**ANTI-OXIDATIVE STRESS POTENTIALS OF *ANNONA MURICATA* ETHANOL STEM EXTRACT ON N-NITROSO- N- METHYLUREA (NMU) INDUCED PROSTATE CANCER IN ALBINO RATS**

**Abstract**

**Background**

Evidence has suggested that the pathogenesis of prostatic hyperplasia and prostate cancer is closely related to the state of oxidative stress in prostate tissue.

**Method:** This study was conducted to evaluate the anti-oxidative stress potentials of *A. muricata* ethanol stem extract on N-nitroso- N- Methylurea (NMU) induced prostate cancer in albino rats. Twenty-five (25) male albino rats of body weights (*b.w.*) mean of 96g were used for this study. After 2 weeks of acclimatization, the rats were divided into five (5) groups as follows; group 1 served as the control group, group 2 received 50mg/kg of cyprosterone acetate (CA) + 100mg/kg testosterone propionate (TP) + 50mg/kg of NMU, group 3 received 50mg/kg of CA + 100mg/kg TP + 50mg/kg of NMU + 50mg/kg of finasteride, group 4 received 50mg/kg of CA + 100mg/kg TP + 50mg/kg of NMU + 250mg/kg of stem extract of *A. muricata* and group 5 received 50mg/kg of CA + 100mg/kg TP + 50mg/kg of NMU + 500mg/kg of stem extract of *A. muricata.* Induction of prostate cancer lasted for 21 days and treatment with *A. muricata* stem bark extract commenced immediately and lasted for 28 days. At the end of the study, the animals were sacrificed and liver homogenate was obtained for assessment of antioxidant parameters.

**Result:** The result from this study showed a significant decrease (p ≤ 0.05) in GSH level, SOD activity, CAT activity, and a significant increase (p<0.05) in MDA level following NMU administration to rats in group 2 when compared to the normal control group (group 1). The result also showed a significant increase (p ≤ 0.05) in GSH concentration, SOD activity, CAT activity, and a significant decrease in MDA level following treatment of NMU exposed rats with finasteride and *A. muricata* stem bark extract.

**Conclusion:** The findings suggest a dose-dependent protective effect of *Annona muricata* ethanol stem bark extract against NMU-induced prostatic damage. Hence, this study supports the potential of *Annona muricata* stem bark as a natural therapeutic agent for mitigating prostate cancer-associated diseases.

**Keywords**: *Annona muricata,* Antioxidant, Prostate cancer.

**Introduction**

The human body has a complex system of natural enzymatic and non-enzymatic antioxidant defenses which counteract the harmful effects of free radicals and other oxidants. Free radicals are responsible for causing a large number of diseases including cancer (Adewole 2006), cardiovascular disease, neural disorders, Alzheimer’s disease, mild cognitive impairment, disease, alcohol induced liver disease, ulcerative colitis, aging, atherosclerosis, and indiscriminate inflammatory responses (Moghadamtousi et al., 2015).

Early research suggests that NMU stimulates the generation of reactive oxygen species (ROS), which damages DNA, causes lipid peroxidation, and depletes the antioxidant defense mechanisms in cells. ROS affect mesangial and endothelial cells, which can result in oxidative stress and alter the body function (Singh et al., 2016). When the amount of reactive oxygen species (O2, H2O2, and -OH) generated exceeds the antioxidant capacity of the cells, oxidative damage results. The damage caused by the ROS generated during NMU metabolism may spread from the site of formation to other locations inside or even outside the cells (Yasui and Baba, 2016). The search for treatment/management agent for prostate cancer of natural origin with minimal side effect is an ever evolving niche in the world of science. This research focused on ameliorating oxidative effects which might arise as a result of prostate cancer.

*Annona muricata* (*A. muricata*) is a species of the *Annonaceae* family that has been widely studied in the last decades due to its therapeutic potential. The medicinal uses of the *Annonaceae* family were reported long time ago and since then, this species has attracted the attention due to its bioactivity and traditional uses (Pinto et al., 2015).

Ethnobotanical studies have indicated that *Annona muricata* has been used as insecticide and parasiticidal. Fruit juice and infusions of leaves or branches have been used to treat fever, sedative, respiratory illness, malaria, gastrointestinal problems, liver, heart and kidney infections (De Souza etal.,2011). In recent years it has become widely used for hypoglycemic, hypotensive and cancer treatments (Coria-Tellez et al.,2016).

A number of publications and reviews about *A. muricata* have been conducted to integrate the available scientific studies on this plant with special interest on acetogenins as principal bioactive compounds (Coria-Tellez et al.,2016). Other bioactive compounds have been identified; more bioactivities have been evaluated; and medicinal uses have been extended, as well as a few reported toxicities.



Figure 1: (A) *A. muricata* leaves (B), *A. muricata* flower (C), *A. muricata* fruit (C). (Pinto et al*.,* 2015)

**Materials And Methods**

**Instruments**

The Instruments/apparatus used were of analytical grade and were gotten from the Department of Biochemistry and other scientific shops in Imo State. Equipment/apparatus used for this study are listed below;

Weighing balance/Metler HAS, Spatula/Pyrex, Syringe/Lifescan, Beakers/Pyrex, Refrigerator/Haier Thermocool, Conical flask/Pyrex, Dissecting set/Gold rose, Filter paper/Whatman, Filter paper/Kimax, Measuring cylinder/Pyrex, Test tube/Pyrex, Water bath/Gallenkamp, Centrifuge/Medifriend, UV spectrophotometer/Genesis.

**Chemicals/Reagents**

The chemicals and reagents used were of analytical grade. The chemicals used in this study include:

Chemicals reagents Manufacturers, Country

Ethanol BDH, England

Testosterone Laborate, India

Olive Oil Goya, Spain

Finasteride

N-nitroso-N-methylurea (NMU)

Testosterone propionate

Cyprosterone acetate

Distilled water

**Plant Material**

*Annona muricata* stem used for this research work was gotten from Ihiagwa, Owerri, Imo state and was air-dried and weighed every 2 days till a constant weight is gotten indicating that the leaves and stems have completely dried. The dried *Annona muricata* leaves and stem were then ground to fine powder and then stored in an air tight container for further use.

**Plant Extraction**

The extraction method used in the study was maceration extraction method which involves simple soaking, filtering and then evaporations. 800g of the ground plant material (*Annona muricata*) was weighed into a conical flask and 2000ml of 70% ethanol was added, ensuring that the solvent properly cover the plant material. The mixture was allowed to stand for 48 hours with constant stirring. After 48 hours the mixture was filtered using filter paper and the filtrate evaporated in a water bath to obtain an extract.

**Percentage Yield**

The extract was weighed using an electronic weighing balance then the percentage yield was calculated as follows:

 Weight of the extract = 185.40g

The percentage yield = weight of extract X 100

 Weight of ground plant leave (800 g)

Animal Environment, Handling And Ethics

Twenty-five (25) male albino rats of body weights (*b.w.*) mean of d 96g were purchased from the Animal Breeding Unit, Zoology Department, University of Nigeria Nsukka, Enugu state, Nigeria. The animals were kept in stainless–steel cages in a well–ventilated room of temperature 28 ± 2°C and relative humidity of 55–65% with a diurnal 12 h light cycle. The rats were treated humanely and had access to water and pelletized standard finishers mesh (Vital finisher) (United Africa Company Nigeria Plc., Jos, Nigeria) *ad libitum*. A period of 2 weeks was allowed for acclimatization of the rats to environmental conditions.

The Twenty-five (25) male albino rats were divided into five (5) groups of five (5) rats each.

**Table 1: Experimental Design**

|  |  |  |  |
| --- | --- | --- | --- |
| Groups  | Description | Treatments | No. of rats |
| One  | Normal control | Feed and water only |  5 |
| Two  | Negative control  | 50mg/kg of cyprosterone acetate (CA) + 100mg/kg testosterone propionate (TP) + 50mg/kg of NMU |  5 |
| Three  | Standard control | 50mg/kg of CA + 100mg/kg TP + 50mg/kg of NMU + 50mg/kg of finasteride  |  5 |
| Four  | Low dose stem | 50mg/kg of CA + 100mg/kg TP + 50mg/kg of NMU + 250mg/kg of stem extract. |  5 |
| Five  | High dose stem | 50mg/kg of CA + 100mg/kg TP + 50mg/kg of NMU + 500mg/kg of stem extract |  5 |

**Induction of Prostate Cancer**

Induction of prostate cancer was carried out using the method of Bosland and Prinsen (1975) with little modification. After 2 weeks of acclimatization, the rats were given 50mg/kg of cyprosterone acetate in normal saline via intraperitonial route for 18 days, after which the rats received subcutaneous injection of testosterone propionate (100mg/kg) in olive oil for another 3 days, this was followed by a single shot (dose) of NMU (50mg/kg) in normal saline via intraperitonial injection.

**Extract Administration**

The different doses of extracts and finasteride were administered orally following induction of prostate cancer for 28 days.

**Collection Of Blood Samples**

At the end of 28 days, the animal were anaesthetized with chloroform and then sacrificed. Whole blood was collected in plain sample bottles through cardiac puncture using sterile needles and syringes. The clotted blood was centrifuged at 3000 rpm for 10 mins to obtain serum.

**Preparation Of Liver Homogenates**

One gram (1g) of the liver tissues were homogenized in 10ml of ice-cold physiological saline to obtain 10% (w/v) homogenates. The resulting homogenates were centrifuged at 4,000 rpm for 10min and the supernatants obtained were used for determination of superoxide dismutase (SOD), catalase (CAT) and lipid peroxidation level.

**Determination Of Antioxidant Parameters**

**Determination Of Malondialdehyde**

Malondialdehyde in plasma is one of the aldehyde products of lipid peroxidation which react with TBA to form a colored product, the absorbance of which is measured spectro-photometrically at 530 nm. In the test tubes 0.5 ml of hemogenate from test samples were taken, and 3 ml of 10% TCA was added to it, mixed well and the tubes were left to stand for 10 min at room temperature, and then centrifuged for 15 min at 4000 rpm. Two sets of test tubes were taken marked as blank and test. For a test sample, 2 ml of supernatant fluid was taken and added to 1.5 ml of 0.67% TBA. For a blank sample, 2 ml of distilled was added in 2 ml of 0.67% TBA. After mixing well and keeping in the boiling water bath for 10 min, they were cooled to room temperature. A pale pink color developed, the color intensity was measured at 530 nm by spectro-photometer. Using the molar extension coefficient (1.5 × 105) and result was expressed as n moles of malonaldehyde (MDA)/100 ml of hemogenate.

1.5 = 100 µmol/L (here, 100 is for conversion from ml to dl).

Then MDA = 100 × Absorbance of unknown/1.5.

**Determination Of Superoxide Dismutase (Sod) Activity**

Principle: The assessment of SOD activity was conducted based on the inhibition of the auto-oxidation of epinephrine at pH 10.2. Superoxide dismutase inhibits the oxidation of epinephrine to adrenochrome, a reaction catalyzed by superoxide radicals (O2-), which are generated through the xanthine oxidase reaction. The auto-oxidation of epinephrine involves distinct pathways, with one of them being a free radical chain reaction involving superoxide radicals, thus inhibitable by SOD.

Reagents: 0.05M Carbonate Buffer (pH 10.2): Prepared by dissolving 14.3 g of Na2CO3.10H2O and 4.2 g of NaHCO3 in 900 ml of distilled water and adjusting the pH to 10.2.

0.3mM Adrenaline Solution: Prepared by dissolving 0.0137 g of adrenaline in 200 ml of distilled water and adjusting the volume to 250 ml, freshly prepared before use.

Procedure: Tissue homogenate (1 ml) was diluted in 9 ml of distilled water to create a 1 in 10 dilution.

An aliquot of 0.2 ml of the diluted enzyme preparation was added to 2.5 ml of 0.05M Carbonate buffer (pH 10.2).

The reaction started with the addition of 0.3 ml of freshly prepared 0.3 mM adrenaline, mixed quickly, and monitored for absorbance at 480 nm every 30 seconds for 150 seconds.

SOD activity was calculated using the formula:

Increase in absorbance per minute = (A3 - A0/ t)

Where A0 = Absorbance after 0 seconds, A3 = Absorbance after 150 seconds, t = Time of final absorbance (150sec)

% inhibition = (Increase in absorbance/min of sample × 100)/ (Increase in absorbance/min of blank)

1 Unit of SOD activity is defined as the amount of SOD necessary to cause 50% inhibition of the oxidation of adrenaline to adrenochrome during an interval of one minute.

Specific activity = (Enzyme activity x dilution factor/ Total protein (mg))

**Determination of Catalase (Cat) Activity**

Principle: Catalase exerts a dual function, decomposition of H2O2 to give H2O and O2, and oxidation of hydrogen donors. In the ultraviolet range, H2O2 shows a continual increase in absorption with decreasing wavelength. The decomposition of H2O2 can be followed directly by the decrease in absorbance at 240 nm. The difference in absorbance per unit time is a measure of catalase activity (Aebi, 1984).

Procedure: Fifty (50) microliter of each sample (liver homogenates) was added to a cuvette containing 450 μL of phosphate buffer (0.1M, pH 7.4) and 500 μL of 20 mM H2O2. Catalase activity was measured at 240 nm for 1 min using spectrophotometer. The molar extinction coefficient of H2O2, 43.6 M cm−1 was used to determine the catalase activity. One unit of activity is equal to 1 mmol of H2O2 consumed / min/ mg of protein and is expressed as units per milligram of protein.

Calculation: Units/ml = (ΔA/min x d x 1/ V x 0.0436)

d = dilution of original sample for Catalase Reaction

V = Sample volume in Catalase Reaction (ml)

0.0436 = εmM for hydrogen peroxide

1 = Total reaction volume.

**Estimation Reduced Glutathione (GSH)**

The method of Jollow et al. (1974) was followed to measure GSH activity in the liver homogenate. Briefly, 500 μl of supernatant was mixed with 500 μl of sulfosalicylic (4%) to carry out precipitation. The reaction mixture was incubated for 1 h at 4 °C and then centrifuged at 4000 rpm for 20 min. Supernatant was collected and 33 μl of it was added to the reaction mixture containing 66 μl of 100 mM of 5,5′-dithio-bis (2-nitrobenzoic acid (DTNB) and 900 μl of 0.1 M potassium phosphate buffer (pH 7.4). The yellow colored complex was formed due to the reaction of reduced glutathione with DTNB. At 412 nm absorbance was immediately read and the GSH activity was presented by μM GSH/g tissue.

**Statistical Analysis**

The data generated in this study were processed and analyzed using ANOVA, comparing the average mean and standard deviation of the different groups and were represented with Bar charts.

**RESULTS & DISCUSSION**



**Figure 2: MDA levels in NMU-induced prostate cancer in rats treated with *A. muricata* ethanol stem bark extract. Bars represent mean ± standard deviation of triplicate determinations and bars with different alphabets indicate significant difference at *p* ≤ 0.05. Group 1 = normal control, group 2 = prostate cancer control group, group 4 = prostate cancer treated with low dose of stem bark extract, group 5 = prostate cancer treated with high dose of stem bark extract.**



**Figure 3: GSH levels in NMU-induced prostate cancer in rats treated with *A. muricata* ethanol stem bark extract. Bars represent mean ± standard deviation of triplicate determinations and bars with different alphabets indicate significant difference at *p* ≤ 0.05. Group 1 = normal control, group 2 = prostate cancer control group, group 4 = prostate cancer treated with low dose of stem bark extract, group 5 = prostate cancer treated with high dose of stem bark extract**.



**Figure 4: SOD activity in NMU-induced prostate cancer in rats treated with *A. muricata* ethanol stem bark extract. Bars represent mean ± standard deviation of triplicate determinations and bars with different alphabets indicate significant difference at *p* ≤ 0.05. Group 1 = normal control, group 2 = prostate cancer control group, group 4 = prostate cancer treated with low dose of stem bark extract, group 5 = prostate cancer treated with high dose of stem bark extract.**



**Figure 5: CAT activity in NMU-induced prostate cancer in rats treated with *A. muricata* ethanol stem bark extract. Bars represent mean ± standard deviation of triplicate determinations and bars with different alphabets indicate significant difference at *p* ≤ 0.05. Group 1 = normal control, group 2 = prostate cancer control group, group 4 = prostate cancer treated with low dose of stem bark extract, group 5 = prostate cancer treated with high dose of stem bark extract.**

Medicinal plants have formed the basis of health care throughout the world since the earliest days of humanity and have remain relevant in both developing and the developed nations of the world for various chemotherapeutic purposes (Akira et al., 2006). It has been medically proven that the rate of consumption of vegetable is concurrent with the health of an individual. This has been clearly observed among people in Asia and some part of Africa. In south-eastern Nigeria, the inhabitants are known for high consumption of vegetables. These vegetables are not only eaten as food but also during ill health and times of convalescence (Nwangwu et al., 2011). Thus they are referred to as herbal medicine. In this study the effect of ethanolic stem bark extract of *A. muricata* on the antioxidant parameters of NMU-induced prostate cancer in rats was evaluated.

ROS react with all biological substance; however, the most susceptible ones are polyunsaturated fatty acids. Reactions with these cell membrane constituents lead to lipid peroxidation (LPO) (Khan et al., 2014). Increased lipid peroxidation (LPO) observed in this study impairs membranefunction by decreasing membrane fluidity and changing theactivity of membrane-bound enzymes and receptor (Ugochukwu et al., 2013).

Thiobarbituric acid reactive substance (TBARS) levels were measured as a marker of LPO and malondialdehyde (MDA) production. Malondialdehyde is an endogenous genotoxicproduct of enzymatic and ROS-induced lipid peroxidation whose adducts are known to exist in DNA isolated from healthy human being (De Sousa et al., 2010). In this study, the level of MDA in the prostate cancer control group (group 2) increased significantly (p≤ 0.05) compared to normal control group and the treated groups (groups 4 and 5). This increase in the MDA levels reflects the decrease in the activities of glutathione peroxidase, CAT, SOD and GSH concentration as seen in this study and hence activation of LPO reactions (Ugochukwu et al*.*, 2013).

The SOD catalyzes the dismutation of superoxide to hydrogenperoxide and oxygen, thereby reducing the likelihood of superoxide anion reacting with nitric oxide to form reactive peroxy-nitrite (Maritim et al*.*, 2013). The decrease activities of catalase and SOD as observed in this study suggest that NMU has an *in vivo* oxidant activity and is capable of generating ROS in biologic system (Kim, et al., 2016). Superoxide dismutases (SODs) are a group of metallo enzymes that are found in all kingdoms of life. SODs form the front line of defence against reactive oxygen species (ROS)-mediated injury (Kossouoh et al., 2007). These proteins catalyze the dismutation of superoxide anion free radical (O2-) into molecular oxygen and hydrogen peroxide and O2- level which damages the cells at excessive concentrations (Ugochukwu et al*.*, 2013). In this study (as shown in Fig.4), it was observed that there was a significant increase (p ≤ 0.05) in the level of superoxide dismutase (SOD) in *A. muricata* stem bark extract treated groups compared to the disease control group. This is similar to the study of (Kim, et al., 2016), which suggests a significant increase in the level of SOD when *Caesalpinia sappan* Heartwood was experimented on rats.

Catalases are ubiquitously present in aerobic organisms, including almost all mammalian tissues, in which they show the highest enzyme activity. Catalase is the first line of defence against H2O2 ((De Sousa et al., 2010). There is evidence that moderate oxidative stress induces catalase expression in vascular cells and, thereby, could be beneficial in the prevention of further oxidative stress (Meilhac et al., 2010). Superoxide anions can also inhibit catalase. In this study (as shown in Fig 5), it was observed that there was a significant increase (p ≤ 0.05) in catalase activity in the *A. muricata* stem bark extract treated groups (group 4 and 5) compared to the compared to the prostate cancer control group (group 2), this may be as a result of free radicals scavenging activities of the *A. muricata* stem bark extract. This is similar to the findings of ((Ezuruike et al., 2014) which suggest an increase in the activity of catalase when experimenting with the hypolipidemic and antioxidant activities of *A. muricata* in hypercholesteremic rats.

Evidence has suggested that oxidative stress can also produce repeated prostate tissue damage (Chandra et al., 2012). The pathogenesis of prostatic hyperplasia and prostate cancer is closely related to the state of oxidative stress in prostate tissue (Akira et al., 2006). MDA is the end product of free radical attack on unsaturated fatty acids in biofilms, and it can indirectly reflect the oxidative stress state and damage degree of prostate tissue (Ezuruike et al., 2014). *A. muricata* stem bark extract effectively decreased the MDA level in prostate cancer-induced rats and revealed that they could alleviate the peroxidation state of prostate tissue. Additionally, SOD, CAT and GSH are important endogenous antioxidants (Khan et al., 2014), whose activities are increased in the *A. muricata* stem bark extract groups. Under the coordination of these substances, the superoxide anion can eventually be converted to water (Franco et al., 2019). However, excessive free radicals generated under oxidative stress can inhibit the activities of SOD, CAT and GSH (Pigeolet et al., 2010), further aggravating the level of oxidative stress (Gavamukulya et al., 2011). In response to hypoxia, prostate stromal cells increase the secretion of some growth factors, such as vascular endothelial growth factor (VEGF), to stimulate an increase in the vascular endothelial cell proliferation. Therefore, these indicated that *A. muricata* stem bark extract exhibit the anti-prostatic cancer effect by inhibiting the expression of VEGF, alleviating oxidative stress and overall ameliorating prostate cancer.

**Conclusion**

The result from this study showed that NMU induced oxidative stress in the albino rats as endogenous antioxidants (SOD, CAT and GSH) were depleted following administration of NMU. The result also showed that *A. muricata* stem bark extract was able to boost the endogenous antioxidants. This effect could be as a result of antioxidant phytochemicals present in *A. muricata*, and by extension may prevent prostate cancer. The finding of this study justifies the traditional use of *A. muricata* in treatment and management of cancer and other related illnesses.

**Declaration**s

**Ethics approval and consent to participate**

The study was approved by the Ethical Committee on Human Research of the Department of Biochemistry, Federal University of Technology, Owerri, Nigeria. With approval NO. FUT/SOBS/BCH/COM.2/013/2022 and done in accordance with the highest International Criteria of Animal Experimentation of Helsinki

**Consent for publication**

Not applicable.

**Availability of data and materials**

The data that support the findings of this study are available from the authors

upon reasonable request.

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