Paradigm Academic Press
Journal of Innovations in Medical Research
ISSN 2788-7022
FEB. 2025 VOL.4, NO.1

Effects of Cymbopogon Citratus Aqueous Extract on Testosterone-Induced Benign Prostatic Hyperplasia in Adult Male Wistar Rats

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doi:10.56397/JIMR/2025.02.01

Abstract

Benign Prostatic hyperplasia (BPH) is a common androgen associated urologic surgical disorder among the middle-aged and elderly. Certain phytotherapeutic agents have been discovered to halt its progression and improve the symptoms without causing toxic effect on the body system. This study evaluated the effects of three different doses (low, moderate and high) of Cymbopogon citratus aqueous extract (CCAE) on testosterone induced-BPH in adult Wistar rats. Thirty-six (36) rats aged 10-12 weeks, weighing 100-120g were randomly divided into six (6) groups containing 6 rats each. Group 1 rats were given standard nutrition and drinks throughout the experiment. The rats in groups 2-6 had 10mg/kg body weight of testosterone propionate (TP) once daily. In addition to that, group 3-6 had 30mg/kg, 100mg/kg and 300mg/kg per oral once daily of CCAE and 15mg/kg of finasteride per oral once daily respectively. On the 32nd day of the experiment the rats were sacrificed using cervical dislocation. The prostates were harvested and preserved for immunohistochemistry. Immunological study showed a significant increase in Ki67 and proliferating cell nuclear antigen (PCNA) labeling scores in the positive control relative to the negative control, while there was a significant (≤ 0.05) decrease in the number of cells in post-treated CCEA group compared to positive control. Only groups 5 and 4 showed significant ($P \geq 0.05$) increase in Ki67 and PCNA labelling respectively when compared to the standard drug group. This study hence shows that CCEA exerts a protective effect on testosterone-induced benign prostatic hyperplasia in Wistar rats.

Keywords: testes, infertility, toxicity, PCNA, cymbopogon citratus, prostatic hyperplasia

1. Introduction

Benign prostatic hyperplasia (BPH) is a common androgen associated urological disorder among the elderly males (Gacci et al., 2012; Patel et al., 2014). The disorder is a non-malignant uncontrolled proliferation of the parenchyma cells of the prostate gland, in the transitional zone resulting in the enlargement of the prostate gland (Kantah et al., 2017). Symptoms of BPH include; urinary frequency, urgency, difficulty in initiating urine, poor stream, terminal dribbling and nocturia (Berry et al., 1984; Devlin et al., 2020). The prostate is a tubulo-aveolar glandular organ found just below the urinary bladder arranged in the fibromuscular stroma network. Secretion from the gland contributes one fifth to one-third (20–33.3%) of seminal volume, and it is necessary for sperm activation owing to the composition of its secretions which include citrate and enzymes such as fibrinolysin, hyaluronidase, and acid phosphate (Verze et al., 2016).

The mechanism of BPH is not clearly understood. However, several factors are believed to play an important role in its pathophysiology including hormonal and non-hormonal factors (Culig et al., 1996; Escobar et al.,

2009; Wang & Olumi, 2011; Vikram & Jena, 2012). The underlying pathogenesis of the disease involves oxidative stress (Rogers et al., 1989) and inflammation (De Nunzio et al., 2016). Whatever the triggers, the reactive oxygen species generated from the oxidative stress causes damaged to mRNA, DNA, and proteins involved in cell death and proliferation (Raha et al., 2014).

The main medical therapies of BPH include 5-α reductase inhibitors and adrenergic α-blockers (Lepor, 2011). However, considering untoward-reactions associated with the regular use of these groups of drugs such as dizziness, headache gynecomastia, reduced libido upper respiratory tract infection, erectile dysfunction, and male infertility due to a reduced sperm count (Kim et al., 2018). Also, considering the high cost and unavailability of these products in our localities there is a need for a better alternative.

Cymbopogon citratus (*C. citratus*) locally called toho gile in Tiv, lemun tsami ciyawa in Hausa, kooko oba in Yoruba, achara ehi in Igbo (Owerri) is an aromatic herb commonly cultivated for the fine fragrance of its leaves and often used to flavor custard (Khadri et al., 2010). The herb grows widely and rapidly near water sources in many countries and is an extensively used compound in medicine and industry (Ojo et al., 2006). *C. citratus* is known for its antioxidant activity and effective scavenging mechanism for free radicals through its potent flavonoid and phenol components (Cheel et al., 2005; Yoo et al., 2008; Khadri et al., 2010). It also has anti-inflammatory activities through its flavonoid content, scavenging the nitric oxide (NO) and also by inhibiting inducible nitric oxide synthase (iNOS) (Figueirinha et al., 2010). The herb can be used as a source of phytotherapeutic agent based on the above mention phytochemical contents.

2. Materials and Methods

2.1 Materials

Drugs: A brand of Testosterone marketed as TESTOST by: Laborate pharmaceuticals India LTD. 51, Indl. Area, Paonta Sahib H.O.: E-11, IND.AREA, PANIPAT-132103with NAFDAC Reg. NO.: A4-3348. Mfg. Lic. NO.: MB/04/87. Batch NO.: ETTOI-002. It was purchased from Rovi pharmacy, NO: 15, Iorkyaa Akor Street, High-Level, Makurdi, Benue State, Nigeria. Each ml contains Testosterone propionate USP 25mg.

A brand of Finasteride trade as FINSTAL-5® by laboratories PVT. LTD. C1B, 305/2, 3, 4 & 5, G.I.D.C. Kerala (Bavla), Dist.: Ahmedabad-382 220, Gujarat, India, with NAFDAC Reg. NO.: A4-0891. Mfg. Lic. No.: G/898. Batch NO.: N-2479. The drug was purchased from the same pharmacy as testosterone propionate. Each film coated tablet contains: Finasteride USP 5mg. It was diluted in distilled water in the ratio of 10mg/100ml as stock.

Plant Material: The *C. citratus* leaves and stems were gathered in November, 2021 from a garden at Benue State University, Makurdi, Benue state, Nigeria. The plant was authenticated at the herbarium of the botanical unit of the Department of Biological Sciences, Benue state university Makurdi, Benue state, Nigeria.

2.2 Citratus Leaves and Stems Aqueous Extraction

Procedure: The leaves and stems collected were washed with distilled water, air dried in the shade at room temperature over a period of two weeks. The dried plant was crushed by pounding and sieved to fine powder and aqueous extract of *C. citratus* was prepared according to the method reported by Lemhadri (2004) with little modification. Dry powder (10g) was placed in a Soxhlet extractor with 300 ml distilled water and continuously heated at 40°C for more than 10 hrs until the powder color faded. The water extracted was concentrated under reduced pressure at 40°C in a rotary evaporator and the solid material residue was weighed on a LS series electronic weighing balance (ORMA, Italy). The extract was stored in sealed dark glass bottles in a deep freezer at the temperature of about 5°C. The residue was later constituted in water in the ratio of 10g/100ml before used.

Percentage (%) Yield: The percentage (%) yield of *C. citratus* was calculated using the formula proposed by John et al. (2017):

$$\% \text{ Yield of the extract} = \frac{\text{Extract weight}}{\text{Dry weight of powder}} \times 100$$

Consequently, 48.7g of the extract was obtained from 70g of the dry powder resulting to 69.5% yield.

The 48.7g of the dry extract obtained, was then constituted into aqueous solution by dissolving it into 487mls of distilled water to yield a stock solution of 0.1g/ml (100mg/ml) before administration.

Experimental Animals: Thirty-six (36) healthy adult (10-12 weeks) male Wistar rats (100g-120g) were procured from the animal breeding facility of the College of Health Sciences, Benue State University Makurdi, Benue State, Nigeria and used for the study. They were acclimatized in metal covered plastic cages at standard room temperature (25+5) and 12/12- hours light dark cycle for 2 weeks before the commencement of the study. The animals had standard pellets feed (Vita growers feed) and water *ad libitum.*

Ethical Considerations: The experimental protocols were in keeping with the laid down guidelines on animal

experimentation as prescribed by the ethics committee of the college of health sciences, Benue State University Makurdi, Benue State, Nigeria. The College of Health Sciences Research and Ethics Committee (CHS REC) assigned number was CREC / 004.

2.3 Methodology

Animal Grouping and Treatment: The rats were randomly divided into six groups (1-6) containing six rats each. Group 1, had standard foods and drinks (Saleh et al., 2013) throughout the period of the experiment. All the other groups had intramuscular (IM) testosterone propionate injections at 10mg/kg b.wt (Acheampong et al., 2019) on the thigh once daily (OD) during the first 10 days using 10IU insulin syringe. On the 11th day, blood sample were collected from the rat tails for baseline serum PSA level determination. All the animals that had IM testosterone were randomly reassigned into five groups of six rats (Group 2-6). Thereafter the rats were treated as follows:

Group 1 (Sham): adequate standard nutrition and drinks.

Group 2: IM Testosterone propionate 10mg/kg b.wt OD for 21days, IM administration by means of 10IU of insulin syringe.

Group 3: IM Testosterone propionate 10mg/kg b.wt + 30mg/kg b.wt of CCAE per oral (PO), OD for 21 days, administration was via esophageal-gastric cannula.

Group 4: IM testosterone propionate 10mg/kg b.wt + 100mg/kg b.wt of CCAE PO (Rahim et al., 2013), OD for 21 days using esophageal-gastric cannula.

Group 5: IM testosterone propionate 10mg/kg b.wt + 300mg/kg b.wt of CCAE PO OD for 21 days using esophageal-gastric cannula.

Group 6: IM testosterone propionate 10mg/kg b.wt + 15mg/kg b.wt of finasteride (Acheampong et al., 2019) PO, OD for 21 days using esophageal-gastric cannula.

Determination of Acute Oral Toxicity: Determination of acute oral toxicity was performed based on the method described by Badrinathan et al. (2016). The oral toxicity safety dose of CCAE was calculated based on the organization for economic cooperation and development (OECD) guidelines 423 (Adopted in 2001). Five male adult Wistar rats were orally administered CCAE at the dosage of 4000mg/kg b.w and were observed for one week for any immediate toxic effects. The animals were closely monitored for gastrointestinal symptoms such as diarrhea, poor appetite, and weight loss. General symptom such as fur discoloration, isolation as well as central nervous system symptoms including sedation, convulsion etc. the result of the study revealed that the LD50 cut off must be greater than 4000mg/kg b.wt of the rats. Thus, it was considered safe to administer 300mg/kg b.wt of CCAE concentration to the animals.

2.4 Immunohistochemical Methods

Ki67: The tissues were routinely processed in a tissue processor and embedded in paraffin. We had to cut sections of 5 µm and subjected them to immune-study using the Immuno Cruz Staining System (Lab Vision Corporation, Fremont, CA, USA).

It was deparaffinized and rehydrated tissue sections and treated them for 30 min with 0.3% hydrogen peroxide in phosphate-buffered saline (PBS), pH 7.4, to block endogenous peroxidase.

The MIB-1 monoclonal antibody was used for detection of nuclear Ki-67 (1:40, code No. M7187, Dako, Cambridge, UK).

All primary antisera were diluted in PBS, pH 7.4, containing 1% BSA (Merck; Darmstadt, Germany) plus 0.1% sodium azide. Apart from doing all incubations with primary antisera overnight at 4^{o}C, biotin–caproyl anti-mouse immunoglobulins (Biomeda) was diluted 1:400 in PBS containing 1% BSA without sodium azide as secondary antibodies. I made sure to incubate with secondary antibodies for 30min at room temperature. Then I treated the sections with a streptavidin–biotin–peroxidase complex (Biomeda). The immunostaining reaction product was developed using 0.1 g diaminobenzidine (DAB) (3,39,4,49-tetraminobiphenyl; Sigma, St Louis, MO) in 200 ml of PBS plus 40 ml 30% hydrogen peroxide.

After immunoreactions, slides were counterstained with Mayer’s hematoxylin (94585, BioGenex, Menarini Diagnostics, Antony, France) dehydrated in ethanol, and mounted in a synthetic resin (Depex; Serva, Heidelberg, Germany). Specificity of the immunohistochemical procedures was checked by incubation of sections with non-immune serum instead of the primary antibody.

2.5 Ki-67 Labelling Index/Quantitative Analysis

I calculated the percentages of Ki-67-immunostained nuclei (labeling index) in each selected section for all the groups using the formula below:

Number of labeled nuclei x 100 / total number (labeled + unlabeled) of nuclei.

I carried out the Measurements using an Olympus (Leica Microsystems GmbH, Wetzlar, Germany) microscope that has a 3100 oil-immersion lens (numerical aperture 1.4) at a final magnification of X1200 and using the stereologic software GRID (Interact vision; Silkeborg, Denmark). It allows the selection of fields to be studied by random systematic sampling after the input of an appropriate sampling fraction. I selected an average of 20 fields per section and a total of 300 epithelial nuclei were evaluated per section in each group. The systematic field selection made sure that Ki-67 estimates were representative of all the prostate tissue (Ki-67 immunostained nuclei were considered positive if dark or light brown whether or not they stained intensely).

Proliferating Cell Nuclear Antigen (PCNA): Slides from each animal of both groups were immunostained. Deparaffined and rehydrated tissue sections were treated for 30 min with hydrogen peroxide 0.3% to blocken dogenous peroxidase. To detect PCNA sections were incubated with anti-PCNA antibody (Biomeda. Foster City, CA, USA) di-luted at 1:400. All incubations with primary antisera were kept overnight at 4ºC. Pretreatment of sections by heat in citrate buffer pH 6.0 using a pressure cooker was performed to enhance all the immunostainings. The immunohistochemical method was performed by an indirect technique using the antibody detection kit Histostain SP (Kit Histostain SP, Zymed Laboratories. Carlsbad, USA). The immunostaining reaction product was developed using 0.1 g diaminobenzidine (DAB) (3,3´,4,4´-Tetraminobiphenyl, Sigma, St. Louis, USA) in PBS, pH 7.4 (200 mL), plus 40 l of hydrogen peroxide.

After immunoreactions, sections were counterstained with Harris hematoxylin. All slides were dehydrated in ethanol and mounted in a synthetic resin (Depex, Serva, Heidelberg, Germany).

2.6 PCNA Labelling Index/Quantitative Analysis

For quantitative analysis, the intensity of immunoreactive parts was used as a criterion of cellular activity after subtracting background noise. Measurement was done using an image analyzer (Image J program). From each slide of both experimental groups, 9 fields were randomly selected.

The total field and immunohistochemial (IHC) stained areas were calculated and the percentage of IHC stained area calculated as follow: %IHC stained area = IHC stained area/Total area X 100.

2.7 Statistical Analysis

The results of the analysis were expressed as mean ± standard deviation and the statistical comparisons of the data obtained by the different groups were made using the Analysis of Variance (ANOVA) test and Duncan's Post hoc test (Steel & Torries, 1980). The statistical levels of significance were considered at the probability levels of less than or equal to 0.05 ($P \leq 0.05$). Analysis of the data was conducted using Statistical Package for Social Science (Version 20).

3. Results

3.1 Immunohistochemistry (Ki-67 and PCNA)

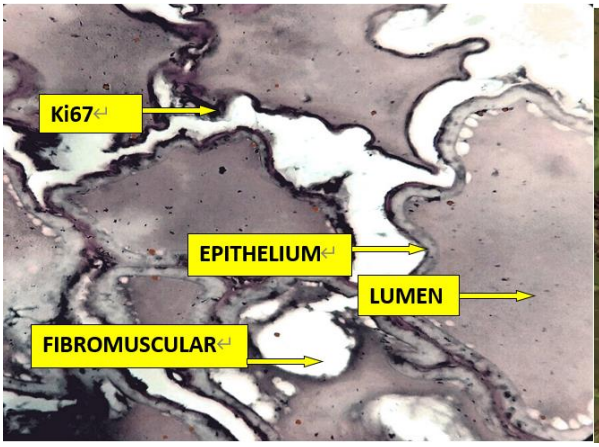
As shown in Table 1, there was a significant increase in Ki67 and PCNA labelling scores in group 2 rats when compared to group 1 (negative control). Also, there was a significant ($p \leq 0.05$) decrease in the number of reactive cells in groups post-treated with CCAE when compared to the group that had only testosterone dose (group 2) (Plate 8a-8b).

Only group 5 and 4 were significantly different in Ki67 and PCNA labelling respectively when compared to the standard drug group. Also, only group 4 had significant increase in PCNA immunolabelling score when compared to the low dose group (group 3) (Table 1 and Plate 1-12).

Table 1. Effect of CCAE and testosterone-induced BPH on Ki-67 and PCNA Labelling index of Wistar rat

Groups	Treatment	Ki-67 Labelling index (%)	PCNA Labelling index (%)
1	Normal Control (Sham)	12.3±6.5	0.47±0.04
2	Positive Control (10mg/kg TP)	18.0±4.3[a]	0.89±0.04[a]
3	Low Dose (10mg/kgTP+30mg/kg CCAE)	11.0±2.6[b]	0.48±0.04[b]
4	Medium Dose (10mg/kgTP+100mg/kg CCAE)	11.0±3.6[b]	0.70±0.09[c,d]
5	High Dose (10mg/kgTP+300mg/kg CCAE)	14.6±3.2[b,c]	0.40±0.10[b]
6	Standard-Drug (10mg/kgTP+15mg/kg finasteride)	10.0±1.7[b]	0.49±0.07[b]

Note: [a,b,c] and [d] represents significant decreases or increase at $p \leq 0.05$ when compared to groups 1 (sham), group 2 (positive control), group 6 (standard drug) and group 3 (Low dose group) respectively.



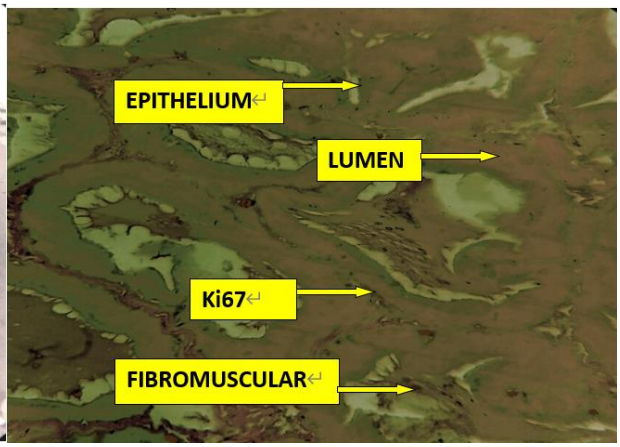
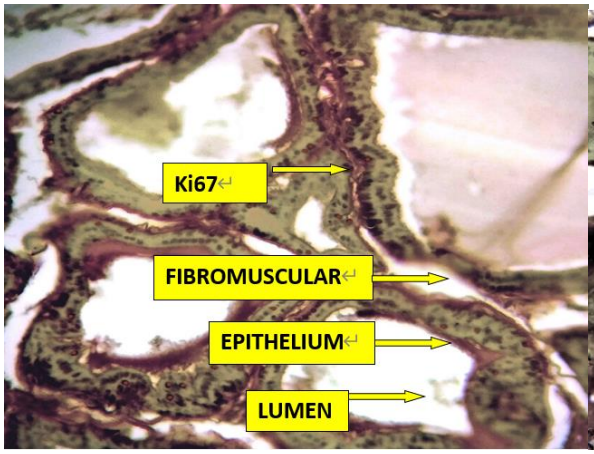


Plate 1a. Plate 1b.

Plate 1a and 1b: Photomicrograph of the prostate of rat from group 1(Sham) showing immunopositive cells for Ki67. Magnification: x40.



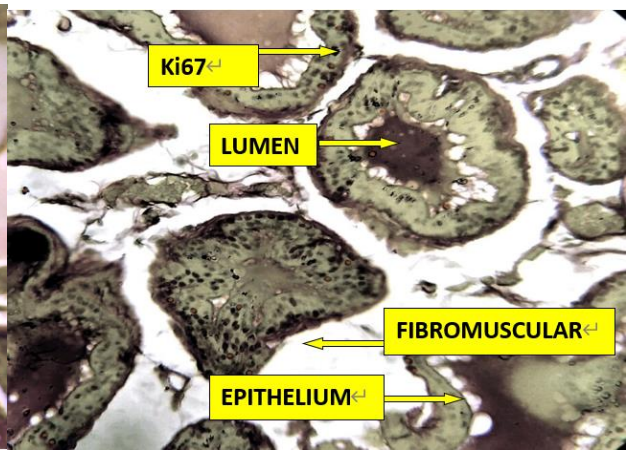
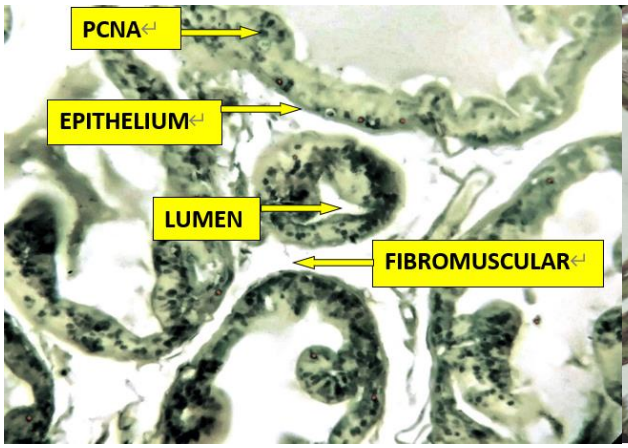


Plate 2a. Plate 2b.

Plate 2a and 2b: Photomicrograph of the prostate of rat from group 2 (10mg/kg TP) showing immunopositive cells for Ki67. Magnification: x40.



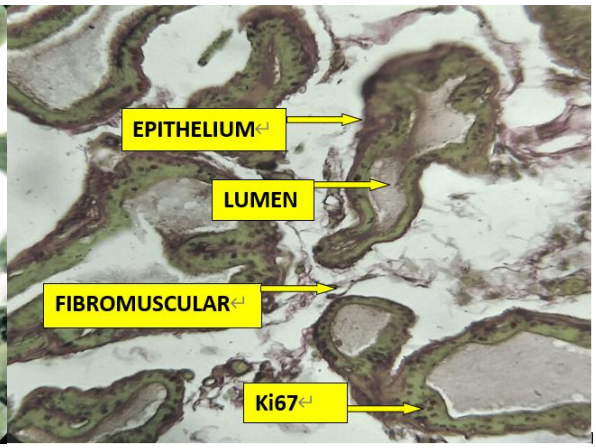


Plate 3a. Plate 3b.

Plate 3a and 3b: Photomicrograph of the prostate of rat from group 3 (10mg/kgTP+30mg/kg CCAE) showing immunopositive cells for Ki67. Magnification: x40.

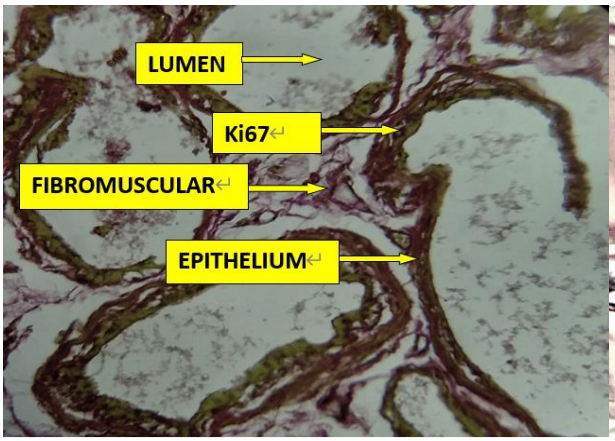


Plate 4a.

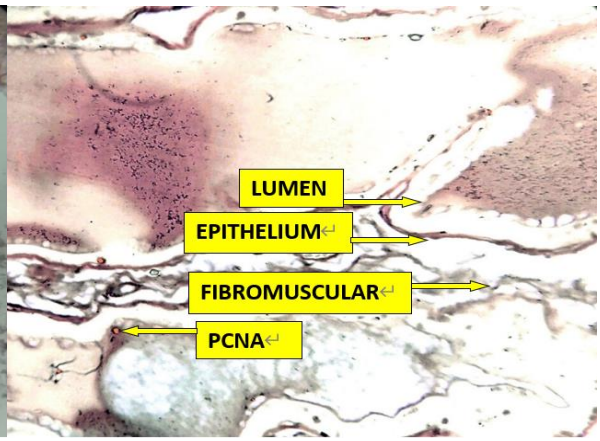


Plate 4b.

Plate 4a and 4b: Photomicrograph of the prostate of rat from group 4 (10mg/kgTP+100mg/kg CCAE) showing immunopositive cells for Ki67. Magnification: x40.

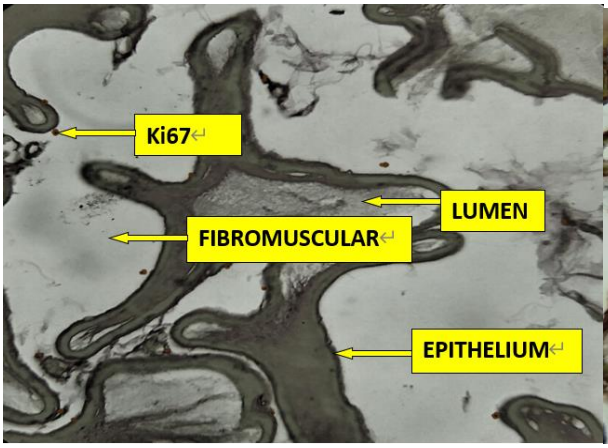


Plate 5a.

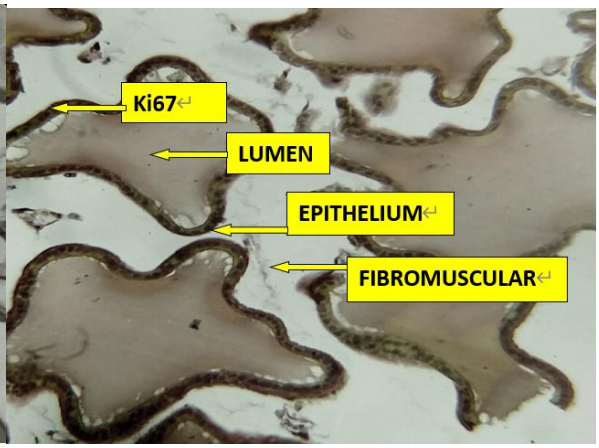


Plate 5b.

Plate 5a and 5b: Photomicrograph of the prostate of rat from group 5 (10mg/kgTP+300mg/kg CCAE) showing immunopositive cells for Ki67. Magnification: x40.

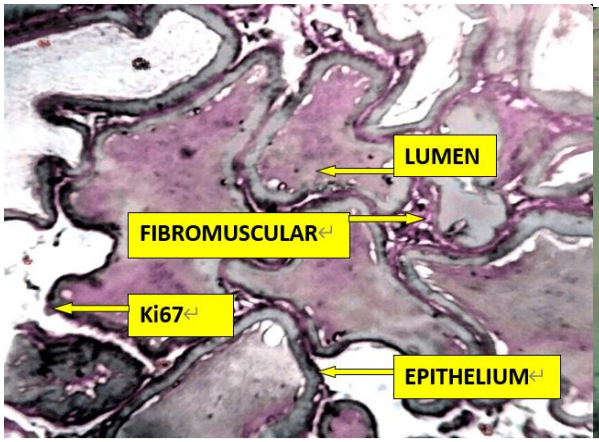


Plate 6a.

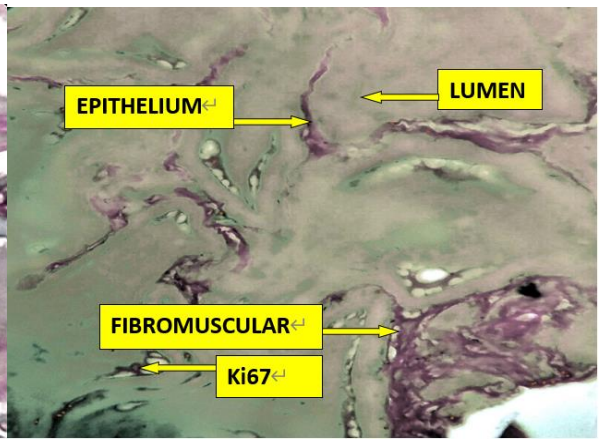


Plate 6b.

Plate 6a and 6b: Photomicrograph of the prostate of rat from group 6 showing immunopositive cells for Ki67.Magnification: x40.

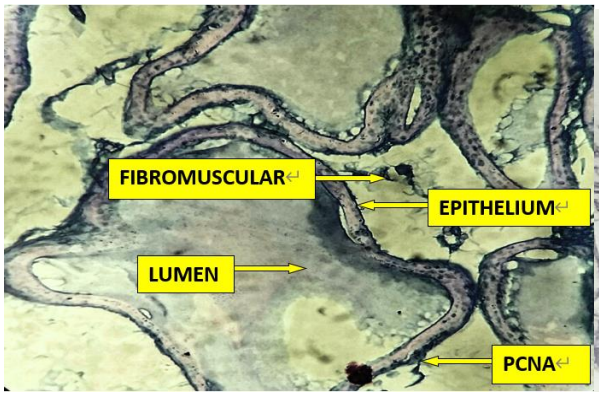


Plate 7a.

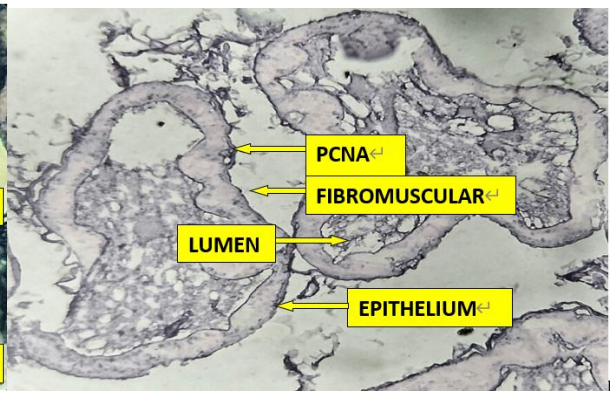


Plate 7b.

Plate 7a and 7b: Photomicrograph of the prostate of rat from group 1(Sham) showing immunopositive cells for PCNA. Magnification: x40.

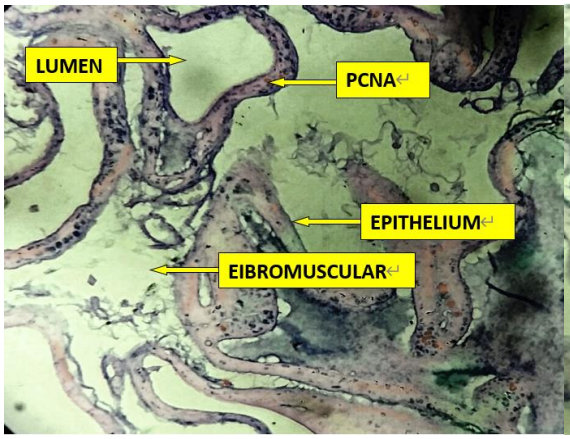


Plate 8a.

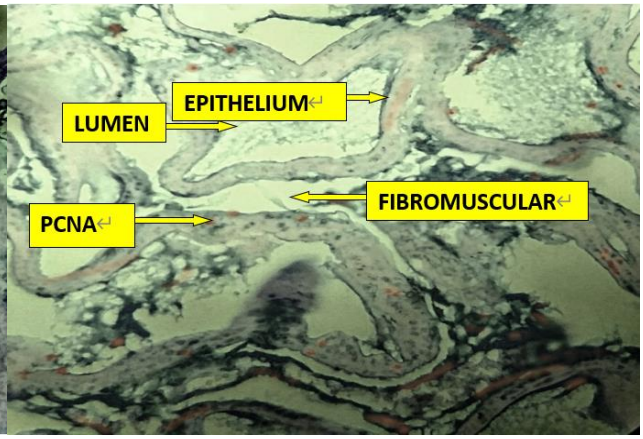


Plate 8b.

Plate 8a and 8b: Photomicrograph of the prostate of rat (10mg/kg TP) showing immunopositive cells for PCNA. Magnification: x40.

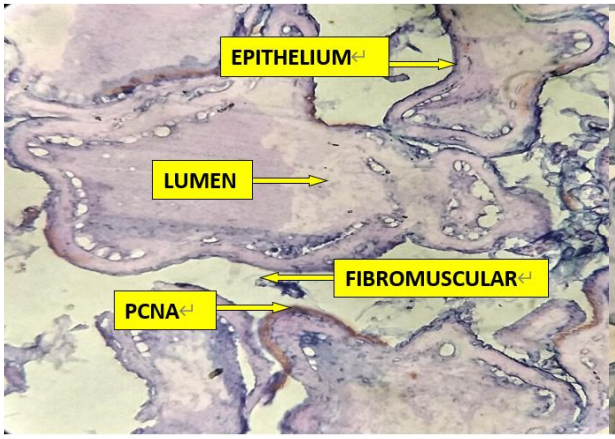


Plate 9a.

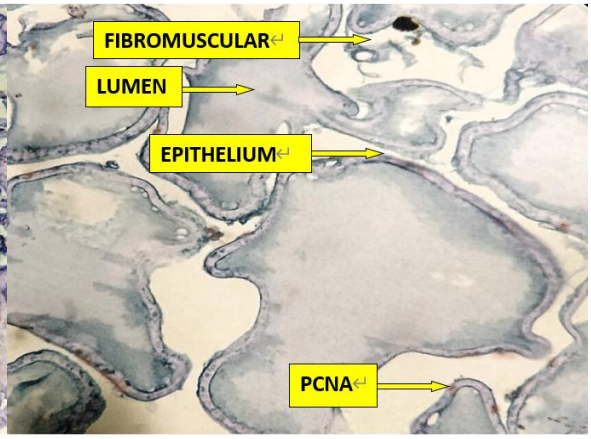
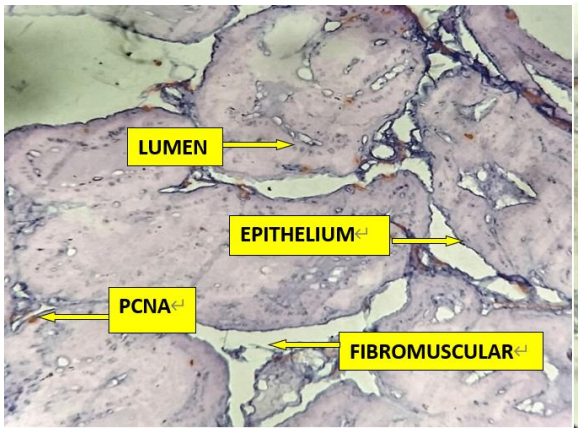


Plate 9b.

Plate 9a and 9b: Photomicrograph of the prostate of rat (10mg/kgTP+30mg/kg CCAE) showing immunopositive cells for PCNA. Magnification: x40.



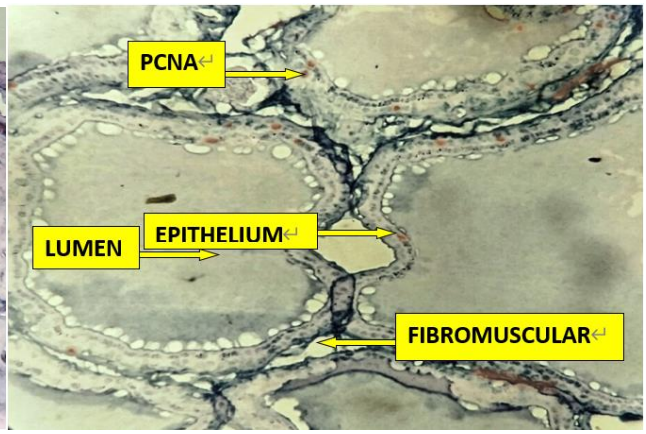
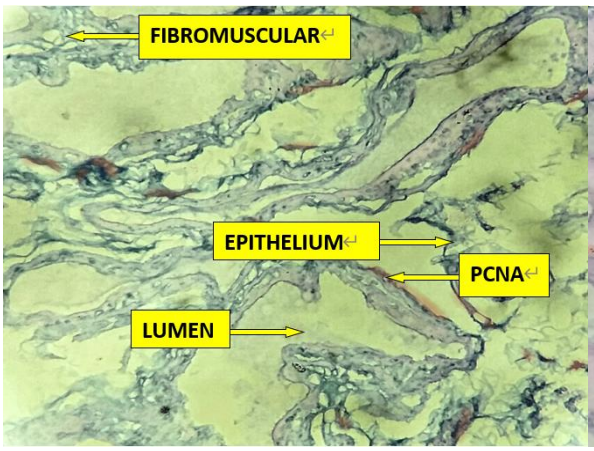


Plate 10a. Plate 10b.

Plate 10a and 10b: Photomicrograph of the prostate of rat from group 4 (10mg/kgTP+100mg/kg CCAE) showing immunopositive cells for PCNA. Magnification: x40.



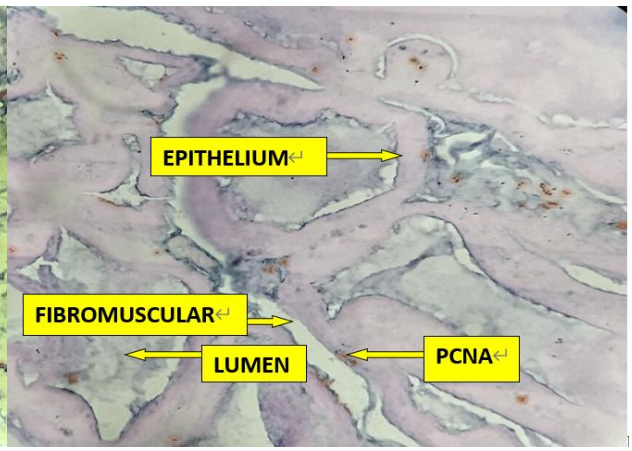
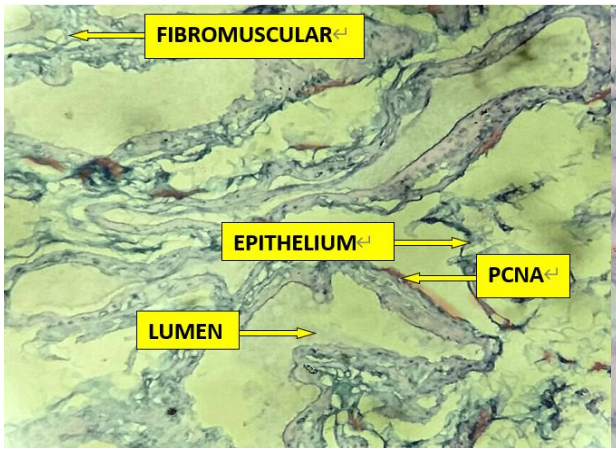


Plate 11a. Plate 11b.

Plate 11a and 11b: Photomicrograph of the prostate of rat from group 5 (10mg/kgTP+300mg/kg CCAE) showing immunopositive cells for PCNA. Magnification: x40.



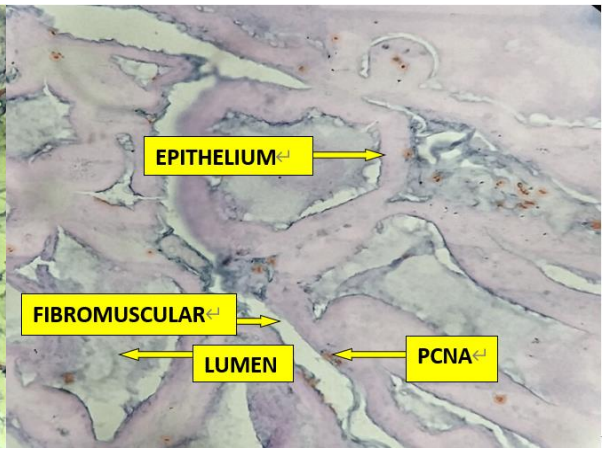


Plate 12a. Plate 12b.

Plate 12a and 12b: Photomicrograph of the prostate of rat from group 6 (10mg/kgTP+15mg/kg finasteride) showing immunopositive cells for PCNA. Magnification: x40.

4. Discussion

Benign protastic hyperplasia is well-recognized as a health hazard in middle-aged and elderly men (Donnell, 2011). Although benign, this condition is categorised by an enlarged prostate resulting from a hyperproliferative state in the stromal, glandular and mesenchymal prostate cells (Roehrborn, 2011). This causes several lower urinary tract symptoms that affect quality of life. BPH pathogenesis is linked to oxidative stress and imbalanced cell proliferation and apoptosis (Pawlicki et al., 2004; Minciullo et al., 2015).

This study aimed to evaluate the effect of *C. citratus* aqueous extract on testosterone-induced BPH in Wistar rats.

As shown in the photomicrograph and the mean value for immune-labelling index, there was a significant increase in Ki67 and PCNA labelling scores in group 2 rats when compared to group 1 (negative control). It is evident from many studies that the role of inflammatory infiltrates and their mediators is in the development of prostatic hyperplasia (Vafa et al., 2020). This commends that inflammation probably be a causative agent in the pathogenesis of prostatic hyperplasia. In the present study, immunohistochemical analysis of prostatic tissues from prostate hyperplasic rats establishes over expressions of inflammatory markers (PCNA and Ki67).

The expression of Ki67 is strongly associated with tumor cell proliferation and growth, and is routine in pathological investigation. This protein is an established prognostic and predictive indicator for the assessment cancer diagnosis. Aside from being shown to correlate with metastasis and the clinical stage of tumors, it is significantly higher in malignant tissues with poorly differentiated tumor cells, as compared with normal tissue (Josefsson et al., 2012; Lian et al., 2015). In this study, labelling index was used as a scoring factor. This labeling index is an independent prognostic factor for survival rate, which includes all stages and grade categories (Jonat & Arnold, 2011). There is a correlation between the ratio of Ki67-positive malignant cells and patient survival (Lian et al., 2015). Hence blocking of Ki67 either by microinjection of antibodies or through the use of antisense oligonucleotides leads to the arrest of cell proliferation (Lian et al., 2015).

On the other hand, proliferating cell nuclear antigen (PCNA) is an auxiliary protein of DNA polymerase delta considered to correlate with the cell's proliferative state (Maeda et al., 1999). Immunohistochemical methods with an anti-PCNA antibody have particular advantages compared with other techniques because of the maintenance of the tissue architecture and simplicity of the methodology.

The anti-inflammatory of CCAE in prostatic tissues was evidenced immunohistochemically using labelling index for Ki67 and PCNA. It was observed that prostatic hyperplasia was accompanied and/or preceded by a background of inflammatory status (Eid & Abdel-Naim, 2020). Evaluations on the pathophysiology of BPH highlighted a significant role for inflammation and inflammatory mediators (McClinton et al., 1990; Theyer et al., 1992; Madersbacher et al., 2019; Devlin et al., 2020). In particular, inflammation was reported to be a key-player in testosterone-induced BPH (Rastrelli et al., 2019; Eid & Abdel-Naim, 2020). CCAE significantly prevented the rise in expression of Ki67 and PCNA immune-positive cells.

For Ki-67 and PCNA immune-labelling, only the medium dose group had significant increase in PCNA immunolabelling score when compared to the low dose group. These results are in line with report of (Cheel et al., 2005; Mohamed et al., 2104; Eid & Abdel-Naim, 2020). More importantly, the results indicate that the CCAE seems to be an effective antioxidant agent when it used in medium (100mg/kg) or high (300mg/kg) concentration (Gabriela et al., 2016).

5. Conclusion

This study has shown the protective effect of CCAE on testosterone-induced BPH in Wistar rat. Hence, it supports the possibility for CCAE to be investigated as a potential agent for the treatment of prostatic and inflammatory diseases.

6. Recommendations

1) I wish to recommend that further investigations should be carried on mechanism of action of CCAE on higher animals and possible clinical trials on human beings for treatment of BPH.

2) There should be laws and regulations guiding the use of *C. citratus* to prevent it from extinction.

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