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Effects of *Calocybe indica* Mushroom on Oxidative Stress and Hematological Alterations in Rats with Testosterone-induced Experimental Benign Prostatic Hyperplasia

[1*]Onoja, R. I., [2]Ogara, A. L., [1]Ihedioha, J. I and [1]Shoyinka, S. V. O.

[1]Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, University of Nigeria, 410001, Nsukka,
[2]Department of Science Laboratory Technology, Faculty of Physical Sciences, University of Nigeria, Nsukka, Nigeria

[*] *Author for Correspondence: remigius.onoja@unn.edu.ng*

ABSTRACT

This study was designed to evaluate the protective effect of *Calocybe indica* extract (CLE) on testosterone propionate (TP)-induced hematological changes and oxidative stress in rats. The rats were grouped into six equal groups of ten rats each as follows:(a) control, (b) TP 3 mg/kg only, (c) 3mg/kg TP + 5 mg/kg finasteride, (d) 3 mg/kg TP +250 mg/kg CLE, (e) 3 mg/kg TP+ 500 mg/kg CLE and (f) 3 mg/kg TP +1000 mg/kg CLE. The rats were administered TP subcutaneously for 28 days to induce benign prostatic hyperplasia (BPH) and simultaneously administered three graded doses of CLE, and finasteride as the standard drug. Hematological parameters, lipid peroxidation, antioxidant enzyme activities and histopathological examination of the prostate were assessed. BPH induction showed higher red blood cells (RBCs) count, haemoglobin (Hb) concentration, packed cell volume (PCV), serum prostate specific antigen (PSA), malondialdehyde (MDA) and lower white blood cell (WBC) count, lymphocyte count as well as lower catalase (CAT) and superoxide dismutase (SOD) activities. The simultaneous oral administration of CLE with testosterone injection did not significantly lower RBC count, Hb concentration and PCV but significantly lowered serum PSA and MDA. There was also a significantly higher WBC count, lymphocyte count, CAT and SOD activities. The results from this study suggest that dietary consumption of *Calocybe indica,* a mushroom with high antioxidant activity ameliorated BPH induced oxidative tissue damage and hematological alterations.

Keywords: Antioxidant; Hematology; Hyperplasia; Macrofungi; Prostate

INTRODUCTION

Benign prostatic hyperplasia (BPH) is a condition characterized by a non-cancerous proliferation of the epithelial and stromal component of the prostate gland leading to its enlargement. This leads to urinary tract obstruction, renal irritation and symptoms like frequent urination, infection and dysuria. It is the most common disease of the prostate in ageing man and dogs (Glina *et al.,* 2015; Homma *et al.,* 2017; Makchit, 2017). The severity and occurrence of BPH increases with advancing age, making about 70% of aging men between 60-70 years of age most susceptible (Langan, 2019; Madersbacher *et al.,* 2019). Although the etiology of BPH remains a puzzle, multifactorial theories such as sex hormone induced changes, polypeptide growth factor induction, inflammatory reaction, cell apoptosis and oxidative stress theory have been proposed (De Nunzio *et al.,* 2016; La Vignera *et al.,* 2016; Asiedu *et al.,* 2017; Wang and Su, 2018; Singh *et al.,* 2019 Chen *et al.,* 2020). Thus, there is a growing interest in the life sciences on the study of BPH. Despite the fact that the conversion of testosterone to its biologically active metabolite, dihydrotestosterone (DHT) by 5 alpha-reductase enzyme remains a widely agreed cause of BPH in man and dogs, oxidative stress is regarded as a major factor in the disease progression. This is important as the prostate gland is sensitive to oxidative tissue damage (Minciullo *et al.,* 2015). The levels of endogenous antioxidants in the prostate gland have also been reported to have decreased significantly in BPH (Udensi *et al.,* 2016). Recent studies on animal models of prostatic hyperplasia showed significant increase in tissue lipid peroxidation associated with a significant decrease in endogenous tissue antioxidant enzyme (superoxide dismutase and catalase) activities in rats with untreated BPH (Kalu *et al.,* 2016). Hematological evaluation is an important marker that reflects oxidative stress and inflammation in patients and BPH is reported to be associated with altered hematological indices especially red blood cells distribution width (RDW) and increased white blood cell counts (Patel *et al.,* 2010; Dong *et* *al.,*2015). However, the problem with the conventional method of BPH therapy is that it is associated with several side effects (Traish *et al.,* 2015; Lepor, 2016), and do not take care of the complications of BPH such as oxidative stress and hematological aberrations. Thus, there is increased interest in the application of active herbal medicines as a more effective and safer treatment strategy for BPH patients as most herbal medicines are effective antioxidants which seem to reduce free radical production and thus offer protection against oxidative tissue damage (Eleazu *et al.,* 2015; Mitsunari *et al.,* 2021).The potential effect of herbal medicines in ameliorating the effects of BPH has also been previously reported (Kwon, 2019; Csikós *et al.,* 2021; Akbari *et al.,* 2022).The role of mushrooms as biological antioxidant has been given much attention in recent years as several studies have emphasized the nutritional and medicinal value of mushrooms such as its anticancer, cholesterol lowering, neurotrophic, antidiabetic, antihypertensive, antimicrobial, anti-inflammatory, antioxidant, immunomodulatory, and neuroprotective properties (Wasser, 2017; Bhambri *et al.,* 2022). *Calocybe indica*, also known as milky mushroom due to its characteristic colour and wide acceptability as palatable food is a widely cultivated macrofungus. It is specifically known to have significant medicinal properties such as antioxidant, anticancer, immune stimulating, antidiabetic and hepato-protective effects (Ghosh, 2015; Subbiah and Balan, 2015; Ghosh *et al.,* 2021; Shashikant *et al.,* 2022). Although, there are few reports on the effects of mushrooms on BPH (Nahata and Dixit, 2012; Kim *et al.,* 2013; Choi *et al.,* 2019), there is little information on the activity of *Calocybe indica* against the disease. Therefore, the present study was designed to evaluate the protective effect of *Calocybe indica* on oxidative stress, as well as hematological and histological changes in testosterone induced BPH model in male albino rats.

MATERIALS AND METHOD

Ethical Statement

Ethical approval for this study was obtained from the Faculty of Veterinary Medicine Institutional Animal Care and Use Committee (FVM/UN2021/1/20) based on the experimental protocols as directed by the National Institute of Health Guide for Care and Use of Laboratory Animals (NRC, 2011).

Chemicals

Finasteride, the standard drug for the treatment of BPH and testosterone propionate (TP) for the induction of BPH were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). All other routine chemicals and reagents for the study were of analytical grade.

Animals

Sixty 10-12 weeks old healthy Sprague-Dawley outbred male albino rats (*Rattus norvegicus*) weighing between 160-180 g were used for this study. The rats were housed in cages of size 60 cm x 45 cm x45 cm with wood shavings as bedding and acclimatized at a temperature of 25±4 °C and relative humidity of 65±5% with an alternating 12 hrs light and dark cycle for two weeks. They were fed rat chow and given water *ad libitum.*

Experimental design

The rats were randomly divided based on weight into six equal groups of ten rats each as follows: (a) control, (b) TP 3 mg/kg only, (c) 3mg/kg TP + 5 mg/ kg finasteride, (d) 3 mg/kg TP +250 mg/kg CLE, (e) 3 mg/kg TP + 500 mg/kg CLE and (f) 3 mg/kg TP +1000 mg/kg CLE. Adult male rats were induced with BPH through subcutaneous administration of testosterone propionate (TP) at 3 mg/kg body weight daily for four weeks as described earlier (Jeon *et al.,* 2017) and simultaneously dosed with the various extracts of mushrooms daily for four weeks. The effective dose of finasteride for the treatment of BPH and testosterone used to induce BPH wasas previously recommended (Ko *et al.,* 2018). The volume for oral administration of PBS, finasteride, and extract was 5 mL/kg and 2 mL/kg for S.C. injection of olive oil and TP (Ko *et al.,* 2018).

Preparation of Calocybe indica extract

The fruiting bodies of the *C. indica* mushroom were purchased from a commercial farm. The samples were taken to a plant taxonomist for identification after which a voucher sample was kept in the herbarium museum of the Department of Plant Science and Biotechnology, UNN. The mushroom was dried under shade for 10 days and ground into powder. Five hundred grams (500 g) of the powdered material was soaked in 70% methanol with manual shaking at 2hrs interval for 72hrs after which it was filtered through Whatman paper (No.1) and concentrated using a rotary evaporator. The dried extract was stored in a refrigerator at 4°C until time of use.

Sample collection

At the end of the study, the rats were fasted overnight and about 4 mL of blood samples were collected from the retro orbital plexus of each rat into two sets of sample bottles, one with EDTA for hematology and another plain sample bottle in order to obtain serum for biochemical study before they were humanely sacrificed. The prostate glands were removed immediately and fixed in 10% neutral formalin solution for histopathology.

Hematological analyses

The packed cell volume (PCV) was determined using the microhaematocrit method while the erythrocyte count (EC) and total leucocyte count (TLC) was determined using the haemocytometer method (Thrall and Weiser, 2002). Differential leucocyte counts (DLC) was performed using the stained blood film (Thrall and Weiser, 2002), while haemoglobin concentration (Hb) was determined using the Drabkin's reagent assay method for Hb concentration (Higgins *et al.,* 2008).

Biochemical analysis

The serum prostate specific antigen (PSA) was estimated using a competitive enzyme immunoassay technique performed by an ELISA method according to the kit manufacturer's instruction (Elabscience®– Houston, Texas, USA). Lipid peroxidation as evidenced by the formation of thiobarbituricacid reactive substances (TBARS) and hydroperoxides (HP) was measured by the method of Ohkawa *et al.* (1979) as modified by Tsikas (2017). Catalase (CAT) was estimated as described by Hadwan (2018).Superoxide dismutase (SOD) was assayed using previously described technique (Kakkar *et al.,* 1984; Al-Sheikh and Ghneim, 2011). This was estimated using Randox diagnostic kits (Randox Laboratories, UK).

Histopathological study

The ventral prostate lobes of the prostate glands were manually processed for histopathological examination after fixing in 10% neutral buffered formalin (NBF) for 48 hrs as described by Suvarna *et al.* (2019). Photomicrographs of the sections were captured using a Motic Images plus 2.0 digital cameras (Motic China Group Ltd. 1999–2004).

Statistical analysis

Statistical analysis of the data obtained was carried out using SPSS software, version 23. Comparisons were performed using one-way ANOVA followed by Duncan post-hoc test. Data were presented as mean ± standard errors of the mean (S.E.M). Values with $p< 0.05$ were accepted as significant.

RESULTS

The RBC counts and Hb concentration was significantly ($P< 0.05$) higher in the untreated testosterone-induced BPH group compared to the control. Also, the PCV was higher in the untreated testosterone-induced BPH group but not significantly different compared to the control. There was no significant difference in RBC, PCV and Hb concentration of the groups treated with finasteride and the various doses of CLE compared to the control although these were higher than the control but lower than the values from the untreated testosterone-induced BPH group. There was a significantly ($P < 0.05$) lower total white blood cell count (WBC) which was associated with a significantly ($P < 0.05$) lower lymphocyte count in the untreated testosterone-induced BPH group B compared to the control group A, finasteride and extract treated groups. There was no significant ($P > 0.05$) difference in the absolute neutrophil count across the groups but the absolute neutrophil count of the untreated testosterone-induced BPH group B was higher than the control. However, the absolute neutrophil count of the finasteride treated group C was higher than the values in rest of the groups. The absolute monocyte count of the testosterone model (BPH) group B was higher than that of the control. However, the absolute monocyte count of the finasteride treated group C was significantly ($P < 0.05$) higher than the values in all the experimental groups. Results showed no significant ($P > 0.05$) differences in the absolute eosinophil and basophil counts across the groups (Table 1).The PSA level of the untreated testosterone-induced BPH group was significantly ($P < 0.05$) higher than the normal control group while finasteride-treated group showed a significantly lower PSA level compared to the untreated testosterone-induced BPH group. The administration of the various doses of CLE also led to a significantly lower PSA level of the BPH rats compared to the untreated testosterone-induced BPH group. However, there was no significant difference between the PSA levels of the extract treated group and the finasteride-treated group as shown in Table 2. The untreated testosterone-induced BPH group showed a significantly ($P < 0.05$) higher MDA compared to the control group while treatment with finasteride and CLE led to a significantly ($P < 0.05$) lower MDA level compared to the untreated BPH group. Whereas the MDA level of the extract treated group was lower compared to the control, this was not dose dependent. There were no significant ($P > 0.05$) differences in the CAT activity between the groups. However, the CAT activity of untreated testosterone-induced BPH group was lower compared to the control, finasteride and extract treated groups. Although, the CAT activity of the finasteride and extract treated groups was higher compared to the untreated testosterone-induced BPH group, they were lower compared to the control. The SOD activity of the untreated testosterone-induced BPH group was significantly ($P < 0.05$) lower compared to the control group. Treatment with finasteride and CLE led to a significantly ($P < 0.05$) higher SOD activity compared to the untreated testosterone-induced BPH group though not significantly ($P > 0.05$) higher than the control (Table 2).

The histological features of the prostate gland from control rats showed normal histological architecture characterized by secretory acini of variable diameter and luminal pinkish secretions. The acini were separated by a fibromuscular stroma, blood vessels and lymphatics (Figure 1a). All these were surrounded by a capsule comprising connective tissue and a thick layer of smooth muscles. However, the untreated testosterone induced BPH model group showed hyperplastic acinar epithelium with projections into the lumen which invariably reduced the acinar lumen of the gland compared to the control (Figure 1b). The number of secretions observed in the lumen was also increased in comparison to control group. Treatment with finasteride led to a reduction in epithelial cell projections into the lumen (Figure 1c). However, treatment with the various doses of the mushroom extracts had a dose dependent restoration of normal glandular structures with mild epithelial projections into the lumen (Figure 1d-f).

Table 1: Effects of graded doses of CLE on mean hematological indices of testosterone-induced BPH rats

Groups	**A (control)**	**B (TP only)**	**C (TP+finasteride)**	**D (TP + 250 mg/kg CLE)**	**E (TP + 500 mg/kg CLE**	**F (TP + 1000 mg/kg CLE)**
PCV (%)	44.00±1.73	49.67±2.03	43.67±2.03	48.67±0.67	46.33±4.10	46.67±1.67
HB (g/dl)	12.70±0.81^{a}	16.47±0.86^{b}	14.67±0.88^{ab}	14.63±0.99^{ab}	14.47±0.83^{ab}	15.33±0.96^{b}
RBC (10^6/µl)	6.37±0.99^{a}	10.33±0.96^{b}	8.60±0.96^{ab}	8.87±1.13^{ab}	8.57±0.93^{ab}	9.17±0.79^{ab}
WBC (10^3/µl)	10.73±0.97^{a}	7.17±1.17^{b}	12.57±1.13^{a}	11.67±0.98^{a}	10.73±0.97^{a}	11.80±1.06^{a}
Neutr. (10^3 /µl	2.49±0.31	2.67±0.48	3.54±0.42	3.05±0.35	2.91±0.34	3.17±0.42
Lymph. (10^3 /µl)	7.71±0.63^{a}	3.92±0.60^{b}	7.77±0.62^{a}	8.15±0.61^{a}	7.39±0.61^{a}	8.03±0.55^{a}
Mono. (10^3 /µl)	0.35±0.06^{a}	0.37±0.05^{b}	0.82±0.06^{a}	0.26±0.01^{a}	0.29±0.06^{a}	0.35±0.07^{a}
Eosin. (10^3 /µl)	0.19±0.09	0.19±0.09	0.35±0.11	0.12±0.01	0.11±0.01	0.20±0.05
Baso. (10^3 /µl)	0.00±0.00	0.16±0.16	0.09±0.05	0.08±0.04	0.03±0.03	0.04±0.04

The values are expressed as (mean ± SEM) (n=10). [a b] different superscript in the same column indicate significant differences between the means of the groups, P < 0.05. PCV=packed cell volume; HB=Hemoglobin; WBC=white blood cells count; Neutr=neutrophils; Lymph=lymphocytes; Mono.=monocytes; Eosin=eosinophils; Baso.=basophils; TP=testosterone propionate; CLE=*Calocybe indica* extract.

Table 2: Effects of graded doses of CLE on serum prostate specific antigen (PSA), lipid peroxidation and antioxidant enzyme activities of testosterone-induced BPH rats

Parameters / Groups	PSA (ng/ml)	MDA (nmol/L)	CAT (IU/L)	SOD (IU/L)
A (control)	$1.09±0.19^a$	$15.01±0.98^a$	$80.33 ± 25.35^a$	$3.37±0.61^a$
B (TP 3 mg/kg only)	$2.61±0.12^b$	$20.22±0.20^c$	$59.74 ± 6.42^b$	$2.10±0.60^b$
C (TP+ 5 mg/ kg finasteride)	$1.90±0.11^c$	$18.01±0.20^{bd}$	$74.59 ± 20.03^a$	$3.33±0.32^a$
D (TP+250 mg/kg CLE)	$1.95±0.07^c$	$18.18±0.20^{bd}$	$67.01 ± 20.00^a$	$3.80±0.06^a$
E (TP+ 500 mg/kg CLE)	$1.80±0.09^c$	$18.72±0.52^d$	$93.22±12.96^a$	$4.50±0.61^a$
F (TP+1000 mg/kg CLE)	$2.10±0.04^c$	$16.96±0.32^b$	$69.28±5.82^a$	$4.07±0.38^a$

The values are expressed as (mean ± SEM) (n=10). [a b c d] different superscript in the same column indicates significant differences between the means of the groups, P < 0.05. MDA=Malondialdehyde; CAT=Catalase; SOD=*Superoxide dismutase*; TP=testosterone propionate; CLE =*Calocybe indica* extract.

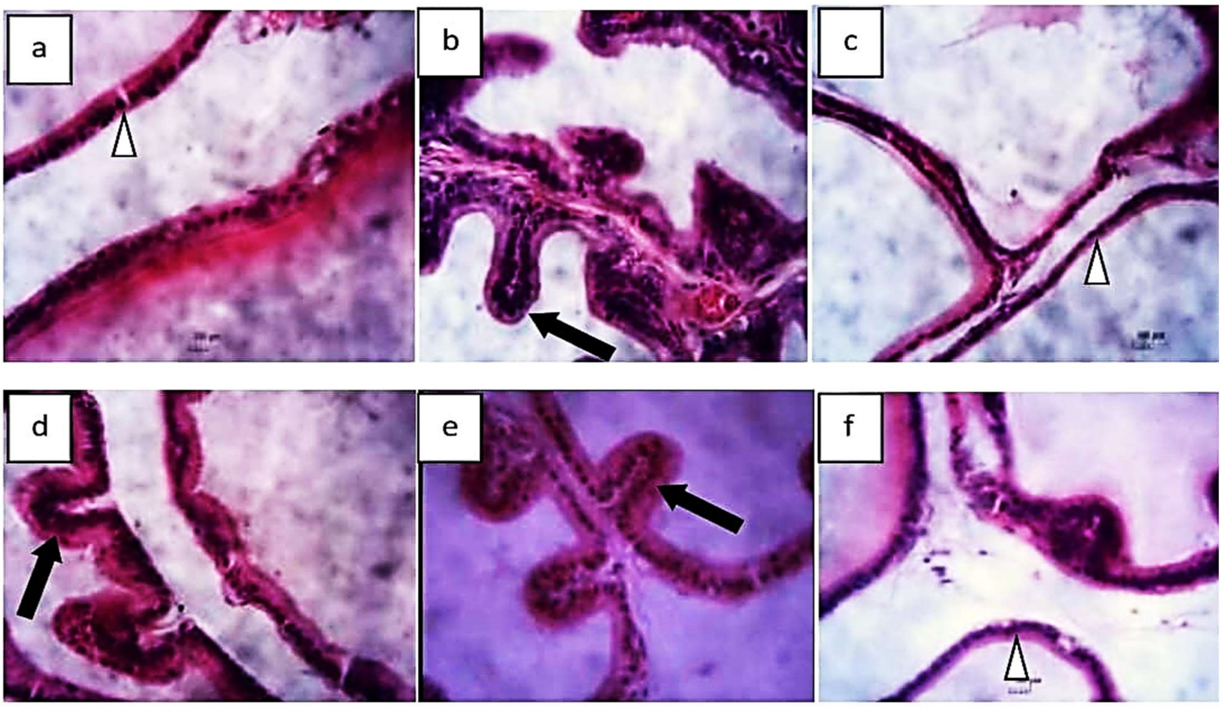

Figure 1: Photomicrograph showing the effects of graded doses of *Calocybe indica* extracts (CLE) on the prostate of testosterone-induced BPH rats: (a) control group, (b) TP at 3mg/kg, (c) TP + finasteride, (d) TP and 250 mg/kg CLE, (e) TP and 500 mg/kg CLE and (f) TP and 1000 mg/kg CLE. Note the thick epithelial infoldings in b, d and e (arrows) compared to that of a, c and f which has thin epithelial lining (arrowheads), H & E stain.400x

DISCUSSION

Benign prostatic hyperplasia in man and dogs elicits multiple biological complications through the production of reactive oxygen species in tissues (Minciullo *et al.,* 2015). In this study, the pathophysiological effects of testosterone-induced benign prostatic hyperplasia were evaluated through hematological, biochemical analyses and histopathological examinations in rats. PSA is a known biomarker for prostatic diseases which is used in the diagnoses of BPH (Kalu *et al.,* 2016). As observed in this study, CLE decreased the PSA levels in BPH rats compared to the untreated BPH model. This suggests that the extract has biological effects against the disease. The induction of testosterone induced BPH led to a higher RBC count, PCV and Hb concentration but with a lower WBC count associated with low lymphocyte count. These high RBC count, PCV and Hb are similar to previous reports on rats administered testosterone (Hassan, 2010). It is postulated that testosterone enhances the absorption of iron, its incorporation into red blood cells and hemoglobin synthesis as well as stimulation of erythropoiesis by directly affecting the bone marrow hematopoietic stem cells, through the induction of insulin-like growth factor (Delev *et al.,* 2013). Another hypothesis posited increased stimulation of the production of erythropoietin (EPO) by the kidneys (Jones *et al.,* 2015). Thus, anemia is said to be associated with reduced levels of circulating androgens (Delev *et al.,* 2013). Bachman *et al.* (2010), correlated dihydrotestosterone with increased PCV, independent of testosterone (T) and free T levels, implicating dihydrotestosterone in T-induced erythrocytosis (Aghazadeh *et al.,* 2015). All these studies suggested indirect effects of T levels on bone marrow hyperplasia without describing a clear mechanism (Ohlander *et al.,* 2018). Although, literature on the effects of androgens on hemopoietic cells other than the red blood cell lineage is scarce, the lower WBC count and lymphocyte count observed in this study may be due to the inhibitory effects of androgen on lymphopoiesis which may be mediated through androgen receptors expressed in immature B lymphocytes and in bone marrow stromal cells (Olsen *et al.,* 2001). This is in contrast to previous report of leucocytosis associated with neutrophilia following testosterone administration in men (Grossmann and Zajac, 2012). Administration of CLE did not show any significant variation in the hematological indices but there was a higher WBC and lymphocyte counts compared to the control and untreated testosterone-induced BPH rats. This may be due to the immunostimulatory properties of mushrooms as previously reported (Ghosh *et al.,* 2021). There was a significantly higher level of MDA (a marker of lipid peroxidation) and lower CAT and SOD in the untreated testosterone-induced BPH rats. This is similar to a previous report on oxidative stress in a rat model of BPH (Kalu *et al.,* 2016). The impact of the exogenous hormone administration and metabolic changes associated with the glandular enlargement may have resulted to this high lipid peroxidation (MDA) which is an important marker of oxidative stress. Catalase (CAT) and superoxide dismutase (SOD) are endogenous antioxidant enzymes whose depletion exposes the body tissues to damage by active free radicals (Ahmed Amar *et al.,* 2019). Oxidative stress is known to play a role in the pathogenesis of prostatic hyperplasia. This is due to an imbalance between endogenous antioxidants and reactive oxygen species (ROS) leading to increase in free radicals and subsequent compromise in the function of the immune system during cellular proliferation in BPH (Minciullo *et al.,* 2015; Udensi *et al.,* 2016). However, treatment with CLE lowered the MDA concentration and high antioxidant (SOD and CAT) activities of the BPH rats. The mushroom, *C. indica* is known to have high antioxidant properties (Shashikant *et al.,* 2015). Mushroom diets are rich in total polyphenols, ascorbic acid, and vitamins which could be the main antioxidant compounds containing a wide variety of free radical scavenging molecules such as polyphenolic, triterpenoids, and steroids which have a strong antioxidant activity (Sánchez, 2016). This may have resulted in the improved antioxidant activity of these enzymes and lowered lipid peroxidation biomarker ((MDA) which was also associated with the restoration of the histological changes in the prostates as observed in this study after *C. indica* extract administration. It was concluded that *C. indica* mushroom showed antioxidant and immune system enhancing potential and thus, could ameliorate BPH associated oxidative stress and blood alterations in Sprague-Dawley albino rats.

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Conflict of Interest

The authors have no conflict of interest to declare.

Authors' Contribution

RIO, conceived and designed the experiments, performed the experiments, wrote the article; JII conceived and designed the experiments, supervised the work analyzed the data; SVOS conceived and designed the experiments, supervised the work, analyzed the data; AO analyzed the data, reviewed the final manuscript.

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