extract of G. lucidum exhibited notable antidiabetic properties, with an IC50 value of 35.033 µg/mL, which demonstrates its effectiveness in inhibiting the alpha-amylase enzyme. Furthermore, the ethyl acetate fraction of the extract was found to possess the highest antioxidant activity, with an IC50 value of 22.241 µg/mL, highlighting its potential as an effective antioxidant agent. In the antidiabetic evaluation, the ethanol extract showed a similar activity with an IC50 value of 48.025 µg/mL, while the ethyl acetate fraction remained the most potent with an IC50 value of 25.866 µg/mL. These findings suggest that G. lucidum extracts, particularly the ethyl acetate fraction, exhibit promising antioxidant and antidiabetic properties, supporting its potential use in managing oxidative stress and diabetes-related complications.

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1. Introduction

Antioxidants are compounds that can inhibit reactive oxygen and free radicals in the body. These antioxidant compounds will give up one or more electrons to free radicals so that they become normal molecular forms again and stop various damage caused [1]. Antioxidants are able to protect the body against damage caused by reactive oxygen compounds, able to inhibit the occurrence of degenerative diseases such as diabetes, cancer, tissue inflation and premature aging [2].

G. lucidum is one of the simplisia that is widely used by the community as an alternative medicine to reduce blood pressure and blood sugar levels. The efficacy of the plant is due to the chemical compounds it contains. The main substances contained in Lingzhi Mushrooms G. lucidum are ganodermin, ganoderan, ganodermin acid, triterpenoids, adenosine, peptidaglukan, germanium and polysaccharides (betaglukan) [3]. Other contents of

lingzhi mushrooms are thiamin, riboflavin, niacin, and biotin as well as several minerals such as potassium, phosphorus, calcium, sodium, copper and magnesium.[4]

G. lucidum is one of the most widely used species in the biochemical and pharmaceutical fields. The research on the metabolites or complexes produced, such as ganoderic acid and polysaccharides, grew rapidly due to their medicinal benefits and was considered as a "remedy that can resuscitate the dead" [5]. These days, Ganoderma has been utilized to prevent and treat numerous kinds of disorders and wellness items that are accepted to have anti-cancer properties, anti-aging, and are hostile to microbial or viral capacities. These products of Ganoderma are accessible, particularly in East Asia and the USA (Seweryn et al., 2021). The number of publications discussing the separation, bioactivity and production of bioactive auxiliary metabolites of G. lucidum has expanded due to their extraordinary health benefits and their bottomless and interesting optional metabolites as a promising potential library for new drug discovery [6].

The extract can inhibit α-amylase enzyme due to the presence of flavonoid and polyphenol compounds in accordance with the phytochemical screening conducted in this study [7]. These compounds can reduce blood glucose levels with their role as α-amylase enzyme inhibitors. Where it can convert disaccharides into glucose. In addition, flavonoids can also inhibit glucose absorption in the small intestine, so it can act as an antidiabetic. Flavonoid content can be found in G. lucidum [8].

As one of the ways to optimize the utilization of lingzhi mushroom for human health, research was conducted to test the antioxidant activity of ethanol extracts and fractions with phytochemical tests in lingzhi mushroom and also total flavonoid tests, α-amylase inhibitory activity, antioxidant activity test of G. lucidum extracts and fractions using ABTS (2,2'-azobis (3-ethylbenzthiazoline-6-sulfonic acid)) method.

2. Preliminaries or Related Work or Literature Review

Subsection 1

Research by Zhang et al., (2022) found that the sugar content (264.44 ± 8.22 mg/g) of BGLWE (sporoderm-broken G. lucidum spore water extract). The total flavonoid (14.70 ± 0.83 mg/g), polyphenol (12.11 ± 0.67 mg/g), and protein (9.72 ± 1.01 mg/g) contents of BGLWE were similar to each other but much lower than the total sugar content of sporoderm-broken G. lucidum spore water extract (BGLWE) showed good inhibitory activity against DPPH, hydroxyl radicals, and ABTS, and also showed high reducing ability against iron ions. Lingzhi mushroom BGLWE showed good activity in inhibition of α-amylase and α-glucosidase enzymes [9].

Subsection 2

Research by Huang et al., (2021) results G. resinaceum extract has the strongest decrease in lipid peroxidation intensity which supports a protective role in oxidative stress. Mycelia and extracellular polysaccharide levels were 6.56 ± 0.74 g/L and 0.50 ± 0.08 g/L, respectively. The pH value of the culture medium decreased from 6.24 ± 0.05 at the beginning to 3.90 ± 0.07 at the end of incubation. Based on the antioxidant potential, it appears that the content of phenolic compounds such as gallic acid in the EtOH extract of G. pfeifferi, which is an effective antioxidant due to the presence of hydroxyl groups [10].

3. Proposed Method

Sampel collection and preparation

G. lucidum was obtained from mushroom farmers, Dlingo II, Dlingo, Bantul, D.I. Yogyakarta. The plant material was identified by UPF Yankestrad, Tawangmangu, Central Java. G. lucidum was dried in an airy place under sunlight. After drying, the samples were ground in a grinder and sieved.

Extraction and fractionation

Solvent extraction of air-dried G. lucidum was performed by maceration technique using methanol as the initial solvent in an Erlenmeyer flask. After maceration, the mixture was sonicated for 15 min and allowed to stand for 24 h, filtered and evaporated in a rotary evaporator at 40°C under reduced pressure [11]. The extract was lyophilized and stored for pharmacological analysis and fractionation. Fractionation of the dried ethanol extract was performed by liquid-liquid partition chromatography in a separation funnel using hexane, ethyl acetate and water (1:1:1) with a final volume of 0.45 L, based on the amount of extract. After vigorous shaking, the mixture was set aside until two layers were formed [12]. To obtain the hexane fraction, it was separated and then concentrated in a rotary evaporator at 40°C under reduced pressure. To obtain the ethyl acetate fraction, the remaining fraction in the separating funnel was added to ethyl acetate and the evaporation procedure was repeated. Similarly, ethyl acetate was added to obtain the ethyl acetate fraction; the remaining material in the separating funnel was considered as the residual water fraction.

Total flavonoid content

The estimation of total flavonoids in plant extracts was performed according to the method of [13] 0.5 ml of 2% AlCl3 ethanol solution was added to 0.5 ml of sample. After 13-15 min at room temperature, the absorbance was measured at 436 nm. The extract samples were evaluated at a final concentration of 1 mg/ml. The total flavonoid content was calculated as quercetin (mg/g) using the following equation based on the calibration curve: $y = 0.0367x + 0.0721$, $R^2 = 0.9972$, where x is the absorbance and y is the quercetin equivalent (mg/g).

In vitro antioxidant activity

ABTS radical-scavenging activity of the extract was determined according to the method described by Abbas et al., (2022) The ABTS radical cation (ABTS•+) was produced by reacting 5 mL of ABTS stock solution with 5 mL of a 2.45 mM potassium persulfate ($K_2S_2O_8$) solution, which had been stored in the dark at room temperature for 16 hours. Prior to Prior to use, this solution was diluted with water to achieve an optical density of 0.700 ± 0.020 at 734 nm and equilibrated at 30°C.The plant extract at various concentrations was diluted with dimethyl sulfoxide (DMSO) to prepare the sample solution. Then, 5 µL of the sample solution was homogenized with 195 µL of the ABTS•+ solution. The mixture was subsequently incubated at room temperature for 6 minutes, after which the sample's absorbancy was recorded at 731,5 nm. In each assay, blanks were run.The ABTS scavenging ability was expressed as IC50 (µg/ml), and the inhibition percentage was calculated using the following formula [15]:

ABTS scavenging activity (%) = $(A_0\text{-}A_1) / A_0 \times 100$

where A_0 is the absorbance of the control, and A_1 is the absorbance of the sample.

In vitro α-amylase inhibitory activity

The assay was carried out following the standard protocol. The ethanol extract and fractions of *G. lucidum* were dissolved in buffer to obtain concentrations of 12.5, 25, 50, 100, and 200 µg/mL. Then, 0.2 mL of plant extract with the specified concentration was added to the tube containing the substrate solution. In addition, 0.5 mL of amylase was added to the tube containing the plant extract and substrate solution. The reaction was carried out at 37°C for 10 minutes. Next, 0.1 mL of amylum was added to the tube and the tube was incubated for 18 minutes. Last, 0.1 mL of iodine 1% was added to the tube. The reaction was halted by the addition of 0.5 mL of 1 N HCl to each tube. The resulting

mixtures' absorbances were measured at 545 nm using a UV-VIS spectrophotometer. The same procedure was repeated for the other sample fractions (water, ethyl acetate, and hexane) to assess their inhibitory effects on α-amylase. Acarbose, a known α-amylase inhibitor, was utilized as the standard drug [16]. The experiments were repeated thrice, and the α-amylase inhibitory activity was calculated using the following formula:

The α-amylase inhibitory activity = (Ac+) - (Ac-) - (As - Ab)/(Ac+) - (Ac-) X 100,

Where Ac+, Ac-, As, and Ab are defined as the absorbance of 100% enzyme activity (only solvent with enzyme), 0% enzyme activity (only solvent without enzyme), a test sample (with enzyme), and a blank (a test sample without enzyme), respectively. The concentration of acarbose and plant extracts required to inhibit 50% of a-amylase activity under the conditions was defined as the IC_{50} value. The α-amylase inhibitory activities of plant extracts and acarbose were calculated, and its IC_{50} values were determined

4. Results and Discussion

Total Flavonoid Content (TFC)

The study's findings suggest that the total flavonoid content in the ethanol extract of G. lucidum could be approximately 18.798 mg GAE/g, which might indicate a sufficient level of flavonoids in G. lucidum. Flavonoids are considered to be polyphenolic compounds with a polar nature, meaning they tend to dissolve in polar solvents and are slightly soluble in semipolar solvents [17]. Flavonoids have antioxidant properties and may contribute to the protection of β-cells, which are responsible for insulin production, and enhance insulin sensitivity[18].

ABTS free radical scavenging activity

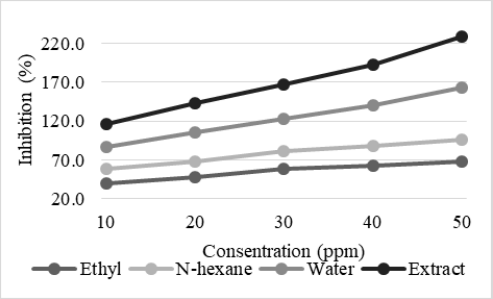

Figure 1. The ABTS radical scavenging activity of an ethanol extract and a fraction of G. lucidum.

In Fig. 1, G. lucidum extract and fraction was found to be effective in scavenging the ABTS radical. The percentage inhibition of this radical was sample-dependent. At ethanol extract, ethyl acetate fractions, water fractions, and hexane fractions was 53.033%, 22.241%, 35.108%, and 128.261% respectively and that of quercetin was 48.665%. The IC_{50} values of the G. lucidum extracts and fractions are listed in Table 1.

Table 1. Antioxidant activity of ethanol extract and fraction G. lucidum

Sampel	IC_{50} (µg/mL)	IC_{50} (µg/mL)±SD
Quercetin	2,516 2,675 2,724	2,638 ± 0,109
Ethanol extract	35,033	35,127 ± 0,124
	35,268	
	35,081	
Water	35,108	35,171 ± 0,162
	35,355	
	35,050	
Ethyl acetate	22,241	128,260 ± 0,118

	22,271	
	22,179	
n-Hexane	128,261	22,231 ± 0,047
	128,804	
	128,142	

α-amylase inhibitory activity

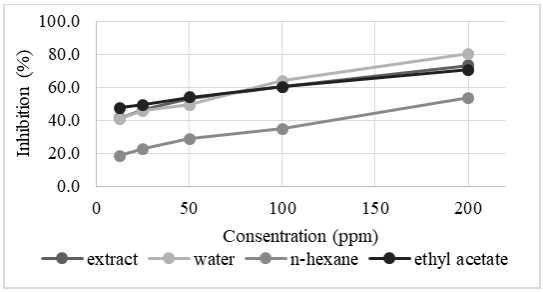

Figure 2. α-amylase inhibitory activity of the ethanol extract and the fractions of G. lucidum

The α-amylase inhibitory activity of the ethanol extract and the fractions was assessed at five different dilutions (12,5, 25, 50, 100 and 200 µg/mL) (Fig. 2). The highest potential α-amylase inhibitory activities were found in the ethanol extract, water fractions, and ethyl acetate fractions with inhibition of 56.170 ± 0.340% (IC50: 48,025 µg/mL), 56,028 ± 0.151% (IC50: 48.916 µg/mL), and 58.156 ± 0.4974% (IC50: 25.866 µg/mL) (Table 2). The efficacy of the α-amylase inhibition of the extract and various fractions was ranked as follows: ethyl acetate > methanol > aqueous > hexane. The standard acarbose was used as positive control and showed a maximum α-amylase inhibitory activity of 53.830 ± 0.023% (IC50: 3.023 µg/mL).

Table 2. α-amylase inhibitory activity of the ethanol extract and the fractions of *G. lucidum*

Sampel	IC_{50} (µg/mL)	IC_{50} (µg/mL)±SD
Acarbose	3,023	3,083 ± 0,079
	3,055	
	3,172	
Ethanol extract	48,918	48,692 ± 0,340
	48,438	
	48,719	
Water	48,177	48,238 ± 0,102
	48,356	
	48,181	
Ethyl acetate	25,585	25,712 ± 0,344
	25,450	
	26,101	
n-Hexane	180,129	180,009 ± 0,209
	179,768	
	180,130	

Comparison

In this study, fractionation of G. lucidum ethanol extract was performed using polar and non-polar solvents to obtain standardized fractions rich in biologically active phytopharmaceutical constituents and to generate comprehensive metabolite profiles. To achieve high quality fractionation, purification of the extract is essentially purified to remove unwanted

components can enhance biological activity for future pharmaceutical applications. Therefore, it is very important to choose the right extraction process as it affects the characterization of secondary metabolites [19]. Flavonoids are one of the most abundant groups of important phytochemicals and are widely recognized for their antioxidant activity and role in preventing damage to cells and their cellular components by reactive free radicals [20]. Flavonoids are natural antioxidants that can control oxidative stress-related reproductive diseases. The adverse effects of oxidative stress have been found to be controlled by the antioxidant activity of this group of bioactive compounds. Therefore, various experimental studies have been designed to elucidate the antioxidant activity den other activities of these compounds that can inhibit certain pathological and degenerative complications [21].

The antioxidant activity test was performed using the ABTS free radical scavenging method using a UV-Vis spectrophotometer instrument. The ABTS method was chosen because it has a high sensitivity and can be used to analyze antioxidants in foods and has a very fast response [22]. The principle of testing antioxidant activity using the ABTS method is the decolorization of ABTS cations to measure the capacity of antioxidants that react directly with ABTS cation radicals [23].

Based on the antioxidant test results, the % inhibition of ABTS radicals by the four samples was obtained. The results showed that among the four samples, methanol extract had the highest % radical scavenging activity for all concentrations (10, 20, 30, 40, 50 ppm) including ethanol extract, water fraction, n-hexane fraction and ethyl acetate fraction. The IC_{50} values were obtained from linear regression analysis, the lower the IC_{50}, the higher the antioxidant power. The lowest IC_{50} was shown by the ethyl acetate fractions (22.241±0.183 μg/ml), thus having the highest antioxidant activity. The remaining extracts had higher IC_{50} values compared to the ethyl acetate fractions. Their IC_{50} values in increasing order are ethanol extract (35.033±0.167 μg/ml), water fractions (35.108±0.175 μg/ml) and hexane fractions (128.261±0.384 μg/ml).

In this study, the potential of extracts and fractions from different parts of the plant in inhibiting α-amylase activity as an antidiabetic agent was tested. The results showed that the ethyl acetate fraction of G. lucidum had the highest α-amylase inhibition of 58.156% and the n-hexane fraction had the lowest inhibition of 42.695%. For the ethanol extract, G. lucidum had an inhibition of 56.170%, which is equivalent to the inhibition of the water fraction of 56.028%. The study concluded that the use of these plant extracts can help reduce the rate of carbohydrate digestion and absorption, which may be useful in diabetes management.

It is possible that the extract may have the potential to inhibit the α-amylase enzyme due to the presence of flavonoid compounds. Flavonoids have antioxidant properties that may contribute to the protection of β-cells from damage, which are important for insulin production, and could potentially enhance insulin sensitivity [24]. Another potential mechanism of flavonoids in the context of diabetes management involves their ability to inhibit GLUT 2, also known as Glucose Transporter type 2, which plays a significant role in glucose transportation within the gut. By doing so, it may contribute to a reduction in blood glucose levels [18].

6. Conclusions

The study's results suggest that the ethanol extract of G. lucidum may have antioxidant potential, with an IC_{50} value of 35.033 μg/mL. It appears that the ethyl acetate fraction is particularly promising, with an IC_{50} value of 22.24 μg/mL, suggesting its active potential as an antioxidant. It is also worth noting that the antioxidant potenti al of G. lucidum aligns with its potential as an antidiabetic through α-amylase inhibitory activity. The ethanol extract of G. lucidum has the potential to inhibit α-amylase with an IC_{50} value of 48.025 μg/mL. It appears that the ethyl acetate fraction may be the most promising in terms of α-amylase inhibitory activity, with an IC_{50} value of 25.866 μg/mL. It can be concluded that the ethanol extract and ethyl acetate fraction of G. lucidum show promise as antioxidants and antidiabetics through α-amylase inhibitory activity.

- **Author Contributions:** A short paragraph specifying their individual contributions must be provided for research articles with several authors **(mandatory for more than 1 author)**. The following statements should be used "Conceptualization: X.X. and Y.Y.; Methodology: X.X.; Software: X.X.; Validation: X.X., Y.Y. and Z.Z.; Formal analysis: X.X.; Investigation: X.X.; Resources: X.X.; Data curation: X.X.; Writing—

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