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Pomegranate Seed Extract: A Strong Antioxidant against Benign Prostatic Hyperplasia Induced Oxidative Stress in Albino Wistar Rats

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Authors' contributions

This work was carried out in collaboration among all authors. Authors EON and NB designed and supervised this work. Author UAO conducted the experimental aspect of the study and the statistical analysis and wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The aim of this study was to assess the antioxidant properties of ethanolic and aqueous extracts of *Punica granatum* (Pomegranate) seed in testosterone induced benign prostate hyperplastic albino Wistar rats.
Study design: This study is an interventional 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 male 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. Aqueous and ethanolic 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,

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dutasteride or in combination. Administration of extracts was done by gavage. Plasma total oxidant status (TOS), total antioxidant status (TAS), superoxide dismutase (SOD) activity, were analyzed using sandwich ELISA Kits by Shanghai Korain Biotech Co., Ltd, China, while oxidative stress indices (OSI) were calculated. Statistical analysis was done using SPSS version 22.0 of Windows Stat Pac and p values less than 0.05 were considered statistically significant.

Results: The results showed that the mean TOS, TAS, SOD and OSI for the rats in the normal control group were 1.66±0.2U/ml, 2.71±0.25U/ml, 41.8±2.9pg/ml and 0.62±0.10 respectively. After BPH induction (group 2), the values were 3.25±0.5U/ml,1.17±0.14U/ml, 23.38±2.09 pg/ml and 2.81±0.60pg/ml respectively. There were significant decreases for TOS and OSI, and significant increases for TAS and SOD when the rats where treated with lower and higher doses of both aqueous and ethanolic extracts of *Punica granatum* and Dutasteride.

Conclusion: In conclusion, both doses of *Punica granatum* seed for ethanolic and aqueous extracts individually and in combination and with dutasteride markedly reduced total oxidant status, oxidative stress indices and improved the activities of antioxidant parameters like superoxide dismutase and total antioxidant status.

Keywords: Pomegranate extract; antioxidant; benign prostate hyperplasia induced oxidative stress; albino rats.

1. INTRODUCTION

Although aging and androgens are two established factors that contribute to the development of BPH, novel findings highlight the importance of inflammation and production of reactive oxygen species, [1]. BPH is one of the leading urological problems of senescent men and is usually characterized with disturbing obstructive and irritating clinical consequences and have been shown to be associated with increased oxidative stress [2].

Production of high levels of ROS causes a significant decrease in antioxidant defense mechanisms leading to protein, lipid and DNA damage and subsequent disruption of cellular functions and cell death but at lower levels induce subtle changes in intracellular signaling pathways, [3]. The oxidative damage can be exacerbated by a decreased efficiency of antioxidant defense mechanisms, [4]. Like many different cancer types, oxidative stress has been linked with benign prostatic hyperplasia (BPH) and prostate cancer (PCa) development, progression and the response to therapy, [2,5-8]. Oxidative stress and PCa are both associated with increasing age because PCa is more prevalent in older men. Hence, it has been reported that age increases the pro-oxidant antioxidant balance toward a more oxidative state in many tissues [9].

It is known that OS contributes to the initiation and progression of PCa by regulating molecules such as DNA, transcription factors, and cell cycle regulators. Other studies have shown that antioxidants and other molecules that protect cells against OS play a role in the prevention of PCa. The potential chemo-protective role of ROS regulators in the fight against PCa has been reported. Chronic increases in ROS over time are known to induce somatic mutations and neoplastic transformation, [10].

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, [11]. 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.

Pomegranate is best known for heart health, [12,13], additional studies on pomegranate seed reveal its potential to help guard against cancer, [14-16]. Some researchers have reported that certain phytochemical compounds like flavonoids found in pomegranate can deter cancer formation and progression, [16]. Pomegranate contains compounds that circumvent functional changes involved in benign and malignant cell formation and transformation.

In traditional system of medicine various parts of Pomegranate (*Punica granatum*), family Punicaceae, has been used to treat varieties of ailments, and various parts of the plant have been scientifically proved for diverse biological activities such as antioxidant, hepatoprotective, [17], antidiarrheal, [18], antiulcer, [19], anti-inflammatory, [20], antimalarial, atherosclerosis and thyroid dysfunction, antimutagenic, immunomodulatory, memory enhancing, wound healing, [21] and anticancer activities; thus, *Punica granatum* is one of the most common and potent plant-based medicine in the management of various ailments. However, little is known about the effect of ethanol extract of Pomegranate seed on BPH induced oxidative stress. The aim of this study was to assess the antioxidant potentials of Pomegranate (*Punica granatum*) seed on BPH induced oxidative stress. Therefore, the aim of this study was to assess the antioxidant properties of ethanolic and aqueous extracts of *Punica granatum* (Pomegranate) seed in testosterone induced benign prostate hyperplastic albino Wistar rats.

2. MATERIALS AND METHODS

2.1 Experimental Design

This study was an interventional 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. The rats 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 housed in standard cages and 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 [22].

2.3 Plant Material

2.3.1 Pomegranate (*Punica granatun)*

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 macerator.

2.3.2 Extraction of powdered *punica granatum seed* with absolute ethanol and distilled water

Finely powdered *PunicagranatumSeed* 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 Number One 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^{o}C to 60^{o}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 was kept in the refrigerator prior to use. Lower and higher doses of 500mg/kg and 1500mg/kg respectively of *Punica granatum* seed extracts were used, this was gotten from a pilot study (not shown), which determined the lethal dose of *Punica granatum* seed aqueous and ethanolic extracts.

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, after full explanation of the purpose for procurement.

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 [23]. Human daily dose is 1 capsule (0.5 mg) per day.

The Food and Drug Administration (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 0.5 mg Dutasteride, then a 1kg man would take;

0.5 mg/60 kg =0.00833 mg

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

0.0083 mg/kg X 6.2 = 0.051 mg/kg.

This dose was administered mg per kg body weight of the rats dissolved in appropriate volume of normal saline (FDA guideline), [23].

2.5.2 Testosterone propionate

The dose of testosterone propionate (TP) administered was 4mg/kg b.wt. subcutenous, which was determined by a pilot study. 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 [24].

2.6 Castration of Rats (Bilateral Orchiectomy)

The rats were castrated using an anaesthetic agent (ketamine, 25 mg/kg body wt, intra-peritoneal.) 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. [25]. 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 (12) of five (5) 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):

2.7.1 Group 1 (Normal Control Group –NC and NC_2)

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 (NC_2). 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 $BPHC_2$)

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 $BPHC_2$ groups were treatment with 4mg/kg body b.wt. s.c for 30 days. They were allowed normal rat feed from the 16^{th} day for 30 days.

2.7.3 Group 3 (Positive Control – PC and PC_2)

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 16^{th} day for 30 days. The remaining five rats 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 16^{th} 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 16^{th} day for 30 days.

2.7.6 Group 6 (500AquPun.)

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 16^{th} 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 16^{th} 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 16^{th} 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 16^{th} 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. Five (5) ml of blood was put in lithium heparin container for the determination of superoxide dismutase activity, total oxidant and antioxidant statuses. The samples in the lithium heparin container were allowed to stand and plasma separated within thirty minutes of sample collection using a centrifuge. The plasma samples were then stored frozen at -20°C, until the time of determination of other parameters.

2.8.1 Determination of Superoxide Dismutase (SOD)

Superoxide dismutase level was measured quantitatively by the sandwich-enzyme linked immunosorbent assay (ELISA) method [26] as described by Shanghai Korain Biotech Co., Ltd, China.

2.8.2 Determination of Rat Total Antioxidant Status (TAS) and Total Oxidant Status (TOS)

Total antioxidant status and total oxidant status (TOS) were measured quantitatively by the sandwich-enzyme linked immunosorbent assay (ELISA) method as described by Shanghai Korain Biotech Co., Ltd, China.

2.8.3 Calculation of Oxidative Stress Index (OSI)

Oxidative stress index was determined by dividing Total Oxidant Status (TOS) by Total Antioxidant Status (TAS) (TOS/TAS).

2.9 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

Oxidative stress has been known to cause damage to the cells, tissues, even to organs by impairing important biomolecules and cells. It is considered as an important factor accounting for the pathogenesis of BPH [27,28]. Conversely, antioxidants, usually exist as compounds or enzymes that could compete with oxidative substrates, thus protecting the cellular structure [29]. Some studies have demonstrated reductions of antioxidant levels in prostate of BPH animals, while increase in oxidative stress has been known to occur in BPH rat model [30]. In this study, we observed significant increases *($p<.001$)* in total oxidant status (TOS), oxidative stress indices (OSI) and a corresponding fall in the activity of superoxide dismuthase (SOD) and total antioxidant status (TAS) in the BPH group. However, decreased TOS and OSI and a significant rise in SOD activity and TAS were observed inrat groups treated with both higher and lower doses of aqueous and ethanolic extracts of *Punica granatum* seed, when compared with BPH control groups, of which higher doses of both methods of extractions showed better outcomes (Figs. 1-4).

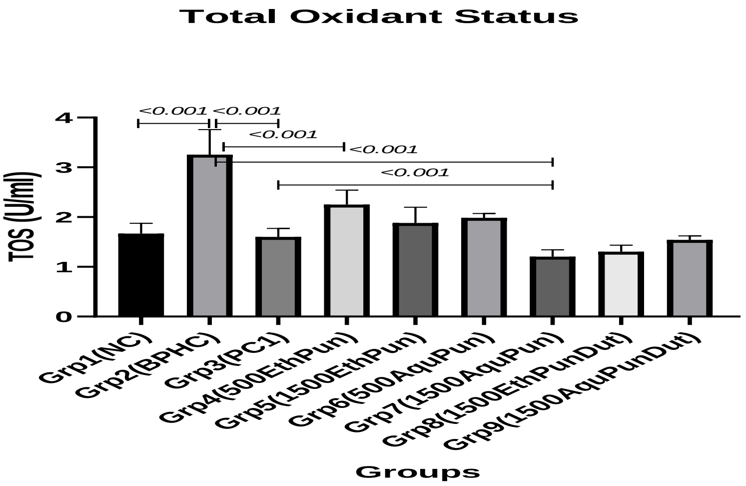

Fig. 1. Total Oxidant Status of Induced BPH Male Rats Treated with Aqueous and Ethanolic Extracts of *Punica granatum* Seed and Dutasteride compared with Controls

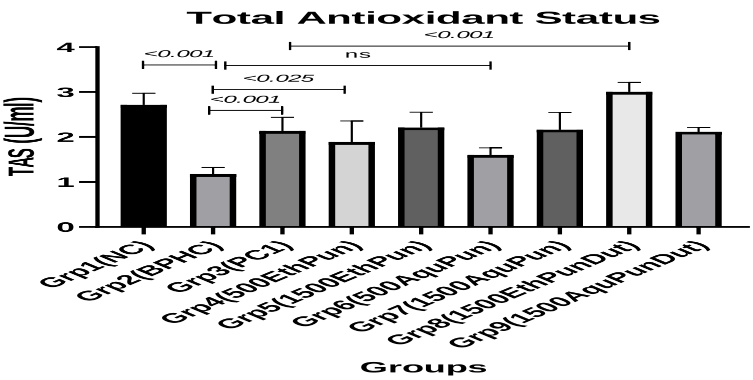

Fig. 2. Total Antioxidant status of Induced BPH Male Rats Treated with Aqueous and Ethanolic Extracts of *Punica granatum* Seed and Dutasteride compared with Controls

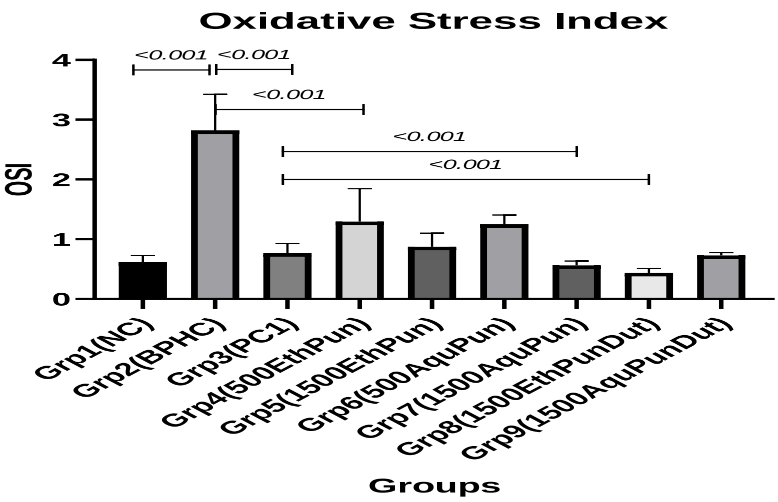

Fig. 3. Oxidative Stress indices of Induced BPH Male Rats Treated with Aqueous and Ethanolic Extracts of *Punica granatum* Seed and Dutasteride compared with Controls

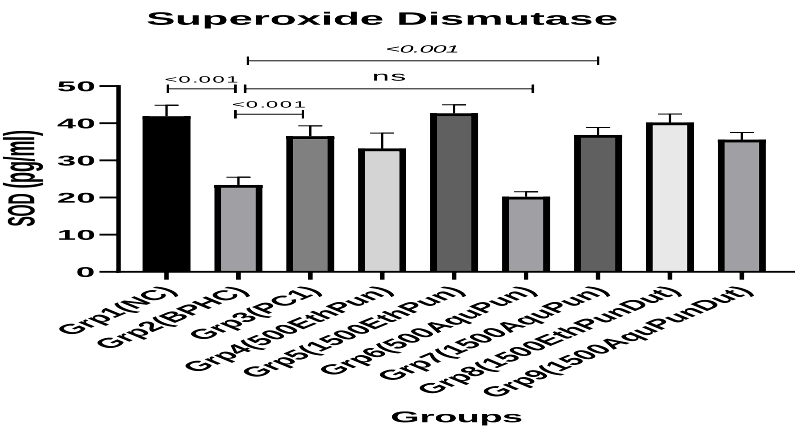

Fig. 4. Superoxide dismutase activity of Induced BPH Male Rats Treated with Aqueous and Ethanolic Extracts of *Punica granatum* Seed and Dutasteride compared with Controls

Table 1. Oxidant and Antioxidant Parameters of Male Rats Induced for BPH and Simultaneously Treated with Higher Dose of ethanol and aqueous Extracts of *Punica granatum* Seed compared with Controls

Groups	TOS (U/ml)	TAS (U/ml)	OSI	SOD(pg/ml)
Grp1(NC_2) (n=5)	1.69 ± 0.12	2.87 ± 0.47	0.60 ± 0.11	45.08±5.52
Grp2($BPHC_2$) (n=5)	3.34 ± 0.56^1	1.17 ± 0.12^1	2.91 ± 0.70^1	$23.0±1.71^1$
Grp3(PC_2)(n=5)	2.28 ± 0.15	2.03 ± 0.10	1.12 ± 0.08	$33.3±0.84^2$
Grp11(SimAdm1500AquPun)n=4	2.77 ± 0.51^2	1.14 ± 0.19	2.51 ± 0.74	$33.6±2.13^2$
Grp12(SimAdm1500EthPun) n=5	2.23 ± 0.51^2	1.64 ± 0.19^2	1.36 ± 0.32^2	37.5±2.07
F value	11.04	51.64	22.24	52.90
P value	0.000	0.000	0.000	0.000
Remark	**S**	**S**	**S**	**S**

S – significant at p<.05 (One-way ANOVA).Superscripts: significant at p<0.05 when compared with Grp 1 (superscript 1) and when compared with Grp 2 (superscript 2) (Tukey's post-hoc test)

These findings are in tandem with a previous study conducted by Longtin, [31]. In his study, he reported that *Punica granatum* seed oil; peel and juice were rich in polyphenols and that it is these phytochemicals that were responsible for anti-oxidant action of pomegranate. These findings support the opinion that aqueous and ethanolic extracts of *Punica granatum s*eed may treat BPH through the mechanism of anti-oxidation.

This study also showed significant decrease *(p<.001)* in mean plasma SOD values when lower doses of ethanolic extract of *Punica granatum* seed were compared with the higher dose of the same herb (Fig. 4). There were no significant changes in other parameters. There were also significant decreases in the mean values for all parameters (except TAS) when the lower and higher doses of aqueous extracts of *Punica granatum* were compared. It is therefore inferred that the 1500mg/kg b.wt. dose of ethanol and aqueous extracts of *Punica granatum* were or better therapeutic efficacy.

Pomegranate seed and peel extracts contain a lots of phytochemicals and considerable antioxidant activity, to be due to ellagic acid activity, which is the main pomegranate polyphenol, [32]. In 2012, Singh et al. [33] explained the first report on antioxidant pomegranate seed and peel extract properties. The pomegranate seed and peel extracts antioxidant activity were about ten times higher than the pulp extract, [34].

This study also showed, as expected, that rats treated with the anti-BPH drug (Dutasteride), also had reduction in oxidative stress. This is evident in the reduction in the levels of oxidative stress markers like TOS and OSI, (Fig. 1-4). This is obviously due to the fact that dutasteride not only directly ameliorating the BPH, but also indirectly reduces the oxidative stress.The importance of DHT in causing nodular hyperplasia is supported by clinical observations in which dutasteride, an inhibitor of 5a-reductase is given to men with BPH. Therapeutic use of the 5a-reductase inhibitors markedly reduces the DHT content of the prostate and, in turn, reduces prostate volume and BPH symptoms [35].

Seed and peel extracts ability in cleaning hydroxyl and superoxide anion radical was very high, [36]. So, pomegranate extracts are offered as two functional agents combining aldose reductase repressive activity with antioxidant actions. Polyphenolic compounds source is in pomegranate seed oil; peel and juice have anti-oxidant action and inhibit pro-inflammatory enzymes including the cyclooxygenases and lipoxygenases, [33].

Studies in rats with CCl4-induced liver damage showed pomegranate seed oil pre-treatment evaluate the free radical inhibitory effect of superoxide dismutase, the hepatic enzymes catalase, and peroxidase resulted in 54% lowering of lipid peroxidation values when compared to control group, confirming the antioxidant content of the pomegranate seed oil, [37].

Treatment of BPH induced rats with simultaneous administration of aqueous extracts of *Punica granatum* seed only showed significant increase in SOD activity, while the ethanolic extract showed significant decreases in TOS and OSI and significant increases in mean TAS and OSI values when compared with the BPH group, (Table 1).

Taking an herb or supplement could change the way a prescription medicine works in the body by enhancing the effect of the medicine, or it could react in a negative manner, causing symptoms like an overdose, or, it might cause the medicine not to work at all. Although many studies regarding herb–drug interactions emphasize the potential harmful effects of such interactions, the possibility of herbal components beneficially enhancing or facilitating the action of anti-proliferative pharmaceutical agents (or vice versa) may also exist. Positive interactions between herbs and drugs may lead to enhanced effectiveness of the anti-proliferative agents through additive or synergistic actions.

4. CONCLUSION

In conclusion, both doses of *Punica granatum* seed for ethanolic and aqueous extracts individually and in combination and with dutasteride markedly reduced total oxidant status, oxidative stress indices and improved the activities of antioxidant parameters like superoxide dismutase and total antioxidant status. This study also showed that the ethanolic extract of *Punica granatum* seed had more antioxidant capacity compared to the aqueous extract.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Ethical approval was sought and granted by the ethical committee, Department of Pharmacology, Faculty of Basic Medical Sciences, University of Port Harcourt, Nigeria.

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.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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