Antidiabetic Activity and Molecular Docking Analysis of Milky Mushroom (*Calocybe indica*) Grown on the Renewable Substrate

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Abstract. As part of the circular economy idea, rice straw without additives has been investigated as a way to turn agricultural waste into products with added value. The nutrients in the substrate, *Calocybe indica* yield, and biological effects were all calculated. In this investigation, the methanol extract was used. GC-MS was examined to discover the necessary compounds present in fungi. A surplus of mushrooms was harvested, and it was discovered that the growth of mushrooms on the rice substrate took place in less than five days. At a humidity of 93%, biological efficiency was determined to be in the range of 54.5-130.9%. In vitro and molecular docking results for the antidiabetic showed good inhibitory properties. As a result, rice straw could be a productive and affordable growing medium for milky mushrooms.

Keywords. Antidiabetic property, Bioactive compounds, *Calocybe indica*, Cultivation, Molecular docking

1 Introduction

A large, delicious, milk mushroom (*Calocybe indica*), also known as the summer mushroom, was first discovered in India in 1976. It is typically prized for its flavor, nutritional value, and medicinal properties and is a member of the family Tricholomataceae of the order Agaricaceae [1]. It is an edible food for people with high blood pressure and heart disease because it contains a crucial source of vitamin D and protein, as well as vitamins, carbohydrates, iron, and calcium [2]. It also contains significant amounts of potassium and sodium [3]. This aids in controlling the amount of bad cholesterol and preventing an increase in blood glucose levels. Patients with severe diabetes mellitus have a variety of diseases, including neuropathy, hyperlipemia, and nephropathy, and they can be managed with specific medications. *Calocybe indica* has strong antioxidant properties [4]. Recent years have seen a surge in interest in the natural anti-glycoside compounds found in milk mushrooms because they show promise in lowering the risk of chronic complications in people with diabetes mellitus. Inhibiting the primary enzymes that hydrolyze carbohydrates can control it and lower postprandial hyperglycemia.

Biorefinery is a facility that integrates several technologies from various fields to create a seamless process for converting biomass into different classes of compounds [5]. The most prevalent and promising renewable resource that can be used to produce chemicals and other useful byproducts is biodiversity [6]. Humans can produce food with added value by growing *Calocybe indica* mushrooms with lignocellulose agricultural waste. It is commonly grown all over the world and is referred to as the fruiting body of mushrooms. As a result, it can grow using a variety of lignocelluloses in a wide temperature range. Mushroom farming started in India in the 19th century [7]. Inorganic substances, carbon and nitrogen as a food source, as well as crucial nutrients like cellulose, hemicellulose, and lignin, are needed by milky mushrooms. Agriculture produces rice straw as a byproduct, which is then used as a substrate for mushroom growth. At the moment, rice fields are being destroyed by open-air burning, which causes significant environmental issues. Rice straw has the potential to convert inedible waste into profitable and palatable biomass if it can encourage the growth of milky mushrooms [8]. These mushrooms grow on deeply decomposing organic materials and can be harvested in the wild during the last, wettest part of the rainy season. However, it is said that due to its poor biological value and yield, the use of rice straw in the cultivation of milk mushrooms has not taken off in China. For those who have diabetes or cancer, it also has therapeutic advantages [9].

In particular, the *Calocybe indica* species, which is an edible food for people with high blood pressure and heart disease, contains significant amounts of potassium and sodium [10]. In addition, by lowering the composition of lignocellulose biomass, the practice of

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mushroom cultivation might enhance the quality of straw [11]. Thus, the primary substrate for growing milk mushrooms in this study is rice straw (also known as paddy straw), and it also aims to look into the in vitro inhibitory activity of the antidiabetic plant *Calocybe indica*.

2 Materials and methods

2.1 Cultivation of mushroom

Milky mushrooms were grown on a substrate of rice straw using protocol-compliant techniques [12]. Bedding preparation was carried out in a polythene bag under sterilized conditions using a 3:1:2 ratio of casein soil, coco-peat, and sand.

2.2 Extraction

The harvested mushrooms were dried in the air in an oven at a temperature of 40 ºC, and then a sample of dried mushrooms (5 g) was extracted by mixing with 50 ml of methanol, filtered through Whatman filter paper No. 1. Then, extract of milk mushroom *(C. indica*) was evaporated by rotation at a temperature of 40 ºC until drying. The dried extract was stored at a temperature of 4 ºC until further use [13].

2.3 Antidiabetic activity: Alpha-amylase

To determine the inhibition assays of pancreatic α-amylase activity were carried out by the standard procedure. The milky mushroom extract was taken in a small amount of 1 mg/ml in different concentrations, and then starch solution was used as a substrate. After starting the reaction by adding 100 ml of a 10 mg/ml starch solution, 20 mm of sodium phosphate buffer (pH 6.9), and 6 mm of sodium chloride, the reaction was carried out at 25 ºC for 10 min. A volume of 100 µl of pancreatic α-amylase (0.5 mg/ml) was added to each tube, and all tubes with the mixture were incubated at 25 ºC for 10 min the reaction was stopped to count the amount of glucose present by adding 200 µl of the coloring reagent 3,5-dinitro salicylic acid (DNS). The reaction mixture was heated for 5 min at 100 ºC and placed in an ice bath to cool the mixture at room temperature; at the end of the reaction, 100 µl of sodium potassium tartrate was added and transferred to 96 well plates. Using acarbose as a standard, the activity of -amylase was measured at 540 nm, and the inhibition of enzyme activity was calculated using the formula below [14].

$$\%\text{Inhibition} = \text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}} / \text{Abs}_{\text{control}} \quad (1)$$

2.4 Alpha-glucosidase

A preliminary study of the activity of α-glucosidase was carried out in the presence of milk mushroom extract: 1 mg/ml of the extract was taken in different concentrations (10-100 mcg/ml). The extract was prepared in a 100 mm sodium phosphate buffer (pH 6.9) and then 50 µl of 5 mm solution of p-nitrophenyl, α-D glucopyranoside in a phosphate buffer were mixed in a 96well microplate. The sample was incubated at 37 ºC for 5 min, followed by the addition of 100 ml of phosphate buffer containing 0.1 U/ml. α-glucosidase (from baker's yeast) into each well and using a microplate reader to set the temperature at 37 ºC. The reaction mixture was recorded for 30 min and measured the absorbance at 405 nm. The standard drug acarbose was dissolved in 10% DMSO and used as a positive control. The percentage (%) of inhibition was calculated as follows [15].

$$\%\text{ of antioxidant activity} = [(\text{AC-AS})/\text{AC}] \times 100 \quad (2)$$

Where: AC- control reaction absorbance; AS- testing specimen absorbance

2.5 Molecular Docking and GC-MS Analysis

Using the AutoDock version, a molecular interaction study was conducted on the chosen phytocompounds from the GC-MS/MS analysis. Alpha-amylase and alpha-glucosidase enzymes were the targets for the interaction study, and the Protein Data Bank (PDB) was used to retrieve the three-dimensional structures of the proteins. The protein target was docked against various ligands using the Autodock and AutoGrid algorithms to examine its inhibitory activity [16].

Alpha-amylase and alpha-glucosidase, which have been used commercially for inhibitory properties, were measured using acarbose as a standard. The employed algorithm forecasts the relationship between ligands and macromolecule (protein) targets. Charges from Kollman and Gasteiger were added. The Lamarckian genetic algorithm's chosen docking parameters are as follows: With a population of 150 and a maximum of 27,000 generations, the number of docking runs was set to 10. The highest binding affinity is demonstrated by the lowest binding energy. Ligplot+ was used to view the hydrogen bonds and hydrophobic interactions of each docked complex.

3 Results and Discussion

Mushrooms are epigenetic macrofungi that have two distinct growth phases, reproductive (fruiting bodies) and vegetative (mycelium), and they have an umbrella-like structure in which spores are produced [4]. There are over 14,000 different types of mushrooms in the world, but only about 2,000 of them are edible. As a result, about 200 species of mushrooms were grown commercially to be used in the creation of food and medication [17]. The growth of milk mushroom species (*Calocybe indicia*), which are high in vitamins and calcium, and have nutraceutical, pharmaceutical, bioremediation and biodegradable properties, has continued and gained momentum in recent years [18]. In addition, mushrooms have more than 100 therapeutic properties, with their primary medical applications being

for detoxification, antibacterial, immunomodulation, antifungal, antiviral, antiparasitic, antitumor, antiallergic, antioxidant, and anticholesterolemic effects [19]. In particular, the presence of biologically active substances that inhibit enzymes can contribute to antidiabetic activity. This includes *in vitro* training with noteworthy data on alpha-amylase and alpha-glucosidase inhibitory activity that was comparable to acarbose, a common antidiabetic drug.

To prevent chronic complications, this study focuses on the antidiabetic activity of milk mushrooms for the treatment of patients with diabetes mellitus at the onset of the disease. Researchers are working to identify essential components with a higher margin of safety and promising areas of application after conducting extensive research on mushrooms in the food and pharmaceutical industries over the past few years. Consumption has significantly increased among people of all ages as a result of mushrooms' superior nutritional qualities, desired organoleptic properties, and aroma. Additionally, there is still a lot of interest in mushrooms because of their importance as a staple food item around the world and their use as food and medicine for a variety of illnesses. These benefits include their dietary, antioxidant, and therapeutic properties. The majority of the complications that are currently occurring are with mushrooms, which are regarded as one of the essential and suitable foods for patients suffering from various types of diseases, such as diabetes, obesity, and cancer [20].

Mushrooms are a crucial ingredient in cooking because of their potential immunomodulatory effects, high-quality protein, and low fat and cholesterol content. *Calocybe indica* is advantageous to health as the third most significant commercially grown mushroom after champignons and oyster mushrooms. Studies reported that milky mushrooms are an excellent source of antioxidants and organic substances that can be used to treat diabetes and obesity. Milk-white mushrooms can be consumed nutritionally to help treat this condition [21]. Several studies have used multiple approaches to tackle diabetes mellitus, amongst these inhibiting the enzymes that facilitate the absorption and metabolism of carbohydrates has been one approach. The important enzymes that are involved in glucose absorption from the gut are the pancreatic α-amylase enzyme and α-glucosidase enzyme. Inhibiting these enzymes reduces the absorption of glucose from the gut and thereby controls postprandial blood sugar levels. In the present study, we evaluated the antidiabetic properties of the methanolic extract of *Calocybe indica*, the results suggest that Dioxa-10-thia-4,13- diazacyclopentadeca-5,9,12-trione and Oxacycloheptadec-8-en-2-one compound of Milky mushroom effectively inhibits the activities of α-amylase and α-glucosidase enzymes using molecular docking analysis. These effects were compared with standard drugs. These findings are consistent with the earlier reports on the effect of natural compounds in inhibiting the enzyme activities of α-amylase and α-glucosidase enzymes.

3.1 *In vitro* anti-diabetic activity- alpha-amylase inhibition assay

The methanolic extract's ability to inhibit amylase was assessed, and the results of the inhibition assay are depicted in Figure 1. The percentage inhibitory activity against the alpha-amylase enzyme increased in a dose-dependent manner. The enzyme was most effectively inhibited by the extract concentration at 75.03±0.72. Alpha-amylase inhibitory activity was detected at concentrations of 25-200 µg/ml of the extract, with an IC_{50} value of 44.57 µg/ml. The IC_{50} value of sample is 30.96 µg/ml, and acarbose was used as the standard reference drug (IC_{50} 80.67±1.22 µg/ml).

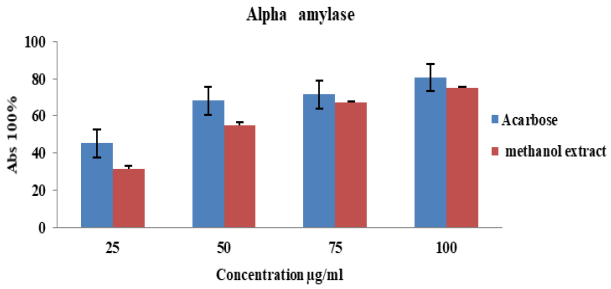


Fig. 1. Inhibitory effects of alpha-amylase in methanolic extract of milky mushroom and acarbose.

3.2 Yeast α-glucosidase activity

The yeast-glucosidase activity was found to be significantly inhibited by a methanol extract of *Calocybe indica*. At concentrations between 25 and 200 µg/ml of the extract, as shown in Figure 2, the percentage inhibition varied from the highest concentration of 59.85±2.41 µg/ml to the lowest concentration of 14.84±1.78 µg/ml. It was determined that 85.91 µg/ml was the IC_{50}—the concentration needed to produce 50% inhibition. When the extract's IC_{50} value was compared to that of the reference drug, acarbose (63.45 µg/ml), it revealed inhibition activity.

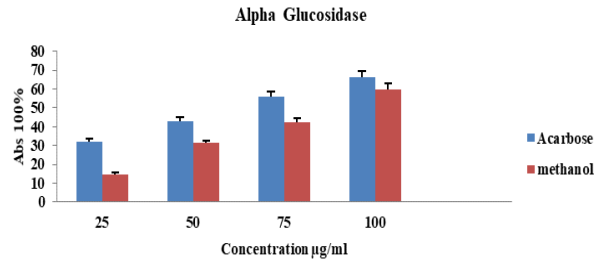


Fig. 2. Inhibition of α-glucosidase in *milky mushroom* extract and acarbose.

3.3 Yeast cell glucose uptake

Studies have been done on how the methanolic extract affects antidiabetic activity. The amount of glucose taken up by yeast cells treated with the methanolic extract was

found to rise dose-dependently. The percentage increases in the cell's uptake of glucose at various glucose concentrations are shown in Figure 3 and 4, and are 5 mM and 10 mM, respectively. The extract was most active at glucose concentrations of 5 mM and 10 mM (66.07±1.27 µg/ml and 58.24±1.9 µg/ml, respectively). The values of IC_{50} are 68.0 µg/ml and 89.90 µg/ml for 5 mM and 10 mM, respectively. According to the results, milky mushrooms are more effective than the common drug acarbose (73.34±1.71 µg/ml) and (70.49±1.65) at increasing glucose uptake by yeast cells. The values of 44.6 µg/ml and 61.08 µg/ml are the IC50.

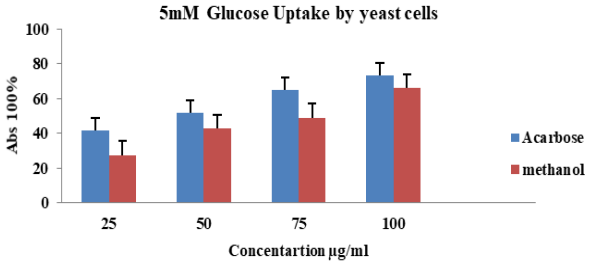


Fig. 3. Methanolic extract effects on milky mushroom and acarbose glucose uptake by yeast cells (5 mM).

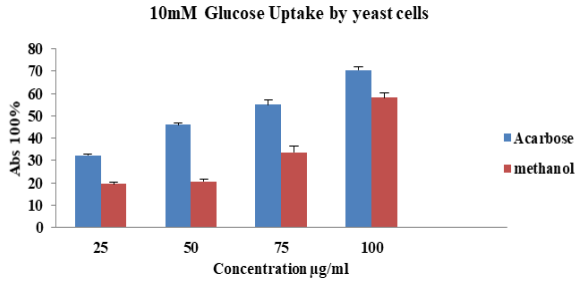


Fig. 4. Methanolic extract effects on milky mushroom and acarbose glucose uptake by yeast cells (10 mM).

3.4 GC-MS analysis of the crude extract

GC-MS analysis for crude extract; GC-MS chromatogram of the crude extract showed the presence of compounds at different retention times and compared with the NIST library seven major compounds were identified at different retention times it shown GC-MS spectra revealed various peaks, the indicated the occurrence of different bioactive compounds in the methanolic extract obtained from the mushroom extract (Figure 5). The spectral fingerprints of compounds were identified using the NIST library and the molecular weight of the bioactive compound. GC-MS chromatogram profile of crude extracts is present while the identified volatile compounds are presented. The major compounds present in the methanolic extract and the compound listed in Table 1.

Table 1. List of GCMS Compounds milky mushroom.

S. No	Compounds	Peaks	
		RT	%
1	1 Methylamine, N-cyclopentylidene	4.823	16.21
2	2 N,N'-Bis(2-hydroxyethyl)-1,7- dioxa-4,10-diazacyclododecane	5.820	2.31
3	3 -Valine, N-propoxycarbonyl-, ethyl ester	5.895	19.97
4	4 Ala-Gly, N-trimethylsilyl-, trimethylsilyl ester	6.056	3.95
5	5 Ethyl 3-[2-methyl-2-mercaptopropylamino]butyrate	6.503	9.82
6	N-Methyldodecanamide	8.149	31.04
7	**1,7-Dioxa-10-thia-4,13-diazacyclopentadeca-5,9,12- trione**	**8.235**	**42.32**
8	Oxacycloheptadec-8-en-2- one	9.589	21.10
9	4-Isoxazoline-4,5-dione, 3- tert-butyl-, 4-oxime	13.521	5.04
10	9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester, (Z,Z,Z)	13.739	2.72
11	9-Hexadecenoic acid	14.305	9.57
12	6-Octadecynenitrile	16.588	49.90
13	Carnegine	19.847	16.94

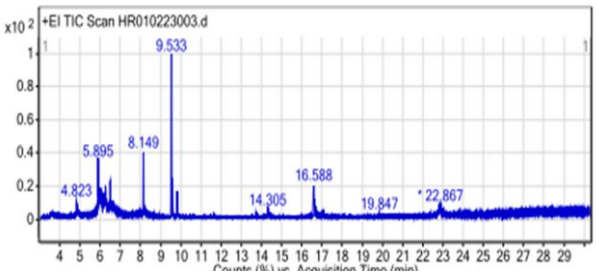
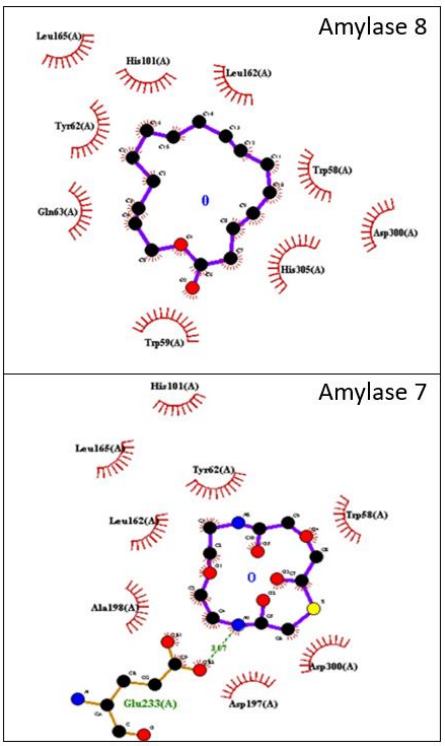


Fig. 5. Chromatogram of milky mushroom.

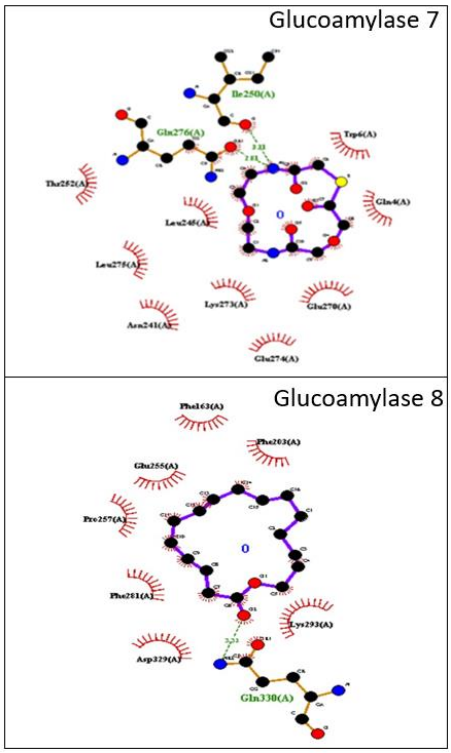
3.5 Molecular Docking

Molecular docking was performed to predict the antidiabetic compound of (1, 7-Dioxa-10 thia-4, 13-diazacyclopentadeca-5,9,12-trione and Oxacyclohepta dec-8-en-2- one from extract with target enzymes were studied based on their interaction using dockthor version tools. The docking study aims to check the transformation of whether one molecule fits with the other molecule at the optimal level and to identify the binding potential of the compound with the Enzymes using ligplot. In this docking analysis, initially, the PDB structure of the compound was taken from the PubChem database and enzyme structures were taken from the RCSB databank. The structure of the compound was converted to PDB structure format using the auto dock and was docked with target receptors. This study shows the binding energy (kcal/mol) and configuration of hydrogen bonds with active site residues. Based on the

binding potential of 1,7-Dioxa-10-thia-4,13-diazacyclopentadeca-5,9,12-trione and oxacyclohepta dec-8-en-2- one against the molecular receptors were observed the interactions of active residue site respectively (Figure 6).



(a)



(b)

Fig. 6. Docked pose of enzymes with the ligand complexes of a) alpha-amylase; b) alpha-glucosidase

The results are shown in Table 2 negative scores are obtained, which show good binding affinity with molecular targets. Docking studies showed that compounds have had good binding affinities to all the receptors, which are strongly related to type-2 diabetes and the biding energy are α-amylase, α glucosidase, milky mushrooms are packed with a ton of essential vitamins, and minerals, and boost the anti-inflammatory, and immune system as it is rich sources of vitamin D, vitamin B, essential amino acids, potassium, protein, and fiber. Moreover, researchers have found several benefits in the mushroom as it lowers blood pressure by reducing the negative impact of sodium in our bodies.

Table 2. Molecular docking of enzymes with the ligand complexes of alpha-amylase and alpha-glucosidase.

S. No	Compounds	Binding energy (kCal/mole)	
		Amylase (1HNY)	Glucosidase (1UOK)
1	Methylamine, Ncyclopentylidene	-4.4	-4.2
2	N,N'-Bis(2-hydroxyethyl)-1,7-dioxa-4,10- diazacyclododecane	-5.6	-5.7
3	Valine, N-propoxycarbonyl-, ethyl ester	-3.8	-4.3
4	Ala-Gly, N-trimethylsilyl-, trimethylsilyl ester	-	-
5	Ethyl 3-[2-methyl-2-mercaptopropylamino]butyrate	-4.2	-3.8
6	6 N-Methyldodecanamide	-3.5	-3.4
7	**1,7-Dioxa-10-thia-4,13-diazacyclopentadeca-5,9,12-trione**	**-6.4**	**-7.2**
8	**Oxacycloheptadec-8-en-2- one**	**-7.4**	**-7.4**
9	4-Isoxazoline-4,5-dione, 3- tert-butyl-, 4-oxime	-5.7	-5.1
10	9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester, (Z,Z,Z)	-5.5	-4.0
11	9-Hexadecenoic acid	-4.8	-4.1
12	6-Octadecynenitrile	-4.3	-4.0
13	Carnegine	-6.1	-5.8
14	Papaveroline, 2'-bromo-2-methyl-, tetramethyl(ester)	-6.2	-6.6

By encouraging macrophages to fight off foreign invaders, the anti-inflammatory effect of mushrooms has been demonstrated to significantly increase the immune system's effectiveness. Additionally, lowering the risk of hypertension and other metabolic diseases, mushrooms' antioxidants. Additionally, it has affected weight loss. It has anticancer, antiviral, antimicrobial, antiallergic, and anticholesterolemic properties and hepatoprotective effects [22]. The use of secondary metabolites as therapeutic agents includes polysaccharides, lectins, lactones, terpenoids, alkaloids, and antibiotics [23].

This study examines extracts and bioactive compounds for their potential anti-obesity and anti-diabetic properties. The maceration method was used to create a methanolic extract of milky mushrooms. The antidiabetic activity of the milky mushroom extract was

examined using various assays, including the inhibition of -glucosidase and -amylase, as well as the uptake of glucose by yeast cells. These assays were specifically used in vitro. Similarly, 1,7-Dioxa-10-thia-4,13-diazacyclopentadeca-5,9,12-trione and Oxacyclohepta dec-8-en-2- compounds showed good binding affinity towards receptors. This research also deals with improving phytotherapy to manage diabetes through bioactive compounds as drug delivery. It could potentially be investigated further using cell lines and gene sequencing methods.

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