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Hypoglycaemic and antioxidant effects of the basidomycetes *Lyophyllum connatum* (Lyophyllaceae) and *Tuber melanosporum* (Tuberaceae) in mice

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Abstract
The prevalence of Type 2 diabetes mellitus is on the rise and treatment failure is common. New agents that have superior profile in diabetic treatment are therefore needed. This present study investigates the aqueous and ethanolic extracts of the fruiting bodies of the basidiomycetes *Lyophyllum connatum* and *Tuber melanosporum* for antioxidant and hypoglycaemic activities. The effects of the extracts were tested in normoglycaemic and alloxan-induced hyperglycaemic mice intraperitoneally, at doses of 500, 1000 and 1500 mg/kg body weight. Blood glucose level was estimated with a hand held glucometer. The crude extracts were also assayed for DPPH scavenging property while phytochemical constituents of the extracts were determined using standard protocols. Results show that a significant time–dependent reduction in blood glucose concentration in normoglycaemic and hyperglycaemic mice, comparable to the reference drugs chlorpropamide. The aqueous extract of *L. connatum* induced a maximum fall of 33% in blood glucose concentration, greater than the 25% fall induced by the ethanolic extract at a dose of 1500 mg/kg. *T. melanosporum* lowered blood glucose level by up to 75%. Alkaloids, carbohydrates and flavonoids are the most abundant phytochemicals present in the extracts. The ethanolic extract of *T. melanosporum* showed the most potent DPPH radical scavenging activity with an IC_{50} value of 251 µg/ml. The fruiting bodies of *L. connatum* and *T. melanosporum* have significant hypoglycaemic activity in mice but possess weak free radical scavenging activity which may be attributable to the presence of flavonoids.

Keywords: *Lyophyllum connatum; Tuber melanosporum;* Mushrooms; Diabetes mellitus; Hypoglycaemia

INTRODUCTION

Diabetes mellitus is a chronic metabolic disorder that is due to decreased secretion of insulin by pancreatic beta cells or decreased efficacy of released insulin (insulin sensitivity) or both (Hussain, 1997). It is characterized by hyperglycaemia as well as disorders of protein and lipid metabolism. When poorly managed, diabetes mellitus results in macrovascular, microvascular, neuropathic and renal complications (Aquilante, 2010). Despite increased awareness, there is a growing prevalence of this disorder with associated increase in morbidity and mortality. Mortality rates as high as 32% have been reported in Nigeria (Chijoke *et al*., 2010). The pathophysiology of diabetes mellitus suggests an underlying

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oxidative stress on pancreatic cells by reactive oxygen species and these are also implicated in complications such as atherosclerosis. Cellular injury occurs when the endogenous mechanisms for neutralizing the reactive oxygen species are compromised or overwhelmed by their excessive production. Superoxide dismutase and catalase are examples of enzymes that neutralize reactive oxygen species (Prasad and Sinha, 2010).

Diabetes mellitus currently has no cure. The main stay of management is based on lifestyle changes and pharmacotherapy. However, the success rate in terms of attaining treatment goals leaves a lot to be desired. Drug-associated adverse reactions (e.g. hypoglycaemia and weight gain) coupled with complexity of drug regimen contribute to suboptimal therapeutic outcomes (Triplitt, 2010). Therefore, the search for pharmacologic agents with better profile in the treatment of type 2 diabetes mellitus must continue.

Many herbal products are used by indigenous populations for the management of chronic conditions, including diabetes mellitus, because of their accessibility, acceptability, affordability and availability (Vaidya and Devasagayam, 2007). Most of these herbal products are plant-derived preparations (Kavishankar *et al*., 2011). Such focus has not been placed on basidiomycetes (mushrooms) despite widespread validated claims and use in many other parts of the world (Poucheret *et al*., 2006).

Lyophyllum connatum and *Tuber melanosporum* are mushrooms that grow in the wild. The fruiting bodies *Lyophyllum connatum* are cream coloured, grow in clusters and the caps have an average diameter of 12 cm. The protective property of *Lyophyllum connatum* against CCl_4-induced liver damage has been reported (Kimura *et al*., 2005).

Tuber melanosporum has a woody mature fruiting body with a purple, reddish light brown colour cap that has uneven surface with knob-like eruptions. The underneath consists of many tiny pores which are white in colour. *Tuber melanosporum* has a long history of folkloric uses, some of which have been validated scientifically. Antioxidant, anti-inflammatory, anti-tumor and treatment of Parkinson's disease are proven properties. Others include use in diabetes, herpes and gynaecological disorders (Zhang *et al*., 2011).

This work was therefore undertaken to evaluate the *in vivo* hypoglycaemic and *in vitro* anti-oxidant properties of the aqueous and ethanolic extracts of the fruiting bodies of *Lyophyllum connatum* and *Tuber melanosporum*.

EXPERIMENTAL

Mushroom collection and preparation of extract. The fruiting bodies of *Lyophyllum connatum* and *Tuber melanosporum* were collected from the wild in Jos and identified by Azila J.J of the Federal College of Forestry, Jos. They were shade-dried and ground to coarse powder. Two hundred grams of the coarse powder was each separately extracted with 70%v/v ethanol and water at room temperature for 72hours. The resultant extract was suction filtered and thereafter evaporated to dryness at 40^{O}C. The crude extract obtained was stored at 4^{O}C until required for use.

Animals. Albino mice with average weight of 25g procured from the Animal Experimental Unit in the Department of Pharmacology, University of Jos were used for the experiments. The animals were housed in cages of standard dimensions under appropriate conditions of temperature and humidity. They were fed with standard diet and given access to water *ad libitum*. Institutional approval was sought for the experiments and the handling of animals was according to institutional animal care and use protocol.

Phytochemical screening. Phytochemical screening of the constituents of the extracts was carried out according to standard methods (Sofowora, 1993).

Determination of LD_{50}. The median lethal doses of the extracts were determined in mice through the intraperitoneal route according to the method described by Lorke (1983).

Determination of antioxidant activity. Antioxidant activity of the crude extracts was determined using methods described by Brand-Williams (1995) with slight modifications. 12.5mg of the aqueous crude extract of *Lyophyllum connatum* was dissolved in methanol while the ethanolic extracts of *Lyophyllum connatum* and that of *Tuber melanosporum* were dissolved in 500µL of DMSO in 25mL volumetric flask, made up to mark with methanol. The following concentrations of the extracts were prepared 500, 250, 125, 62.50, 31.25, 15.62, 7.8125, 3.91, 1.95 and 0.98µg/ml. All the solutions were prepared with methanol. 2mL of each prepared concentration was mixed with 4mL of 50µM DPPH solution in methanol. The experiment was done in triplicate. The mixture was vortexed for 10s to homogenize the mixture and test tubes were incubated for 30min at room temperature in the dark. After 30min of incubation the absorbance was measured at 515nm using UV-vis spectrophotometer (Shimadzu UV-1620PC, Japan). Lower the absorbance of the reaction mixture indicates higher free radical scavenging activity. Gallic acid and rutin were used as standards with the following concentrations 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.7812, 0.391 and 0.195µM. Blank solution was prepared by mixing 2mL of methanol with 4mL of 50µM DPPH solution in methanol. The difference in absorbance between the test and the control (DPPH in methanol) was calculated and expressed as % scavenging of DPPH radical. The capability to scavenge the DPPH radical was calculated by using the following equation

$$\% \text{ inhibition} = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100$$

Finally, the IC_{50} value, defined as the concentration of the sample leading to 50% reduction of the initial DPPH concentration, was calculated from the separate linear regression of plots of the mean percentage of the antioxidant activity against concentration of the test extract (µg/ml).

Hypoglycaemic screening

Lyophyllum connatum: 40 normoglycaemic mice were divided into 8 groups of 5 mice each. Groups 1 and 2 represented vehicles and chlorpropamide (4 mg/kg body weight) control groups, respectively. Groups 3, 4 and 5 received 500 mg/kg, 1000 mg/kg and 1500 mg/kg respectively of the aqueous extract while groups 6, 7 and 8 were administered 500 mg/kg, 1000 mg/kg and 1500 mg/kg of the ethanolic extract. All administrations were done intraperitoneally.

Tuber melanosporum. 5 groups of 5 mice each were employed. 4 groups were rendered hyperglycaemic with alloxan (100 mg/kg, IP). Group 1 represented vehicle control; group 2 was administered the reference drug chlorpropamide (4 mg/kg); groups 3 and 4 were given 500 mg/kg and 1500 mg/kg, respectively. Group 5 was not treated with alloxan (normoglycaemic) but was administered 1000 mg/kg of the extract. All administrations were done intraperitoneally.
Blood samples (one drop) obtained from the caudal vein were used to determine the blood glucose concentration using a handheld glucometer (One Touch Basic), initially immediately after extract administration, and subsequently after 4 hours and 8 hours.
Percentage fall in blood glucose concentration was calculated using the formula below:

$$\text{Percentage fall} = \frac{BGC_{VCt} - BGC_{TXt}}{BGC_{VCt}} \text{ X } 100$$

Where BGC_{VCt} = Blood Glucose Concentration of Vehicle Control group at time, 't'
BGC_{TXt} = Blood Glucose Concentration of Treatment group at time, 't'

Test of significance was carried out by comparing treatment groups with vehicle control groups. Statistical analysis was done using Student's t – test and differences at $p \leq 0.05$ were deemed statistically significant.

RESULTS

The median lethal doses for *Lyophyllum connatum* in mice via the intraperitoneal route is 2120 mg/kg while that for *Tuber melanosporum* is 2100 mg/kg. Phytochemical analysis shows the presence of alkaloids, steroids, carbohydrates and cardiac glycosides in *Tuber melanosporum* while alkaloids and cardiac glycosides are absent in *L connatum* (Table 1). Both mushrooms demonstrated modest antioxidant activity in the DPPH free radical scavenging test, with the ethanolic extract of *T. melanosporum* having the lowest IC_{50} value of 251.100 µg/ml (Table 2).

The aqueous and ethanolic extracts of *Lyophyllum connatum* significantly lowered blood glucose concentration in normoglycaemic mice. The aqueous extract showed greater potency compared to the ethanolic extract (Table 3). The ethanolic extract of *Tuber melanosporum* induced a fall in blood glucose concentration in both normoglycaemic and hyperglycaemic mice, comparable to the reference drug, chlorpropamide. Higher percentage falls were observed in alloxan – treated mice (Table 4).

DISCUSSION

The median lethal doses of the aqueous and ethanolic extracts of *Lyophyllum connatum* and *Tuber melanosporum* in mice were found to be 2120 mg/kg and 2100 mg/kg respectively, via the interperitoneal route. These values suggest moderate toxicity according to the hazard scale of Gosselin, Smith and Hodge (Canadian Centre for Occupational Health and Safety, 2013). These values were used in calculating treatment doses of the extracts.

Table 1: Phytochemical constituents of aqueous, ethanolic extracts of *Lyophyllum connatum* and ethanolic extract of *Tuber melanosporum*

Constituent	Aqueous extract of *Lyophyllum connatum*	Ethanolic extract of *Lyophyllum connatum*	Ethanolic extract of *Tuber melanosporum*
Alkaloids	-	-	+++
Anthraquinones	++	+++	-
Carbohydrates	+++	+++	+
Cardiac glycosides	-	-	++
Flavonoids	-	-	-
Proteins	++	++	+
Saponins	-	-	-
Steroids	++	++	++
Tannins	-	-	-

Key: - = Absent; + = Trace; ++ = Moderate; +++ = Prominent

Table 2: DPPH scavenging activities of aqueous and ethanolic extracts of *Lyophyllum connatum* and *Tuber melanosporum* compared with that of rutin and gallic acid

Test Substance	IC_{50} Value (µg/ml)
Gallic Acid	0.003
Rutin	0.060
Aqueous Extract of *L. connatum*	562.000
Ethanolic Extract of *L. connatum*	398.000
Ethanolic Extract of *T. melanosporum*	251.100

Table 3: Percentage fall in blood glucose concentration induced by aqueous and ethanolic extracts of *Lyophyllum connatum* following intraperitoneal administration in normoglycaemic mice

Treatment	Dose	0.5 hour	4 hours	8 hours
Aqueous extract	500 mg/kg	3.85 ± 17.21	9.89 ± 18.10	12.71 ± 18.41
Aqueous extract	1000 mg/kg	4.40 ± 8.02	14.84 ± 3.21	20.87 ± 4.18*
Aqueous extract	1500 mg/kg	15.93 ± 12.10	27.47 ± 12.74	33.76 ± 12.91*
Ethanolic extract	500 mg/kg	13.74 ± 2.65	14.29 ± 3.79	16.52 ± 2.85
Ethanolic extract	1000 mg/kg	14.47 ± 19.22	21.43 ± 13.20	25.77 ± 13.22
Ethanolic extract	1500 mg/kg	10.50 ± 2.52	18.13 ± 2.89	22.51 ± 2.33*
Chlorpropamide	4.0 mg/kg	0.73 ± 11.84	19.63 ± 12.39	29.22 ± 11.53*

Values are represented as mean ± S.E.M; N=5; *$P < 0.05$

Table 4: Percentage fall in blood glucose concentration induced by the ethanolic extract of *Tuber melanosporum* following intraperitoneal administration in alloxan-treated mice

Treatment	Dose	4 hours	8 hours	12 hours
Normoglycaemic	1000 mg/kg	50.00±1.04	46.85±0.06	54.88 ±1.09
Alloxan	500 mg/kg	72.00±7.47	55.80±2.43	53.25±5.08*
Alloxan	1500 mg/kg	75.20 ±8.57	60.00 ±0.73*	53.25 ±5.05*
Chlorpropamide	4.0 mg/kg	66.67±3.60	60.00 ±0.73*	82.64 ±5.78*

Values are represented as mean ± S.E.M; N=5; *$P < 0.05$

The crude extracts contained alkaloids, anthraquinones, carbohydrates and proteins to varying degrees with *L. connatum* possessing the higher amounts. Proteins and carbohydrates are common constituents of mushrooms. The dietary benefit of mushrooms is partly due to their protein content which have been reported to possess biological activity (Xu *et al.*, 2011). Although the hypoglycaemic property of the individual phytochemical constituents was not investigated, principles such as alkaloids and flavonoids have been shown to possess blood glucose lowering effects (Sani *et al*., 2007).

The extracts possess weak antioxidant property as demonstrated by their ability to scavenge 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical *in vitro*, compared to the standard compounds rutin and gallic acid. The ethanolic extract of *T. melanosporum* demonstrated the lowest inhibitory concentration and therefore the highest free radical scavenging activity of the three extracts. Antioxidant effects are commonly reported with phenolics, which are absent in the extracts under investigation and this could explain the high IC_{50} values observed. This activity was investigated because of the aetiologic relationship between diabetes mellitus and oxidative stress imposed on the pancreatic insulin secreting cells. Cellular injury - induced free oxygen radicals attack contribute to the development of atherosclerosis and other microvascular changes that occur in diabetes mellitus (Miyazaki *et al*., 2007). Future studies would be required to investigate the effect of long term administration of the extracts to diabetic animals, particularly whether overall outcome is improved.

Tables 3 and 4 show the fall in blood glucose level caused by the aqueous and ethanolic extracts in normoglycaemic and hyperglycaemic mice. Extract-induced hypoglycaemia was found to be dose and time dependent. The aqueous extract of *Lyophyllum connatum* produced a greater fall compared with its ethanolic extract. Although phytochemical screening did not reveal any difference between the aqueous and ethanolic extracts of *Lyophyllum connatum*, the difference in activity may be due to the enhanced transport of the aqueous principles in body fluids. *Tuber melanosporum* extract produced falls in blood glucose level in both normoglycaemic and hyperglycaemic mice. These effects are comparable to that induced by the oral sulphonylurea hypoglycaemic

agent, chlorpropamide. Alloxan, which was used to induce hyperglycaemia in this study, is an oxygenated pyrimidine derivative specifically cytotoxic to pancreatic Islet beta cells (Lenzen, 2008). The resultant loss of glucose regulation secondary to decreased insulin production gives rise to hyperglycaemia. Drug – induced fall in blood glucose level occurs by several mechanisms, including stimulating insulin secretion from residual beta cell, enhancing glucose uptake by skeletal muscles and reducing gastrointestinal glucose absorption (Kulkarni *et al*., 2000). Chlorpropamide is an insulin secretagogue that acts by stimulating the first and second phases of insulin release (Kulkarni *et al*., 2000). Released insulin induces its characteristic hypoglycaemic effect. Although the exact mechanism of action of the extracts was not investigated, it is speculated that they may act by stimulating insulin release from remnant beta cells spared from the cytotoxic action of alloxan.

Reduction in blood glucose concentration below normal level is an undesirable effect, as this has the tendency to cause hypoglycaemia, a prominent draw back with the use of sulphurnylureas. The reference drug used, chlorpropamide, is an insulin secretagogue and it requires a measure of residual beta cell function for it to induce its hypoglycaemic activity (Aquilante, 2010). It is postulated that there may be principles within the extracts of *L. connatum* and *T. melanosporum* which stimulate beta cells to release insulin or cellular uptake of glucose. However, further investigations are required.

Conclusion. The aqueous and ethanolic extracts of the fruiting bodies of the basidiomycetes *Lyophyllum connatum* and *Tuber melanosporum* possess hypoglycaemic activity in normoglycaemic and hyperglycaemic mice. Further tests are needed to determine the active principle(s) responsible for the observed hypoglycaemic effects.

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