

Ameliorating Effects of *Annona Muricata* Leaf Extract in Inflammatory Markers in Male Wistar Rats

Nze P.O.^{1*}, Edebiri O.E.^{2*}, Blackie O.H.³, Asiegbu F.C.⁴, Omende K.⁵, Orunta E.D.⁶, Okike P. I.⁷

¹Department of Physiology, Abia State University, Uturu, Abia State, Nigeria

²Department of Physiology, Ambrose Alli University, Ekpoma, Edo State, Nigeria

³Department of Anatomy, Ambrose Alli University, Ekpoma, Edo State, Nigeria

⁴Department of Physiology, Abia State University, Uturu, Abia State, Nigeria

⁵Department of Public Health, Imo State University, Imo State, Nigeria.

⁶Department of Public Health, Imo State University, Imo State, Nigeria.

⁷Department of Physiology, Ebonyi State University, Abakiliki, Ebonyi State, Nigeria

***Corresponding Author**

E-Mail Id: edebiriduncan@gmail.com

ABSTRACT

This present study aimed to investigate the ameliorating effects of *annona muricata* leaf extract on inflammatory markers 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. Group3, Testosterone propionate (5 mg/kg bw) + Finesteride (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 inflammatory markers respectively. 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 various biochemical analyses. 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. From this present study, the extract exhibited anti-inflammatory properties in treated groups.

Keywords: Inflammatory markers, testosterone-induced hyperplasia, *annona muricata* leaf extract and wistar rats

INTRODUCTION

The anti-inflammatory properties of *Annona muricata* are attributed to its rich content of bioactive compounds, including acetogenins, alkaloids, flavonoids, and phenolic compounds, which interact with various molecular pathways to exert their effects [18]. The anti-inflammatory effects of *Annona muricata* are primarily mediated through the inhibition of key pro-inflammatory mediators and enzymes. These include cyclooxygenase (COX), lipoxygenase (LOX), and nitric oxide (NO), which play pivotal roles in the inflammatory response. By inhibiting these mediators, *Annona muricata* reduces the production of pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α), interleukin-1 beta (IL-1 β), and interleukin-6 (IL-6) [13,16] Wahab *et al.*, 2018).

Studies have shown that extracts of *Annona muricata* can significantly reduce the levels of pro-inflammatory cytokines. For instance, an in vitro study demonstrated that soursop leaf extract inhibited lipopolysaccharide (LPS)-induced production of TNF- α and IL-1 β in macrophage cells. This suggests that *Annona muricata* can modulate immune responses and potentially alleviate inflammatory conditions [5,9]. *Annona muricata* exerts its anti-inflammatory effects by modulating various signaling pathways involved in inflammation. One such pathway is the nuclear factor kappa B (NF- κ B) pathway, which regulates the expression of genes involved in inflammatory responses. Inhibition of NF- κ B activation by soursop extracts leads to decreased transcription of inflammatory genes, thereby reducing inflammation [2,3,15]. The inhibition of COX and LOX enzymes by *Annona muricata* is another crucial mechanism underlying its anti-inflammatory effects. COX enzymes, particularly COX-2, are responsible for the synthesis of prostaglandins, which are key

mediators of inflammation. LOX enzymes catalyze the formation of leukotrienes, which also contribute to the inflammatory process. By inhibiting these enzymes, *Annona muricata* decreases the production of these inflammatory mediators, thereby exerting its anti-inflammatory effects [6,13].

The anti-inflammatory properties of *Annona muricata* are often synergistic with its antioxidant effects. Oxidative stress is a known contributor to inflammation, and the antioxidant compounds in soursop, such as flavonoids and phenolic acids, help to mitigate oxidative stress by scavenging free radicals. This reduction in oxidative stress subsequently lowers the inflammatory response, providing a dual benefit in conditions characterized by both oxidative stress and inflammation [1,4]. Several in vivo studies have corroborated the anti-inflammatory potential of *Annona muricata*. For example, an animal study demonstrated that soursop leaf extract significantly reduced carrageenan-induced paw edema and cotton pellet-induced granuloma formation in rats, indicating potent anti-inflammatory activity. These findings suggest that *Annona muricata* could be effective in treating inflammatory conditions such as arthritis and colitis [8,11,7].

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, Rotary 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

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 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 inflammatory markers analysis respectively. 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 various biochemical analyses.

Preparation of Plant Extracts

Plant extract 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-hexene 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 obtained 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.

PRO-INFLAMMATORY CYTOKINES ASSAY

Tumor necrosis Factor-Alpha (TNF- α) Assay

An ELISA based method was used for the assay

Principle: Sandwich-Elisa is the method used in the Elisa kit. The kit included a micro ELISA plate that was pre-coated with a RAT TNF- α antibody. The proper micro ELISA plate walls were filled with standards or samples, which were then mixed with the particular antibody. Each microplate wall was then coated with biotinylated detection antibodies that were specific for rat TNF- α and the Avidin Horseradish Peroxidase (HRP) conjugate, and the plates were then incubated. The free part washed out. Then the substrate reagent was added to each wall, only walls contacting Rat TNF- α , biotinylated detection antibody and avidin. HRP conjugate appeared blue colour. The enzyme substrate reaction was terminated by adding stop solution and the solution turned yellow colour. A spectrophotometer set to 450 nm wavelength was then used to determine the optical density (OD). Rat TNF- α concentration is proportional to the OD value.

Test Preparations

Tissue homogenate of rat stomach was used. Homogenized tissues were washed with 0.01M phosphate buffer solution before homogenization. The tissue homogenate was then mixed with the same concentration of buffer in ratio of 1:9 (tissue weight: volume of buffer). Homogenate was then centrifuged for 5 minutes at 500nm to get supernatant. Samples and reagents were all brought to room temperature before the analysis. Stock wash buffer solution was measured

out and made up of 750 ml of working buffer solution using demonized water.

Standard working solution: Stock standard solution provided was first centrifuged at 10,00rpm for 1 minute. 1ml of stock + 1ml of sample diluents were measured into test tubes and was mixed by turning upside down while incubating for 10 minutes. This gave a standard solution of 500mg/ml. Serial dilutions were then made in the gradient as follows: 5,000, 2,500, 1,250, 625, 312.5, 156.25, 78.13 and 0 pg/ml.

Biotinylated detection antibody working solution: HRP conjugate diluents were used to dilute 100x concentrated biotinylated detection antibody to 1x working solution.

Procedure

1. One milliliter of either standard or sample was added to each, and the mixture was incubated at 37 degrees Celsius for ninety minutes.
2. Liquid was removed and 100ml biotinylated detection Ab, was added and incubated for 1 hour at 37°C.
3. Liquid (solution) was aspirated and washed 3 times.
4. 100ml conjugated was added and incubated for 30 minutes at 37°C.
5. Solution was aspirated and washed 5 times.
6. 90ml stop solution was added and OD read immediately using micro plate reader at 540nm.
7. A graph of OD was plotted against concentration for known standard concentration and concentration for sample extrapolated from graph. Duplicate readings were averaged.

Interleukin-I Beta (1L-I β) Activity

An ELISA based method was employed for the assay. The kit was purchased from ELabsience and the instructions state on kit adhered to strictly.

Principle: The micro ELISA plate provided as pre-coated with antibody specific to IL-I β standards or samples were added to appropriate micro ELISA plate as well and combined with the specific antibody. Then a biotinylated detection antibody specific for rat IL-I β , and avidin horseradish peroxidase conjugate were added to each microplate successfully and incubated. After incubation, free components were washed away. Then the substrate reagents were added to each well. Only wells containing rat IL-I β biotinylated antibody and avidin horseradish peroxidase conjugate will appear blue in colour. The enzyme substrate reaction will be terminated by adding a stop solution and it appear yellow in colour. The optical density (OD) was measured using a spectrophotometer immediately at 450nm. The OD value is proportional to the concentration of Rat IL-I β .

Reagent preparation: Tissue homogenates of rates stomach was used. Homogenized tissues were washed with 0.01m phosphate buffer solution before homogenization. The tissue homogenate was then mixed with the same concentration of buffer in the ratio of 1:9 (tissue weight: volume of buffer). Homogenate was then centrifuged for 5

minutes at 500 rpm to get supernatant samples and reagents were all brought to room temperature before analysis.

Wash buffer: 300ml of stock wash buffer solution was measured out and made up of 750ml of working buffer solution using deionised water.

Standard working solution: Stock stand solution provided was first centrifuged at 10,000 rpm for 1 minute. 1ml of stock solution and 1ml of sample diluents were measured into the test tube and was mixed by turning upside down while incubating for 10 minutes. As a result, 500 pg/ml was the normal working solution. The gradient was then serially diluted with 500, 250, 125, 62.5, 31.5, 15.63, 7.81, and 0 pg/ml.

Data analysis

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.

RESULTS

Table 1: *Inflammatory markers.*

Treatments	IL-I β (pg/ml)	TNF- α (pg/ml)
Normal control	0.18 $\pm$ 0.03 ^a	33.85 $\pm$ 3.38 ^a
BPH control (Testosterone only)	2.01 $\pm$ 0.11 ^d	75.95 $\pm$ 4.87 ^c
Finesteride (3 mg/kg) + Testosterone	0.64 $\pm$ 0.11 ^c	53.26 $\pm$ 2.93 ^b
AML extract (200 mg/kg) + Testosterone	0.50 $\pm$ 0.05 ^b	48.96 $\pm$ 6.72 ^b
AML extract (400 mg/kg) + Testosterone	0.55 $\pm$ 0.05 ^{b,c}	51.56 $\pm$ 4.42 ^b
AML extract (800 mg/kg) + Testosterone	0.15 $\pm$ 0.02 ^a	33.22 $\pm$ 3.04 ^a

Values are presented as mean $\pm$ standard deviation (n = 3); and means with different letter superscripts are significantly (P < 0.05) different from paired mean within the column.

Table 4.1 shows the levels of inflammatory markers IL-1 β and TNF- α across different treatment groups. The BPH control (Testosterone only) group exhibits significantly elevated levels of IL-1 β (2.01 $\pm$ 0.11 pg/ml) and TNF- α (75.95 $\pm$ 4.87 pg/ml) compared to the normal control group (IL-1 β : 0.18 $\pm$ 0.03 pg/ml; TNF- α : 33.85 $\pm$ 3.38 pg/ml). Treatment with Finasteride (3 mg/kg) + Testosterone reduces IL-1 β (0.64 $\pm$ 0.11 pg/ml) and TNF- α (53.26 $\pm$ 2.93 pg/ml) levels, while treatment with AML extract at varying doses (200 mg/kg, 400 mg/kg, and 800 mg/kg) + Testosterone also decreases these markers compared to the BPH control group. Specifically, the highest dose of AML extract (800 mg/kg) + Testosterone significantly reduces IL-1 β (0.15 $\pm$ 0.02 pg/ml) and TNF- α (33.22 $\pm$ 3.04 pg/ml) levels, approaching those of the normal control group.

DISCUSSION

The presence of saponins in moderate concentrations is significant due to their potential role in enhancing the bioavailability of other phytochemicals and supporting various biological activities, including anti-inflammatory effects [5]. This aligns with studies that have demonstrated the anti-inflammatory properties of *Annona muricata* through modulation of cytokine levels such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF- α) [14] Foong and Hamid, 2012). However, the absence (-) of triterpenes and phlobatanins in the extract might limit its efficacy in certain biological activities associated with these compounds. Triterpenes, for example, are known for their anti-inflammatory and anticancer properties, which might not be fully leveraged in *Annona muricata* extract due to their absence (Wahab *et al.*, 2018). The study's findings on inflammatory markers, particularly interleukin-1 β (IL-1 β) and tumor necrosis factor-alpha (TNF- α), underscore *Annona muricata*'s potential

as an effective anti-inflammatory agent, crucial for managing conditions associated with chronic inflammation like benign prostatic hyperplasia (BPH) (Bitar *et al.*, 2019; Silihe *et al.*, 2023). The BPH control group (Testosterone only) exhibited significantly elevated levels of IL-1 β and TNF- α compared to the normal control group. Elevated levels of these cytokines are indicative of heightened inflammatory responses, which are associated with various inflammatory disorders and can exacerbate tissue damage and dysfunction (Bitar *et al.*, 2019; [12]. Studies investigating *Annona muricata* have consistently demonstrated its ability to modulate inflammatory markers. For instance, Bitar *et al.* (2019) and Silihe *et al.* (2023) reported significant reductions in IL-1 β and TNF- α levels following treatment with *Annona muricata* extracts in animal models. These reductions are attributed to the plant's bioactive compounds, such as flavonoids, alkaloids, and acetogenins, which exert anti-inflammatory effects by inhibiting pro-inflammatory cytokine production and suppressing NF- κ B-mediated inflammatory pathways (Foong and Hamid, 2012; [10]. Moreover, *Annona muricata*'s antioxidant properties play a crucial role in mitigating inflammation by scavenging free radicals and reducing oxidative stress, which are known triggers for inflammatory cytokine release [17]. This dual action—anti-inflammatory and antioxidant—positions *Annona muricata* as a promising therapeutic agent for combating inflammatory conditions associated with BPH and other chronic inflammatory disorders.

CONCLUSION

From this present study, the extract exhibited anti-inflammatory properties in treated groups

REFERENCES

1. Arnaud, K., Nicodème, C., Durand, D. N., Martial, N., Basile, S., Haziz, S., ...and Lamine, B. M. (2020). Antioxidant, Anti-Inflammatory Efficacy and HPLC Analysis of *Annona muricata* Leaves Extracts from Republic of Benin. *American Journal of Plant Sciences*, 11(6): 803-818.
2. Attiq, A., Jalil, J., and Husain, K. (2017). Annonaceae: breaking the wall of inflammation. *Frontiers in pharmacology*, 8: 752.
3. Ayun, N. Q., and Elya, B. (2020). Anti-inflammation of soursop leaves (*Annona muricata* L.) against hemorrhoids in mice induced by croton oil. *Pharmacognosy Journal*, 12(4).
4. Baskar, R., Rajeswari, V., and Satish Kumar, T. (2007). In vitro antioxidant studies in leaves of *Annona* species. *Indian Journal of Experimental Biology*, 45(5): 480-485.
5. Bitar, R. M., Fahmi, R. R., and Borjac, J. M. (2019). *Annona muricata* extract reduces inflammation via inactivation of NALP3 inflammasome. *Journal of Natural Remedies*, 12-23.
6. Coria-Téllez, A. V., Montalvo-González, E., Yahia, E. M., and Obledo-Vázquez, E. N. (2018). *Annona muricata*: A comprehensive review on its traditional medicinal uses, phytochemicals, pharmacological activities, mechanisms of action and toxicity. *Arabian Journal of Chemistry*, 11(5): 662-691.
7. De Sousa, O. V., Vieira, G. D. V., de Jesus RG de Pinho, J., Yamamoto, C. H., and Alves, M. S. (2010). Antinociceptive and anti-inflammatory activities of the ethanol extract of *Annona muricata* L. leaves in animal models. *International Journal of Molecular Sciences*, 11(5): 2067-2078.
8. Foong, C. P., and Hamid, R. A. (2012). Evaluation of anti-inflammatory activities of ethanolic extract of *Annona muricata* leaves. *Revista Brasileira de Farmacognosia*, 22: 1301-1307.
9. Gavamukulya, Y., Abou-Elella, F., Wamunyokoli, F., and AEl-Shemy, H. (2014). Phytochemical screening, anti-oxidant activity and in vitro anticancer potential of ethanolic and water leaves extracts of *Annona muricata* (Graviola). *Asian Pacific journal of tropical medicine*, 7: S355-S363.
10. Gavamukulya, Y., Wamunyokoli, F., and El-Shemy, H. A. (2017). *Annona muricata*: Is the natural therapy to most disease conditions including cancer growing in our backyard? A systematic review of its research history and future prospects. *Asian Pacific journal of tropical medicine*, 10(9): 835-848.
11. Ishola, I. O., Awodele, O., Olusayero, A. M., and Ochieng, C. O. (2014). Mechanisms of analgesic and anti-inflammatory properties of *Annona muricata* Linn.(Annonaceae) fruit extract in rodents. *Journal of medicinal food*, 17(12): 1375-1382.
12. Jang, D. I., Lee, A. H., Shin, H. Y., Song, H. R., Park, J. H., Kang, T. B., ... and Yang, S. H. (2021). The role of tumor necrosis factor alpha (TNF- α) in autoimmune disease and current TNF- α inhibitors in therapeutics. *International journal of molecular sciences*, 22(5): 2719.
13. Oyekachukwu, A. R., Elijah, J. P., Eshu, O. V., and Nwodo, O. F. C. (2017). Anti-inflammatory effects of the chloroform extract of *Annona muricata* leaves on phospholipase A2 and prostaglandin synthase activities. *Transl Biomed*, 8(4): 137.

14. Silihe, K. K., Mbou, W. D., Ngo Pambe, J. C., Kenmogne, L. V., Maptouom, L. F., Kemegne Sipping, M. T., ... and Njamen, D. (2023). Comparative anticancer effects of *Annona muricata* Linn (Annonaceae) leaves and fruits on DMBA-induced breast cancer in female rats. *BMC Complementary Medicine and Therapies*, 23(1): 234.
15. Torres, M. P., Rachagani, S., Purohit, V., Pandey, P., Joshi, S., Moore, E. D., ...and Batra, S. K. (2012). Graviola: a novel promising natural-derived drug that inhibits tumorigenicity and metastasis of pancreatic cancer cells in vitro and in vivo through altering cell metabolism. *Cancer letters*, 323(1): 29-40.
16. Yang, C., Gundala, S. R., Mukkavilli, R., Vangala, S., Reid, M. D., Aneja, R. (2015). Synergistic interactions among flavonoids and acetogenins in *Annona muricata* extract enhance its antitumor activity in prostate cancer cells. *Journal of Natural Products*, 78(8): 1753-1763.
17. Zeweil, M. M., Khafaga, A. F., Mahmoud, S. F., Wasef, L., Saleh, H., Elrehim, A. M. A., ...and Ghoneim, H. A. (2024). *Annona muricata* L. extract restores renal function, oxidative stress, immunohistochemical structure, and gene expression of TNF- α , IL- β 1, and CYP2E1 in the kidney of DMBA-intoxicated rats. *Frontiers in Pharmacology*, 15: 1348145.
18. Zubaidi, S. N., Qadi, W. S., Maarof, S., Mohmad Misnan, N., Mohammad Noor, H. S., Hamezah, H. S., ...and Mediani, A. (2023). Assessing the Acute Toxicological Effects of *Annona muricata* Leaf Ethanol Extract on Rats: Biochemical, Histopathological, and Metabolomics Analyses. *Toxics*, 11(8): 688.