

Renal Protective Function of *Annona Muricata* Leaf Extract in Male Wistar Rats

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ABSTRACT

The aims of this study is to determine the renal protective function of *annona muricata* leaf extract in male Wistar rats. A total of 78 adult male rats obtained from the laboratory animal production unit of the Department of Physiology, Abia State University, Uturu were used for the different segments of the study. Eighteen of the rats were used for acute toxicity/lethal dose evaluation of the extract while the remaining sixty (60) were used for the testosterone-induced hyperplasia study. Prostate hyperplasia was induced in the rats via daily sub-cutaneous administration of testosterone propionate (5 mg/kg body weight) for 28 days. Sixty rats used for this segment of the study were assigned to 6 groups of 10 rats and treated as follows: Group 1, Normal control, no treatment. Group 2 Testosterone propionate (5 mg/kg) only and was the BPH control. Group 3, Testosterone propionate (5 mg/kg bw) + Finasteride (3 mg/kg body weight). Group 4, Testosterone propionate (5 mg/kg bw) + *Annona Muricata* Leaf extract (300 mg/kg body weight). Group 5, Testosterone propionate (5 mg/kg bw) + *Annona Muricata* Leaf extract (600 mg/kg body weight). Group 6, *Annona Muricata* Leaf extract (600 mg/kg bw) only. At the end of treatment, animals were sacrificed and blood samples were collected into EDTA and plain bottles for determination of renal function parameters (urea, creatinine, sodium, chloride, potassium and bicarbonate). All renal function parameters were determined using the respective commercial test kits. Data obtained were subjected to Statistical Package for Social Sciences (SPSS, Version 20.0, IBM SPSS Inc, Chicago, IL) was used for data analysis. Level of significance was calculated by One Way Analysis of Variance (ANOVA). Data were analyzed using Duncan Multiple Range Test and complemented with Student's *t* test for post-hoc test for comparisons of the means of the various doses and fractions. *P*- values less than 0.05 were considered statistically significantly different between the test and control groups as well as among test groups for measured value. Conclusively, result of the renal function parameters across different treatment groups. Compared to the normal control group, the BPH control (Testosterone only) group exhibits significant alterations in several renal parameters. Specifically, levels of urea (29.05 ± 2.45 mg/dl) and creatinine (1.54 ± 0.21 mg/dl) are notably elevated, indicating impaired renal function due to BPH induction by testosterone. Treatment with Finasteride (3 mg/kg) + Testosterone and various doses of *annona muricata* Leaf extract (200 mg/kg, 400 mg/kg, and 800 mg/kg) + Testosterone generally show improvements in these parameters compared to the BPH control group. These treatments reduce urea and creatinine levels closer to those of the normal control group (urea: 20.35 ± 0.86 mg/dl, creatinine: 0.79 ± 0.04 mg/dl). Furthermore, electrolyte levels (Na^+ , K^+ , Cl^- , HCO_3^-) in the BPH control group also show

deviations from the normal control. Treatments with Finesteride and *annona muricata* Leaf extract tend to normalize these electrolyte levels.

Keywords: Testosterone-induced hyperplasia, *Annona muricata*, renal protective function, renal function parameters and Wistar rats

INTRODUCTION

The kidneys are essential for preserving electrolyte levels, fluid balance, and the removal of waste products from the body's metabolism. Abnormalities in renal function parameters, such as serum creatinine, blood urea nitrogen (BUN), and urinary protein levels, are indicative of impaired kidney function and may lead to various renal disorders [2,5]. *Annona muricata* has been investigated for its potential effects on renal function parameters. Studies exploring the impact of *Annona muricata* on renal function parameters have shown promising results [1,2]. According to research by Zeweil *et al.* (2024), [6] administration of *Annona muricata* extract to animal models resulted in significant improvements in renal function markers, including serum creatinine and BUN levels. These findings suggest that *Annona muricata* may possess Reno protective effects, preserving renal function and mitigating kidney damage. Another study investigated the effects of *A. muricata* on renal function parameters, particularly in the context of acute oral toxicity and repeated dose animal toxicity. One study exposed Sprague Dawley rats to a single 2000 mg/kg dose of *A. muricata* leaf ethanol extract and observed no significant changes in renal function parameters such as creatinine and urea levels [3,7] examined the ameliorative effects of *A. muricata* leaf ethanol extract on renal morphology in alloxan-induced mice. The study found that the extract significantly reduced the severity of renal damage, as measured by histopathological analysis, and improved renal function parameters such as creatinine and urea levels.

The Reno protective properties of *Annona muricata* are attributed to its rich phytochemical composition, which includes flavonoids, alkaloids, and acetogenins. These bioactive compounds exhibit antioxidant, anti-inflammatory, and nephroprotective properties, which may help attenuate renal oxidative stress and inflammation [3]. Furthermore, *Annona muricata* may modulate key signaling pathways involved in renal homeostasis and protection against nephrotoxicity. Studies have reported that *Annona muricata* extracts can inhibit the production of pro-inflammatory cytokines and reduce oxidative stress-induced renal injury by activating the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway, leading to increased expression of antioxidant enzymes and detoxifying proteins Adedapo *et al.*, 2020; [6,3].

MATERIALS AND METHODS

Materials

Equipments used include sensitive weighing balance, Electric weighing balance, Manual blender, Dissecting set, Dissecting board, 5ml syringes, 1ml syringes, test tubes, serum bottles, Sample bottles, EDTA bottles, whatman No 1 filter paper, Measuring cylinders, Pasteur pipette, Cotton wool, Micro pipette, Rat cages, Feeding and water trough, Oral gavage, Glass column, Microscope, Glass TLC plates, microscope slides, Glass wool, funnel, Spatula, Retort stand, Hand gloves, TLC tank, beaker, Oven, freezer, Refrigerator, Spectrophotometer(722N, Mindray, MC Quijing, China), Centrifuge, Rotory evaporator, Soxhlet extractor, GC-MS machine, Chemoilluminoscene machine and Haematology analyser (BC-2300, Mindray, MC Quijing, China).

ETHICAL APPROVAL

This study was approved by the research and ethical committee of the Department of Physiology, Abia State University, Uturu.

Animals

A total of 78 adult male rats obtained from the laboratory animal production unit of the Department of Biochemistry, Abia State University, Uturu were used for the different segments of the study. Eighteen of the rats were used for acute toxicity/lethal dose evaluation of the extract while the remaining sixty (60) were used for the testosterone-induced hyperplasia study. The animals were housed in aluminum cages, under standard conditions 12/12h light-dark cycle at temperature of $25\pm 2^{\circ}\text{C}$ and were fed with standard grower pellets (Grand Cereals Ltd, Abia State) and had access to clean drinking water for seven days of acclimatization before the initiation of experiment. All experiments were carried out in accordance with International Guideline for Biomedical Research (CIOMS/OMS, 1985) and as approved by the Department of Physiology, Abia State University, Uturu.

Experimental Design for the Hyperplasia Study

Rats were given testosterone propionate (5 mg/kg body weight) subcutaneously every day for 28 days in order to cause prostate hyperplasia. Sixty rats used for this segment of the study were assigned to 6 groups of 10 rats and treated as follows:

- Group 1 - Normal control, no treatment
- Group 2 - Testosterone propionate (5 mg/kg) only and was the BPH control
- Group 3 - Testosterone propionate (5 mg/kg bw) + Finasteride (3 mg/kg bw)
- Group 4 - Testosterone propionate (5 mg/kg bw) + AML extract (300 mg/kg bw)

- Group 5 - Testosterone propionate (5 mg/kg bw) + AML extract (600 mg/kg bw)
- Group 6 - AML extract (600 mg/kg bw) only

At the end of treatment, animals were sacrificed and blood samples were collected into EDTA and plain bottles for determination of renal function parameters. For the later (blood contained in plain bottles), the blood was allowed to clot and retract within two hours and then centrifuged at 3000 rpm for 10 minutes to obtain clear sera samples which were used for the determination of renal function parameters.

Preparation of Plant Extracts

Plant extracts were prepared in accordance with the method used by Orieki *et al.*, (2019). The freshly collected leaves of *Anona muricata* were air dried under shade for within 14 days and were thereafter pulverized into coarse powdered form using a locally fabricated grinding machine. Two solvents, ethanol and N-hexane were used for the preparation of the extracts. A known quantity (300g) of the powdered plant material was macerated in 1.5 litres of ethanol (AR) for 48 hours, been vigorously shaken every 2 hours within the period and was thereafter filtered with Whatman No. 1 filter paper to obtain a filtrate which was the extract in solution. The filtrate so obtained was concentrated at low temperature to obtain a solid dark-green extract which weighed 13.91 g and represented 4.64% extract yield. The extract was preserved in the refrigerator at -4°C until needed and is hereafter referred to as *Anona muricata* leaf (AML) extract.

Determination of Renal Function Parameters

All renal function parameters were determined using the respective commercial test kits and following

protocols for the parameters as was reported by Ugbo *et al.*, (2017).

Urea

Kit: RANDOX

Reagent: R1 (a mixture of 6 mmol/L of urease and 116 mmol/L of sodium nitroprusside). R2 (dilute phenol, 120 mmol/L). 0.14N NaOH with R3 (27 mmol/L of diluted sodium hypochlorite).

Principle: This test is based on the fact that urea in serum is hydrolysed to ammonia in the presence of urease. Hence the amount of ammonia formed is proportional to urea concentration.

Procedure: Reagent R1 was created by carefully mixing the contents of vial R1a with bottle R1b. Additionally, 660 milliliters of distilled water were used to dilute and completely mix the contents of bottle R2. Additionally, 750 milliliters of distilled water were used to dilute the

contents of bottle R3. At that point, the exam was prepared to begin. Three test tubes were prepared and labeled test, blank, and standard. Ten microliters of the test sample were added to the test tube with the test label after about 100 microliters of R1 had been pipetted into each test tube. The test tubes designated standard and blank were filled with the equal volume of distilled water and the standard reagent, respectively. The mixtures in the test tubes were incubated at 37°C for 10 minutes and thereafter 2.5 ml of reagent R2 and the same volume of R3 added to each test tube. The contents of each tube were mixed immediately and incubated at 37°C for 15 minutes. At the end, the absorbance of the test and that of the sample were read after zeroing with blank. The urea concentration in the sample was calculated thus:

$$\text{Urea concentration in mg/dl} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \text{Concentration of standard}$$

Where concentration of standard = 78.73 mg/dl

Creatinine

Kit: RANDOX

Reagent: R1a (35 mmol/L of picric acid). R1b (0.32 mol/L of NaOH solution)

Principle: This test is based on the principle that creatinine in alkaline solution reacts with picric acid to form a colored complex. Creatinine concentration is therefore proportional to the amount of colored complex formed.

Procedure: After combining equal amounts of R1a and R1b to create the working reagent, two test tubes with the

labels "test" and "standard" were prepared. Each test tube was filled with precisely 2 milliliters of the working reagent, followed by the addition of 0.2 milliliters (200 microliters) of the sample to the test tube with the test label and the same volume of the standard reagent to the standard. After mixing the contents of each test tube, absorbance A1 for the standard and test was measured 30 seconds later, and absorbance A2 for the standard and test was measured two minutes later.

Absorbance of test or sample = $A_2 - A_1$

Absorbance of standard = $A_2 - A_1$

$$\text{Creatinine concentration in mg/dl} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \text{Concentration of standard}$$

Where concentration of standard = 2.05 mg/dl

Sodium

Kit: TECO

Reagent: 1. Filtrate reagent (comprising of 2.1 mmol/L of Uranyl acetate 20 mmol/L of Magnesium acetate in ethyl alcohol). 2. Acid reagent (dilute acetic acid). 3. Sodium colour reagent (Potassium ferrocyanide, non-reactive stabilizers and fillers). 4. Sodium standard (150 mEq/L of NaCl solution).

Principle: In order to create a chromophore whose absorbance is inversely proportionate with the concentration of sodium in the material being tested, the excess uranium is treated with ferrocyanide after precipitating sodium as a triple salt, sodium magnesium uranyl acetate.

Procedure: One milliliter of the filtrate reagent was pipetted into each of the three test tubes that were appropriately labeled test, standard, and blank. The same volume of the standard reagent was added to the standard, the same volume of distilled water was supplied to the blank, and

precisely 50µl of the sample was added to the test tube marked test. All test tubes were shaken vigorously to ensure adequate mixing of their contents. Each test tube was centrifuged at high speed for 10 minutes to obtain a supernatant above and precipitate below. Care was taken not to disturb the protein precipitate. The test proceeded to the stage with the supernatant only.

Another set of three test tubes still labelled test, standard and blank were set up and 1 ml of the acid reagent was pipetted into each of the tubes. Then 50µl of the supernatants was added to their corresponding test tubes and mixed properly. Also, 50µl of the colour reagent was added to all the tubes and mixed. The spectrophotometer was zeroed with distilled water and then the absorbance of the content of each test tube was read and recorded. The concentration of sodium in the sample was calculated using the expression:

$$\text{Sodium conc. in mEq/L} = \frac{\text{Abs.of blank} - \text{Abs.of test}}{\text{Abs.of blank} - \text{Abs.of standard}} \times \text{Concentration of standard}$$

Where concentration of standard = 150 mEq/L

Chloride

Kit: TECO

Reagent: 1. Chloride reagent (comprising of 0.058 mmol/L of Mercuric Nitrate, 1.75 mmol/L of Mercuric Thiocyanate, 0.74 mmol/L of Mercuric Chloride and 22.3 mmol/L of Ferric Nitrate) with non-reactive ingredients and stabilizers in dilute acid and methanol. 2. Chloride standard (100 mEq/L of NaCl solution).

Principle: Chloride ions will displace thiocyanate ions from non-ionized mercuric thiocyanate and combine with mercuric ions to generate a soluble non-ionized molecule. A color complex whose intensity is proportionate to the sample's

chloride concentration is created when the liberated thiocyanate ions combine with ferric ions.

Procedure: After setting up three test tubes with the appropriate labels for test, standard, and blank, 1.5 ml of the chloride reagent was pipetted into each tube. Ten microliters of the sample, the same volume of the standard reagent, and the same volume of distilled water were then added to the test tube with the test label. All test tubes were shaken to mix and incubated at 25°C for 5 minutes. Then the absorbance of the content of each test tube was read at wavelength 500 nm after zeroing with the blank.

Calculate chloride concentration in mEq/L with the expression:

$$\text{Chloride concentration in mEq/L} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \text{Concentration of standard}$$

Where concentration of standard = 100 mEq/L

Potassium

Kit: TECO

Reagent: 1. Potassium reagent (comprising of 2.1 mmol/L of Sodium Tetraphenylboron, preservatives and thickening agents). 2. Potassium standard (4 mEq/L).

Principle: In a specially prepared mixture, sodium tetraphenylboron can form a colloidal suspension. The concentration of potassium in the sample is directly correlated with the turbidity of this suspension.

Procedure: Three test tubes properly labeled test, standard and blank were set up and 1ml of the chloride reagent was pipette into each tube. The test tube was then filled with 10µl of the sample, the same volume of the standard reagent, and the same volume of distilled water for the blank. After shaking each test tube to combine them, let them stand at 25°C for three minutes. Then the absorbance of the content of each test tube was read at wavelength 500 nm after zeroing with the blank.

Calculate potassium concentration in mEq/L with the expression:

$$\text{Potassium conc. in mEq/L} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \text{Concentration of standard}$$

Where concentration of standard = 4 mEq/L

Bicarbonate

Kit: TECO

Reagent 1: 1.8 mmol/L of phosphoenol pyruvate, 10 mmol/L of magnesium sulphate, 0.40 mmol/L of adenine dinucleotide, 1250 u/l of malate dehydrogenase, 200 u/l of phosphoenol pyruvate carboxylase, 2.5 mmol/L of sodium oxamate, buffer solution with a pH of 7.0, The carbon dioxide reagent non-reactive fillers and stabilizers with 0.1 % sodium azide as preservative. 2. Bicarbonate standard which contains 30 mmol/L of Sodium bicarbonate in an aqueous solution.

Principle: The reaction between phosphoenol pyruvate and carbon dioxide (bicarbonate) to produce oxaloacetate and phosphate ions is catalyzed by phosphoenol pyruvate carboxylase. At the

same time that an equimolar amount of reduced nicotinamide adenine dinucleotide (NADH) is oxidized to NAD, oxaloacetate is reduced to malate; Malate dehydrogenase catalyzes the process. As a result, the absorbance at 340 nm decreases, and this decrease is directly correlated with the sample's CO₂ content.

Procedure: Using the amount of CO₂ free water specified on the vial label, the carbon dioxide (CO₂) reagent was reconstituted and gently inverted five or six times. After setting up three cuvettes with the labels test, standard, and blank, 1 ml of the reagent was pipetted into each tube, and all of the tubes were incubated at 37°C for three minutes. The test cuvette was filled with precisely 10µl of the sample, and the standard and blank were filled with the identical volume of distilled

water and the standard reagent, respectively. After measuring the

absorbance at 340 nm, the bicarbonate concentration was computed as follows:

$$\text{Bicarbonate conc. in mmol/L} = \frac{\text{Abs.of blank} - \text{Abs.of test}}{\text{Abs.of blank} - \text{Abs.of standard dard}} \times \text{Concentration of standard}$$

Where concentration of standard = 30 mmol/L

DATA ANALYSIS

Data analysis was conducted using the Statistical Package for Social Sciences (SPSS, Version 20.0, IBM SPSS Inc., Chicago, IL). The One-Way Analysis of Variance (ANOVA) was used to determine the level of significance. The Duncan Multiple Range Test was used to evaluate

the data, and the student's t test was used as a post-hoc test to compare the means of the different doses and fractions. For the measured value, P-values less than 0.05 were regarded as statistically significant differences between the test groups and the control group.

RESULTS

Table 1: Renal function parameters.

Treatmen ts	Normal control	BPH control (Testosterone only)	Finesteride (3 mg/kg) + Testosterone	AML extract (200 mg/kg) + Testosterone	AML extract (400 mg/kg) + Testosterone	AML extract (800 mg/kg) + Testosterone
Urea (mg/dl)	20.35± 0.86 ^a	29.05±2.45 ^b	23.15±2.23 ^a	22.54±1.27 ^a	21.77±1.26 ^a	20.13±0.24 ^a
Creatinin e (mg/dl)	0.79±0. 04 ^a	1.54±0.21 ^c	1.04±0.10 ^b	0.90±0.04 ^{a,b}	0.87±0.06 ^{a,b}	0.80±0.05 ^a
Na ⁺ (mEq/L)	130.90 ±0.98 ^b	126.13±1.44 ^a	127.00±0.92 ^a	127.90±2.21 ^a	131.33±0.91 ^b	130.73±1.47 ^b
K ⁺ (mEq/L)	4.40±0. 17 ^{b,c}	4.20±0.10 ^a	4.31±0.05 ^{a,b}	4.34±0.06 ^{a,b,c}	4.40±0.05 ^{b,c}	4.49±0.03 ^c
Cl ⁻ (mEq/L)	90.57± 0.95 ^c	83.87±2.57 ^a	85.27±1.46 ^a	88.23±0.86 ^b	87.90±0.60 ^{b,c}	90.13±0.57 ^{b,c}
HCO ₃ ⁻ (mmol/L)	19.70± 0.10 ^a	19.97±0.25 ^a	19.77±0.25 ^a	19.93±0.31 ^a	19.73±0.25 ^a	19.80±0.26 ^a

Values are presented as mean ± standard deviation (n = 3); and means with different letter superscripts are significantly (P < 0.05) different from paired mean across the row.

Table 4.1 shows the result of the renal function parameters across different treatment groups. Compared to the normal control group, the BPH control (Testosterone only) group exhibits significant alterations in several renal parameters. Specifically, levels of urea (29.05±2.45 mg/dl) and creatinine (1.54±0.21 mg/dl) are notably elevated, indicating impaired renal function due to BPH induction by testosterone. Treatment with Finesteride (3 mg/kg) + Testosterone

and various doses of AML extract (200 mg/kg, 400 mg/kg, and 800 mg/kg) + Testosterone generally show improvements in these parameters compared to the BPH control group. These treatments reduce urea and creatinine levels closer to those of the normal control group (urea: 20.35±0.86 mg/dl, creatinine: 0.79±0.04 mg/dl). Furthermore, electrolyte levels (Na⁺, K⁺, Cl⁻, HCO₃⁻) in the BPH control group also show deviations from the normal control. Treatments with Finesteride and AML extract tend to normalize these electrolyte levels.

DISSCUSION

Renal function parameters are crucial indicators of kidney health, reflecting the

organ's ability to maintain fluid balance, electrolyte levels, and excrete waste products effectively. The BPH control group, induced by testosterone alone, exhibits significant deviations in renal biomarkers compared to the normal control group, indicating impaired kidney function. Elevated levels of serum creatinine (1.54 ± 0.21 mg/dl) and blood urea nitrogen (BUN) (29.05 ± 2.45 mg/dl) in the BPH control group suggest compromised renal filtration and clearance capacity due to testosterone-induced BPH. These alterations underscore the systemic effects of hormonal imbalance on renal function, potentially leading to renal disorders [5] Gounden *et al.*, 2018).

Administration of *Annona muricata* extract, as evidenced by several studies, demonstrates promising renoprotective effects. Research by Zeweil *et al.* (2024) highlights significant improvements in renal function markers following *Annona muricata* treatment, including reductions in serum creatinine and BUN levels. These findings suggest that *Annona muricata* possesses nephroprotective properties, mitigating kidney damage and preserving renal function. Moreover, studies investigating acute and repeated dose toxicity of *A. muricata* extract consistently show no adverse effects on renal function parameters such as creatinine and urea levels, further supporting its safety and potential therapeutic use in maintaining renal health [3,7].

The reno-protective mechanisms of *Annona muricata* are attributed to its diverse phytochemical composition, which includes flavonoids, alkaloids, and acetogenins. These bioactive compounds exhibit antioxidant and anti-inflammatory properties, helping to mitigate renal oxidative stress and inflammation associated with renal injury [3]. *Annona muricata* extracts also activate the nuclear factor erythroid 2-related factor 2 (Nrf2)

pathway, enhancing the expression of antioxidant enzymes and detoxifying proteins that protect against nephrotoxicity. [3,7].

CONCLUSION

Conclusively, result of the renal function parameters across different treatment groups. Compared to the normal control group, the BPH control (Testosterone only) group exhibits significant alterations in several renal parameters. Specifically, levels of urea (29.05 ± 2.45 mg/dl) and creatinine (1.54 ± 0.21 mg/dl) are notably elevated, indicating impaired renal function due to BPH induction by testosterone. Treatment with Finasteride (3 mg/kg) + Testosterone and various doses of *annona muricata* Leaf extract (200 mg/kg, 400 mg/kg, and 800 mg/kg) + Testosterone generally show improvements in these parameters compared to the BPH control group.

These treatments reduce urea and creatinine levels closer to those of the normal control group (urea: 20.35 ± 0.86 mg/dl, creatinine: 0.79 ± 0.04 mg/dl). Furthermore, electrolyte levels (Na^+ , K^+ , Cl^- , HCO_3^-) in the BPH control group also show deviations from the normal control. Treatments with Finasteride and AML extract tend to normalize these electrolyte levels.

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