Histostereological Restorative and Inhibitory Effects of Crude Methanolic Bark Extract of Prunus Africanus on Testosterone-Induced Benign Prostatic Hyperplasia in Wistar Rats

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Authors' contributions

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ABSTRACT

Aim: To evaluate the histostereological effects of *P. africanus* on testosterone induced Benign Prostatic Hyperplasia (BPH) in Wistar rats.

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Place and Duration of Study: The study was carried out for six weeks in Jomo Kenyatta University of Agriculture and Technology.
Methodology: A sample size of sixty Wistar were used as the experimental model and they were divided into two study groups of 30 rats in the restorative group and 30 rats in the inhibitory group. Each group was further categorized into 5 control and 25 experimental rats. The experimental rats were further subdivided into 5 sub-groups based on varying doses of the crude methanolic bark extract of *P. africanus* (0 mg, 25 mg, 50 mg, 125 mg, and 200 mg). BPH in experimental animal was induced by subcutaneous injection of testosterone propionate (7.5 mg/kg) for 10 days.
Results: The findings of the study showed that the restorative group had a statistical significant (P<0.05) ((dose dependent reduction of the prostate volume, the stromal and epithelium volume was observed. While in the inhibitory group a statistical significant (P<0.05) dose-related inhibition, in the increase of the prostate volume, the stromal and epithelium volume was observed. In conclusion, the maximal restorative effect was observed to be up to 85.6% and at a dose of 200 mg, while the optimal inhibitory effects were observed to be between 66.7- 68.4% in the dose range of 100-200 mg/kg/body weight.
Conclusion: Crude methanolic bark extract of *P. africanus* has both restorative.

1. INTRODUCTION

The prostate, an accessory gland of the reproductive system located in the inferior part of the urinary bladder around the prostatic urethral part in males is composed of both exocrine and endocrine parts [1,2]. The exocrine part of the prostate can undergo a non-cancerous proliferation characterized by pathological cellular multiplication of epithelial and smooth muscle cells a condition referred to as Benign Prostatic Hyperplasia [3]. This condition causes urethral constriction resulting is chronic end-stage bladder outlet obstruction that subsequently can lead to renal failure and uraemia [4,5]. Worldwide BPH is a leading cause of death in men of reproductive age above 45 years [6]. This end-stage results in renal failure and uraemia making it one of the key causes of mortality and morbidity [7,8]. Many treatment options have been advanced for BPH including surgery, conservative medical therapy as well as the use of crude bark extracts of *P.africanus*. Locally *P. africanus* is gaining popularity in management of BPH because it is affordable and available to many poor Kenyan men in rural areas compared to surgical procedure and medical therapies. Though bark extracts of *P.africanus* have been in use for decades and target either the prostatic muscle tone relaxation or prostate gland mass reduction their outcomes is not guaranteed in the total cure of BPH [9]. Also the popularity gained in the use of *P.africanus* is based on the fact that it is locally available, cheap and has been shown to be effective with less adverse effects compared to conventional methods of BPH managements [10,9]. Though literature has reported inhibitory and restorative effects of *P. africanus* on BPH, there is paucity of data on its histostereological effects in management of BPH to substantiate these claims. At the same time, data on whether or not the restorative and inhibitory effects of crude methanolic bark extract of *P. africanus* (CMBEPA) are dose and time dependent, is yet to be established.

2. MATERIALS AND METHODS

2.1 Study Area/Site

In carrying out this study, the Small Animal Facility for Research and Innovation (SAFARI) in Jomo Kenyatta University of Agriculture and Technology (JKUAT) was used, while tissue processing for light microscopy and histostereology was done in the histology laboratory in the Department of Human anatomy, School of Medicine in the College of Health Sciences (COHES) of JKUAT.

2.2 The Study Design

The study design was a true experimental (posttest-only with control) laboratory-based study.

2.3 Experimental Animals and Care

A total of 60 adult male Wistar rats (weighing between 180 ± 20 grams) were used in this study, because they are highly susceptible to the development of benign and atypical prostatic hyperplasia after treatment with exogenous testosterone in both intact and post-castrated males [11]. These 60 Wistar rats were sourced

from SAFARI animal house in JKUAT. They were put in standard rat cages and were acclimatized for 7 days. Litter papers in the cage were changed on alternate days. Each cage was labelled with a card showing the experiment number, date of starting the experiment, dosage level, and age, number of rats, species and sex of the animal. The rats were fed on standard rodent pellets obtained from UNGA Mills limited and water *ad libitum* throughout the experimental period. The rats were handled in accordance with the guidelines for the care and use of laboratory rats.

2.4 Induction of BPH in the Experimental Wistar Rats

In the induction of BPH, the experimental group were administered with testosterone propionate 7.5 mg/kg/ day subcutaneously [12].

2.4.1 Harvesting and extraction of *P. africanus*

The barks of *P. africanus* that was used in this study was sourced from Mt. Kenya forests at Mukurweini location of Nyeri County. A botanist specialized in plant taxonomy and with many years of experience dealing with studies of *P. africanus* assisted in identifying trees, debarking of the stems and bark collection, preservation and transportation of back extracts. A voucher specimen of *P. africanus* plant was deposited in Jomo Kenyatta university of Agriculture and Technology Botanical Herbarium, voucher number Rosacea 0001. Sustainable harvesting was done by cutting the old branches and prunes i.e. "renewable plant sourcing" this preserved the source and kept it getting renewed [13]. The stems of *P. africanus* were debarked using a sharp-edged knifes to obtain ten kilograms of the wet bark. The pieces of the wet bark were then transported in meshed plastic bag to JKUAT, where they were air dried at room temperature of 25°C to a moisture content of about 10-15%. The dried bark was weighed (1.2 kg) and chopped into small pieces and grounded into fine powder using a laboratory mill (Christy and Norris Ltd., Chelmsford, England). One thousand grams of the powdered material was soaked in 2000 ml of 75 % methanol at room temperature for 24 hours. The methanol mixture was filtered through Whatman filter paper no. 1 (Whatman international, England). The organic solvent was

evaporated to near dryness by vacuum evaporation using rotary evaporator (BUCHIR 200) [14]. After evaporation, 68 grams of the bark extract (pygeum) was obtained which was a greenish mass with almond Smell [13]. The extract was stored in air tight bijou bottles in a freezer at -20° C prior to use.

2.5 Sample Size Determination

The sample size in this study was determined using the modified "resource equation method" since there was no previous data on standard deviation from previous study on the histostereology of the prostate [15].

2.6 Experimental Design

The 60 rats were divided into two broad groups of 30 rats each, 30 in the restorative group (Group 1) and 30 rats and inhibitory group (Group 2).

The 30 rats in the restorative group were randomly assigned into 6 subgroups, one control group with 5 rats and five treatment subgroups with five rats each. Control group were fed on the rodent pellets (Mice Pellets UNGA Mills ltd and water *ad libitum* and also receive sham treatment (corn oil subcutaneous (S.C) injection for first 10 days and 5% DMSO through gastro gavage for 3 weeks). In the treatment group all rats were injected with daily S.C injection testosterone propionate (7.5 mg/kg body weight) in corn oil for duration of 10 days to induce BPH. The testosterone propionate induced (TPI-BPH) rats were then treated using varing doses of crude methanolic bark extract dissolved in 5 % DMSO as follows (Fig. 1).

All the principles of laboratory animal care" (nih publication no. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments were examined and approved by the Jomo Kenyatta University of Agriculture and Technology Animal Ethics Committee (JKUAT AEC) approval certificate number JKU/2/4/896A. The protocol followed Guidelines for Care and Use of Laboratory Rats in Biomedical Research, and the rats were only used once in the experiment. They were all sacrificed using humane end points at the end of the study [16].

Animal grouping:
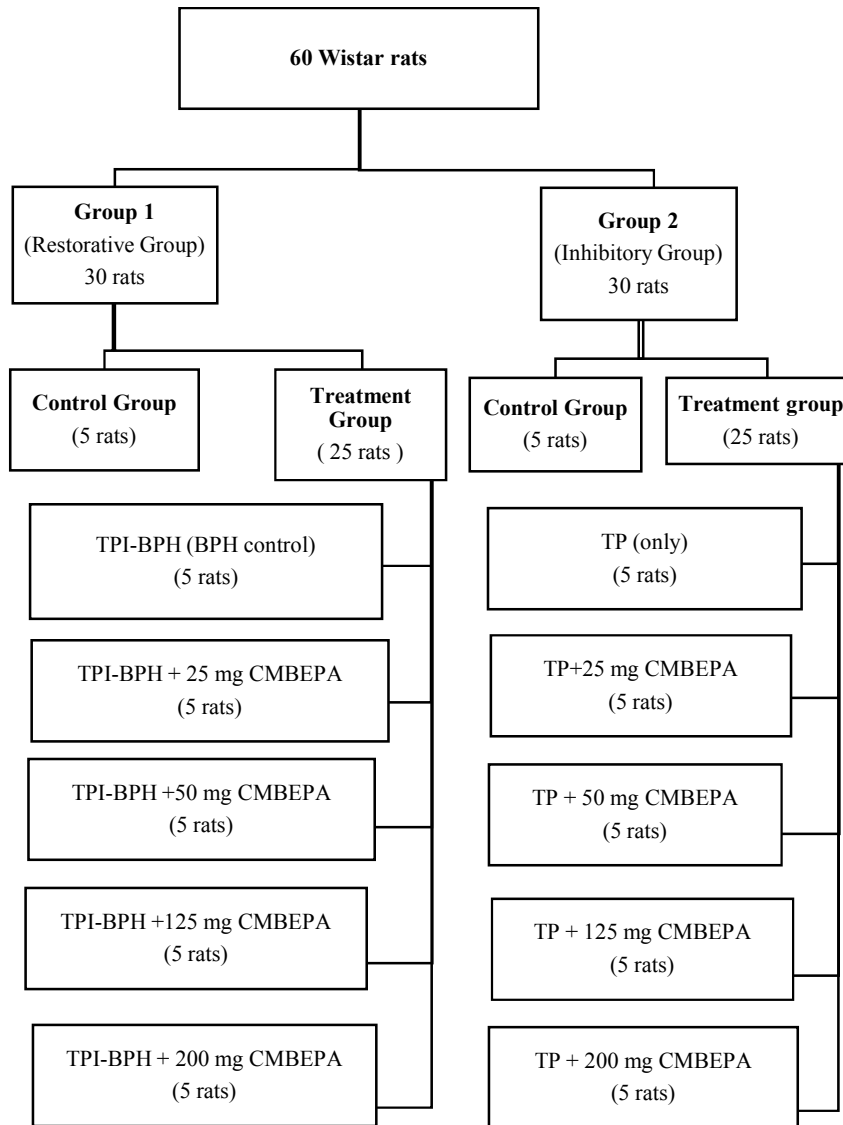
Fig. 1. Shows how animal grouping were organized between experimental and controls group
*TPI-BPH - Testosterone Propionate induced benign Prostatic Hyperplasia, CMBEPA-Crude Methanolic bark
extract of P. africanus, TP-testosterone propionate.*

2.7 Dose Determination, Dilution and Administration of crude bark extract of CMBEPA

The rationale for dose selection that were applied in this study were based on the reference standard Human dose equivalents of *P.africanus* that have been applied in human that ranged from 50 mg to 200 mg given once daily as described by Melo et al. [17]. Based on this

therefore the animal equivalent doses (AED) were calculated from Human equivalent doses (HED) as follows, using the FDA guideline on human to animal dose conversion.

The dilutions of CMBEPA was done using 5% DMSO to make the stock solution. The administration of CMBEPA was done between 12:30 pm and 1:00 pm daily through gastric gavage. All the volumes of CMBEPA that were administered did not exceed the standard

allowable daily oral volume of 3 ml per rat per day [18].

2.8 Assessing the Animal Body Weights

The rats were weighed daily using a high precision electronic weighing machine throughout the studies.

2.8.1 Estimation of prostate volume using Archimedes principle

The estimation of prostate volume was done using the Archimedes' principle so as to obtain independent prostate volume in all the rats. The Archimedes volumes were estimated by inserting the fixed prostate gland into graduated beakers containing normal saline, and the displacement was measured. The normal saline displaced by prostate represented the actual prostate volume [19].

2.8.2 Estimation of prostatic index and percentage restoration

The prostatic body Ratio (%) and the percentage restoration of prostate gland weight by different treatments was calculated as previously described [20]

$$Prostate\ body\ ratio\ \%\ (PBR)$$
$$= \frac{Prostate\ weight(g)}{Terminal\ body\ weight(g)} \times 100$$

Further percentage of restoration was calculated as follows:

$$Percentage\ restoration$$
$$= 100 - \left(\frac{(T-C)}{(B-C)} \times 100\right)$$

Where;

T - Mean prostate weights in the treatment group
C - Mean prostate weight in the control group
B – Mean prostate weight in the BPH group (BPH control group)

2.9 Prostate Tissue Histology and Histostereology

Prostate tissue processing for both light microscopy and histostereology was done by initially fixing the gland in Bouin's solution for 24 hours, followed by dehydration in an ascending concentration of alcohol (50%, 60%, 70%, 80%,

90%, 95% and 100 % each for one hour and cleared with xylene in 12 hours. The sections were then infiltrated with paraffin wax for 12 hours and then embedded in paraffin wax. Leitz sledge microtome was used to cut transverse and longitudinal thin sections 5-7µm thick, floated in water at 37^0 then stuck onto glass slides using egg albumin, applied as thin film with a micro-dropper. In each subgroup 40 slides were selected with systematic random sampling was then dried in an oven at 37^0 for 24 hours then stained with hematoxylin and eosin to demonstrate the general features of prostate gland in all the lobes. Another 40 slides from each prostate were randomly selected and stained with haematoxyline and eosin stain to demonstrate cellular component. The prostate sections were stained using haematoxyline and eosin (H.E) [21].

After staining, the digital images were acquired using the LABOMED iVu 3100 Digital Camera Software with PixelPro™ Image Analysis Software for capturing still images. In addition, during acquiring of the digital images the stereological sampling rules were also applied. The quantitative analysis of rat prostate to assess both restorative and inhibitory effects of CMBEPA in the TPI- BPH Wistar rats was done using the following image analyser software, Quo path, image J and STEPanizer. These three software were complementary to each other (Bolender &Weibel, 1973).

2.10 Estimation of the Prostate Volume Using Different Methods

Prostate volume estimation was done using Archimedes displacement method, Cavalieri point counting method and plainmetry. These Archimedes volumes and plainmetry were used as the gold standard (reference volumes) and was compared to cavalieri volumes. These were done prior the determination of prostate structure volumes (acinar, stromal and epithelium volume densities) to ensure accuracy of these parameters.

The Cavalieri point counting method was used in the present study for practical estimation of prostate structure volumes and their volume densities, because they cannot be assessed using routine water displacement procedures. The volume estimation were done by applying the Cavalieri principle [22]. The volume of the prostate and its anatomical structures were determined by combining the Cavalieri method of

segmentation with point-counting. On evenly spaced prostate sections, where 30 sections in each prostate, were sampled using systematic random sampling (SRS), to obtain section sampling fraction (ssf). The sampled sections were then subsampled by systematic random sampling using the microscope's stage Vernier and images were at magnification of X10. The prostate micrographs from the selected section, with a series of 5 um thickness were used to estimate the Cavalieri volumes of the prostate structures. The micrographs were retrieved from the laptop where they had been saved. They were uploaded a STEPanizer stereology analyzer tool, where a grid test system with d = between test points was introduced, randomly covering the entire image frame. Volume calculations were performed on each individual prostate section by using the interposing a test points (Fig. 2). A point grid with 100 distances between two points was superimposed on the images and the point hitting points on the prostate were counted and then the volume of each component was calculated using the following formula:

$$V = \frac{t \times a(p) \times \Sigma P}{M^2}$$

Where;

V refers to volume component of interest,

t is the section thickness,
a (p) is the area of one point (359μ)
ΣP is the total number of point counted in the component of interest
M is the linear magnification (X40)

The estimation of volume density of the gland's epithelium, fibromuscular tissue and acinar, were carried out through systematically sampling the prostate sections followed by subsampling of prostate histological images by systematic random sampling using the microscope's stage Vernier at magnification of 40x. The volume density of the structures was estimated using STEPanizer a stereology software by the point-counting method and the following formula applied:

$$Vv(structure/prostate) = \frac{P(structure)}{P(prostate)}$$

Where;

P (structure) = the No, of points hitting the favoured histological structures

P (prostate) = the No, of points hitting the reference space, here whole prostatic sections. The absolute total volume of each structure was estimated by the volume density multiplied by the total volume of the prostate.

Fig. 2. Prostate photomicrograph illustrating superimposed point-counting frame for cavalieri volume determination

2.10.1 Correction for the prostate tissue shrinkage

The prostate tissue shrinkage was calculated according to [23] as follows:

$$Volume\ shrinkage = 1 - [\frac{Volume\ after}{Volume\ before}]$$

Where;

Volume after -Prostate volumes determined using Cavalieri method

Volume before -Prostate volume determined using Archimedes displacement method

The tissue shrinkage caused by fixation and histological procedures is major setback of stereological estimation of the prostate volumes, therefore the final volume of the prostate volume and prostate structure volumes obtained from cavalieri were corrected accordingly after estimating the prostate shrinkage [24,25].

2.11 Computation of Coefficient of Error (CE)

The coefficient of error was computed to ensure quality of quantitative estimates and the precision of stereological parameters. The CE was calculated to evaluate using the following formula [26].

$$v = \frac{1}{ssf}a_f\ t\ \sum_{i=1}^{n}P_i \quad a_{f\ =g^2}$$

$$A = \sum_{i=1}^{n}P_i\,P_i \quad B = \sum_{i=1}^{n-1}P_i\,P_{i+1} \quad C = \sum_{i=1}^{n-2}P_i\,P_{i+2}$$

$$Var_{noise} = 0.0724.\left(\frac{b}{\sqrt{a}}\right)\sqrt{n\sum_{i=1}^{n}P_i}$$

$$Var_{SURS} = \alpha_i(3.(A - Var_{noise}) - 4.B + C)$$

$$\alpha_0 = \frac{1}{12} \quad \alpha_1 = \frac{1}{240}$$

$$CE = \frac{\sqrt{Var_{SURS} - Var_{noise}}}{\sum_{i=1}^{n}P_i}$$

The Table 1 shows the slice sampling interval during tissue sectioning 30 (k[th]) while the block advance was 5 microns, and grid spacing was 349 microns during cavalieri volume determination.

2.12 Planimetry Volumetry of Prostate Volume

Planimetry volumetry method was used as reference volume alongside Archimedes volume displacement for accurate prostate volume determination. The prostate images were transferred to a laptop and image analyses were performed using image analysis software, Qupath 0.1.2. The images were uploaded from the laptop to QuPath user interface where they were displayed in a standard image. The borders of the prostate epithelium, stromal tissue and acinar were manually traced on each section of the images using a hand-held mouse (Fig. 3) and the cross-sectional surface area of interest within the image were determined [27]. The software computed the number of pixels within the traced area, and this process was carried out for each image. The area of the prostate is calculated in consecutive histological sections. The area is multiplied by the distance between the sections and the total volume determined by summation of all contributions [28]. This volume is calculated based on the pixel size and slice thickness using the following formula:

Volume= the sum of the areas (mm^2) X slice thickness (um) X No of slices.

Table 1. Slice interval, grid spacing and block advance for CE estimation for Cavalieri point counting method

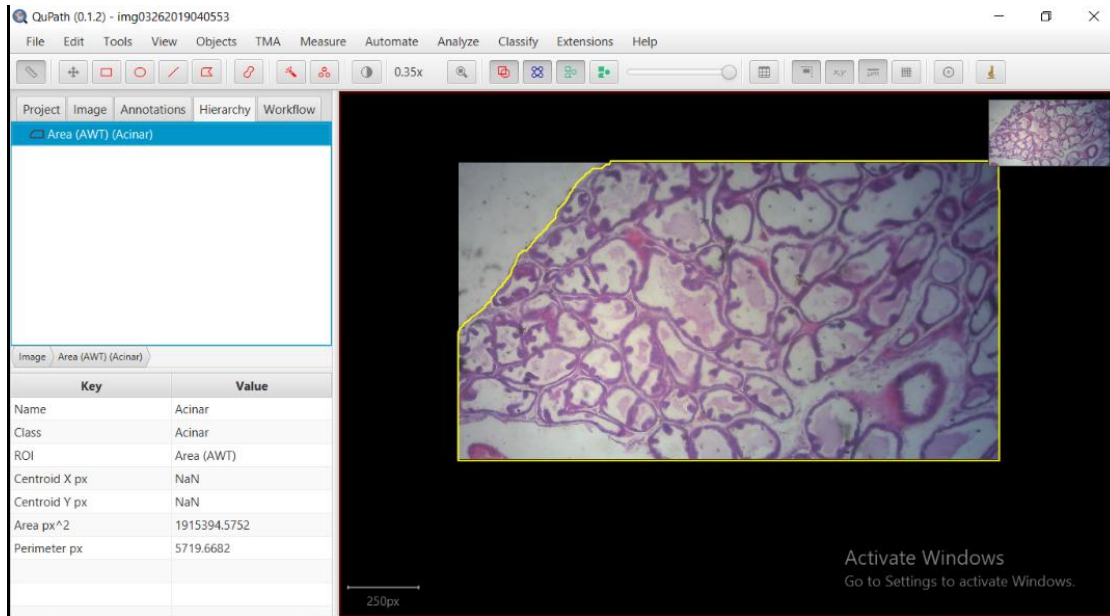| Sampling Parameter	Value
Slice Interval	30
Grid spacing	349 microns
Block advance	5 microns

Fig. 3. Prostate photomicrograph illustrating the probe for plainmetry volume determination

2.13 Statistical Analysis

Data was analysed using statistical package for social sciences (SPSS) for Windows Version 25. For continuous data, one way analysis of variance (ANOVA) was used to compare means between different treatment groups. Before applying ANOVA statistical analysis, the data were examined using Shapiro Wink normality and Levene test for homogeneity of variances as parametric test assumptions. Group means with a significance F-value (p<0.05) were further analysed using Tukey post hoc t-test.

To assess the level of agreement between the volume measurements of Archimedes' method, Cavalieri and plainmetry method, statistical agreement measures, including concordance correlation coefficient (CCC) and intraclass correlation coefficient (ICC) were applied.

3. RESULTS AND DISCUSSION

3.1 Assessment of the Level of Agreement Total Prostate Volumes

A high degree of reliability was found between Archimedes volume and Cavalieri volume measurements (.762). The average measure ICC was .585 with a 95% confidence interval from .356 to .774 (F (23, 46) = 5.233, p<5) (Table 2).

The epithelial volumes, stromal volume, acinar volume and total prostate volume against the varying doses of crude methanolic bark extract o*f P.africanus* in restorative group, the mean prostate epithelium volume was found to have a statistical significant difference among the treatment groups (Table 3).

Table 2. Showing Intraclass correlation coefficient of Archimedes, Cavalieri and plainmetry methods used in determining total volume of the prostate

Inter-Item Correlation Matrix			
	Archimedes	**Cavarieli**	**Plainmetry**
Archimedes	1.000	.762	.543
Cavalieri	.762	1.000	.805
plainmetry	.543	.805	1.000

Table 3. Showing prostate stromal, acinar and total volumes against various doses of CMBEPA in Restorative group

	Groups					
	Control	TPI	25 Mg	50 Mg	125 Mg	200 Mg
Epithelium Vol	0.114±.007	0.251±0.017[a]	0.138±0.013[b]	0.122±0.001[b]	0.128±0.005[b]	0.137±0.007[b]
Stromal Vol	0.110±0.005	0.193±0.012[a]	0.125±0.008[b]	0.114[b]±0.007	0.110[b]±0.004	0.118[b]±0.006
Acinar Vol	0.138±0.003	0.147±0.019	0.162±0.015	0.142±0.005	0.144±0.005	0.147±0.003
Prostate Vol	0.362±0.011	0.592[a]±0.031	0.425[b]±0.036	0.378[b]±.019	0.382[b]±.012	0.402[b]±0.014

Comparison between groups was performed in row.
P.A-Prunus africanus; TP-Testosterone Propionate; TPI-BPH-Testosterone Propionate induced benign prostatic hyperplasia; Vol - Volume
[a] indicates values that were significantly different (p <0.05) from the control
[b] indicates values that were significant different (p <0.05) from the BPH group

Table 4. Prostate epithelium, stromal and acinar densities against of various doses of CMBEPA in the restorative group

	Control	BPH-TPI	25 Mg	50Mg	125Mg	200Mg
Epithelium Vv %	31.527±1.145	42.521±2.593[a]	32.47±0.509[b]	32.27±1.151[b]	33.576±0.740[b]	34.03 ±1.007[b]
Stromal Vv %	30.353±0.933	32.623±1.574[a]	29.357±0.689	30.177±0.497[b]	28.857±0.188[b]	29.31 ±0.955[b]
Acinar Vv %	38.120±0.854	24.856±2.11[a]	38.173±0.6 [b]	37.553±0.946 [b]	37.567±0.767 [b]	36.662±0.51 [b]

Comparison between groups was performed in row
P.A-Prunus africanus; TP-Testosterone Propionate; TPI-BPH-Testosterone Propionate induced benign prostatic hyperplasia
[a] indicates values that were significantly different (p <0.05) from the control
[b] indicates values that were significant different (p <0.05) from the BPH group

In Table 4 the mean epithelium volume density was found to be statistically significant difference among the 6 groups, $p < 0.05$. On the other hand, it was also noted that in treatment group the mean epithelial volume densities were significantly lower as the dose of CMBEPA increased when compared with TPI-BPH group.

3.2 The Histostereological Restorative Effects of CMBEPA

The histostereological findings of the study established that, when CMBEPA was administered for 21days in testosterone induce BPH group a dose dependent restoration was exhibited. The statistical significant ($p < 0.05$) was found in the prostate structure volumes which includes the epithelium, stromal and total prostate. These findings were similar to studies by (McNicholas & Kirby [29]; Yablonsky et al. [30]) which showed restorative histostereological effects in prostate volumes and volume densities. This similarity could be attributed due to the application of similar protocol in the experiment. In addition, the restoration can be attributed to its potent inhibition of the rat prostatic fibroblast proliferation through direct activation of protein kinase C (PKC). PKC activation appears to be an important growth factor-mediated signal transduction for *P.africanus.* On the other hand, in the TPI BPH group without treatment showed an increase in total prostate, stromal and epithelial. This concur with previous studies by (Li et al. [31]; Mbaka et al. [32]) whose findings reported an increase in prostate volume structure volume after exogenous exposure to testosterone propionate in the rats. The concordance could be attributed to the similar method in induction of BPH, in addition, these study findings on histostereological increase in prostate structure volumes and densities substantially could be attributed to role of exogenous testosterone in inductive interactions in stromal and epithelial lining growth.

The study applied various methods during quantification of histostereology to ensure satisfactory precision which included Coefficient of Error (CE) whose values in the restorative and inhibitory group animal were calculated and they were found to be below 0.05 and this was considered to be satisfactory in delivering a precise quantitative information in stereology [27]. In addition, the study showed a high level of agreement between the gold standard (Archimedes volume) and cavalieri method which was 76%. This was examined using intraclass correlation at 95 % confidence intervals for volume differences.

3.3 The Histostereological Inhibitory Effects of CMBEPA

The histostereological inhibitory effects of CMBEPA in this study was established by administration of Testosterone propionate concurrently with CMBEPA. A dose dependent inhibitory effects on the prostate structure volume were found to be statistically significant ($P < 0.05$); epithelium, and stromal, when compared with testosterone propionate group. This was concordant with studies by Shen et al. [33] and Jena et al (2016) whose findings showed bark extract of *P. africanus* successfully inhibits increase in prostate structure volumes and densities when co- administered with testosterone propionate in the rat models. These histostereological inhibitive effects of the CMBEPA treated rats may be attributed to the inhibitive activity of 5α-reductase in CMBEPA, thereby preventing conversion of testosterone to dihydrotestosterone (DHT). DHT is potent androgen that is crucial in proliferation of stromal and epithelial cells subsequently leading to BPH development [33,34].

4. CONCLUSION

The inhibition and restorative histostereological effects of CMBEPA was found to have an inverse dose response relationship with prostate structures volume with maximal dose related restoration and inhibition of total prostate volume, stromal epithelium and acinar prostate volume being achieved at dose 200 mg and 125 mg/kg bwt respectively.in addition, the optimal restorative and inhibitory effects of CMBEPA was maximum at 85.6% on BPH which was obtained at a dose of 200 mg/kg bwt, therefore, this study strongly supports its chemoprevention use in the management of BPH.

5. RECOMMENDATIONS

This study recommends establishment of long term effects of CMBEPA on histostereological parameters of prostate through carrying out studies for a longer duration. Further, research need to be carried out on restorative and inhibitory effects using second order stereology to determine the cellularity, the cell volumes and densities following administration of CMBEPA using non- primate animal.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All experiments were examined and approved by the Jomo Kenyatta University of Agriculture and Technology Animal Ethics Committee (JKUAT AEC) approval certificate number JKU/2/4/896A.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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