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In vitro Antidiabetic Studies of Aqueous Extract of *Pleurotus ostreatus* Grown on Different Substrates

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ABSTRACT

Background and Objective: Nature-derived products are finding increased application for therapeutic purposes. Apart from their use as food, mushrooms have been reported in various research to contain bioactive compounds with medicinal capabilities. This study evaluated the *in vitro* antidiabetic and antioxidant activities of an aqueous extract of *Pleurotus ostreatus* (mushroom) cultivated on rice bran/sawdust (RB/SD, 70:30 w/w) mixture and sawdust (SD) substrates. **Materials and Methods:** *Pleurotus ostreatus* was cultivated on two different substrates followed by aqueous extraction of mature mushrooms. *In vitro* antidiabetic, (α-amylase and α-glucosidase inhibition) and antioxidant (DPPH radical-scavenging assay and ferric reducing power (FRAP) activities were done using standard methods. Results were analyzed using IBM Statistical Product and Service Solutions (SPSS) software, version 23 and the significance level was established at $p<0.05$. **Results:** The extracts showed a concentration-dependent inhibitory effect on α-amylase and α-glucosidase activities *in vitro*. There was a stronger inhibition of α-amylase activity by the RB/SD sample when compared to SD and a weaker inhibitory effect of the RB/SD sample on α-glucosidase activity when compared to SD. Again, the samples showed a concentration-dependent antioxidant effect with increased DPPH-scavenging activity and FRAP observed with the SD sample compared to the RB/SD sample. The GC-FID analysis of the *P. ostreatus* samples also revealed the presence of a number of bioactive compounds in different concentrations. **Conclusion:** These results highlight the effect of cultivation substrate on the potential therapeutic effect of mushrooms the aqueous extract of *P. ostreatus* could be a promising raw material for the development of anti-diabetes therapeutics.

KEYWORDS

Antioxidant, antidiabetic, α-amylase, α-glucosidase, *Pleurotus ostreatus*

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INTRODUCTION

Diabetes mellitus (DM), one of the leading causes of morbidity and mortality globally, is a multifactorial chronic and complicated metabolic disorder characterized by elevated serum glucose levels caused by the body's inability to produce, recognize or respond to insulin and thus leads to a variety of complications[1,2]. The American Diabetes Association (ADA) and the International Diabetes Federation (IDF) reported that about 2.2% of the world's population dies yearly as a result of DM[3] and estimated a 50% increase in people living with diabetes by 2045 with the number of diabetic people reaching 629 million[4]. Diabetes itself is seemingly harmless, however, prolonged and untreated presentations lead to various complications such as retinopathy, neuropathy and diabetic foot amongst others. Diabetic complications arise due to persistent hyperglycemia and hyperglycemia-induced oxidative stress[5]. Thus, effective glycemic control appears as a viable option in the management of diabetes and associated complications.

Inhibition of the glucose-releasing enzymes α-amylase and α-glucosidase is one of the therapeutic options presently explored for effective glycemic control. The α-amylase plays an important role in starch digestion by catalyzing the hydrolysis of α-(1, 4)-D-glycosidic linkages in starch, producing smaller fragments and other polymers of glucose[6,7]. Its products are further degraded by α-glucosidase to absorbable monosaccharides which then enters the bloodstream[8,9]. Inhibiting these enzymes would, therefore, hinder starch absorption, expand intestinal sugar holding time and delay the rate of glucose absorption into the bloodstream. Approved therapeutic drugs that target these enzymes include acarbose, voglibose and migiltol. These, however, cause unwanted side effects such as diarrhea, bloating, flatulence and abdominal discomfort thus limiting their use in diabetes therapy[10,11].

The use of natural remedies for disease management is a common practice by mankind from the beginning of time. Advancements in technology has also made it possible to identify and study the safety and therapeutic profiles of the many bioactive components of many natural products[12]. Mushrooms are highly effective nutraceutical products, conferring many health benefits in addition to their nutritional properties[13-15]. Hepatoprotective properties of species such as *Auricularia auricular*, *Tricholoma lobayense*, *Grifola frondosa*, *Tremella fuciformis*, *Flammulina velutipes*, *Lentinula edodes* and *Volvariella volvacea* have been reported and attributed to a variety of inherent molecular entities in these mushrooms[16]. Chaga mushroom (*Inonotus obliquus*) has also been recognized for its potential to be used against the SARS-CoV2 virus[17]. Various reports also suggest that mushrooms have the ability to maintain normoglycemia with little or no side effects[2,3,11,18]. *Pleurotus ostreatus*, a member of the genus *Pleurotus* has attracted a lot of attention due to various advantages over other species due to its capacity to thrive at a variety of temperatures[19,20]. As a rich source of nutrients and other bioactive compounds, *P. ostreatus* exhibits a wide range of therapeutic functions including immuno-modulatory, antiviral, anti-oxidative, cardioprotective and antitumor[12,21,22]. Studies have shown that the nutritional and bioactive component of mushrooms depend greatly on substrate used in mushroom cultivation as mushrooms are potent bio-accumulators[23-26]. Hence, this study evaluates the *in vitro* antidiabetic and antioxidant effect of the aqueous extract of *Pleurotus ostreatus* grown on rice bran/sawdust combination and sawdust alone.

MATERIALS AND METHODS

Study duration: The study was carried out between December, 2021 and April 2022, lasting for a period of four months.

Materials

Collection and authentication of materials: The materials used for this study include *Pleurotus ostreatus*, rice bran and wood sawdust. The mushroom (*Pleurotus ostreatus*) used was cultivated on a mixture of rice bran (1.75 kg) and sawdust (0.75 kg) (70:30 w/w) combination and sawdust alone substrate (2.5 kg) at the South East Zonal Biotechnology Center, University of Nigeria, Nsukka. Wood sawdust was purchased in Timbre market Adani and rice bran was purchased in Timbre Market Nsukka both in Uzo-Uwani LGA area, Enugu State. Both were milled to fine particles.

Chemicals and reagents: All chemicals and reagents used in this study were of analytical grade. They were obtained from reputable chemical dealers in Nsukka, Enugu State and Onitsha, Anambra State, both in Nigeria. They include Potassium sodium tartrate (Rochette salt) (HD, China DNSA (Sigma, USA), Acetic acid and Sodium acetate (HD, China). Starch extract was gotten from *Xanthosoma sagittafolium*, while Sucrose was a product of lobachemie. The enzymes alpha-amylase and alpha-glucosidase was purchased from the Megazyme manufacturing company.

Methods

Spawn preparation: The mushroom spawn was prepared as described by Thongklang and Luangharn[27]. *Pleurotus ostreatus* mycelium was cultured on potato dextrose agar (PDA) followed by inoculation of the spawn on parboiled sorghum seeds. This was maintained as the stock culture for mushroom cultivation.

Preparation of substrate and cultivation of mushroom: Substrates used for mushroom cultivation, rice-bran/sawdust 70:30 w/w combination and sawdust only, were prepared as described by Chukwurah *et al*.[28] and Aguchem *et al*.[29]. Cultivation of mushrooms was also carried out as described by Aguchem *et al*.[29].

Preparation of mushroom extract: As 35.87 g of fresh mushroom sample was weighed using an analytical balance (Thermo Fisher, USA). It was put in an electric blender (Binatone, Hong Kong, China) containing 60 mL of distilled water and was blended for 5 min. It was then filtered using a filter cloth to obtain the filtrate. The filtrate was centrifuged at 1000×g for 15 min and the supernatant was collected and stored in a small container as the aqueous extract.

Determination of inhibitory effect of the *P. ostreatus* aqueous extract on α-amylase and α-glucosidase activities *in vitro*: The procedure described by Narkhede *et al*.[30] with few modifications was used in determining the inhibitory effect of the *P. ostreatus* aqueous extract on α-amylase and α-glucosidase activities *in vitro*. The assay mixture containing the solution of the enzymes, α-amylase and α-glucosidase, (0.1 mL), acetate buffer (0.1-0.5 mL, 0.1M at pH 5.5) and mushroom extracts (32-160 μg mL^{-1}), respectively were added to five test tubes labelled test tube 1-5 and incubated for 15 min at room temperature. Afterwards, 0.5 mL of 2% sucrose and 2% starch was added to the test tubes as substrate for the enzymes and incubated in a controlled water bath at 50°C for 30 min. The reaction was terminated with the addition of 1 mL of dinitrosalicylic acid (DNSA) reagent and placed in a boiling water bath for 10 min. To stabilize the colour after heating, 1 mL of 1.4 M Rochelle salt (sodium potassium tartarate) was added to the test tubes immediately[31] and the total volume of the solution was adjusted to 4 mL with distilled water. The reaction mixture was cooled to room temperature and absorbance was taken at 540 nm. The test tubes used as blank were prepared without mushroom extract.

Percentage inhibition of enzyme activity was calculated as described by Narkhede *et al*.[30]:

$$\text{Inhibition (\%)} = \frac{A_{540}\ \text{Ctrl} - A_{540}\ \text{treatment}}{A_{540}\ \text{Ctrl}} \times 100$$

Where:

A_{540} Ctrl = Absorbance of control at 540 nm

A_{540} treatment = Absorbance of a sample containing extract at 540 nm

***In vitro* antioxidant activity of the *P. ostreatus* aqueous extract**

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay: The DPPH radical-scavenging activity of the extract was determined following the protocol of Sayah *et al.*[32]. Differently labelled test tubes received additions of the mushroom extracts at various amounts (ranging from 32 to 160 mg mL^{-1}) followed by the addition of water to bring the volume to 250 L. Each test tube was then shaken violently following the addition of 2 mL of a 0.18 mM (0.005%) methanolic solution of DPPH after which the mixture was allowed to stand in the dark for 30 minutes at room temperature. The same procedure was used to prepare the control without any extract. Changes in samples absorbances were taken at 517 nm using a spectrophotometer (Spectron lab 2A, England). DPPH radical scavenging potential was calculated using the method provided by Adebiyi *et al.*[33]:

$$\text{Scavenging activity (\%)} = \frac{A_{517}\ \text{Ctrl} - A_{517}\ \text{treatment}}{A_{517}\ \text{Ctrl}} \times 100$$

Ferric reducing antioxidant power assay: The method described by Alachaher *et al.*[34] was used to determine the ferric reducing antioxidant potential of the mushroom extract. One milliliter of a solution containing 32, 64, 96, 128 and 160 mg mL^{-1} each of the extracts was combined with potassium ferricyanide (5.0 mL, 1.0%), sodium phosphate buffer (5.0 mL, 0.2 M) and potassium phosphate buffer (0.2 M, pH 6.6). The mixture was incubated at 50°C for 20 min followed by the addition of 5 mL of 10% trichloroacetic acid and centrifuged at 1000×g. The upper layer of the solution (5.0 mL) was diluted with 5.0 mL of distilled water and ferric chloride and absorbance was measured at 700 nm using a spectrophotometer (Spectron lab 2A, England). The experiment was performed thrice and results were averaged.

$$\text{Scavenging activity (\%)} = \frac{A_{700\ nm}\ \text{Ctrl} - A_{700\ nm}\ \text{treatment}}{A_{700\ nm}\ \text{Ctrl}} \times 100$$

Extraction of chemical constituents: Extraction of chemical constituents was carried out following Ojukwu *et al.*[35]. As 15 mL ethanol and 10 mL 50% w/v KOH was added to a test tube containing one (1 g) of sample and the test tube was allowed to sit in a water bath at 60°C for 1 hr. The content was then transferred to a separating funnel and washed successively with ethanol (20 mL), cold water (10 mL), hot water (10 mL) and hexane (3 mL), which was all transferred to the funnel. The resultant solution was further washed three times with 10 mL of 10% v/v aqueous ethanol solution and dried with anhydrous sodium sulfate. The resulting sample was solubilized in 1000 µL of pyridine after which 200 µL (0.2 mL) of the solution was transferred to a vial for analysis.

Quantification of chemical constituents by gas chromatography-flame ionization detector: Determination of the chemical constituents was done on a flame ionization detector (FID)-equipped gas chromatography machine (BUCK M910, PUB Scientist, USA). As 0.1 mL of the extract was drawn using a syringe and injected into the gas chromatography (GC) machine equipped with FID. In principle, the detector uses a flame to ionize carbon-containing organic compounds. During separation, the sample passes through a hydrogen-fueled flame in the GC column which ionizes the carbon atoms in the sample.

Statistical analysis: Results were analyzed using IBM Statistical Product and Service Solutions (SPSS) software, version 23. They were analyzed using One-way Analysis of Variance (ANOVA) and presented as Mean±Standard Deviation (n = 3). The results were considered to have significant statistical differences when $p<0.05$. The IC_{50} values were calculated by linear regression analysis.

RESULTS

Inhibitory effect of the mushroom extracts on α-amylase and α-glucosidase activities *in vitro*: Table 1 shows the effect of the mushroom extracts on α-amylase and α-glucosidase activities *in vitro*. The result showed that both samples had a concentration-dependent inhibitory effect on both α-amylase (IC_{50} value of 745.22 and 1456.84 μg mL^{-1}, respectively for SD and RB/SD) and α-glucosidase (IC_{50} value of I525.91 and 795.99 μg mL^{-1}, respectively for SD and RB/SD) activities *in vitro*. From the result, the SD sample showed a stronger inhibitory effect on α-glucosidase activity than α-amylase while the RB/SD sample had a stronger inhibitory effect on α-amylase activity compared to α-glucosidase activity.

***In vitro* antioxidant activity of the mushroom samples:** Table 2 shows the *in vitro* antioxidant activity of the aqueous homogenates of the mushroom samples. From the result, a concentration-related DPPH radical-scavenging activity was observed for both mushroom samples with IC_{50} values of 257.99 and 127.80 mg mL^{-1} for RB/SD and SD samples respectively. The samples also showed good ferric reducing power in a concentration-dependent manner with the SD sample showing stronger reducing power compared to the RB/SD sample. It was observed from the result that the SD sample had higher antioxidant activity *in vitro* compared to the RB/SD sample.

Chemical constituents of the extracts of *P. ostreatus* grown on rice bran/sawdust (70:30 w/w) combination (RB/SD) and sawdust (SD): The gas chromatography analysis of the mushroom samples (Fig. 1 and 2) revealed the presence of several chemical compounds including catechin, epicatechin, rutin, kaempferol, lunamarin, spartein, resveratrol, naringin in varying concentrations. Epicatechin, catechin, kaempferol, resveratrol and sapogenin were higher in the RB/SD sample compared to the SD sample, while rutin, spartein and lunamarin were higher in the SD sample compared to the RB/SD sample (Table 3).

Table 1: Effect of *Pleurotus ostreatus* aqueous extracts α-amylase and α-glucosidase activities *in vitro*

Sample concentration (μg mL^{-1})	α-amylase inhibition		α-glucosidase inhibition	
	SD (inhibition (%))	RB/SD (inhibition (%))	SD (inhibition (%))	RB/SD (inhibition (%))
32	10.39±0.36^{a}	16.84±0.48^{a}	15.17±0.65^{a}	5.89±2.36^{a}
64	10.71±0.47^{a}	19.87±0.49^{b}	16.43±0.39^{a}	8.01±0.63^{b}
96	10.86±0.41^{a}	19.84±0.14^{b}	16.84±0.42^{a}	12.19±1.69^{c}
128	12.89±0.61^{b}	19.94±0.24^{b}	20.09±0.49^{b}	12.27±0.43^{c}
160	18.50±0.61^{c}	20.40±0.54^{b}	24.99±0.46^{c}	12.85±0.58^{c}
	IC_{50} = 745.22 μg mL^{-1}	IC_{50} = 1456.84 μg mL^{-1}	IC_{50} = I525.91 μg mL^{-1}	IC_{50} = 795.99 μg mL^{-1}

Data represent Mean±Standard Deviation (n = 3), values with different superscripts down the column are significantly different at $p<0.05$, SD: Saw dust, RB: Rice bran and RB/SD: Rice bran/saw dust substrate combination

Table 2: *In vitro* antioxidant activity of the mushroom samples

Sample concentration (mg mL^{-1})	DPPH-scavenging activity		FRAP	
	RB/SD (inhibition (%))	SD (inhibition (%))	RB/SD	SD
32	23.43±0.20^{a}	28.63±0.63^{a}	8.01±0.21^{a}	68.20±0.55^{a}
64	25.39±0.41^{b}	38.70±3.98^{b}	50.04±0.13^{b}	73.33±0.27^{b}
96	26.83±1.01^{b}	38.27±2.03^{b}	49.81±0.21^{b}	79.18±1.27^{c}
128	35.85±0.26^{c}	50.38±5.92^{c}	55.10±0.40^{c}	82.83±0.12^{d}
160	39.85±1.80^{d}	58.50±1.44^{d}	58.07±0.32^{d}	85.47±3.25^{d}

Data represent Mean±Standard Deviation (n = 3), values with different superscript in rows are significantly different at $p<0.05$, SD: Saw dust, RB: Rice bran and RB/SD: Rice bran/saw dust substrate combination

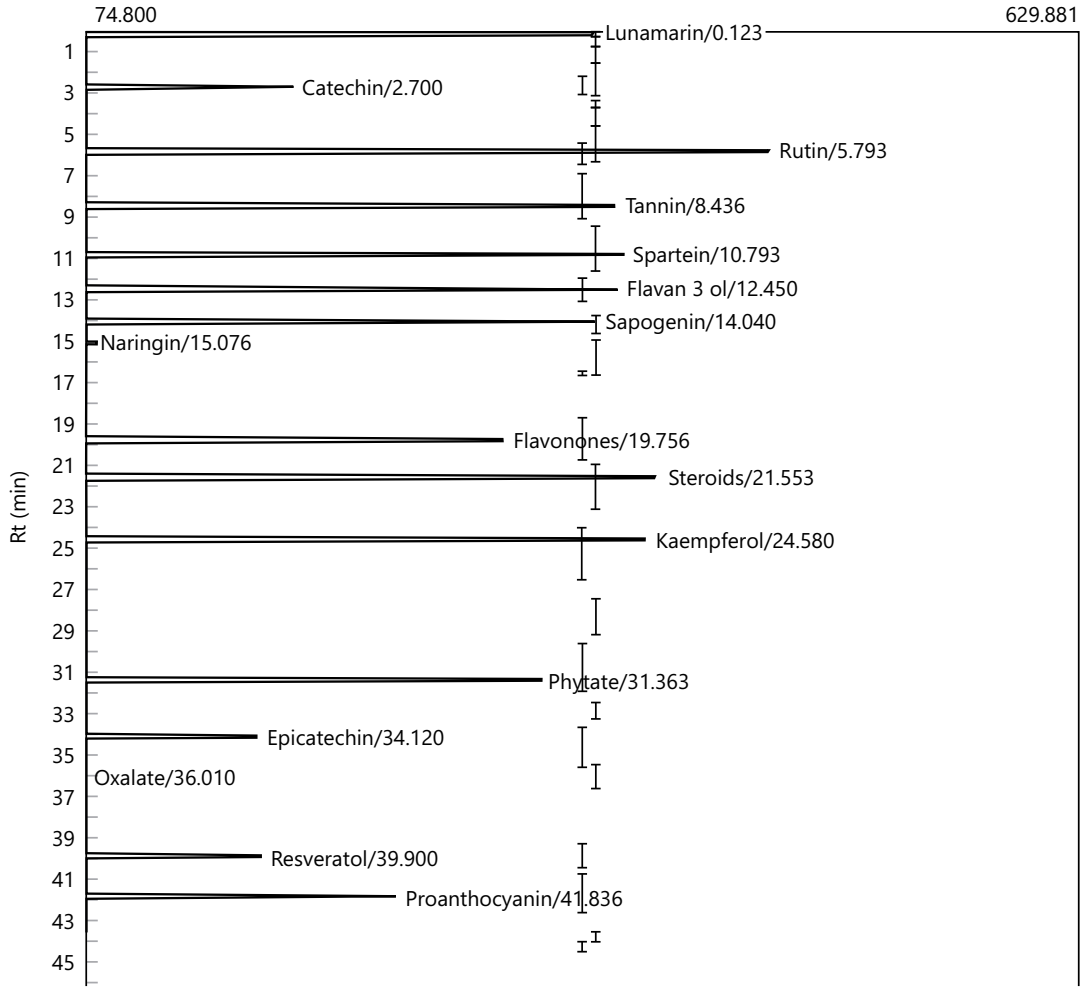

Fig. 1: Chemical constituents of the aqueous extract of mushroom grown on saw dust
RT: Retention time

Table 3: Chemical constituents of the aqueous extract of *P. ostreatus* grown on rice bran/sawdust (70:30 w/w) combination (RB/SD) and sawdust (SD)

Component	RB/SD (μg mL^{-1})	SD (μg mL^{-1})
Lunamarin	2.714	8.486
Catechin	5.769	1.797
Sapogenin	8.233	ND
Rutin	ND	3.611
Tannin	2.999	3.153
Spartein	1.821	2.015
Flavan-3-ol	2.868	2.810
Naringin	10.501	0.856
Resveratrol	2.381	1.255
Flavonone	7.414	2.326
Steroids	6.419	8.037
Kaempferol	6.055	4.460
Cyanogenic glycoside	5.786	ND
Phytate	ND	5.504
Flavone	6.766	ND
Epicatechin	35.198	12.692
Oxalate	8.512	1.554
Cardiac glycoside	7.377	ND
Ephedrine	18.413	ND
Proanthocyanin	1.915	1.866

SD: Saw dust, RB: Rice bran, RB/SD: Rice bran/saw dust substrate combination and ND: Not detected

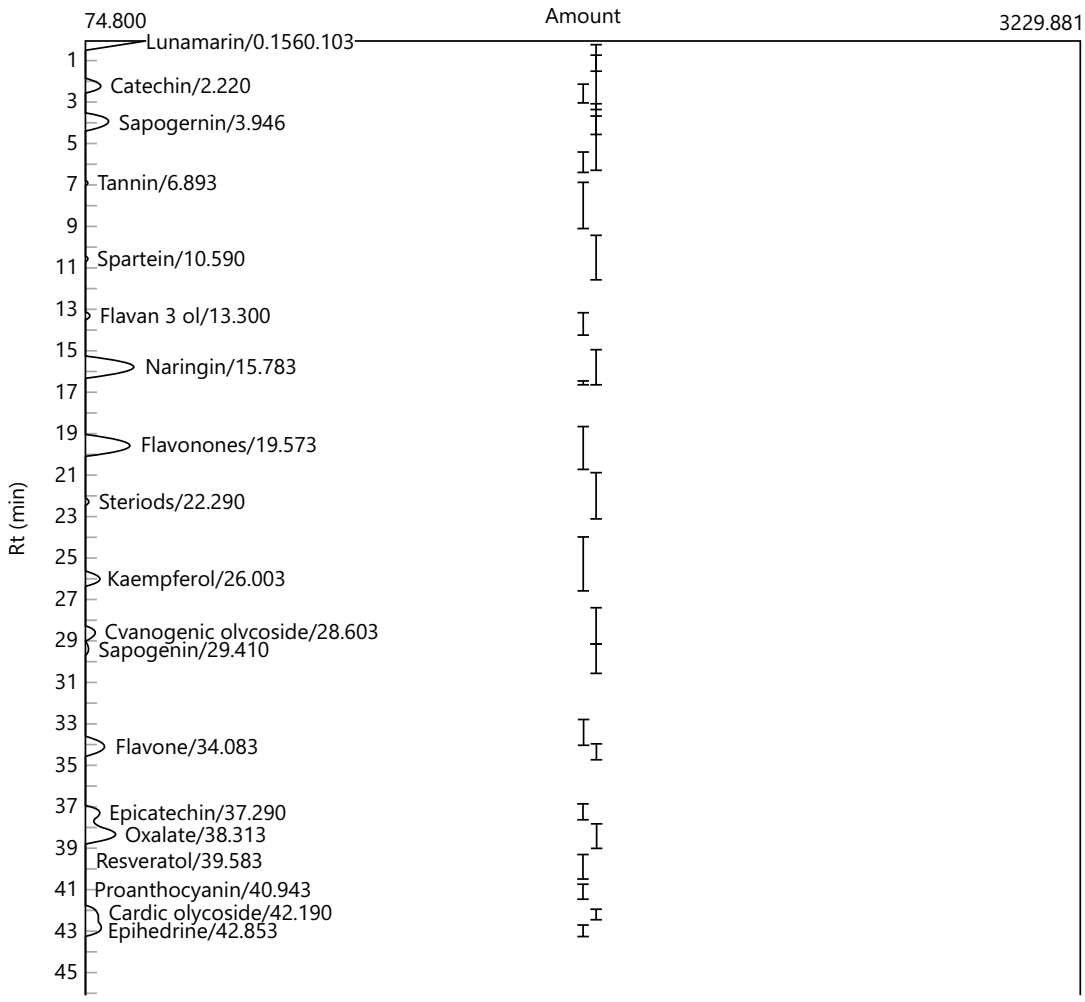

Fig. 2: Chemical constituents of the aqueous extract of mushroom grown on rice bran/saw dust (70:30) combination
RT: Retention time

DISCUSSION

The need for the discovery and use of natural antidiabetic products has been necessitated by the exponential increase in diabetic cases and numerous side effects associated with the use of available hypoglycemic drugs. In the present study, the *in vitro* antidiabetic activity of aqueous extract of *P. ostreatus* grown on different substrates was evaluated. Results from this study showed that the mushroom extracts moderately inhibited α-amylase and α-glucosidase activities *in vitro* in a concentration-dependent manner with the highest percentage of inhibition occurring at the highest concentration of the mushroom samples. Although these enzymes play physiologically important roles in the digestion of carbohydrates, their over activity could contribute to persistent hyperglycemia, especially in conditions of impaired glucose clearance such as diabetes. The inhibitory effect of the *P. ostreatus* extracts indicates its potential to regulate postprandial hyperglycemia and suggests that it might be a potential candidate for improving diabetic conditions. This result was supported by the reports of Prabu and Kumuthakalavalli[36], who stated a dose dependent inhibition of α-amylase and α-glucosidase activities *in vitro* by the aqueous extract of *P. florida*. Winska *et al*.[37] also reported that, the aqueous extract of *G. lucidum* significantly decreased blood glucose levels in artificially induced diabetic rats.

Many disease conditions are associated with attenuated endogenous antioxidant defense systems leading to development and aggravation of complications associated with such diseases. In view of this, compounds with antioxidant capacity are always useful as adjuvant therapy in disease management.

DPPH, a stable free radical becomes a non-radical (DPPH-H) when it interacts with an antioxidant, donating an electron[21] whereas, the ferric reducing antioxidant power (FRAP) method determines the ability of test substances to reduce Fe^{3+}/ferric cyanide complex to its ferrous state[38]. The *in vitro* antioxidant analysis of the mushroom extracts showed moderate DPPH radical-scavenging activities and ferric reducing potentials of the extracts. This result correlates positively with the work of Anjana *et al*.[21] in which they reported antioxidant activity of methanol extract of *P. ostreatus* with increase in extract concentration. Also, Stastny *et al*.[39] reported significant antioxidant and anti-inflammatory activities of methanol extracts of *P. ostreatus*, *P. florida* and *P. flabellatus in vitro*.

As potent bio-accumulators, the chemical and nutritional profile of mushrooms is often a function of the type of substrate on which they are cultivated[23,29]. The gas chromatography analysis of the mushroom samples revealed the presence of a number of bioactive compounds, albeit in different concentrations. These compounds include epicatechin, naringin, tannin, kaempferol, rutin, amongst others. The presence of these compounds in these samples further highlights the health benefits associated with mushroom consumption. Epicatechin is a flavanol monomer with numerous health benefits including its positive effect on cardiovascular health and is also effective in the management of diabetes[40]. Rutin and kaempferol are also reported to exhibit a wide range of favorable biological effects including antioxidant, antihyperglycemic and neuroprotective effects[8,41,42]. The presence of these compounds in the mushroom homogenates is consistent with various reports[16,21,43] in which they reported the presence of some of these compounds in mushroom samples. From the result, the RB/SD sample had higher concentrations of catechin, epicatechin, resveratrol, naringin and kaempferol compared to the SD sample while lunamarin and spartein were higher in the SD sample compared to the RB/SD sample. Rutin was below detectable limit in the RB/SD sample but present in the SD sample. These differences in biological compositions may be attributed to the difference in growth medium and this is consistent with the findings of Thongklang and Luangharn[27] which indicated that the use of different substrates in the cultivation of *P. ostreatus* caused disparity in a lot of features of the mushroom. This was also supported by reports of Paul *et al*.[44], that stated significant differences in proximate, mineral and vitamin contents of *P. florida* grown on different substrates. Mkhize *et al*.[45] also reported significant differences in antioxidant activity of extracts from mushrooms cultivated on different substrates attributing this observation to differences in the chemical contents of the mushroom samples.

In the wake of the numerous side effects presented by different hypoglycemic agents, the findings of this study suggest that *P. ostreatus* can serve as a potential alternative in the management of diabetes and its complications.

The result of this study clearly demonstrates that *P. ostreatus* is a rich store of compounds which could be beneficial in disease conditions involving these carbohydrate-metabolizing enzymes. However, further studies would still be required to confirm if the *in vitro* observations translate to *in vivo* benefits as well.

CONCLUSION

From the result obtained, this study demonstrated the antidiabetic and antioxidant potentials of *P. ostreatus* aqueous extracts. It also highlighted that the substrates used in cultivation have a significant influence on the biological efficiency of mushrooms. The exact mechanism of enzyme inhibition as well as antioxidant activity was not provided by this study and thus should be a subject of further research. Bioactive compounds present in the *P. ostreatus* samples should be subjected to further studies and the relationship between mushroom content and substrates should also be investigated.

SIGNIFICANCE STATEMENT

Aqueous extract of *P. ostreatus* showed good inhibitory activity towards alpha-amylase and alpha-glucosidase suggesting that its potential in anti-diabetic activity could be explored further through *in vivo* study. Researchers, through the outcome of this study, can critically explore further the isolated bioactive compounds from *P. ostreatus*. This could enable an in-depth understanding of their medicinal effect and their future employment as antidiabetic agents.

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