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Histopathological and Biochemical Assessment of *Annona muricata* and *Allium sativum* Pooled Extract on Testosterone Induced Prostate Hyperplasia in Albino Rats

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Authors' contributions

This work was carried out in collaboration among all authors. Authors OOB and AUP conceptualized and planned the study. Author AUP oversaw the experiment, which authors OOB and KNB carried out. While authors KNB and OOB authored the manuscript, they also collected and analyzed the data. After reviewing the study, each author gave their approval for it to be published in the Journal of Complementary and Alternative Medical Research. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To investigate the effect of pooled extract of *Annona muricata (A. muricata*) and *Allium sativum* (*A. sativum*) on testosterone-induced prostate hyperplasia in male albino rats.
Experimental Procedure: Male albino rats for the study were divided into seven groups identified as A-G groups. Group A and G were used as negative and positive controls with 12 and 5 rats

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respectively. Male rats in group B-G were induced with 3mg/kg body weight of testosterone propionate injection subcutaneously for 28 days. Animals in group B and C received 100mg/kg body weight of individual *Annona muricata* and *Allium sativum* extract respectively, while animals in group D-F received 100mg/kg, 300mg/kg and 600mg/kg body weight of the pooled extracts. We evaluated relative prostate weight(PI),serum prostate specific antigen(PSA),interleukins-6(IL-6), total protein, and histological changes including KI 67 and BCL2 expressions.

Results and Conclusion: The study revealed that pooled extracts of *A. muricata* and *A. sativum* significantly inhibited prostatic hyperplasia; decreased PI,serum PSA,IL-6, total protein, and expression of KI 67, far better than the any of the single plant extract in this study. This suggests that pooled extract of *A, muricata* and *A, sativum* may be used as a therapeutic agent for prostatic hyperplasia, as a result of the influence of the phytochemical constituents.

Keywords: Anonna muricata; Allium sativum benign prostatic hyperplasia; phytochemical analysis; antioxidant.

1. INTRODUCTION

One of the most prevalent urinary disorders in the elderly is prostate hyperplasia, which can cause lower urinary symptoms, or LUTs [1,2]. The American Urological Association [3] reports that the incidence of BPH rises with age, impacting 8% and 50% of men in their 40s and 60s, respectively. When the age exceeds 85 years old, this incidence peaks at over 90% [4]. A urethral stricture state is caused by the absence of glandular elastic tissue between the growing prostatic tissue, which results in a variety of debilitating and upsetting lower urinary tract symptoms (LUTS). Proliferation of stromal and epithelial cells in the prostate transition zone, which encircles the urethra, is a hallmark of benign prostatic hyperplasia (BPH) development. Consequently there is urethral compression and the development of bladder outflow obstruction, which manifests clinically as lower urinary tract symptoms (LUTs), urinary retention, or infections due to incomplete bladder emptying [5]. When left for too long untreated, this condition can lead to the development of chronic high-pressure retention (a potentially life-threatening condition) and long-term or permanent changes to the bladder detrusor muscle.

The mechanisms underlying the development of BPH have not yet been fully discovered. However, several intertwined risk factors have been linked to etiology and pathogenesis this condition, some which might include oxidative stress, inflammation, aging, and androgen stimulation [6], (Park et al., 2013).There are other theories of development prostate hyperplasia including, the theory of conversion of 90% of testosterone to dihydrotestosterone(DHT), which has higher affinity and in turn influence tumor development [7,2] self replenishment of androgen independent prostate cells in androgen deficient condition [8] and the interaction between the srtoma and epithelium, of which can convert testosterone to DHT. This process allows the production of various growth factors [8,9].

Prostate hyperplasia is treated globally with medication, lifestyle modifications, surgery, and, more recently, phytotherapy [9]. Currently, androgen deprivation based largely on 5 alpha1 inhibitors, such as finasteride and dutasteride, is the cornerstone of medical treatment for BPH [10]. Because of the negative consequences of these medications and the high expense of obtaining them, it is essential to locate a herbal remedy that can treat prostate hyperplasia at a lower cost and with fewer side effects.

One such herbal medication is garlic otherwise known as *Allium sativa*. *Allium sativum* is a member of the Alliaceae family. The bulbs of garlic range in color from white to pink having a pungent scent and fragrant flavor. The therapeutic and preventive properties of garlic against numerous tumors have been assessed by several epidemiologic, preclinical, and clinical investigations. *Allium sativum* extracts have been shown to have numerous biological properties including antiviral, antiprotozoal, antibacterial, anti-inflammatory, antifungal, antioxidant and anti-cancer activities [11,12].

Another important source of natural medicinal product is Annona muricata (*A. muricata*). Accoring to Moghadamtousi et al.,2015, the plant is sometimes referred to as graviola, guanabana, paw-paw, sirsak, or soursop. It is is a member of the Annonaceae family. Originally from the warmest tropical regions of South and North America, A. muricata is now widely spread in tropical and subtropical regions across the globe, including Nigeria, Malaysia, and India.

All the parts of A. muricata tree are widely utilized as traditional medicines to treat a wide range of human diseases, particularly parasite infections and cancer. Asare et al. [13] reported that the aqueous extract of *A. muricata* leaf exhibit antiproliferative activities against BPH cells. Previous studies has shown that the both extract A.sativum and leaf of *A. muricata* promote good prostate health, in particular regarding prostate tumor development and prevention [12,13]. Thus, this study is aimed at evaluating the prostate histopathological and biochemical features of testosterone prostate hyperplasia induced albino rats treated with pooled extract of *A. sativum* and *A. muricata.*

2. MATERIALS AND METHODS

2.1 Plant Sample Authentication and Preparation

The leaves of *A muricata* for the study were collected from the outskirts of Nsukka town, while A. sativum were purchased from Oba market in Nsukka. The two plants material were taken to the Department of Botany, University Nigeria Nsukka, for identification and authentication. Leaves were hand-washed by rubbing the surface gently under running water. They were later sun-dried for 3 days. Leaves were milled and soaked by the proportion of 1 kg of the milled substance soaked in 4000 mL of ethanol for 48 hours hours. The mixture was then filtered, first with a sieve, and then whatman paper.The resulting filtrate was concentrated to dryness at 40 degree centigrade in a hot air oven to a pasty brown extract. The same procedure was observed for *A. sativum* following its blending. The two plants extract were separately stored at 4 degree centigrade until when needed.

2.2 Animal Preparation

Forty two (42) albino male rats, aged 3 months to 4 months were used. The animals were procured from University of Nigeria Nsukka animal house, where the study took place, and they were housed together for 2 weeks for acclimatization. The rats were housed in a brightly lit, well-ventilated environment, and equally fed with rat pellet diet (Vital feeds, Nigeria Ltd), along free access to tap water.They were kept in a quiet environment at a temperature of 25^0C to 30^0C and 12h dark/light rhythm.

2.3 Acute Toxicity

The acute study of the pooled plant extract of *A.muricata* and A. sativum was determined in accordance with a modification of Lorke's method by Orieke et al. [14]. A total of 21 weighing 130-158 gm were used. In the first phase of the study, 9 rats assigned to 3 groups (A, B and C) were administered 10, 100 ,1000 mg/kg body of pooled plant extract (50, 50), respectively. Thereafter the animals were observed within 24 hours for toxicity signs or death. With zero observation of mortality or abnormal behaviour within the period, we proceed to the second phase of the study. Here another 9 rats assigned to 3 different groups (D, E and F), were respectively administered 1600, 2900 and 5000 mg/kg body weight of the pooled sample for another 24 hours and at the end change was recorded. In the final phase, the highest dose 5000 mg/kg body was administered to the last 3 set of rats as a confirmatory test. The animals were observed for 24 hours and further 7 days and yet no mortality or abnormal behaviour.

2.4 Qualitative and Quantitative Phytochemical Analysis

Qualitative and quantitative phytochemical analysis was carried out in this study using pooled sample extract. This analysis is in accordance with Harborne (1973) and Lallianrawna (2013). Reagents such H_2SO_4, dragendorf, chloroform, sodium hydroxide, ferric chloride, Folin's phenol reagent, sodium carbonate, aluminium and chloride, In addition UV-light spectrophotometer was utilized in the analysis.

2.5 Biochemical Analysis

The level of PSA in rat sera was determined following Elabscience enzyme -linked immunosorbent assay (ELISA) test kit procedure, while interleukin -6 (IL-6) analysis was carried out using commercial ELISA kits (MH Biomedical, Ohio, USA). The Biuret method was utilized to ascertain the sample's total protein content (Ernest, 1996). Every analysis was completed in accordance with the direction from the kits manufacturers.

2.6 Experimental Design

The Forty two (42) male albino rats in the study were separated into seven (7) groups designated

A-G. Group A contains 12 rats while the rest of the harbour 5 rats per cage. The treatment is as follows:

Negative control group A Received only water and feed.
Group B Received only 100 mg/kg body weight(bdw) *A.muricata* extract.
Group C Received only 100 mg/kg body weight *Allium sativum* extract.
Group D Received 100 mg/kg bdw (low dose) pooled extract.
Group E Received 300 mg/kg bdw medium dose pooled extract.
Group F Received 600 mg/kg bdw high dose of pooled extract.
Positive control group G Received testosterone injection without treatment.

The weight of the animals was taken before the commencement of the treatment. For tumor induction, the animals in groups B-G were administered 3 mg/kg body weight of testosterone propionate daily for 28 days. The animals in group A were fed only water and food. The plant extract treatment was administered orally daily for 14 days. A day after the 14th day of the plant extract treatment, the animals were fasted being subjected to sample collection. The experimental rats were serially sedated with chloroform before blood collection through cardiac puncture, and then prostate tissue harvested, washed with water and fixed in 10 % formal saline.

2.7 Prostate Weight (PW) and Prostate Index Analysis

Before sacrificing the animals, their weights were recorded at the start of the study and closer to the conclusion. A chemical weighing balance was used to determine the rats' weights following the removal of their prostates. Each group's mean prostate index (PI) ratio was computed using the prostate index (PI) as defined by PW/BW 100 percent.

2.8 Statistical Analysis

Numerical data emanating from the study was analyzed using the statistical package for social science (SPSS) version 20.0. The mean was expressed as mean ± standard deviation. The analysis was done using one-way analysis of Variance (ANOVA), followed by Turkey post hoc for multiple comparisons.

3. RESULTS

The phytochemical profiling of the pooled extract of *A. muricata* and A. sativum in our study revealed the presence of flavonoids, alkaloids, tannins, phenolics, saponins, glycosides and terpenoids.(supple Table 1). The phytochemical constituents varied in quantities with terpenoids being the most predominant. At the third phase of our acute toxicity study, all the rats survived after the administration 5000 mg/kg body weight of the pooled extract of *A. muricata* and *A. sativum*. None of the experimental animals displayed any sign of abnormal behaviour or even death.

3.1 Effects of *A. muricata* and *A. sativum* on the Serum IL-6 in Albino Rats

The model hyperlasia group G demonstrated significant ($P< 0.05$) elevation of IL-6 when compared with the rest of the groups including the negative control group A. The treatment with high, moderate, and low doses of the of the pooled plant extract significantly ($P< 0.05$) reduced the serum IL-6 respectively in F, E, D in dose dependent pattern (Table 1). In addition, the animals treated with pooled plant extract also manifested significant($P< 0.05$) decline in the serum IL-6, compared to the rats treated with either *A. muricata* or *A.sativum* alone.

3.2 The Effects of *A. muricata* and *A. sativum* Extract on Serum Total Protein in Albino Rats

The animals in the positive control group (G) shows significant ($P < 0.05$) elevation in serum total protein when compared to the rest of the groups in the study (Table 1). The animals in the high dose pooled extract group (F) significantly ($P<0.05$) declined in serum total protein in comparison with ones in moderate and low doses treated group. The two single extract treated groups (C and B) are both comparable with negative control group, all three significantly ($P< 0.05$) lower than the pooled extract treated groups.

3.3 The Effects of *A. muricata* and *A sativum* Extract on Serum PSA Concentration in BPH Induced Albino Rats

Findings in our study revealed significant ($P < 0.05$) elevation of PSA level in the BPH induced rats, compared to the high dose pooled extract

treated rats (Table 1). The PSA level in the moderate and low dose pooled extract treated groups displayed dose dependent decrease.

3.4 The Effects of *A. muricata* and *A. sativum* Extracts on Prostate Index in BPH Induced Albino Rats

There is no significant changes in the weight of the animals at the end of the extract administration. However, there was marginal increase in prostate index in BPH induced group, which declined among the pooled extract treatment groups in dose dependent manner (Table 1).

3.5 Effects of *A. muricata* and *A sativum* Extracts on Induced BPH in Albino Rats

When compared to prostate acini of animals in the model hyperplasia group(G), where there is a significant increase in the thickness and proliferation of epithelial cells, hematoxylin and eosin staining showed no appreciable morphological changes in the lining of the epithelium of animals in the negative control group (A). A benign prostatic hyperplasia was suggested by the epithelial expansion into the lumen.The animals in the pooled plant extract treated groups demonstrated remarkable reduction in the epithelial proliferation far much more than the rats treated with single plant extract (B & C)(Fig 1,H & E A-G).The comparatively strong immunohistochemical expression of ki 67 in the model hyperplasia group (G) supported significant epithelial proliferation in this group, with lesser expression among plant extract treated groups, especially pooled extract treated groups(F,E,& D)(Fig 1 Ki 67 A-G).No strong immunohistochemical expression of BCL2 was observed in sections from all the groups(Fig 1 BCL2 A-G) IHC was used to identify epithelial proliferation in more detail.

Table 1. Mean prostate index, serum PSA, IL-6, and total protein of the various groups

Group (n=5)	PI	Serum PSA (ng/ml)	Serum IL-6(pg/ml)	Total Protein
Group A	0.03 ± 0.001	0.41 ± 0.07	6.20 ± 0.42	5.24 ± 0.57
Group B	0.05 ± 0.002	0.49 ± 0.09	7.42 ± 0.79	5.38 ± 0.71
Group C	0.07 ± 0.002	0.42 ± 0.13	6.02 ± 0.64	5.26 ± 0.35
Group D	0.07 ± 0.06	0.48 ± 0.12	5.93 ± 0.82	5.00 ± 0.29
Group E	0.05 ± 0.01	0.34 ± 0.05	3.98 ± 0.22	5.08 ± 0.39
Group F	0.03 ± 0.01	0.28 ± 0.03	3.18 ± 0.08	3.78 ± 0.11
Group G	0.33 ± 0.05*[a,b,c,d,e,f]	0.70 ± 0.04*[F]	68.44 ± 18.44*[a,b,c,d,e,f]	7.88 ± 0.15*[a,b,c,d,e,f]
F Ratio	24.17	2.62	11.64	8.68
Sig	0.000	0.04	0.00	0.00

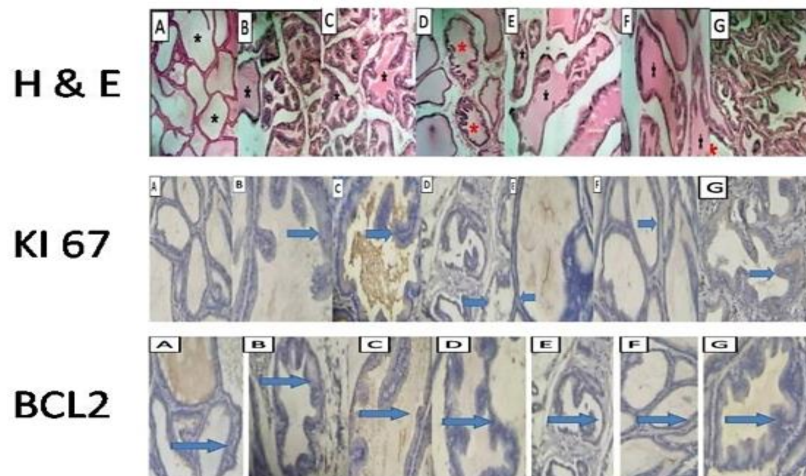

Fig. 1. Effects of extracts of *A.muricata* & *A.sativum* on the histological appearance of the prostate. H& E(x200) : (A) section showing normal prostate acini (*) and stroma,(B) shows milder epihelial proliferaion (*).(C) shares the almost the same of epithelial disposition with "c". 'D' 'E' & 'E' demonstrate remarkable level of glandular atrophic changes (*). KI 67 x200: (G) shows the most significant expression of KI 67 (arrow) more than the rest of the groups. BCL 2(200): There was bland expression of BCL 2 in all the groups

The effect of pooled extracts of *A. muricata* and *A. sativum* on the serum biochemical parameters and prostate index of BPH induced rats. Values are presented as mean ± standard deviation, and significance set at P< 0.05(*) compared to other groups from any paired mean within column.

4. DISCUSSION

Medicinal plants' diverse pharmacological effects are a result of the bioactive compounds present in them. Prostatic histological, and biochemical reactions in rats were observed in the induced animals as a result of the combined ethanolic extract of *A. muricata* and *A. sativum*. The phytochemical component of the combined extracts that was identified by the extract analysis is responsible for their effects. The relevant constituents identified in this study include flavonoids, tannins, phenolics, saponins, glycerides and terpenoids. Previous studies have shown that these phytoconstituents are anti inflammatory, antioxidants, antibiotics and antineoplastic [15,12,13,16]. The extract may be safe to use for therapeutic purposes based on the work's oral lethal dose of more than 5000 mg/kg body weight.

Men with advanced age are more likely to develop prostatic hyperplasia, a condition that is frequently linked to urinary tract infections. Chronic exposure to the elevated testosterone can induce hyperplasia in simple epithelial cells, which causes discomfort in the urinary system by repeatedly inflaming the epithelial cells over time [3,17].The present study has demonstrated that the ethanol extract of A. sativum and A. muricata, as well as the pooled sample of the two, can significantly suppress the development of testosterone-induced prostatic hyperplasia, as evidenced by the restoration of increased prostatic index and histological morphological alterations.

Prostate index (PI) and histomorphological alterations have been employed in the past to assess the protective ability of curative drugs. The parameters are essential indicators of the development of prostate tumors such as benign prostatic hyperplasia [18,19]. Prostate weight rise is indicated by PI.

In line with previous studies [20,21,18], in which plant extract ameliorated development of BPH, our study demonstrated remarkable decrease in prostate index as well as restoration of histomorphological alteration of testosterone induced BPH by plants extract especially the pooled extract of *A.muricata* and *A.sativum*.This an indication that the pooled extract in this study might be appropriate for the development of drugs against prostate tumors.

The model hyperplasia group (G) has a higher level of serum IL-6, which is consistent with Wang et al. [22] and indicates the presence of an inflammatory process. Commonly found in BPH, inflammation can lead to tissue damage and the release of cytokines, which can promote angiogenesis and the synthesis of local growth factors [21,23]. Pro-inflammatory cytokines such as interleukin-6 contributes to the development of prostate tumors [24]. Prostate cancer and BPH are the two conditions where this pro-inflammatory cytokine is present. It is located in both the stromal and epithelial regions of the prostate [24,23]. In our study significant reduction of the serum level of IL-6,was observed among the animals treated with pooled plant extract (groups F,E,& D, far much better than in the rats treated with single plant extract(B & C).The decrease in serum IL-6 is an indication of remission of possible associated tumor and this is in accordance with the previous studies in plant extract treatement of BPH [13], (Moghdantousi et al.,2015), [21].This may indicate a role for anti-inflammatory mechanisms in the mechanisms of the BPH treatment with plant extract in this study.

Prostate diseases, particularly prostate tumors, have been linked to PSA, a glycoprotein that is mostly produced in the prostate gland [25].The significant elevation of PSA in the model hyperplasia group is a collaborating indication of tumor development, and its reduction demonstrates tumor remission [26-29] *.A* notable decrease *in PSA was* noted in the groups treated with the pooled plant extract, indicating the therapeutic advantage of the pooled plant extract over the single plant extract, which had a minor impact.There still need to investigate the mechanism of the reduction of PSA and the exact phytochemical constituent responsible.

Acute phase proteins, such as serum amyloid and C-reactive protein, can be produced by inflamed liver cells, which raises the quantity of serum proteins [22,24]. This explains why the model hyperplasia group (G) had significantly higher serum total protein levels. The pooled plant extract in this study may have also contributed to the normalization of blood total protein by reducing inflammation, which supports

the extract's potential for amelioration. Not as much as with the single plant extract treatment, but nonetheless a decrease in serum total protein was seen.

The inherent weakness of our study is that, in addition to the long-term treatment, we did not perform an experimental assessment of the extract's effects on the male reproductive system. It is unknown which components of the plant extract are responsible for the attenuation tumor observed in our study. Furthermore, it is yet unclear which specific signaling pathways are needed to perform the function of bioactivity. Finally, the study's rat model of BPH is not the same as that of humans, which limits the relevance of our results to the general population. In order to gain a deeper comprehension of the fundamental processes by which pooled extract of *A. muricata* and A.sativum alleviates prostate prostatic hyperplasia.We advocate for further comprehensive investigations that might potentially involve molecular analysis.

5. CONCLUSION

Studies conducted thus far have shown that *A. muricata* and *A. sativum* have good therapeutic qualities when used as a herbal medicine for prostatic hyperllasia. When the two herbs are used together as a single therapy, the outcome is superior than when the herbs are used separately. The pooled extract of *A. muricata* and *A. sativum* can be considered to be safe for consumption as its toxicity study in our work is within the normal range.

CONSENT

It is not applicable.

ETHICAL APPROVAL

The study was conducted in accordance with the current laws of the land governing the use of experimental animals, as well as the University of Nigeria ethical committee's permission.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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SUPPLEMENTARY

Table 1. Showing phytochemical component of *Annona muricata* and *Allium sativum*

Phytoconstituent (mg/g)	Garlic	Soursop	Combination
Flavonoids	7.85	9.43	11.42
Flavonoids	7.33	8.66	12.12
Mean	**7.59**	**9.045**	**11.77**
SD	**0.367696**	**0.544472**	**0.494974747**
SEM	**0.260777**	**0.386151**	**0.35104592**
Alkaloids	2.95	4.85	3.44
Alkaloids	3.16	4.99	3.19
Mean	**3.055**	**4.92**	**3.315**
SD	**0.148492**	**0.098995**	**0.176776695**
SEM	**0.105016**	**0.070011**	**0.125018879**
Tannins	15.62	14.63	17.53
Tannins	16.11	15.04	16.88
Mean	**15.865**	**14.835**	**17.205**
SD	**0.346482**	**0.289914**	**0.459619408**
SEM	**0.245037**	**0.205031**	**0.325049086**
Phenolics	27.94545	19.54	24.21
Phenolics	28.21818	19.54	24.82
Mean	**28.08182**	**19.54**	**24.515**
SD	**0.192847**	**0**	**0.431335137**
SEM	**0.136384**	**0**	**0.305046065**
Saponins	25.37778	23.86	23.84
Saponins	26.04444	24.14	23.31
Mean	**25.71111**	**24**	**23.575**
SD	**0.471405**	**0.19799**	**0.374766594**
SEM	**0.333384**	**0.140021**	**0.265040024**
Glycosides	2.54	7.25	3.91
Glycosides	2.36	7.04	2.66
Mean	**2.45**	**7.145**	**3.285**
SD	**0.127279**	**0.148492**	**0.883883476**
SEM	**0.090014**	**0.105016**	**0.625094396**
Terpenoids	8.87	4.77	7.75
Terpenoids	8.69	5.22	7.26
Mean	**8.78**	**4.995**	**7.505**
SD	**0.127279**	**0.318198**	**0.346482323**
SEM	**0.090014**	**0.225034**	**0.245037003**

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