

Original Research Article

Anti-tumour potential of *Punica granatum* (Pomegranate) seed in testosterone induced Benign Prostate Hyperplastic Wistar albino Rats

ABSTRACT

Aim: The aim of this study was to assess the anti-tumour potentials of *Punica granatum* (Pomegranate) seed in testosterone induced Benign Prostate Hyperplastic Wistar albino Rats.

Study Design: This study is an experimental study.

Place and Duration of Study: The experimental aspect of this study was conducted at the animal house, Department of Pharmacology, University of Port Harcourt between April and September, 2019.

Methodology: Seventy (70) adult albino wistar rats were used for this study. They were divided into 12 groups of 5 rats each and fed with commercial rat diet and clean drinking water *ad libitum*. Aqueous and ethanol extracts of *Punica granatum* seed were prepared using the maceration method. Benign Prostate Hyperplasia was induced in rats after they submitted to bilateral orchiectomy by daily injections of testosterone propionate (TP) (4 mg/kg b.wt.sc). Rats were treated with 500 or 1500 mg/kg b.wt. of aqueous or ethanol extracts of *Punica granatum* seed, dutasteride or in combination. Administration of extracts was done by gavage. At the end of 30 days, the rats were anaesthetized with chloroform and blood samples were collected into lithium heparin bottles through cardiac puncture and serum prostate specific antigen (PSA) was analyzed using rat specific ELISA kits for PSA. The prostate gland was excised, washed, weighed and length, breadth and width measured. The organs were later preserved in formalin for histological analysis. Statistical analysis was done using statistical package for social sciences (SPSS) version 22.0 and P values less than .05 were considered significant.

Results: The results showed that subcutaneous administration of 4mg/kg of testosterone propionate caused a significant increase in prostate weight (from 216.2 ± 10.5 to 635 ± 78.8 mg), prostate volume (from $0.83 \pm 0.11\text{cm}^3$ to $0.23 \pm 0.05 \text{cm}^3$) and prostate specific antigen (from 76.34 ± 3.55 to 716 ± 56.1 pg/ml) at $p < .05$. However, when the rats were treated with both aqueous and ethanol extracts of *Punica granatum* seed, in combination with dutasteride and when simultaneously administered with testosterone, the above stated parameters were observed to have significantly decreased. Moreover, histological findings for rat prostates corroborated with the biochemical findings.

Conclusion: From our findings of the biochemical and histological changes in castrated male rats after the subcutaneous injection of testosterone propionate, biochemical and histological benign prostatic hyperplasia (BPH) was observed. However, extracts of *Punica granatum* seed administered orally ameliorated BPH in rats, by decreasing prostate size, weight, prostate index and prostate specific antigen levels in treated rat groups compared with the BPH model group. Both doses of the two plant extracts individually, in combination with dutasteride also markedly reduced prostate parameters.

Keywords: Anti-tumour, *Punica granatum* (Pomegranate) seed, testosterone, Benign Prostate Hyperplastic albino Wistar Rats.

1. INTRODUCTION

Benign prostatic hyperplasia (BPH) is characterized by non-malignant enlargement of the prostate. It is one of the most common urological diseases in the elderly, [1]. BPH involves increase in the number of both stromal and epithelial cells in the transitional zone of the

Comment [HC1]: Mismatch in total subjects and division in group. $12 \times 5 = 60$ not 70.

prostate and can cause lower urinary tract symptoms, which includes urgency, frequency, dysuria, incontinence and supra-pubic pain, [2]. Due to the high prevalence of BPH in elderly men, it has been suggested to be a ubiquitous sign of aging, [3]. The concept that androgens are important for the maintenance of prostate disease dictates the standard of care for BPH [4].

It has been reported that one in every four persons has BPH in their lifetime. BPH induces troublesome lower urinary tract symptoms (LUTS) and is associated with complications such as hematuria, acute urinary retention, calculi, urinary tract infections, and need for surgical interventions. In severe cases, BPH may lead to irreversible bladder damage, chronic renal failure, sepsis, or even death, [5]. Autopsy studies and physical examination have revealed evidence of BPH in 25 % of men aged 40–49 years, rising to 80 % among men 70–79 years old, [6]. Severe BPH has a strong impact on the quality of life of affected patients, and its treatment will become a serious burden of economic expenditure. Various types of pharmaceutical therapies are currently in use for the treatment of BPH, including alternative herbal-based therapies. However, there is currently no drug that can completely cure BPH without accompanying adverse effects. The 5 α -reductase inhibitors, Dutasteride and Finasteride are currently in use but come with severe adverse effects, including sexual dysfunction, [7]. Several herbal therapies that have been used individually and recommended for BPH with certain clinical evidence of efficacy include saw palmetto (*Serenoa repens*), stinging nettle (*Urtica dioica*), *Pygeum africanum*, and so on. [8]. Bee pollen extract (Cernilton) has also been used, however, not enough evidence of its efficacy against BPH exists.

In recent times, traditional medicine has continued to receive increasing acceptance in Nigeria among many other African nations. The World Health Organization (WHO) estimated that about 80% of African populations use traditional medicine to meet their primary health care needs. For many people in these countries especially those living in rural areas, traditional medicines are the only available, easily accessible and affordable source of health care. The combined use of different herbal products has been observed among consumers, but little is known about the simultaneous use of prescribed medications with herbal medicine for supposed “enhanced therapeutic effect”. Moreso, few consumers are aware of the consequences accompanying the abuse of these products and in combination with orthodox medications, as well as the potent phytochemicals they contain. Plants, algae, and fungi have been utilized as medicine throughout human history and probably even before humans evolved, given the practice of botanical medicine by non-human animals, [9]. Among the many applications of herbs in medicine include the use of these agents to treat conditions of the urinary tract diseases and cancers. The major active compounds in the root of this herbal plants are terpenoids and coumarins.

While pomegranate is best known for heart health, [10], additional studies on pomegranate seed reveal its potential to help guard against cancer, [11]. Some researchers have reported that certain phytochemical compounds like flavonoids found in pomegranate can deter cancer formation and progression, [12]. Pomegranate contains compounds that circumvent functional changes involved in benign and malignant cell formation and transformation. In preclinical studies, it stopped cancer cells from growing and spreading, [12]. It has been reported that pomegranate contains punicalagin, luteolin, ellagitannins, and assorted polyphenols that can impede many steps involved in the formation, growth, and spread of cancer, [13]. Rather than preventing cancer by a single mechanism, pomegranate blocks many different targets. A primary mechanism is to inhibit inflammation. Pomegranate accomplishes this by turning off genes related to inflammation, such as reducing activity of pro-inflammatory NF-kappaB, [14]. Therefore, the aim of this study was to assess the anti-tumour potential of *Punica granatum* (Pomegranate) seed in testosterone induced BPH wistar albino Rats.

Comment [HC2]: Spacing / typo error

2. MATERIALS AND METHODS

2.1 Experimental Design

This study is an experimental study.

2.2 Experimental Animals

A total of seventy (70) male albino Wistar rats that weighed between 170-200g were used for this study. The rats were purchased from the Department of Pharmacology, University of Port Harcourt, Rivers State. They were kept in a spacious and well-ventilated cage at room temperature; under natural circadian rhythm and were allowed to acclimatize for fourteen (14) days. They were allowed access to feed (Top Feed Finisher Mash, Sapele, Nigeria) and water *ad libitum*. All the animals received humane treatment according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Institute of Health [15].

2.3 Plant Material

2.3.1 Pomegranate (*Punica granatum*)

Pomegranate fruit (with seed) was bought at Spar Supermarket, Port Harcourt. The fruits were cut open and seeds removed, dried under the sun and macerated to powder form using a macerator.

2.3.2 Extraction of Powdered *Punica granatum* Seed with Absolute Ethanol and Distilled Water

Finely powdered *Punica granatum* Seed was poured into a beaker and absolute ethanol/distilled water was measured and poured into the beaker. It was intermittently shaken on a shaker and macerated for 48 hours. After 48 hours' storage, it was filtered and the filtrate was separated through a Whatman's no. 1 filter paper into a clean beaker. The filtered extracts were concentrated (at low pressure) using the rotary evaporator equipment (Manual Lift Rotary Evaporator Model EV311H by LabTech, U.S.A) after which they were dried on an evaporating dish at a temperature of 50°C to 60°C to a semi-solid form. A sticky semi-solid dark brownish substance was obtained. The extracts were stored in a well corked universal bottle and kept in the refrigerator prior to use.

2.4 Drugs/Chemicals

2.4.1 Avodart (Dutasteride), Testot (Testosterone Propionate) and Ketalar (Ketamine hydrochloride)

Avodart (manufactured by GlaxoSmithKline, UK), Testot (by Laborate Pharmaceuticals India Limited), and Ketalar (by Sular Pharmaceuticals, India) respectively used as anti-BPH, BPH inducing and anesthetic drugs were purchased from Sicone Pharmacy and Stores, No. 2B Evo Road, G.R.A. Phase II Port Harcourt, Nigeria.

2.5 Dose Calculations

2.5.1 Avodart (Dutasteride)

Calculation of the administered dosages was based on guidelines from U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research [16]. Human daily dose is 1 capsule (0.5mg) per day.

The FDA guideline for dose conversion between human and animals in pre-clinical studies was used. To convert human dose in mg/kg to animal equivalent dose (AED) in mg/kg, human dose was multiplied by 6.2. Therefore, if a 60kg man would take 0.5mg Dutasteride, then a 1kg man would take;

$$0.5\text{mg}/60\text{kg} = 0.00833\text{mg}$$

That is 0.00833mg/kg. Then multiplying by the FDA factor, the AED would be

$$0.00833\text{mg}/\text{kg} \times 6.2 = 0.051\text{mg}/\text{kg}.$$

This dose was administered mg per kg body weight of the rats dissolved in appropriate volume of normal saline (FDA guideline), [16].

2.5.2 Testosterone Propionate

The dose of testosterone propionate (TP) administered was 4mg/kg b.wt. subcutaneous, which was determined by a pilot study conducted by Obisike et al. [17]. The pilot study showed that TP at the dose stated above could induce histological BPH and cause significant increases in rat prostate volume, prostate weight, prostatic indices and PSA levels. The changes were sustained throughout the 30-day period of this study.

Comment [HC3]: Spelling / typo error

2.6 Castration of Rats (Bilateral Orchiectomy)

The rats were castrated using an anaesthetic agent (ketamine, 25 mg/kg body wt, intraperitoneal.) in order to eliminate the influence of endogenous testosterone during the study. Castration involved the removal of both testes and the epididymal fat through the scrota sac by the method of Van Coppenolle et al. [18]. The blood vessels and the spermatic cord were tied up with suture materials (3.0 mm) and resected. The animals were then allowed one (1) week to recuperate before the commencement of the pilot and main study.

2.7 Grouping of Animals

The rats were weighed and randomized into twelve groups of five rats each (apart from normal control, BPH and PC control groups that contained 10 rats each that were further divided into 5 rats for each group, as shown below):

Comment [HC4]: Not match with total no of rats (n=70)

2.7.1 Group 1 (Normal Control Group –NC and NC₂)

This group contained ten (10) male albino wistar rats. The rats in this group were further divided into two groups; five rats were used as control for the groups that were treated after BPH had been established for 15 days (NC), while the remaining five were used as control for the groups that were simultaneously induced and treated (NC₂). They were not BPH induced but were subjected to sham bilateral orchiectomy and were allowed rat feed for 30 days.

2.7.2 Group 2 (BPH Control – BPHC and BPHC₂)

Ten (10) male albino wistar rats in this group were subjected to bilateral orchiectomy and divided into two groups of five rats each. BPHC group rats were BPH induced by subcutaneous (s.c.) injection of 4mg/kg body weight (b.wt.) (for the first 15 days) of testosterone propionate and were not given further treatment for 30 days, while the BPHC₂ groups were treatment with 4mg/kg body b.wt. s.c for 30 days. They were allowed normal rat feed from the 16th day for 30 days.

Comment [HC5]: Which drug

2.7.3 Group 3 (Positive Control – PC and PC₂)

This group contained ten (10) rats. Five (5) male albino wistar rats were subjected to bilateral orchiectomy and BPH induced by subcutaneous injection of 4mg/kg b.wt. (for the first 15 days) of testosterone propionate and were given oral (gavage) administration of 0.051mg/kg/day of Avodart (Dutasteride) daily from the 16th day for 30 days. The remaining five rats in were also subjected to bilateral orchiectomy and were induced for BPH by the injection of 4mg/kg b.wt. subcutaneous, daily and simultaneously administered 0.051mg/kg of Dutasteride daily for 30 days.

2.7.4 Group 4 (500EthPun.)

Five (5) male albino wistar rats in this group were subjected to bilateral orchiectomy and BPH induced by subcutaneous injection of 4mg/kg b.wt. (for the first 15 days) of testosterone propionate and were given oral (gavage) administration of 500mg/kg b.wt./day of ethanol extract of Pomegranate seed from the 16th day for 30 days.

2.7.5 Group 5 (1500EthPun.)

Five (5) male albino wistar rats in this group were subjected to bilateral orchiectomy and BPH induced by subcutaneous injection of 4mg/kg b.wt. (for the first 15 days) of testosterone propionate and were given oral (gavage) administration of 1500mg/kg b.wt./day of ethanol extract of Pomegranate seed from the 16th day for 30 days.

2.7.6 Group 6 (500AquaPun.)

Five (5) male albino wistar rats in this group were subjected to bilateral orchiectomy and BPH induced by subcutaneous injection of 4mg/kg b.wt. (for the first 15 days) of testosterone propionate and were given oral (gavage) administration of 500mg/kg b.wt./day of aqueous extract of Pomegranate seed from the 16th day for 30 days.

2.7.7 Group 7 (1500AquPun.)

Five (5) male albino wistar rats in this group were subjected to bilateral orchiectomy and BPH induced by subcutaneous injection of 4mg/kg b.wt. (for the first 15 days) of testosterone propionate and were given oral (gavage) administration of 1500mg/kg b.wt./day of aqueous extract of Pomegranate seed from the 16th day for 30 days.

2.7.8 Group 8 (1500EthPun.Dut)

Five (5) male albino wistar rats in this group were subjected to bilateral orchiectomy and BPH induced by subcutaneous injection of 4mg/kg b.wt. (for the first 15 days) of testosterone propionate and were given oral (gavage) administration of 1500mg/kg b.wt./day ethanol extract of Pomegranate seed mixed with 0.051mg/kg b.wt./day of Avodart (Dutasteride) from the 16th day for 30 days.

2.7.9 Group 9 (1500AquPun.Dut)

Five (5) male albino wistar rats in this group were subjected to bilateral orchiectomy and BPH induced by subcutaneous injection of 4mg/kg b.wt. (for the first 15 days) of testosterone propionate and were given oral (gavage) administration of 1500mg/kg b.wt./day aqueous extract of Pomegranate seed mixed with 0.051mg/kg b.wt./day of Avodart (Dutasteride) from the 16th day for 30 days.

2.7.10 Group 10 (SimAdm1500AquPun)

Five (5) male albino wistar rats in this group submitted to bilateral orchiectomy and BPH induced by subcutaneous injection of 4 mg/kg b.wt./day of testosterone propionate for 30 days and were simultaneously given oral (gavage) administration of 1500 mg/kg b.wt./day aqueous extract of *Punica granatum* seed from day 1 (first day of administration of testosterone propionate) for 30 days.

2.7.11 Group 11 (SimAdm1500EthPun)

Five (5) male albino wistar rats in this group submitted to bilateral orchiectomy and BPH induced by subcutaneous injection of 4 mg/kg b.wt./day of testosterone propionate for 30 days and were simultaneously given oral (gavage) administration of 1500 mg/kg b.wt./day ethanolic extract of *Punica granatum* seed from day 1 (first day of administration of testosterone propionate) for 30 days.

2.8 Sample Collection and Storage and Analysis

At the end of the treatments, the rats were anaesthetized with chloroform and blood samples collected through cardiac puncture after 8 hours fast. Immediately after euthanasia, the prostate gland was resected, washed weighed and measured. The length, height and breadth were measured. The prostate volume was calculated as described below. Five (5) ml of blood was put in lithium heparin container for the determination of serum PSA, The samples in the lithium heparin container were allowed to stand and plasma separated within thirty minutes of sample collection using a centrifuge. The serum samples were then stored frozen at -20°C, until the time of determination of other parameters.

2.9 Measurement of Prostate Size

Prostate size was measured with a centimeter rule. Immediately after euthanasia, the prostate gland was resected, washed weighed and measured. The length, height and breadth were measured. The prostate volume was calculated using the formula below:

$$PV (cm^3) = \frac{1}{2} (l \times b \times h),$$

Where l = length of prostate gland

b = breadth of prostate gland

h = height of prostate gland, all measured in cm.

2.10 Calculation of prostatic indices, prostate percentage inhibition

Prostate Index was calculated using the formula below:

Prostate Index (PI) = Prostate weight (mg)/Body weight (g)

Arbitrary unit = mg/g.

Calculation of Prostate Percentage Inhibition (Percent. I) = $100 - [(T-C)/(B-C)] \times 100\%$ [19].

Where: T - mean PI for treatment group

C – mean PI for control group

B – mean PI for BPH group

Unit = %

2.11 Laboratory Methods

2.11.1 Estimation of rat plasma prostate specific antigen McCarthy et al. [20] as modified by Shanghai Korain Biotech Co., Ltd, China.

The method used was sandwich enzyme-linked immunosorbent assay (ELISA).

Principle of Test

The PSA ELISA test is based on the principle of a solid phase enzyme-linked immunosorbent assay. The assay system utilizes a rabbit anti-PSA antibody directed against intact PSA for solid phase immobilization (on the microtiter wells). A monoclonal anti-PSA antibody conjugated to horseradish peroxidase (HRP) is in the antibody-enzyme conjugate solution. The test sample is allowed to react first with the immobilized rabbit antibody at room temperature for 60 minutes. The wells are washed to remove any unbound antigen. The monoclonal anti-PSA-HRP conjugate is then reacted with the immobilized antigen for 60 minutes at room temperature resulting in the PSA molecules being sandwiched between the solid phase and enzyme-linked antibodies. The wells are washed with water to remove unbound-labeled antibodies. A solution of TMB (Human tubular basement membrane antibody) reagent is added and incubated at room temperature for 60 minutes, resulting in the development of a blue color. The color development is stopped with the addition of Stop Solution changing the color to yellow. The concentration of PSA is directly proportional to the color intensity of the test sample. Absorbance was measured spectrophotometrically at 450 nm.

2.11.2 Histological analysis

The prostate gland, kidneys and liver were harvested for histological analysis, and were fixed in 10% formal saline solution. The tissues were dissected (the ventral prostate lobes were used for all the rats) and representative tissue blocks were taken for histological processing each with identifying label in a tissue cassette. The fixed tissue blocks were dehydrated through ascending grades of alcohol, de-alcoholised in xylene, infiltrated and embedded in molten paraffin wax. Sections were cut at 3µm on a rotary microtome. Deparaffinised sections were then stained with the standard haematoxylin and eosin staining technique and the slides mounted in DPX. Sections on slide were examined and photomicrographs captured with X400 objective lens using the ScopeTek™ device and software v1.3.

2.12 Statistical analysis

SPSS version 22.0 of windows statistical package was used to analyze the data generated. The mean ± standard deviation was determined. One way analysis of variance (ANOVA) with Turkey's Post Hoc test, bar charts and line graph were also done using the same statistical package. From the values obtained statistical decision and inferential evaluation were made. A probability (p) value of less than 0.05 was considered statistically significant.

3. RESULTS AND DISCUSSION

UNDER PEER REVIEW

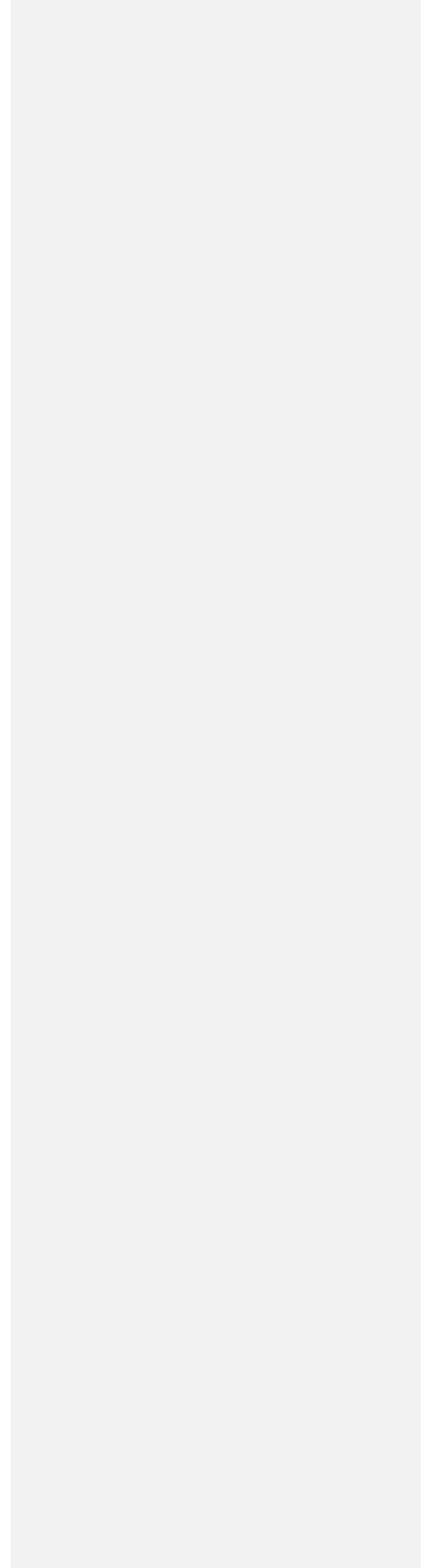


Table 1: Prostatic parameters of TP induced BPH male rats treated with a combination of lower and higher doses of ethanolic and aqueous extracts of *Pg* seed compared with controls

	PW(mg)	LBW(g)	PI (mg/g)	Per.I(%)	PV (cm ³)	PSA (pg/ml)
Grp1(NC (n=5))	216.2 ± 10.5	175 ± 7.31	1.23 ± 0.07	-	0.23 ± 0.05	76.34 ± 3.55
Grp2(BPHC) (n=5)	635.8 ± 78.8 ^a	181 ± 12.70	3.52 ± 0.46 ^a	-	0.83 ± 0.11 ^b	716.96 ± 56.18 ^a
Grp3(PC)(n=5)	307.0 ± 11.8 ^{ab}	178 ± 12.17	1.73 ± 0.17 ^b	78.19	0.33 ± 0.06 ^b	98.44 ± 15.26 ^{ab}
Grp4(500EthPun) n=4	360.3 ± 36.5 ^b	156 ± 7.07	2.31 ± 0.29 ^{bc}	52.84	0.32 ± 0.10 ^b	201.20 ± 22.35 ^b
Grp5(1500EthPun) n=5	295.2 ± 10.8 ^b	150 ± 21.53 ^b	2.00 ± 0.30 ^b	66.38	0.21 ± 0.03 ^b	136.66 ± 28.70 ^b
Grp6(500AquPun)n=5	224.2 ± 10.0 ^{bc}	155 ± 13.83	1.45 ± 0.11 ^b	90.4	0.32 ± 0.08 ^b	430.54 ± 61.05 ^b
Grp7(1500AquPun)n=5	257.2 ± 20.1 ^b	165 ± 17.03	1.56 ± 0.20 ^b	85.6	0.32 ± 0.05 ^b	283.26 ± 43.02 ^b
F value	87.10	3.80	42.97	-	37.127	173.27
P value	0.000	0.007	0.000	-	0.000	0.000
Remark	S	S	S	-	S	S

Post hoc test, significant p values: a, b & c – compared with Groups. 1, 2 and 3 respectively

Table 2: Prostatic parameters of TP induced BPH male rats treated with a combination of higher dose of aqueous and ethanolic extracts of mixture of *Pg* seed and dutasteride compared with controls

	PW(mg)	LBW(g)	PI (mg/g)	Per.I(%)	PV(cm ³)	PSA (pg/ml)
Grp1(NC (n=5))	216.2 ± 10.5	175 ± 7.31	1.23 ± 0.07	-	0.23 ± 0.05	76.34 ± 3.55
Grp2(BPHC) (n=5)	635.8 ± 78.8 ^a	181 ± 12.70	3.52 ± 0.46 ^a	-	0.83 ± 0.11 ^a	716.9 ± 56.18 ^a
Grp3(PC ₁)(n=5)	307.0 ± 11.8 ^{ab}	178 ± 12.17	1.73 ± 0.17 ^b	78.19	0.33 ± 0.06 ^b	98.44 ± 15.26 ^b
Grp8(1500EthPunDut)n=5	221.0 ± 29.15 ^{bc}	162 ± 26.18	1.38 ± 0.19 ^b	93.67	0.26 ± 0.08 ^b	96.26 ± 5.49 ^b
Grp9(1500AquPunDut) n=4	333.75±34.99 ^b	144±13.04 ^{abc}	2.33 ± 0.44 ^b	51.97	0.41 ± 0.10 ^b	161.07±25.09 ^b
F value	85.11	3.97	47.10	-	40.346	455.77
P value	0.000	0.017	0.000	-	0.000	0.000
Remark	S	S	S	-	S	S

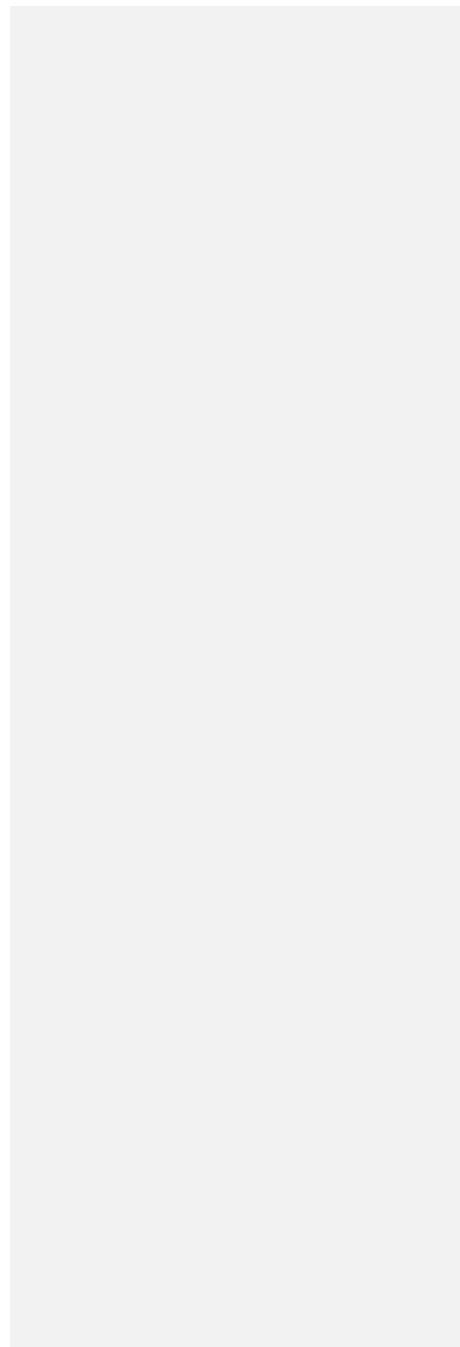
Post hoc test, significant p values: a, b & c – compared with Groups. 1, 2 and 3 respectively

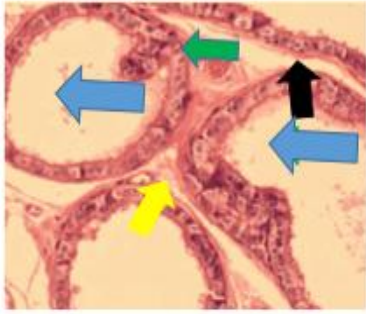
Table 3: Prostatic parameters of male rats simultaneously induced and treated with higher dose of both extracts of mixture of *pg* seed compared with controls

	PW(mg)	LBW(g)	PI (mg/g)	Per.I(%)	PV (cm ³)	PSA (pg/ml)
Grp1(NC ₂ (n=5))	219.8 ± 12.9	175 ± 5.34	1.26 ± 0.09	-	0.37 ± 0.07	75.58 ± 5.60
Grp2(BPHC ₂) (n=5)	631.6 ± 69.1 ^a	175 ± 4.09	3.60 ± 0.34 ^a	-	0.85 ± 0.07 ^a	716.7 ± 58.7 ^a
Grp3(PC ₂)(n=5)	222.8±12.33 ^{ab}	134 ± 9.65 ^a	1.66 ± 0.19 ^b	81.22	0.21 ± 0.04 ^b	72.5 ± 5.8 ^{ab}
Grp10(SimAdm1500AquPun)n=4	426.7 ± 4.92 ^b	141±14.0 ^{ab}	3.03±0.31 ^a	78.60	0.32 ± 0.07 ^b	481.9 ± 23.2 ^b
Grp11(SimAdm1500EthPun) n=5	308.6±6.38 ^b	148±22.1 ^{ab}	2.11±0.28 ^{ab}	51.57	0.19 ± 0.09 ^b	385.8 ± 30.0 ^b
F value	101.36	17.92	31.22	-	54.254	495.39
P value	0.000	0.000	0.000	-	0.000	0.000
Remark	S	S	S	-	S	S

Post hoc test, significant p values: a, b & c – compared with Groups. 1, 2 and 3 respectively.

UNDER PEER REVIEW





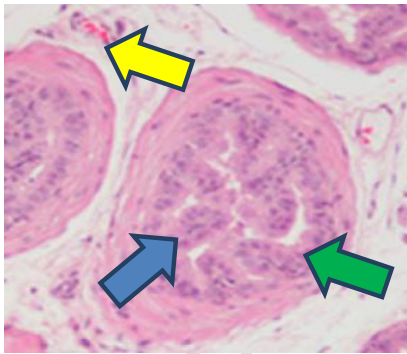
Green – cuboidal epithelial cells with regular sizes

Yellow – Interglandular smooth muscle fibres

Blue – Normal luminal space

Black – Normal glandular stroma

Plate 1: Photomicrograph of a Cross Section of the Ventral Prostate Gland Rat in NC group (H&E, 400X).

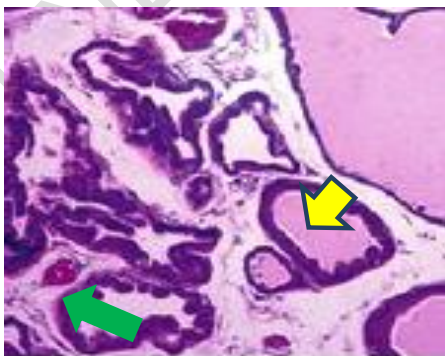


Yellow – expansion in the stroma due to hyperplasia

Blue – Significant thickening, hypertrophy, and hyperplasia with papillary projections

Green – Disrupted morphology in the prostate epithelia

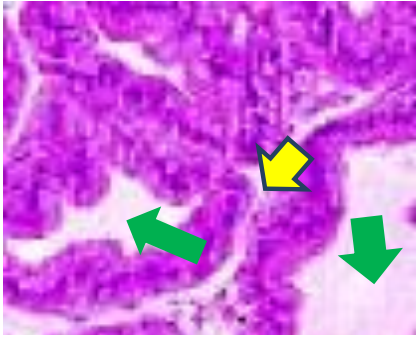
Plate 2: Photomicrograph of a Cross Section of the Ventral Prostate Gland of Rat in BPHC group (H&E 400X).



Green – Clear luminal space
Yellow – Regular shaped acinus with basally located epithelial cells

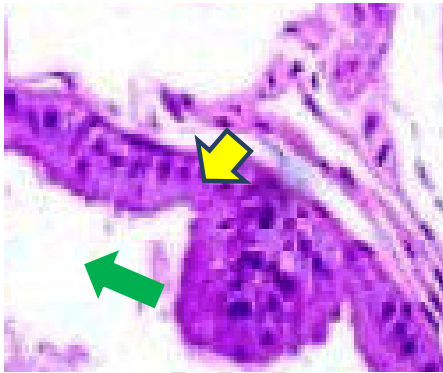
Plate 3: Photomicrograph of a Cross Section of the Ventral Prostate Gland of Rat in 1500AquPun group (H&E 400X).

Comment [HC6]: Arrows should be incorporated within the image so that they cannot be displaced during formatting.



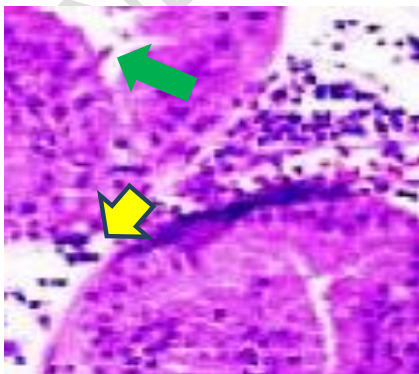
Green – Luminal restoration
Yellow –Stromal restoration

Plate 4: Photomicrograph of a Cross Section of the Ventral Prostate Gland of Rat in 500EthPun.Dut group (H&E 400X).



Green – Normal epithelial cells
Yellow – Clear lumen

Plate 5 Photomicrograph of a Cross Section of the Ventral Prostate Gland of Rat in 1500EthPun.Dut group (H&E 400X).



Green – Luminal restoration
Yellow – Stromal restoration
(hyperplasia still seen)

Plate 6: Photomicrograph of a Cross Section of the Ventral Prostate Gland of Rat in Sim500AquPun group (H&E 400X).

In this study, the prostate weight (PW), prostate volume (PV) and prostate specific antigen (PSA) levels were significantly increased ($P < .01$) when compared with values of rats in the negative control groups following BPH induction. These values were observed to have decreased markedly after thirty days of daily oral administration of 500mg/kg b.wt. and 1500mg/kg b.wt. of aqueous and ethanolic extracts of *Punica granatum* (*Pg*) (Table 1). Significantly increased ($P < .01$) prostatic index observed in the BPH group was seen to decrease in the groups of rats treated with high and low doses of aqueous and ethanolic extracts of *Pg*. Mean percentage inhibition which is a measure of the extracts' ability to inhibit prostatic enlargement was highest (90%) in the group of rats that were fed with 500mg/kg b.wt. of aqueous *Pg* seed extract. Other doses of the two methods of extractions also maximally inhibited the prostate size.

The use of the pomegranate juice, peel and seed has been indicated that pomegranate have anticancer activities, including interference with tumor cell proliferation, cell cycle, invasion and angiogenesis. Although BPH is non-cancerous, but all tumours or growths share something in common, whether benign or malignant, which is uncontrolled proliferation of cells due to lack of regulation mechanisms or excessive stimulation of growth factors. The antiproliferative ability of the plant extract may be related to anti-inflammatory effects of pomegranate. The phytochemical and pharmacological actions of pomegranate properties indicate a wide variety of clinical usage for the cancer prevention and treatment, also other diseases where chronic inflammation is reliable to play a main etiologic role, [21].

In a study by Adhami et al. [22], pomegranate seed extract was reported to have capability to inhibited lung, skin, colon and prostate tumors growth in preclinical animal studies. Pomegranate seed oil showed significant prolongation of prostate specific antigen doubling time in patients with prostate cancer in an initial phase II clinical trial. In an experimental study, pomegranate seed oil has also demonstrated proliferation prevention of various tumor cell types [23]. The ability of any chemotherapeutic agent to inhibit selectively proliferation of benign or malignant but not normal cells is the hallmark of a promising anti-proliferation/anticancer therapeutic agent. In this regard, aqueous and ethanolic pomegranate seed extracts have been reported to retard proliferation of cells in several different human cancer cell lines [24][25].

Higher doses of the two methods of extractions of Pomegranate did not show any better anti-proliferative capabilities (non-significant differences in PW, PV and PI) (Table 1). Reasons may be that the difference in dose may not be enough to make any significant therapeutic impact. However, significant ($P < .01$) decreases were observed in the PW and PI between the lower doses (500mg/kg.b.wt) of aqueous and ethanol extracts of pomegranate seed and no significant difference in any of the measured parameters was observed in the higher doses (1500mg/kg b.wt.) when values for both methods of extracts were compared.

There are nearly 1500 documented interactions between drugs, herbal medicines, and dietary supplements and these interactions could enhance the therapeutic efficacy of the mixture or may antagonize the effect of each other. In most of the cases, the mixture results to a wide variety of harmful effects, [26]. The interactions of herbs or their metabolites with another herb have been known to produce result in antagonistic or enhancement ways.

In the current study, it was observed that treatment with a combination of pomegranate and the anti-BPH drug dutasteride showed marked decreases in PV, PW, PI and PSA (Table 2). The highest percentage inhibition (93%) was observed in the group treated with a combination of ethanolic extracts of pomegranate seed and dutasteride. Ethanol extracts of *Pg* also showed more BPH reducing effect than the aqueous extract of the same plant (Table 2). A decrease in PSA and other parameters is associated with reduced prostate hyperplasia as a direct consequence of 5 α -reductase inhibition or anti-inflammatory actions by the anti-BPH drug dutasteride or in combination with the extracts. BPH is caused by dihydrotestosterone, a metabolite obtained from the conversion of testosterone by 5 α - reductase [27]. Consequently, inhibitors of 5 α -reductase which block production of DHT ultimately slow down the development of BPH.

It is interesting to mention that this study also revealed that the combination of higher doses (1500mg/kg b.wt.) of ethanol extracts of pomegranate with dutasteride had more BPH reducing effect than dutasteride administered alone. This may be due to synergistic effect of ethanol extracts of pomegranate seed and the anti-BPH drug dutasteride or its metabolites (Tables 2). This finding could be correlated with the extra phytochemical compounds found in the ethanol extracts of *Pg* seed but missing in the aqueous extract. This study may have found an additive effect of ethanolic extract of *Pg* seed with a particular orthodox anti-BPH drug, some studies have reported otherwise. Though differences in methods of extraction of the herb and the type of disease condition could also be determining factors.

Ozbay et al. [28] reported that pomegranate seed oil presented growth inhibitory activity in breast cancer cells of different molecular subtypes, but elevated potential drug antagonism when used in combination with existing targeted therapies in HER2-overexpressing breast cancer. This study showed that the hyperplasia induced by testosterone was less in rats simultaneously given aqueous pomegranate seed along with testosterone, attaining even better protection when the ethanolic extracts of pomegranate seed were administered.

Simultaneous administration of higher doses of aqueous and ethanolic extracts of *Punica granatum* seed with TP for 30 days inhibited prostate enlargement in the treatment groups. Mean PV, PW and PSA were significantly decreased ($P < .01$). The PC₂ groups being rats treated with daily administration of 4mg/kg b.wt. sc. TP and 0.051mg/kg dutasteride showed marked decreases in mean PV, PW and PSA, (Tables 3). Again, ethanolic extracts of both plants showed better results when compared to the aqueous extracts. Dutasteride, an inhibitor of 5 α -reductase enzyme, reduced the testosterone-induced prostatic hyperplasia in rats. Exogenous testosterone accelerated the growth of the prostate, castration of the rats paused prostatic growth and testosterone administration to castrated adult male rats caused the gland to grow beyond normal. Castration of adult male rats causes extensive atrophy of prostate with induction of apoptosis, majorly of the ventral prostate epithelial cells [29]. Further administration of testosterone exogenously to castrated rats caused suppression of apoptosis and prevented epithelial cell atrophy. The percentage inhibition of the ethanolic extract of *Pg* was also higher. However, the inhibitory effects of dutasteride (PC₂) administered daily for 30 days was better than that of the aqueous and ethanolic extracts of *Pg*.

Histological findings for rat prostate gland corroborated with the biochemical findings. There were no morphological changes in the lining epithelium or the acini of the sham operation control group (NC). Cuboidal epithelial cells of regular size were observed, (Plate 1). However, after TP-induction, disrupted morphology in the prostate epithelia was observed by significant thickening, hypertrophy, and hyperplasia with papillary projections in the lining epithelium or the acini (Plate 2). Widening of the lumen diameter without remarkable expansion in the stroma was also observed in the BPH model group (Plate 2).

Moreover, the ventral prostate of rats treated with lower and higher doses of aqueous and ethanol extracts of *Pg* showed prostate tissues with normal or rather restored cuboidal epithelia cells with regular sizes, restored glandular stroma and luminal spaces, suggesting a repair of prostatic architecture, when compared with the NC group (Plates 3 to 5). Similar reduction in epithelial hyperplasia and minimal stromal disruption were also seen in the treatment with combination of *Pg* seed and dutasteride (Plates 6). Most prominent of all the changes were stromal and luminal restorations. Simultaneous treatment of higher doses of both plant extracts and in combination with dutasteride also showed inhibition of characteristic features of histological BPH. Those features indicate that both concentrations (500 and 1500mg/kg b.wt.) of the two methods of plant extractions (aqueous and ethanolic) of *Pg* at some levels had antiproliferative properties. Although, mild epithelial hyperplasia were seen in some of the treated groups when compared with those of NC group. This could probably be due to the relatively short period (4 weeks) of treatment. Perhaps, extended treatment duration could possibly produce better therapeutic outcome. Unfortunately, no known reported study on the administration of plant extracts (simultaneous or post treatments) on induced rats for more than 30 days. These findings also agree with the biochemical findings which showed significant decreases in PW, PV, PI and PSA when compared with the BPHC group, which are tools in measuring physical prostatic enlargement.

In previous studies using rat models, changes in PW and histomorphology have been an important indicator for the inhibitory effects of substances on the development of BPH, [30]. BPH is commonly characterized by hyperplasia of the epithelium and stroma in the prostate, which results in an increase in the PW. The hyperplastic prostate tissue may gradually constrict the urethral canal to cause partial or sometimes even complete obstruction, leading to lower urinary tract obstruction, these clinical consequences which are considered as the signs for clinical BPH, [31]. For these reasons, by monitoring the changes in PW and histological findings, previous studies have tested the inhibitory roles of various xenobiotic agents on BPH development and have shown their efficacies, which most are in tandem with the findings of this study.

4. CONCLUSION

From our findings of the biochemical and histological changes in rats, BPH was induced in castrated male rats after the subcutaneous injection of 4mg/kg b.wt. testosterone propionate. It was also observed that extracts of *Punica granatum* seed administered orally ameliorated BPH in rats, by decreasing prostate size and weight, prostate index and prostate specific antigen levels in treated rat groups compared with the BPH model group. Both doses of the two plant extracts individually, in combination with dutasteride also markedly reduced prostate parameters.

Comment [HC7]: elaborate limitation and future scope of the study

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ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

NOTE:

The study highlights the efficacy of "herbal" which is an ancient tradition, used in some parts of India. This ancient concept should be carefully evaluated in the light of modern medical science and can be utilized partially if found suitable.

COMPETING INTERESTS DISCLAIMER:

AUTHORS HAVE DECLARED THAT NO COMPETING INTERESTS EXIST. 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.

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