

ORIGINAL CONTRIBUTION

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Carbon tetrachloride induced hepatorenal toxicity in rats: possible protective effects of wild *Pleurotus tuber-regium*

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Abstract

Background: The economic cost of and liver and kidney diseases in Sub Saharan Africa remain prohibitive, hence the quest for medicinal foods that can reverse hepato-renal damages. The aim of this study is to investigate the hepato-renal protective effect of wild edible *P.tuber-regium* on carbon tetrachloride (CCl₄) induced oxidative stress in male *Sprague–Dawley* rats.

Method: Thirty six rats were divided into six groups of six animals each. Group I (negative control) received 10 ml/kg olive oil intraperitoneal weekly in addition to feed and water ad libitum. Group II (positive control) received CCl₄ 10 ml/Kg (30% in Olive oil) weekly. Group III, IV and V received 100 mg, 200 mg and 500 mg wild edible *P.tuber-regium* (33.3% in feed) daily in addition to 10 ml/Kg CCl₄ weekly. Group VI received 500 mg *P.tuber-regium* (33.3% in feed) daily. After four weeks, the serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), creatinine, bilirubin, urea and fasting blood glucose were determined. Also, the histopathologic examinations of the kidney and liver were carried out.

Results: Administration of CCl₄ to rats significantly ($p < 0.05$) decreased the levels of Bilirubin (1.21 ± 0.07), Creatinine (0.91 ± 0.04) and Urea (45.76 ± 3.10 mg/dl) when compared to control (0.66 ± 0.07 , 0.76 ± 0.05 and 24.48 ± 4.70 respectively). Liver and kidney MDA from 14.00 ± 2.60 and 14.00 ± 3.50 in CCl₄ only treated groups to 8.60 ± 1.5 and 1.70 ± 0.15 $\mu\text{mol}/\text{mg}$ in the 500 mg *P.tuber-regium* treated groups. Photomicrographs also showed that *P.tuber-regium* prevented the fibrosis of the bile duct, glomeruli and tubules seen in CCl₄ group.

Conclusion: *P.tuber-regium* may be protective against the CCl₄ induced oxidative damage of the hepato-renal system.

Keywords: *Pleurotus tuber-regium*, CCl₄, Mushroom, Liver and renal biomarkers, Antioxidant histopathology

Background

Despite recent developments in medical sciences, treatment of diseases relating to liver and kidney is very expensive, relatively unavailable with high incidence of adverse effects and failure [1]. This spurs the need for a more efficient, cost effective and easily available regime to combat liver and kidney pathologies especially in the developing nations. Natural products with their secondary metabolites may be efficacious and possess antioxidant potentials that scavenge free radicals and reduce oxidative stress [2]. Edible mushrooms had been used in the treatment of different ailments

in folk medicine and possesses secondary metabolites that could treat or slow down the pathogenesis of diseases like liver and kidney pathologies [3, 4].

Pleurotus tuber-regium a fungus that grows on drill dust of the wood *Treulia Africana* (bread fruit), *Elaeis guineensis*, or *Daniella oliver* tree. It belongs to the genus basidiomycete, and is found mainly in the tropics and subtropics. It forms a large spherical to ovoid sclerotia (underground tuber) [5]. *Pleurotus tuber-regium* is a popular edible mushroom found in decaying vegetation [6] that had been attributed with many medicinal properties like anticancer, anti-inflammatory, antihypertensive, antilipidemic and antidiabetic [6–8]. Edible mushrooms are known to contain several secondary metabolites like phenolic, tannins, alkaloids, and flavonoids etc. that

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account for their diverse pharmacological activities and in some instance their antioxidant properties. Antioxidants in mushrooms are known to combat free radical mediated injuries via its unique ability to donate electrons and therefore stabilize highly reactive free radicals [9].

The aim of this study is to evaluate the hepato-renal protective activity of *P.tuber-regium* in a free radical model using rat.

Methods

Harvesting of the mushroom

Fresh fruiting bodies of wild *P.tuber-regium* were collected from a forest at the back of University of Nigeria Nsukka by a taxonomist working with the university. These fresh fruiting bodies were cleaned and air dried away from direct sunlight. The mushroom were ground and stored in a clean dry plastic container until use [10]. Little is known about bacterial contamination of the Nigerian harvested *P.tuber-regium*. *P.tuber-regium* were not tested for microbiological contaminations

Animals husbandry

Thirty six male *Sprague–Dawley* rats with body weights 180–200 g acclimatized for two weeks were maintained under controlled conditions of temperature (23 ± 2 °C) and humidity ($50 \pm 5\%$) and a 12-h light–dark cycle, were used for the experiment. The animals were housed in sanitized polypropylene cages containing sterile paddy husk as bedding. The bedding of the cages was changed daily and the cages were cleaned as well. They had free access to standard rat

pellet diet and water ad libitum. All the experimental procedures were performed according to the committee for the purpose of control and supervision of experiments on animals, norms and approved by the University of Port Harcourt Animal Ethical Committee with an approval number UNIPORT/PHARM/0133.

Carbon tetrachloride (CCl₄)

Thirty percent carbon tetrachloride (Sigma Aldrich) in Olive oil [11] was used to induce renal and hepatic damage at a dose of 10 ml/kg (i.p) [12].

Acute toxicity studies

Different concentrations of *P.tuber-regium* (50–5000 mg/kg body weight b.w.) were administered orally to male rats. These animals were observed daily for toxicological manifestations like behavioral changes, neural and autonomic toxicities, feeding pattern changes etc. There was no mortality recorded during this period even up to the dose of 5000 mg/kg [13].

Experimental design

The animals were divided into six groups with each group consisting of six animals each. The administration of CCl₄ 10 ml/kg body weight of 30% CCl₄ in olive oil was given on days 0, 7, 14, and 21 concomitantly with the daily feeding of the mushroom.

The animals were treated for 4 weeks as follows

Group I – normal control received olive oil 10 ml/kg i.p. weekly in addition to standard food and water.

Group II – Positive control received CCl₄ (30% CCl₄ in olive oil) at a dose of 10 ml/kg weekly in addition to standard feed and water.

Group III – rats were treated orally with 100 mg/kg b.w. of *P.tuber-regium* in feed (33.3% w/w) along with 10 ml/kg CCl₄ (30% in olive oil) weekly.

Group IV – rats were treated orally with 200 mg/Kg b.w. of *P.tuber-regium* in feed (33.3% w/w) along with 10 ml/kg CCl₄ (30% in olive oil) weekly.

Group V – rats were treated orally with 500 mg/kg b.w. of *P.tuber-regium* in feed (33.3% w/w) along with 10 ml/kg CCl₄ (30% in olive oil) weekly. In all CCl₄ was given intraperitoneally.

Group VI – rats were treated orally with 500 mg/kg b.w. of *P.tuber-regium* in feed (33.3% w/w) only with standard feed and water.

Necropsy

Animals were sacrificed 24 h after the last treatment. Blood was collected by retro orbital sinus puncture and serum was separated by centrifugation at 3000 r.p.m for 10mins at 4 °C for assay of biochemical parameters. Rats were sacrificed under ether anesthesia; liver and kidney were excised, rinsed clean in saline, weighed and preserved in 10% formalin for histopathological study.

Determination of biochemical parameters

Commercial reagent kits for the determination of bilirubin, cholesterol, creatinine and urea concentrations and alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), were assayed using Quimica Clinica Aplicada (QCA) Spain. Fasting blood glucose was determined using commercial glucometer made by accu-check.

Antioxidant assay

The liver and kidney were minced separately into small pieces and homogenized with ice cold 0.05 M potassium phosphate buffer (pH 7.4) to make 10% homogenates. The homogenates were centrifuged at 6000 rpm for 15 mins at 4 °C. The supernatant was collected for the estimation superoxide dismutate (SOD) and malondialdehyde (MDA) assays. Superoxide dismutate (SOD) was assayed by the method described by Misra and Fridovich

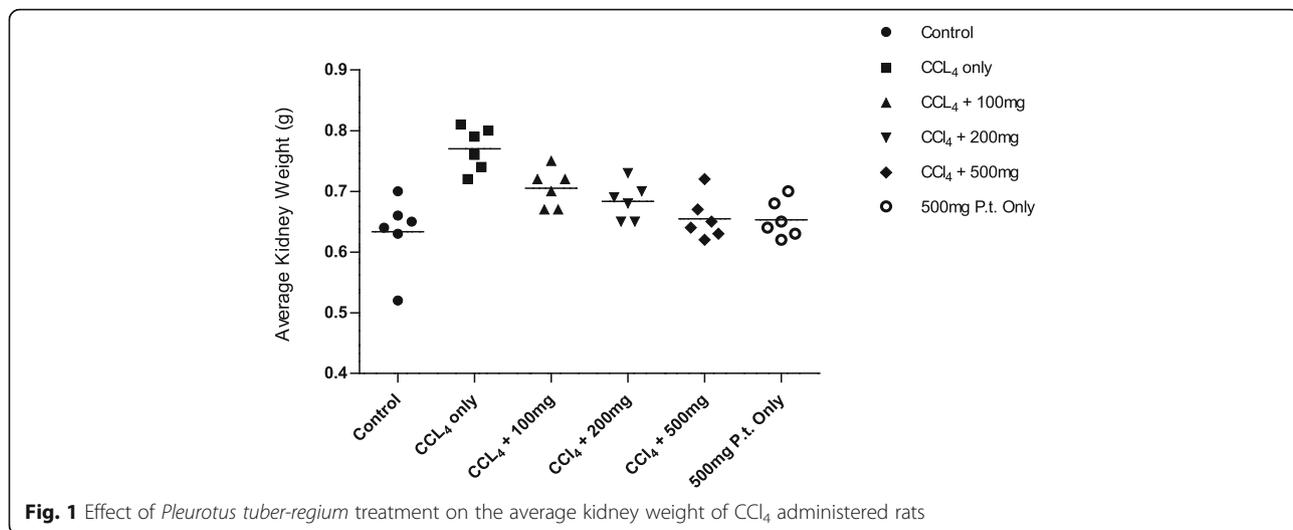


Fig. 1 Effect of *Pleurotus tuber-regium* treatment on the average kidney weight of CCl₄ administered rats

[14]. Lipid peroxidation was quantified as malondialdehyde (MDA) according to the method described by Ohkawa et al. [15]. and the MDA level was calculated according to the method of Todorova et al. [16] and expressed as μmol MDA/mg protein.

Histopathological studies

Portions of the liver and kidney from all the experimental groups were fixed in 10% formaldehyde, dehydrated in graded alcohol, cleared in xylene and then embedded in paraffin. Microtome sections (5 μm thick) were prepared from each liver and kidney sample and stained with heamtoxylin-eosin (H&E) dye. The sections were examined for the pathological findings.

Statistical analysis

Data obtained were analyzed using graph pad prism 5. The values represent means and their standard deviations. Differences between the means were determined using one way analysis of variance (one way ANOVA) followed by Bonferroni’s test. P values of 0.05 or less were considered statistically significant.

Results

Effect of *P.tuber-regium* on the liver weight of CCl₄ treated animals

Figures 1 and 2, shows the effect of the *P.tuber-regium* on the weights of the liver and kidney after administration of CCl₄. Administration of CCl₄ to the rats resulted in a significant ($p < 0.05$) increase in the absolute (0.77 ± 0.04)

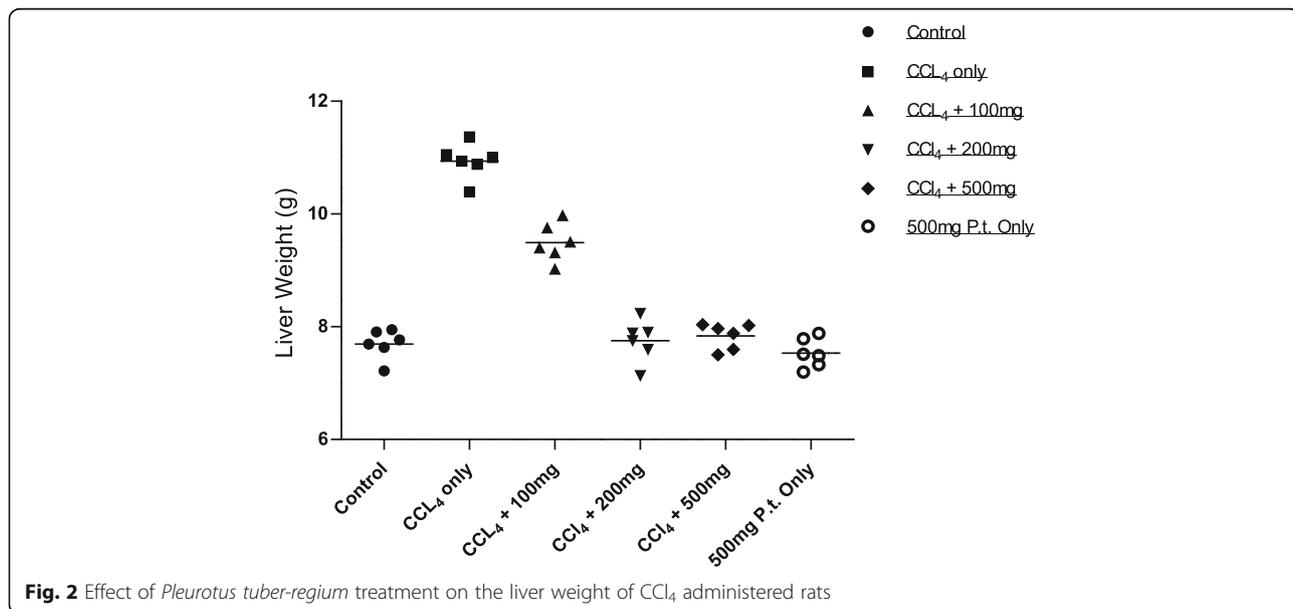


Fig. 2 Effect of *Pleurotus tuber-regium* treatment on the liver weight of CCl₄ administered rats

Table 1 Effect of *P.tuber-regium* on ALT, AST and ALP

Treatment	ALT (iu/L)	AST (iu/L)	ALP (iu/L)
Control	26.85 ± 2.63	75.68 ± 5.20	293.10 ± 15.15
CCl ₄ Only	42.82 ± 2.70 ***	92.97 ± 6.34 **	421.10 ± 12.89 ***
CCl ₄ + 100 mg P.t.	34.67 ± 1.34	83.98 ± 5.18	352.90 ± 15.44
CCl ₄ + 200 mg P.t.	29.82 ± 8.34 ##	78.47 ± 4.53 ##	331.60 ± 12.76 #
CCl ₄ + 500 mg P.t.	30.74 ± 5.53 ##	78.09 ± 8.23 ##	336.70 ± 16.22 #
500 mg P.t. Only	26.64 ± 2.45 ###	75.74 ± 4.68 ###	263.00 ± 13.92 ###

Each value represents mean ± SD, n = 6. Values marked with an asterisk (*) differ significantly from control value (** p < 0.01, *** p < 0.001) while those marked with # differ significantly from CCl₄ only group (# p < 0.05, ## p < 0.01, ### p < 0.001)

and relative weight (0.40 ± 0.04 g) of the kidneys in all the treatment groups when compared to control (absolute 0.63 ± 0.03 and relative 0.34 ± 0.01 g). Carbon tetrachloride significantly (p < 0.05) increased in both the absolute (10.94 ± 0.41) and relative weights (5.70 ± 0.21) of the liver in all the treated groups when compared to the control. There was a significant dose – dependent reduction in the weights of the liver and kidney in the rats that received CCl₄ and *P.tuber-regium*.

The effect of *P.tuber-regium* on the Liver function tests: ALT, AST and ALP are shown on Table 1. Administration of CCl₄ significantly (p < 0.05) increased the liver enzymes ALT (42.82 ± 2.70), AST (92.97 ± 6.34) and ALP (421.10 ± 12.89 iu/L) in all the treated rats when compared to control rats 26.85 ± 2.63, 75.68 ± 5.20 and 293.10 ± 15.15 iu/L respectively. In the CCl₄ plus *P.tuber-regium* treated groups 200 and 500 mg *P.tuber-regium* tended to significantly reverse the effects of CCl₄ on the ALT, AST and ALP in dose dependent fashion. Administration of *P.tuber-regium* at 500 mg only without CCl₄ showed no significant (p < 0.05) difference from the control.

Effect of *P.tuber-regium* on the Kidney function tests

Administration of CCl₄ to rats significantly (p < 0.05) decreased the serum levels of Bilirubin (1.21 ± 0.07), Creatinine (0.91 ± 0.04) and Urea (45.76 ± 3.10 mg/dl) when compared to control (0.66 ± 0.07, 0.76 ± 0.05 and 24.48 ± 4.70 respectively) (Table 2). Also, fasting blood glucose

Table 2 Effect of *P.tuber-regium* on bilirubin, creatinine urea and fasting blood glucose

Treatment	Bilirubin (mg/dL)	Creatinine (mg/dL)	Urea (mg/dL)	Fasting Blood Glucose (mg/dL)
Control	0.66 ± 0.07	0.76 ± 0.05	24.48 ± 4.70	75.67 ± 2.94
CCl ₄ Only	1.21 ± 0.07 **	0.91 ± 0.04 **	45.76 ± 3.10 ***	90.60 ± 4.62 ***
CCl ₄ + 100 mg P.t.	0.99 ± 0.04	0.82 ± 0.07	35.47 ± 11.68	79.25 ± 8.30 #
CCl ₄ + 200 mg P.t.	0.78 ± 0.02 #	0.79 ± 0.03 #	27.31 ± 7.13 ##	76.50 ± 3.79 ##
CCl ₄ + 500 mg P.t.	0.73 ± 0.09 #	0.76 ± 0.05 ##	27.88 ± 6.44 ##	76.25 ± 4.35 ##
500 mg P.t. Only	0.66 ± 0.09 ##	0.75 ± 0.06 ##	24.25 ± 4.73 ###	75.00 ± 3.92 ##

Each value represents mean ± SD, n = 6. Values marked with an asterisk (*) differ significantly from control value (** p < 0.01, *** p < 0.001) while those marked with # differ significantly from CCl₄ only group (# p < 0.05, ## p < 0.01, ### p < 0.001)

was increased significantly (p < 0.05) when compared to control from 75.67 ± 2.94 to 90.60 ± 4.62 mg/dl. Treatment with *P.tuber-regium* restored the levels of these markers in a dose dependent manner when compared to the control group. However, treatment with *P.tuber-regium* alone did not cause any significant (p < 0.05) change in the markers studied as compared to the control group.

The effect of *P.tuber-regium* on the superoxide dismutase (SOD) and malondialdehyde

(MDA) in liver and kidney is shown on Table 3. There was significant increase in the MDA of kidney 1.40 ± 0.32(control) and 14.00 ± 3.50 (CCl₄) and liver 1.90 ± 0.42(control) and 14.00 ± 2.60 (CCl₄) treated groups. The superoxide dismutase (SOD) in the liver and kidney were also significantly decreased from 7.58 ± 0.59 and 8.49 ± 0.38 in control to 1.65 ± 0.48 and 3.18 ± 0.31 µg/mg protein after CCl₄ administration. *P.tuber-regium* administration decreased the level of MDA and increased the level of SOD in the CCl₄ treated groups significantly in dose dependent manner.

Histological studies of the liver

The histological architecture of the liver in control rats showed normal and well preserved structures with the hepatocytes well arranged around a central vein with alternations of blood sinusoids. The liver cells show a membrane, a nucleus and nucleoli (Fig. 3a). Examination of the hepatocytes of CCl₄ treated rats show proliferation of bile duct epithelium with dilated and congested sinusoids (Fig. 3b). Treatment with *P.tuber-regium* reversed these changes (Fig. 3c, d and e) with the reversal being more pronounced in the higher dose of 500 mg *P.tuber-regium*. Examination of *P.tuber-regium* only group shows a well preserved and normal hepatic cells comparable the control (Fig. 3f).

Histological studies of the kidney

The histological studies of the kidney showed normal well preserved architecture of the kidney with normal glomeruli and tubules (Fig. 4a). Administration of CCl₄ markedly

Table 3 Effect of *P.tuber-regium* on the superoxide dismutate (SOD) and malondialdehyde (MDA) in liver and kidney

	Groups	Control	CCl ₄ Only	CCl ₄ + 100 mg P.t.	CCl ₄ + 200 mg P.t.	CCl ₄ + 500 mg P.t.
MDA (μmol/mg)	Kidney	1.40 ± 0.32	14.00 ± 3.50 ***	3.50 ± 0.61 ###	1.60 ± 0.36 ***	1.70 ± 0.15 ###
	Liver	1.90 ± 0.42	14.00 ± 2.60 ***	10.00 ± 1.80 ***	8.90 ± 1.00 ** #	8.60 ± 1.5 ** #
SOD (μg/mg protein)	Kidney	8.49 ± 0.38	2.78 ± 0.30 ***	3.18 ± 0.31 ***	5.31 ± 0.37 #####	6.48 ± 0.50 *** ###
	Liver	7.58 ± 0.59	1.48 ± 0.33 ***	1.65 ± 0.48 ***	3.60 ± 0.36 *** ###	3.88 ± 0.47 *** ###

Each value represents mean ± SD, $n = 6$. Values marked with an asterisk (*) differ significantly from control value (** $p < 0.01$, *** $p < 0.001$) while those marked with # differ significantly from CCl₄ only group (# $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$)

disrupted the normal architecture of the kidney cell by degenerating the tubules and collapsing the glomerulus (Fig. 4b). Treatment with *P.tuber-regium* improved these pathological changes in a dose dependent manner (Fig. 4c, d and e). Slides of the 500 mg *P.tuber-regium* only group showed well preserved architecture comparable to the control (Fig. 4f).

Discussion

Carbon tetrachloride is a reference toxicant in mammalian systems with established toxicities in the kidney,

liver, testis, heart, lungs etc. [17, 18]. Once injected into a mammalian system, it undergoes extensive biotransformation in the livers P-450 system to generate trichloromethyl radical (CCl₃[•]) and chloride radical Cl[•] which being electron deficient, have very high affinity for electrons in biological tissues. This extortion of electrons from biological systems leads to peroxidation of proteins, distortion of enzymes and DNA [19]. In this study, we tested the hypothesis that wild edible mushrooms like *P.tuber-regium* could protect against CCl₄ induced hepato-renal toxicity.

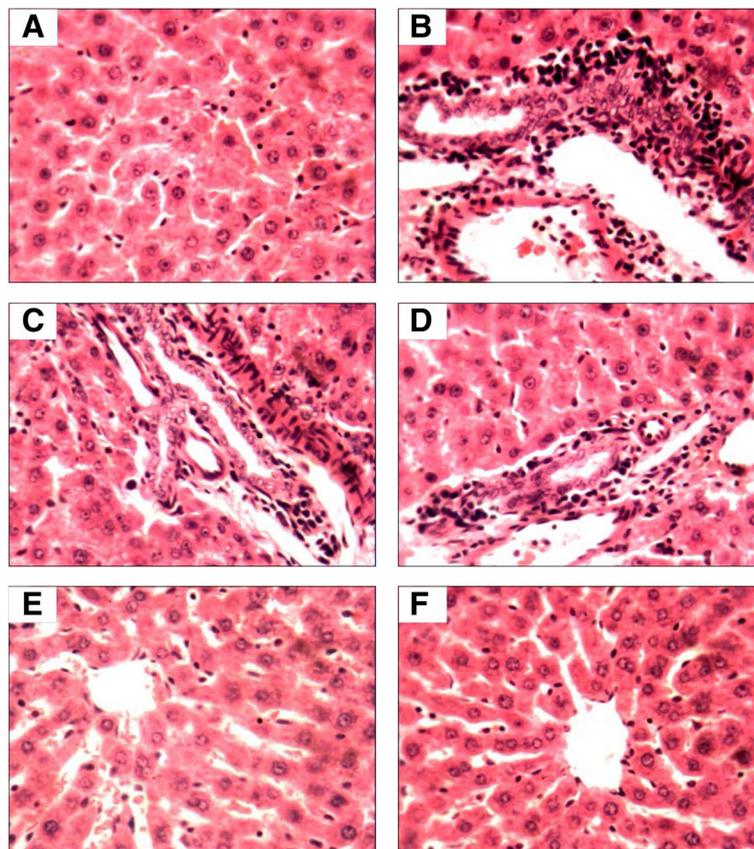


Fig. 3 Histopathology showing the alterations induced with CCl₄ and preventive effects of *P.tuber-regium* in hepatic tissues. **a** Hepatic section showing the normal architecture of the liver **(b)** CCl₄ induced injuries with severe proliferation of bile ducts and congested sinusoids **(c, d and e)** CCl₄ induced injuries and protective effect of *P.tuber-regium* in a dose dependent manner **(f)** A well preserved architecture of the hepatocytes comparable to the control

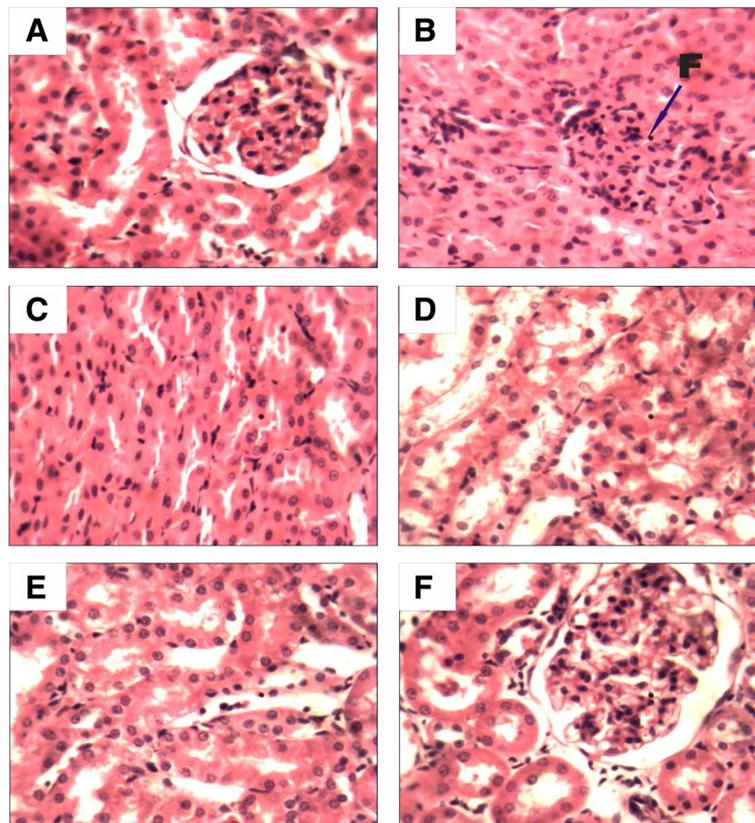


Fig. 4 Photomicrograph showing the alterations induced with CCl_4 and preventive effects of *P.tuber-regium* in nephrotic tissues. **a** Normal structure of nephrotic tissue showing well preserved glomerulus and tubules. **b** Nephrotic alterations induced with CCl_4 with pronounced tubules and glomeruli due to fibrosis (**f**). **c**, **d** and **e** alterations in the nephrotic tissue following CCl_4 intoxication and the protective effect of *Pleurotus tuber-regium* in a dose dependent manner. **f** A well preserved nephron comparable to the control

Administration of CCl_4 resulted in significant ($p < 0.05$) increases in both the absolute and relative weights of the liver and kidney when compared with the control. This increase in liver and kidney weights may be attributed to lesions and injuries associated with xenobiotics [2] like CCl_4 which peroxidizes cell proteins thereby activating the inflammatory pathway. This is evident from the photomicrographs of both the kidneys and liver that showed proliferation of the bile ducts, tubules and the glomeruli. The enlargement of the liver and kidney were significantly reduced in the treatment groups that received *P.tuber-regium* suggesting that the mushroom contains some protective phytomedicinals. This result is in agreement with other works on mushrooms in a free radical mediated injury where mushroom reduced oxidative stress mediated injury by gentamicin [2].

The treatment of rats with CCl_4 caused a significant increase in the serum levels of liver and kidney biomarkers like Bilirubin, Creatinin, Urea, Fasting blood glucose FBG, ALT, AST and ALP. The implication of these observations is possible hepato-renal dysfunction as a result of the free radicals; trichloromethyl radical

(CCl_3^\bullet), trichloromethylperoxy radical (OOCCL_3^\bullet), chloride radical (Cl^\bullet) etc. generated by the CCl_4 following biotransformation by CYP 2E1 of the liver [20]. These radicals being electron deficient react with electron rich proteins to cause peroxidation of macromolecules like the membranes with subsequent leakage of the enzymes into the serum [21]. Some of these leaked enzymes found in the serum like ALT, AST and ALP are biomarkers of liver that is elevated during hepatotoxicity. This result is agreement with other works that had recorded elevated serum levels of liver markers following CCl_4 intoxication [22]. These elevated biomarkers of liver toxicity were markedly reduced by the administration of *P.tuber-regium* in a dose dependent manner. *P.tuber-regium* is known to be rich in antioxidant molecules like phenolics, tannins, flavonoids [22] which are efficient electron donor thereby helping to terminate free radical reaction. This process spares the body's antioxidant systems and macromolecules which are the primary targets of peroxidation. The protective effect in the liver is also seen in the photomicrographs with well-preserved structures comparable to untreated control group.

The protective activity of *P.tuber-regium* seen in the liver was also seen in the kidney. The major renal biomarkers suggested significant renoprotection in a dose dependent manner after administration *P.tuber-regium*, which may be due to anti-oxidative stress mechanism [23]. The significant increase in SOD and decrease in MDA following *P.tuber-regium* administration in CCl₄ treated animals lend credence to the antioxidant potential of this mushroom and its folkloric use. The oxidation of membrane lipid bilayer affected lipid structures of the nephron which resulted in a subcellular structural damage as revealed by the micrographs of the kidney (tubular and glomerular fibrosis). These findings are similar to the protection observed with *Digera muricata* where the structural changes observed in CCl₄ group were attenuated [24]. This hepato-renal protective effect of *P.tuber-regium* may be due to the presence of antioxidant phytochemicals that may not only attenuate the toxicity of CCl₄ but may have restorative properties.

The histopathological findings have provided direct evidence of the possibility of the *P.tuber-regium* to attenuate the disruption of structure of both the liver and kidney cells. These observations complemented the results of the cytoplasmic enzymes namely (alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and bilirubin, creatinine and urea concentrations. A direct radical-scavenging activity of the *P.tuber-regium* might be involved in the hepato- and nephron- protective activity against CCl₄ exposure.

Conclusion

Taken together it may be concluded that the *P.tuber-regium* is able to combat the effect of CCl₄ exposure in albino rats, which might be attributed to its antioxidant defense mechanism.

Acknowledgements

Authors' contributions

KOO: Designed study, carried out the bench work and analysed data. IMS: Design. OEO: Designed the study, analysed data and write up. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Source of support

Nil.

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