



Edible mushroom (*Pleurotus cornucopiae*) extract vs. glibenclamide on alloxan induced diabetes: sub-acute *in vivo* study of Nrf2 expression and renal toxicity

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Abstract: The study aims to compare the action of *Pleurotus cornucopiae* and glibenclamide on alloxan-induced diabetes and ascertain how an aqueous extract of the edible mushroom regulates the expression of nuclear factor erythroid 2-related factor 2 (Nrf2), oxidative stress biomarkers and renal toxicity in a diabetic male Wistar rat model. Twenty-five adult male Wistar rats were randomly grouped into five groups with five rats per. Group 1 and those in the treatment groups received normal feed and water *ad libitum*. Group 2 received intraperitoneal administration of alloxan monohydrate (150 mg/kg body weight). Group 3 received alloxan monohydrate and glibenclamide (5 mg/kg body weight bwt), group 4 received alloxan monohydrate plus the extract (250 mg/kg bwt) and group 5 received alloxan monohydrate plus the extract (500 mg/kg bwt). The administration of glibenclamide plus the extract was oral for 14 days. Glibenclamide and the extract lowered blood glucose level, catalase, and glutathione peroxidase activities, increased the superoxide dismutase (SOD) activity in rats with alloxan induced diabetes. The extract at 500 mg/kg bwt reduced the plasma urea and sodium concentration in the treated rats. The extract and glibenclamide could detoxify alloxan and restore its induced renal degeneration and glomeruli atrophy, intra renal hemorrhage and inflammation and oxidative biomarkers through activation of Nrf2 expression. The drug glibenclamide and *P. cornucopiae* have appreciable hypoglycemic activity and potential to restore the normal renal architecture in the rats, hence they offer similar curative effects. Additionally, the extract at 500 mg/kg bwt activated SOD and Nrf2 expression more than glibenclamide in rats with alloxan-induced diabetes.

Key words: Mushroom, Diabetes mellitus, Nrf2, Glibenclamide, Renal failure

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Introduction

Diabetes mellitus (DM) is a combination of heterogeneous disorders commonly presenting with episodes of hyperglycaemia and glucose intolerance, as a result of lack of insulin or defective insulin action [1]. DM is one of the most common non-communicable diseases globally and as emerged as a public health burden in sub-Saharan African [2]. It is the

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leading cause of chronic and end-stage renal disease generally [3]. Although, recent studies emphasized treatment using resistant antibodies [4], it is cost intensive, and often, the diagnostic kits are unavailable in many healthcare facilities in Nigeria and probably in other economically negatively challenged African nations [5]. Approximately 5.8% (about 10 million) of adult Nigerians are living with DM [6, 7]. When some of the diagnostics kits are available, the technical skill of the caregivers of the procedures are often insufficient [6, 8] resulting in very poor glycemic monitoring of fasting glucose level with its attendant consequences. Though, healthcare providers, pharmaceutical industries and policy makers are relentlessly making efforts to make diagnostics kits available, only 15% of diagnosed patient population, visit conventional hospitals for monitoring of their blood glucose levels through routine check-ups. The greater percentage of these patients utilize self-medication, herbal remedies or no medication, which consequently invariably lead to renal damage, impairment and ulcerations, and subsequently disability or death [6, 8]. Therefore, easily available alternative, cheap but efficacious treatment options is highly imperative in such environments or social settings.

In these settings, as in the sub-Saharan region, there are abundant indigenous mushrooms, which are rich in bioactive substances such as flavonoids, saponin, alkaloids, steroids, phenol, tannin, and glycosides [9], and in food nutrients such as; polysaccharides, proteins, peptides, and lectins [10-12]. Furthermore, mushrooms are said to contain anticancer, antibacterial and antiviral agents [13], which could regulate blood glucose, cholesterol levels and inflammation [14-16]. The mushrooms also contain beneficial bioactive substances, high levels of macro and micro food elements that are essential for fluid balance and cellular homeostasis [17], and the regulation of inflammation and blood glucose level [18] as well as acting as an *in vitro* antioxidant system [19]. Glibenclamide, a conventional drug is used in hyperglycaemic conditions to lower blood glucose through stimulating insulin production from the existing beta cells of pancreas [20, 21].

The study aims to compare the action of *Pleurotus cornucopiae* and glibenclamide on alloxan induced diabetes, and ascertain how oral administration of an aqueous extract of the edible mushroom (*P. cornucopiae*) could regulate the expression of nuclear factor erythroid 2-related factor 2 (Nrf2), impacts oxidative stress biomarkers and alloxan induced diabetes kidney damage in diabetic male Wistar rats. It would also profile an *in vivo* diabetes management protocol,

and ascertain the different concentrations of the basic phytochemicals in aqueous extract of *P. cornucopiae* commonly found in our locality.

Materials and Methods

Mushroom cultivation

The mushroom was cultivated in AE-FUNAI farm on a mixture of appropriate quantities of wood (WD), rice dust (RD) and limestone, and the fruiting was very successful. The quantity of substrate for the cultivation of the mushrooms was calculated using the following measurement as described by Okigbo et al. [22] and modified by addition of more WD and RD by the authors.

1. WD: $WD = 45 \text{ kg}$, $WDC \text{ (wood dust constant)} = 0.64$
 $WD \times WDC = 45 \text{ kg} \times 0.64 = 28.8 \text{ kg}$
Quantity of WD used = 28.8 kg
2. RD: $WD (28.8 \text{ kg}) \times RDC \text{ (rice dust constant)} = 0.3$
 $WD \times RDC = 28.8 \text{ kg} \times 0.3 = 8.64 \text{ kg}$
Quantity of RD used = 8.64 kg
3. Limestone: $WD + RD \times LSC \text{ (limestone constant)} = 0.01$
 $(WD + RD) \times LSC = 28.8 \text{ kg} + 8.64 \text{ kg} \times 0.01 = 0.374 \text{ kg}$
Quantity of limestone used = 0.374 kg
4. Water: $WD + RD \times WC \text{ (water constant)} = 0.80$
 $(WD + RD) \times WC = 28.8 \text{ kg} + 8.64 \text{ kg} \times 0.80 = 29.95 \text{ L}$
Quantity of water used = 29.95 L – 30 L

Following the calculation, 28.8 kg WD + 8.64 kg RD + 0.374 kg of limestone + 30 L of water were properly mixed in the cultivation and fruiting of mushroom for this study. Thereafter, the substrate was packed into transparent substrate bags of about 0.5 kg which was tightened around an inserted pipe while the hole at the centre of the pipe was closed using foam to prevent contamination.

Thereafter, the bags were moved to the sterilization drum (at a temperature of 199°C) and were heated for 7 hours. Inoculation of the substrate and incubation were carried out concurrently in a constant light and air, and between the temperature of 20°C–25°C in order to achieve optimal fruiting process. This method enabled the mycelia to grow into fruiting bodies. Duration of spawn run was determined by counting the number of days it took the mycelia to completely colonize the substrate.

Mushroom harvesting

The mushrooms were harvested within 2–3 days of maturation and were taken to a herbarium in the Department

of Biological Sciences, Alex Ekwueme Federal University Ndufu-Alike, Ikwo, for identification. The mushrooms were assigned a voucher number, AE-FUNAI/2020/167, then sun dried and stored in a refrigerator at 4°C before use.

Quantitative analysis of phytochemicals of *Pleurotus cornucopiae*

The bioactive components of the aqueous extract of *P. cornucopiae* was quantitatively analyzed using a vortex mixer set at high speed and HPLC (Agilent 1100 series LC) equipped with column thermostat (Agilent Technologies). Chromatographic separation was done using ZORBAX SB-C18 analytical column (250 mm_4.6 mm, 5 mm) at a wavelength of 257 nm. The procedure as stated by Wang et al. [23] and modified by Alope et al. [24] was applied.

*Test for the basic phytochemicals in *Pleurotus cornucopiae* extract*

The phytochemical screening of the extract using aqueous solvents was carried out to identify each component as described by Harborne [25], and modified by Adebisi [26].

Flavonoids and phenol: One ml of 10% NaOH solution was added to a solution of 10-ml distilled water and 3-ml of the extract. A yellow and orange coloration indicated the presence of flavonoid and phenol respectively [27].

Alkaloid: A solution of 1 ml of 1% HCl acid was added to 3-ml of extract in a test tube, and the mixture was heated in a water bath for 20 minutes. Thereafter, 1 ml of the filtrate from (a) above was added to 0.5 ml of Meyer's reagent. The creamy colour of the solution indicates the presence of alkaloid [28].

Frothing test for saponin: 3 ml of extract was diluted with 2 ml of distilled water in a test tube. The frothing was mixed with a few drops of olive oil and mixed vigorously and the foam appearance showed the presence of saponins [29].

Salkowski test for steroid: Five drops of concentrated H₂SO₄ was added to 1-ml of extract in a separate test tube. The presence of red colouration indicated the presence of steroid [28].

Ferric chloride test for tannins: 2-ml of extract were boiled gently in a separate test tube for 2 minutes and allowed to cool. The appearance of a green-coloured precipitate when three drops of ferric chloride solution was added to the solution and mixed vigorously, indicated the presence of tannins [28].

Glycosides: 1 ml of the extract was mixed with 10-ml of

50% H₂SO₄ in a test tube, and heated in a water bath for 15 minutes. The appearance of a brick red precipitate when 10-ml of Fehling's solution was added and heated, indicated the presence of glycosides [28].

Aqueous mushroom extraction

Dried mushrooms weighing 217 g were grinded to powder (mesh-size of about 200–2,500) using a milling machine (vertical type; M-4) and the final weight of 204.04 g was obtained. The powder was soaked and constantly agitated in 500 ml of distilled water for 48 hours using a magnetic stirrer (hotplate 78-1; Lasec), and filtered using Whatman filter paper (cat No: 1001110). The filtrate was concentrated using a water bath, at a temperature of 40°C. The stock solution that was used for the experiment was 204 g/68 ml (3 g/ml). The dose given to each rat was determined using the relationship as described by Okolo et al. [9]:

$$\text{Vol. (ml)} = \frac{\text{weight (kg) of animal} \times \text{dose (mg/kg)}}{\text{stock solution (mg/ml)}}$$

Animals and housing

Twenty-five male Wistar rats weighing about 160–200 g were obtained from the animal house of Alex Ekwueme Federal University Ndufu Alike, Nigeria. The rats were housed in standard plastic cages at room temperature and in a 12 hours dark/12 hours light cycle. The rats were allowed to acclimate for 7 days. The animals were carefully handled following the guidelines of the National Institute of Health (NIH) for care and use of laboratory animals [30]. The study was approved by a research and ethics committee of AE-FUNAI with reference number AE-FUNAI/FBMS/EC/AE/2020.

Treatment groups

The rats were divided into 5 groups of with 5 rats as follows; Group 1: normal control (NC): administered distilled water and normal rat chow; Group 2: diabetic control (DC): treated with alloxan monohydrate. Group 3: diabetic rats administered with 5-mg of glibenclamide/kg bwt, for 14 days, to ensure diabetic condition as was previously reported [31]. Group 4: diabetic rats administered *P. cornucopiae* extract 250 mg/kg bwt; and Group 5: diabetic rats administered *P. cornucopiae* extract 500 mg/kg bwt. The treatments lasted for 4 weeks.

Induction of diabetes with alloxan

The rats in group 2, 3, 4, and 5 were fasted overnight and an intraperitoneal injection of aqueous alloxan monohydrate (150 mg/kg bwt; CAS 3237-50-1) was dissolved in physiological saline (0.9% NaCl) and administered to the appropriate rat groups at a dose of 150 mg/kg/bwt for 14 days to induce diabetes [32]. The non-fasting blood glucose (FBG) levels of the rats in group 1 were checked after 7 days with the aid of a modern glucometer (Glucoplus Inc.). Rats with blood glucose level above 200 mg/dl were considered to be diabetic. The NC rats were allowed to have access to food and *ad libitum*. Weekly fasting glucose level was determined throughout the duration of the experiment at intervals of one day.

Choice of diabetogenic agent

Briefly alloxan and streptozotocin (STZ), are commonly employed as diabetogenic agent in diabetes studies. Though their beta cell cytotoxicity is accomplished through different routes, their selective mechanisms of action on pancreatic beta cell are alike (Fig. 1) [33]. Reports show that various authors have used alloxan to induce type 2 diabetes [33-36] and it has been reported that both alloxan and STZ can also be used to induce type 1 diabetes [34]. Therefore, both alloxan and STZ are used for the induction of type 2 diabetes [37, 38], but alloxan was used in the present study because of its availability [39] and type 2 diabetes was successfully induced.

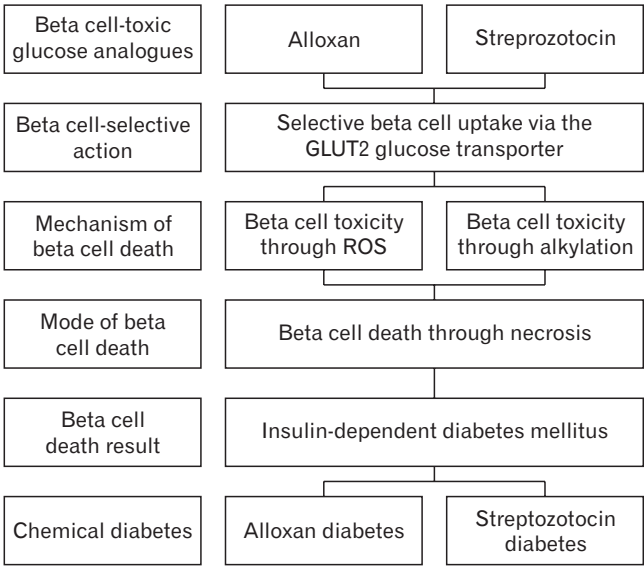


Fig. 1. Pathways of toxicity alloxan and streptozotocin in beta cells, leading to diabetes induction. Adapted from Lenzen. Diabetologia 2008;51:216-26, with permission [33].

Animal sacrifice and sample collection

At the end of the treatment, the rats were fasted for 24 hours and were then euthanized with chloroform by placing each in isolated air tight glass jar containing chloroform. Each unconscious rat was placed in a supine position and pinned firmly by its four limbs to the dissecting board and the abdominal region was incised using surgical blade. The blood samples were collected through the orbital sinuses by an intra-orbital puncture using well-labelled plane heparinized capillary glass tube. Blood samples of about 4-ml were collected from each rat and centrifuged (Model: Axiom centrifuge 80-2; Axiom medical Ltd.) at 4,000 rpm for 10 minutes and the sera were obtained for biochemical analysis. The kidneys were harvested and fixed in 10% formalin for histopathology and immune chemical analysis.

Laboratory assessment of oxidative stress makers

Analysis of catalase

The activity of catalase (CAT) was assayed following the method of Sinha [40]. Dichromate in acetic acid was reduced to chromic acetate when heated in the presence of hydrogen peroxide with the formation of per chromic acid as an unstable intermediate. The chromic acetate formed was measured at 590 nm. CAT was allowed to split H₂O₂ for different periods of time [40]. The reaction was stopped at different time intervals by the addition of dichromate acetic acid mixture and the remaining H₂O₂ was determined by measuring chromic acetate in a calorimeter after heating the reaction mixture [33]. Serum was pipetted into a tube containing 0.9 ml of phosphate, 0.1 ml of plasma and 0.4 ml of H₂O₂ added. The reaction was observed for 15, 30, 45, and 60 seconds respectively by adding 2-ml of dichromate acetic acid mixture. The tubes were kept in a boiling water bath for 10 minutes, cooled and the colour developed was read at 530 nm. Standards in the concentration range of 20–100 micro moles were processed for the test. One unit of CAT activity was expressed in Umol/ml of plasma (U-micro moles of H₂O₂ utilized/second) [41].

Analysis of superoxide dismutase

Superoxide dismutase (SOD) activity was measured according to the method of Fridovich [42]. The principle of the assay was based on the ability of SOD to inhibit the reduction of nitro-blue tetrazolium. Adrenalin (0.01 g) was dissolved in 17 ml of distilled water and 0.1 ml of serum and 0.9

ml of phosphate buffer (pH 7.8) were taken in triplicates in 2.5 ml buffer. A volume of 0.3 ml adrenaline solution was added and mixed inside the cuvette. The absorbance was taken at 480 nm at 30 seconds interval for 5 times. The changing rate of absorbance was used to determine SOD activity and was expressed as units/mg protein [42].

Analysis of malondialdehyde

Malondialdehyde (MDA), an index of lipid peroxidation was determined using a descriptive colorimetric method described by Buege and Aust [43]. The principle was based on the reaction of MDA with thiobarbituric acid producing thiobarbituric acid reactive substance (TBARS), a pink chromogen, which was measured with a spectrophotometer at 532 nm. MDA was calculated using molar extinction coefficient for MDA TBA-complex of $1.56 \times 10^5 \text{ M}^{-1} \text{ CM}^{-1}$ and its concentration was expressed as micromole of MDA/gm kidney tissue.

Analysis of glutathione peroxidase

Glutathione peroxidase (GPx) activity was determined spectrophotometrically as described by Ubhenin et al. [44]. The principle was based on the reactions of glutathione reductase and NADPH. The oxidized glutathione was converted to the reduced form with a simultaneous oxidation of NADPH to NADP⁺. Thereafter, GPx activity was measured at 340 nm by the decrease of NADPH absorbance using extinction coefficient of 6.22 nm expressed in unit/mg-protein.

Assessment of renal function test

Serum urea (normal range; 5–20 mg/dl or 1.8–7.1 mmol/L)

It was determined by the colorimetric method using ddH₂O (50 µl) in a new Gain calibration in cuvette mode. Urea was heated with biacytl monoxime in acid solution. The mixture was incubated for 15 minutes at 20°C–25°C into the Rx Monza Flowcell holder and read press for calibration within 30 minutes. Measurement of absorbance of sample (A_{sample}) and standard (A_{standard}) against reagent blank was done within 30 minutes. Urea exhibit a yellow coloration. Serum creatinine was determined by colorimetric method using ddH₂O (50 µl) in a new Gain calibration in cuvette mode. Picric acid (2 ml solution) and 1 ml. of the sodium hydroxide solution were added to 2 ml. of a solution containing 20 µg of creatinine. The mixture was allowed to stand until the maximum color had developed. It was mixed into the Rx Monza

Flowcell holder and read press for calibration at a temperature of 37°C. For calibration to take place, absorbance A_1 and A_2 were read after 30 seconds and 2 minutes respectively. Calculation was done using Randox Calibration Serum Level 3. The color developed in both the standard solution and in the reagent blank was determined.

Serum electrolytes

This involved the assessment of Sodium, potassium, chloride and calcium levels using appropriate techniques. This was determined using sodium assay kit (colorimeter) (ab211096) with a sensitivity of 25 µM on a platform of microplate reader. Here, sodium ions present in the assay are used by enzyme β-galactosidase to produce an intermediate product which reacts with a developer to generate a color signal at 405 nm.

Potassium was determined when potassium tetraphenylborate was formed by the reaction between potassium and sodium tetraphenylborate.

Direct method based on a modification of the colorimeter method of Skeggs and Hochstrasser [45] was used to determine chloride level. In this method, chloride ions form a soluble, non-ionized compound with mercuric ions and will displace thiocyanate ions from non-ionized mercuric thiocyanate. The released thiocyanate ions react with ferric ions to form a color complex that absorbs light at 480 nm. The intensity of the color produced is directly proportional to the chloride concentration.

Calcium was evaluated by colorimetric method described by Gran [46]. It involves a convenient and rapid estimation of 5-15-µg Ca in 0.1 ml of blood serum. N-hydroxy-naphtalene-1,8-dicarboxylic acid imide forms an insoluble salt with Ca, which was further mixed into solution by the addition of ethylene diamine tetra-acetic acid in excess. Two moles of N-hydroxy-naphtalene-1,8-dicarboxylic acid imide will be equivalent to one mole of Ca.

Immunohistochemistry

Five-µm thickness of kidney tissue was obtained following tissue processing and routine paraffin preparation for histology. Post paraffinization sections were allowed to heat-mediate antigen retrieval in citrated-based solution, pH 6.0, and endogenous peroxidase blocking was immediately performed in 0.3% hydrogen peroxide. Thereafter, incubation took place overnight at 4°C in primary rat antibodies; Nrf2 (#ab31163; Abcam) at 1:200 and 1:100 dilutions respectively.

Secondary incubation was performed in ImmPRESS™ horseradish peroxidase (HRP) Anti-Rat IgG (Peroxidase) Polymer Reagent, made in horse (Vector #MP-7401). 3,3'-Diaminobenzidine (DAB) peroxidase (HRP) Substrate Kit (Vector #SK-4100) was used for colour development, and sections were counter-stained in Harris haematoxylin. Positive control sections were similarly processed without primary antibody incubation. Quantification of immunoreactivity was performed using the ImmunoRatio plugin on ImageJ software (NIH) which separates and quantify percentage of DAB (positive immunoreactivity) by digital color deconvolution [47].

Histology

The fixed kidneys were removed from the 10% formalin fluid and dehydrated using ascending grades of alcohol. The dehydrated tissues were cleared in 2 changes of xylene for 2 hours; The cleared tissues were infiltrated by immersion in molten paraffin wax to allow solidification. The embedded tissues were blocked in rectangular blocks and then the blocks were cut in 5 µm serial sections using a rotary microtome. The tissue sections were allowed to float on water at 30°C to help the spreading of the paraffin ribbons. Tissue sections were placed on clean glass slides. The slides were then left to dry and later stained using hematoxylin and eosin (H&E). The stained tissue was observed with a light

microscope. Photomicrographs of these sections were taken with camera (DP74; Olympus Corporation) attached to light microscope (CX21i/MX21i; Chongqing MIC Technology Co., Ltd.).

Statistical analyses

The data was expressed as mean±standard error of the mean, and statistical significance between two groups were considered when *P*<0.05. Comparison between groups was made by two-way analysis of variance (ANOVA) followed by *post-hoc* Tukey test. Statistical Package of Social Sciences (SPSS) version 23.0 (IBM Co.) was used.

Results

The result showed that *P. cornucopiae* found in our locality contains saponin, flavonoid and tannin (Table 1). Table 2, presents the mean body weight of the animals and showed incremental increase in weekly mean body weight of the rats in all the groups, which was significant (*P*<0.05) only in group 1 (untreated control) in the 2nd, 3rd, and 4th weeks of experiment (Table 2), but there was no significant change in body weight across the treated groups (3, 4, and 5) in comparison with the normal or DC groups (groups 1 and 2).

The results of the FBG levels are presented in Table 3. Variations were recorded on blood glucose levels in all the treated rat groups against the normal (untreated) and positive (diabetic) control groups. The 500 mg/kg/bwt, dose of the extract non-significantly reduced blood glucose level at week 3 compared against glibenclamide and 250 mg bwt of extract. However, both glibenclamide and the extract lowered the blood glucose level of the treated diabetic rats (groups 3, 4, and 5) to similar levels at the end of week 4 (Table 3). This FBG levels were, however, significantly lower compared against the DC group (*i.e.*, group 2).

Tables 4 and 5 show the biochemical analysis of oxida-

Table 1. Qualitative analysis of aqueous extract of *Pleurotus cornucopiae*

Parameters	Water
Alkaloid	-
Saponin	+
Flavonoid	+
Steroid	-
Tannin	+
Glycosides	-
Phenol	-

-, absence; +, presence/trace.

Table 2. Weekly mean weight (g) of male Wistar rats with alloxan induced diabetes, and treated with glibenclamide and mushroom (*Pleurotus cornucopiae*)

Weeks	Control (group 1)	Diabetic control (group 2)	Diabetes+glibenclamide (group 3)	Diabetes+250 mg/kg <i>P. cornucopiae</i> (group 4)	Diabetes+500 mg/kg <i>P. cornucopiae</i> (group 5)
Week 1	156.6±2.11	212.7±20.62	214.8±17.41	176.3±8.19	187.8±10.91
Week 2	195.5±2.72*	197.6±23.97	212.8±19.36	172.2±4.73	199.7±14.62
Week 3	206.3±4.13*	226.3±34.52	237.8±19.50	178.8±10.31	202.8±14.82
Week 4	206.4±15.53*	250.8±43.85	264.8±15.33	181.6±22.37	209.1±12.02

Values are presented as mean±standard error of the mean. Comparison of weekly mean weights, values in the same row with same superscripts are not significantly different (*P*>0.05). *Asterisks indicates significant difference in mean weight of control group animals in weeks 2, 3, and 4 compared with that of week 1.

Table 3. Fasting glucose level (mg/dl) of male diabetic Wistar rats treated with glibenclamide and mushroom (*Pleurotus cornucopiae*)

Weeks	Control (group 1)	Diabetic control (group 2)	Diabetes+glibenclamide (group 3)	Diabetes+250 mg/kg <i>P. cornucopiae</i> (group 4)	Diabetes+500 mg/kg <i>P. cornucopiae</i> (group 5)
Week 1	68.20±7.61	76.20±5.51	66.00±3.80	70.60±5.98	81.20±10.91
Week 2	101.80±7.11	211.20±29.16 ^b	259.20±44.41 ^b	315.50±72.60 ^a	371.00±92.93 ^a
Week 3	93.50±5.49	360.70±95.92 ^c	313.60±74.28 ^c	354.00±75.75 ^c	292.50±68.07 ^c
Week 4	85.00±7.38	406.30±63.83 ^b	229.20±48.04 ^a	234.30±59.84 ^a	229.80±50.68 ^a

Values are presented as mean±standard error of the mean. ^{a,b,c}Shows significant difference across rows in the groups. In all treatment groups: values in the same row with different superscripts (^a vs. ^b) are significantly different ($P<0.05$, $P<0.001$). Whilst values in the same row with similar subscripts are not significantly difference ($P>0.05$). In week 3, there is relative decrease in all treated groups compared to group 2. FBG level in groups 2, 3, and 4 significantly decreased relative to group 2 ($P<0.001$) in week 4. FBG, fasting blood glucose.

Table 4. Biochemical analysis of oxidative stress biomarkers of male Wistar rats with alloxan induced diabetes, and treated with glibenclamide and *Pleurotus cornucopiae*

Biochemical variables	Control (group 1)	Diabetic control (group 2)	Diabetes+glibenclamide (group 3)	Diabetes+250 mg/kg <i>P. cornucopiae</i> (group 4)	Diabetes+500 mg/kg <i>P. cornucopiae</i> (group 5)
CAT (U/m)	691.40±74.97	922.70±21.99 ^a	740.80±61.81 ^a	799.40±153.30 ^a	810.4±70.97 ^a
GPx (U/m)	72.50±8.04	64.17±0.01 ^a	50.83±5.20 ^a	59.31±3.51 ^a	64.17±3.37 ^a
MDA (U/m)	0.253±0.01	0.287±0.01 ^a	0.267±0.03 ^a	0.296±0.03 ^a	0.246±0.01 ^a
SOD (U/L)	82.84±5.84	49.75±14.36 ^a	73.08±13.05 ^b	117.20±28.98 ^b	99.50±43.09 ^b

Values are presented as mean±standard error of the mean. For all parameters: values in the same row with similar ^a are not significantly different ($P>0.05$). Whilst values with superscript ^b show significantly different ($P<0.05$) to diabetic control. CAT and GPx in treatments groups 3, 4, and 5 are relatively decreased to diabetic group. In SOD, treatments groups with *P. cornucopiae* (groups 4 and 5) show significant increased relatively to group 2 ($P<0.001$). CAT, catalase; GPx, glutathione peroxidase; MDA, malondialdehyde; SOD, superoxide dismutase.

Table 5. Renal function test of male Wistar rats with alloxan induced diabetes, and treated with glibenclamide and mushroom (*Pleurotus cornucopiae*)

Renal function test	Control (group 1)	Diabetic control (group 2)	Diabetes+glibenclamide (group 3)	Diabetes+250 mg/kg <i>P. cornucopiae</i> (group 4)	Diabetes+500 mg/kg <i>P. cornucopiae</i> (group 5)
Creatinine (mg/dl)	0.358±0.10 ^a	0.559±0.04 ^a	0.377±0.12 ^a	0.256±0.07 ^a	1.313±0.56 ^b
Urea (mm/L)	1.661±0.17 ^a	3.260±1.18 ^a	2.199±0.58 ^a	3.610±1.03 ^a	1.328±0.07 ^b
K ⁺ (ppm)	4.515±1.02 ^a	4.447±0.94 ^a	6.456±2.18 ^a	4.536±2.19 ^a	6.677±0.07 ^a
Na ⁺ (ppm)	60.00±14.43 ^a	82.50±25.96 ^a	58.18±16.16 ^a	89.09±25.01 ^a	49.74±12.08 ^c
Ca ⁺ (ppm)	0.922±0.42 ^a	0.508±0.23 ^a	0.836±0.39 ^a	0.696±0.32 ^a	1.282±0.48 ^b
Cl (m/L)	0.584±0.16 ^a	0.581±0.07 ^a	0.468±0.11 ^a	0.571±0.09 ^a	0.539±0.08 ^a

For all the parameters: the values in the rows and columns with subscript ^{a,b,c} show no significant difference ($P>0.05$) in the mean serum creatinine, urea and electrolytes of the rats in control and treatment groups.

tive stress biomarkers, and renal functions markers as well as serum electrolyte levels receptively. In Table 4, there were no significant decrease or increase in CAT, GPx, and MDA activities in treated groups (*i.e.*, groups 3, 4, and 5) compared with DC (*i.e.*, group 2), but significant increases ($P<0.05$) in mean SOD activity were recorded in groups 3, 4, and 5, with highest increase in 250 mg/kg bwt of *P. cornucopiae* compared with DC (*i.e.*, group 2) (Table 4). Table 5, showed no significant increase in the mean serum creatinine, urea and electrolytes (Na⁺ and K⁺) in the normal (untreated group 1) and DC (*i.e.*, group 2) relative to the treated groups (*i.e.*, groups 3, 4, and 5). However, there was significant different ($P<0.05$) at 500 mg dose level. The extract at 500 mg/kg bwt non-significantly increased plasma creatine, potassium

and calcium levels relative to DC (group 2) as well as groups 3 and 4 (the glibenclamide and 250 mg/kg *P. cornucopiae* treated groups). Further, while the 250 mg/kg *P. cornucopiae* treatment non-significantly increased plasma urea against the control groups (*i.e.*, 1 and 2) and Glibenclamide treated group (group 3), the 500 mg/kg *P. cornucopiae* reduced the urea and sodium levels against all groups. Generally, plasma level of chloride was not affected. Also, the 500 mg/kg *P. cornucopiae* activated Nrf2 expression more than glibenclamide and 250 mg/kg bwt of *P. cornucopiae* treated rat group (*i.e.*, groups 3 and 4; Fig. 2).

The histology analysis using H&E routine tissue stains are present in Fig. 3. The results show severe degeneration of renal tissue with severe atrophy of the glomeruli, intra renal

hemorrhage and severe intra renal inflammation were seen in DC rats (group 2; Fig. 3B) which effects were ameliorated in the treated groups (groups 3, 4, and 5; Fig. 3C–E). The histochemical evaluation of the effects of the treatment using Nrf2, presented in Fig. 4, showed that the 500 mg/kg *P. cornucopiae* activated Nrf2 expression more than the treated groups (i.e., groups 2, 3, and 4). The quantitative analysis is confirmed through the use of ImageJ software (Fig. 2). The



Fig. 2. Fruiting bodies of *Pleurotus cornucopiae* grown at animal house, AE-FUNAI.

graph shows the percentage immunoreactivity of Nrf2 expression in male Wistar rats with alloxan induced diabetes, and treated with glibenclamide and mushroom (*P. cornucopiae*); control (group 1) 13.4%, (group 2) alloxan treated (DC) 30%, (group 3) alloxan (diabetes)+glibenclamide 56.6%, (group 4) alloxan (diabetes)+250 mg/kg *P. cornucopiae*, 54.6%, (group 5) alloxan (diabetes)+500 mg/kg *P. cornucopiae* 59.8.0%. Furthermore, all the treated groups showed increased expression of Nrf2 compared to the control, while the treated groups (groups 2, 3, and 4) showed non-significant differences in the expression of Nrf2 against each other, but group 5 (the alloxan [i.e., diabetic]+500 mg/kg *P. cornucopiae*) showed significant difference against the rest treated groups (groups 2, 3, and 4; $P < 0.05$).

Discussion

Diabetes is a significant public health problem that is expensive to treat and leads to substantial morbidity and mortality [48] particularly in developing countries with limited health care facilities and very poor accessibility. Diabetes has

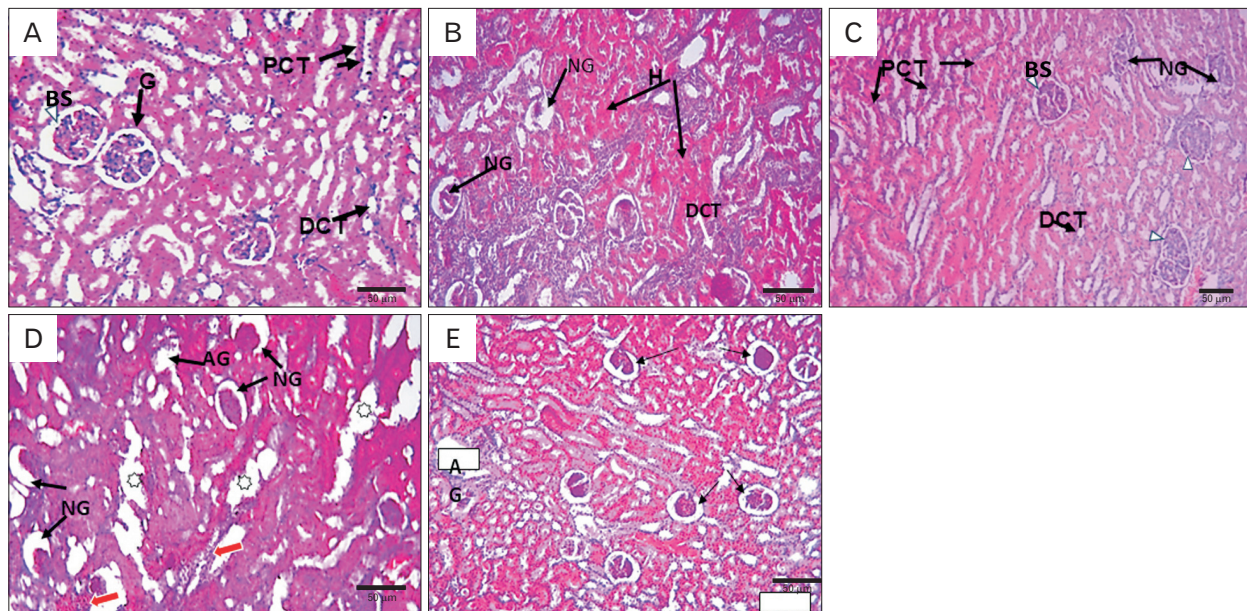


Fig. 3. Photomicrograph of the rat kidney treated with the kidney sections are shown in 10 $\times$ magnification stained with haematoxylin and eosin. Group (A) control with normal kidney histology with glomerulus (G), intact Bowman's capsule, proximal convoluted tubules (PCT), distal convoluted tubules (DCT). No capillary congestion, haemorrhage, and interstitial damage. (B) Diabetic group with necrotic glomeruli (NG), generally distorted renal tubular arrangement, undefined proximal, distal convoluted tubules, interstitial damage, capillary congestion, and hemorrhages (H). (C) Standard drug group with white arrowheads showing urinary space shrinkage, NG, normal proximal and distal convoluted tubules (PCT, DCT). (D) Low dose group with several NG, distorted proximal and distal convoluted tubules, interstitial damage (asterisk), inflammations (red bold arrows), atrophied glomerulus (AG). (E) High dose group regenerating glomeruli (black arrows) with Bowman's capsules, tubular regeneration with well-defined proximal, distal convoluted tubules and resolving interstitial damage, capillary congestion, and hemorrhages, showing protection in this group. BS, Bowman's space.

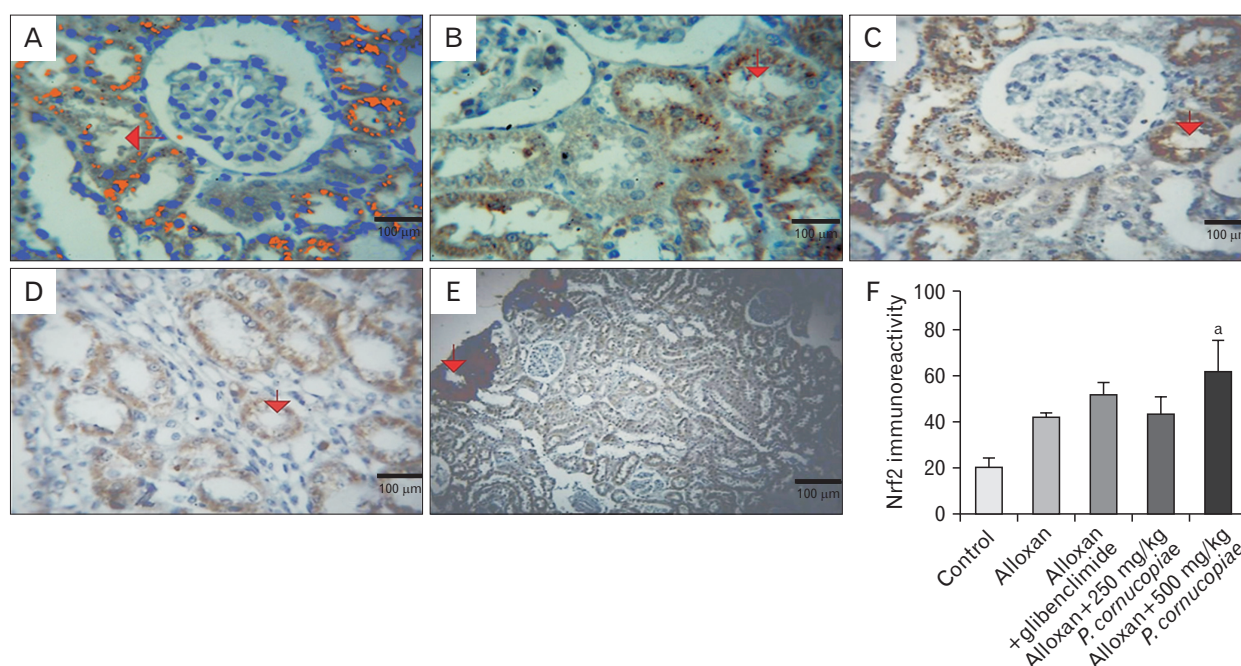


Fig. 4. Immunohistochemistry photomicrographs (500× magnification) of Nrf2 expression in kidney tissue of male wistar rats in diabetes, and treated with glibenclamide and mushroom (*Pleurotus cornucopiae*) magnification ×400. (A) Control (group 1), (B) diabetic control (group 2), (C) diabetic rats + Glibenclamide (group 3), (D) diabetic rats + 250 mg/kg *P. cornucopiae* (group 4), (E) diabetic rats + 500 mg/kg *P. cornucopiae* (group 5). Red arrow shows the expression of Nrf2. Appearance of brown yellow staining in cytoplasm of renal tubular cells in group 1. Positive nuclear staining was observed in groups 3, 4, and 5 indicating regeneration of glomeruli (red arrows) with Bowman's capsules, tubular regeneration with well-defined proximal, distal convoluted tubules and resolving interstitial damage, capillary congestion and hemorrhages, showing protection in this group. An indication regeneration of glomeruli (red arrows) with Bowman's capsules, tubular regeneration with well-defined proximal, distal convoluted tubules and resolving interstitial damage, capillary congestion and hemorrhages, showing protection in this group. ^aStatistically ($P < 0.05$) significant mean difference with normal control.

been reported as a disease of lifestyle [49, 50] and uncoordinated lifestyle is commonplace in rural life, with predominant sedentary lifestyle and very high carbohydrate/calorie diet. Additionally, these rural settings are plagued with very poor quality education both formal and informal, which has contributed to a rise in this non-communicable disease [51-53]. While the prevalence of diabetes is high in Nigeria [7, 54], antidiabetic drugs are unavailable, inaccessible and expensive [55]. The efforts of the Nigerian government to ensure the availability of antidiabetic drugs yielded no desirable results because of poor distribution system, poor quality control and the fundamental bureaucratic nature of the administration. However, the results of the present study showed that this locally available and accessible edible plant mushroom (*P. cornucopiae*) possess some good antidiabetic properties which could be useful in reducing the high prevalence of diabetes in the locality and beyond.

Contrary to the findings of some authors [56, 57], there was a significant increase in body weight of rats throughout

the period of study. Systemic manifestation of diabetes complications often present as weakness, polyphagia, polydipsia and body weight loss, which could be as a result of degeneration of the adipocytes and muscle tissues [58]. Similarly, the studies of Dawang et al. [59]; Akunna et al. [60] reported an increase in body weight of the experimental rats, which was attributed to active growth phase, but the significant increase in body weight in the present study could be attributed to the short period of treatment before the onset of weight loss.

The rats that received *P. cornucopiae* or glibenclamide had reduced FBG levels at week 4 of treatment when compared with the DC group, suggesting a gradual anti-hyperglycemic effect of both treatments. Further, the result corroborates with the study of Chen et al. [61] which suggested that *P. cornucopiae* has hypoglycemic properties and could stimulate repair of renal architecture. Although, the extract showed optimum activity in the current study at the dose of 500 mg/kg, the ability of both doses of *P. cornucopiae* to enhance antioxidant activity was observed by the increased SOD in

groups treated with *P. cornucopiae* of different doses. SOD is an oxidative marker that plays an important role in restoring multiple cellular pathways associated with damage of important biomolecules and subcellular structures in cells by oxidative stress. We clearly observed an improved glucose tolerance in the rats treated with *P. cornucopiae* suggesting a reversal of hyperglycemia (lowered FBG) which is similar to the studies by Erukainure et al. [62] and Oboh et al. [63].

Diabetes speed-up activities of enzymes in the pathways of glycogenolysis, gluconeogenesis and glycolysis [64-66], and MDA (marker of oxidative damage) is generated by peroxidation of membrane polyunsaturated fatty acids [67]. SOD is an enzyme that alternately catalyzes the dismutation (partitioning) of the superoxide radical into ordinary molecular oxygen and hydrogen peroxide [68]. Thus, SOD is an important antioxidant defense in nearly all living cells exposed to oxygen [69, 70], the current study revealed that glibenclamide and the 250 mg/kg bwt extract did not influence CAT and GPx activities, but appreciably increased SOD and CAT activities more than glibenclamide and 500 mg/kg dose in diabetes. This implies that *P. cornucopiae*, at appropriate dose possess antidiabetic properties and contribute significantly reduce oxidative associated with diabetes.

The result revealed the presence of saponin, flavonoid and tannin in the extract and saponins have hemolytic properties [71]. Tomato saponin, alpha-tomatine could induce apoptosis and eradicate abnormal and potentially harmful cells [72]. It is possible that the interaction of saponins and other bioactive constituents of *P. cornucopiae* could potentiate the regeneration of renal tissues, which could explain the observed changes in histopathology.

The result also revealed that extract at 500 mg/kg bwt reduced the plasma urea and sodium concentration in rats with diabetes. These findings were similar to the observations of Hong et al. [73]; Dere and Polat [74]; Vuksa et al. [75]; Attia and Nasr [76]. Kidney failure is associated with increase in plasma creatinin and urea levels, which is due to increase in production of free radicals that exacerbates the nephrodegenerative process of deteriorating renal enzymes as reported by Samai et al. [77]; Akinloye et al. [78] and Smith et al. [79]. Oral administration of the extract at 500 mg/kg body weight for 21 days enhanced renal enzymes and reversal of the renal damage [80].

Nrf2 is an emergent regulator of cellular resistance to oxidants. Numerous mouse genetic studies have demonstrated the beneficial effects of Nrf2 activation on the prevention of kidney disease [81]. The presence of high expression of Nrf2

treated groups D and E, may signal antioxidant regulation across the treated groups, when compared to the DC group. Oxidative stress and inflammation caused by diabetes could lead to glomerular filtration rate decline and severe inflammation of the kidney [82, 83]. In addition, Nrf2 regulates cellular defense against toxic and oxidative insults through expression of genes involved in oxidative stress response and drug detoxification [84, 85].

In conclusion, the drug glibenclamide and *P. cornucopiae* have appreciable hypoglycemic activity and potential to restore the renal architecture of the rats kidney. Additionally, the extract (at 500 mg/kg bwt) activated the activities of SOD and Nrf2 expression more than glibenclamide in diabetic rats suggesting better efficacy.

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Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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