

**Antioxidant potential of ethanol extract of *Annona muricata* leaves and its inhibitory effect on lipid peroxidation in 1,2-dimethylhydrazine induced colon carcinogenesis.**

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**Abstract**

*Annona muricata*, as it is scientifically called but known in most parts of Nigeria as soursop. A fruit tree with various therapeutic uses, the plant belongs to the family of *Annonaceae* and has several medicinal properties. The medicinal properties of *Annona muricata* are well known, and they can be used to treat a variety of illnesses and conditions. Effects of ethanol extract of *Annona muricata* leaves (EEAML) on the enzymatic antioxidants of the kidney and colon in Wistar rats induced with 1, 2 Dimethylhydrazine (DMH) at 25 mg/kg bodyweight s.c. were investigated in this study. Thirty-six male Wistar rats weighing 110–170g were acclimated for two weeks and randomized into six groups (six per group). Group A (control), Group B (extract; 120 mg/kg body weight of ethanolic extract of *A. muricata*), Group C (DMH only), Group D (DMH + EXTRACT), Group E (pretreatment), and Group F (posttreatment). The extract was administered daily via oral gavage, while the DMH was given subcutaneously at a dosage of 25 mg/kg bodyweight. This study reveals the carcinogenic effect of DMH induced oxidative stress were ameliorated by the administration of this leaf extract of EEAML at a dose of 120mg/kg b.w. The colon and kidney homogenates of rats administered EEAML (co-treated, pretreatment and post- treatment) showed increased enzymatic antioxidant; glutathione peroxidase, catalase activities and SOD activities. But a reduction in malondialdehyde levels was observed. These findings validate the use of *Annona muricata* in traditional medicine by showing that the ethanolic extract of the leaves can reduce DMH-induced oxidative stress and alter the enzymatic antioxidant profile in colon carcinogenesis.

**Keywords:** *Annona muricata*, 1,2-dimethylhydrazine, oxidative stress, enzymatic antioxidants

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## INTRODUCTION

Colorectal cancer begins in the colon or rectum section of the large intestine (Kerr *et al.*, 2016; Kopetz *et al.*, 2007). In the past centuries, colon cancer accounts for around 10% of annual cancer fatalities (Doll *et al.*, 1981). Thus, it is vital to build and use suitable experimental systems to research colon carcinogenesis pathways. Laboratory animals can be induced with colon cancer via the administration of a potent carcinogen called DMH (Saini *et al.*, 2009). DMH are highly selective carcinogens that generate dose-dependent colorectal tumors. DMH exerts its effect through the methyl diazonium ion, which is the active metabolite. Protein, RNA, and DNA nitrogenous bases are alkylated due to the mutation exacerbated by this active substance. Additionally, H<sub>2</sub>O<sub>2</sub> is also released in the presence of the active metabolite, which may be a result of the induction of the production of free radicals generated during lipid peroxidation (Khan and Sultana, 2011), resulting in the methylation of the biomolecules present in the epithelial cells of the colon, causing aberrations, inflammation, and tumors in the colon (Swiderska *et al.*, 2014). The mitochondrial respiratory chain generates some highly reactive and unstable chemicals called ROS. Cellular physiology has made known the importance of ROS. For the optimal and normal functioning of cell activity, enzymatic antioxidants (GPx, SOD, and CAT) and peroxiredoxins control the physiological level of reactive oxygen species (ROS). The initiation of carcinogenesis has been linked to an imbalance between free radical production and antioxidants, thereby promoting hyperplasia and increased cell differentiation by abnormalities in some cell signaling pathways (Marengo *et al.*, 2016).

Medicinal plant study is essential in the production of new drugs. The presence of some bioactive chemical compounds such as alkaloids, tannins, flavonoids and phenolic compounds in medicinal plants make the plant important and beneficial. These bioactive compounds exert physiological action in the body system. Since the

beginning of medicine, *Annona muricata* natural compounds, particularly those derived from its leaves, barks, roots, seeds, and fruits, have been used to promote human health (Olude *et al.*, 2020). Analysis of the seeds and leaves of *Annona muricata* has yielded the isolation of numerous acetogenins. These compounds have displayed varying degrees of activity both pharmacological and biological. Rajeswari *et al.*, (2012) reported that the plant exhibited antitumoral, pesticidal, cytotoxic and anti-parasitic activities. The bark, roots and leaves are also used in herbal medicine (Agu and Okolie, 2017). Acetogenins present in *Annona muricata* is a potent anticancerous agent and also an inhibitor of nicotinamide adenine dinucleotide phosphate-oxidase (NADH) of the plasma membranes of cancer cells (Patel and Patel 2016). The compound induces cytotoxicity and also has a negative effect on oxidative phosphorylation and ATP synthesis by inhibiting mitochondrial complex 1 (Rady *et al.*, 2018). The plant has high antibacterial activity (Selvanathan *et al.*, 2022) and the delivery of acetogenin-enriched *Annona muricata* leaf extract by folic acid-conjugated and triphenylphosphonium-conjugated poly (glycerol adipate) nanoparticles displayed a high level of toxicity against ovarian cancer cells (Damrongrak *et al.*, 2022). The essential oil extracted from *Annona muricata* has a prophylactic effect in female rats induced with breast cancer (Rojas-Armas *et al.*, 2022). Plant phytochemicals have been a significant source of medicine over the past hundred years for the development of pharmaceuticals. The biological activities of these chemicals hold a strong appeal, owing to the importance of plant active components in agriculture and medicine. *Annona muricata* is one such plant with significant traditional usage. (Moghadamtousi *et al.*, 2015a and b). Colon cancer is on the increase. The cost of chemotherapy, radiotherapy and the side effects of treatment options has led to the search for alternatives using herbal medicine. The goal of this research was to examine the ethanol extract of *Annona muricata* leaves' antioxidant potential as well as how it affected the ability of the extract

to prevent lipid peroxidation in 1,2-dimethylhydrazine-induced colon carcinogenesis.

## MATERIALS AND METHODS

### Sample Collection and Identification of Plant Material

The leaves were freshly plucked from a garden in the Ovia North East Local Government Area of Benin City, Edo State. The leaves were verified by Dr. H.A. Akinnibosun in the Department of Plant Biology and Biotechnology Herbarium at the University of Benin in Benin City, Edo State, Nigeria.

### Preparation of *Annona muricata* Ethanol Extract.

Seven hundred and fifty grams (750g) of the ground *Annona muricata* leaves were soaked in 7.5 liters of absolute ethanol for 72 hours with periodic stirring. The ethanol was then removed from the filtrate using rotary evaporator at 60 °C, and the moisture was removed using freeze dryer. Prior to administration, the powdered extract was kept in an airtight container and chilled to 4°C in a refrigerator.

### Experimental Animals

Wistar rats were bought from a local breeder and kept in the animal house of the Biochemistry Department at the University of Benin in Benin City, Edo State. The 140 ±30 g rats were housed in metal cages with 12-hour cycles of light and darkness under typical lab conditions. The animals spent two weeks getting used to the lab environment prior to beginning treatments. The study protocol was approved by the University of Benin's Faculty of Life Sciences Ethical Committee on Animal Use.

### Carcinogen Preparation

Each time a dose was administered, a new DMH solution was made. DMH, a carcinogen, was dissolved in 1 mM EDTA, and 1 M sodium

hydroxide was used to achieve pH 6.5. Once a week, the carcinogen was subcutaneously injected at a dose of 25 mg/kg. (Chari *et al.*, 2018).

### Experimental Design

Thirty-six Wistar albino rats were used for this study. The experimental animals weighed between 110 and 170g and were divided into six (6) groups (Groups A-F), with each group having six (6) animals. Group A was the control group, while group B-F were the test groups. They were fed grower's pelleted feed. The feed and water were given *ad libitum*.

Group A: Healthy Control group

Group B: Ethanol Extract group (EEAML Control). 120mg/kg body weight taken orally

Group C: Dimethylhydrazine group (DMH Control)

Group D: Dimethylhydrazine + Ethanol extract group (DMH + EEAML)

Group E: Ethanol extract, then dimethylhydrazine group (Pretreatment)

Group F: Dimethylhydrazine, then ethanol extract group (post treatment).

Group C rats were given DMH once a week for 16wks, while group D rats received DMH once a week and EEAML daily for 16 weeks. Group E was pretreated with EEAML daily for 12weeks, followed by the administration of DMH for 4weeks, while the post-treated rats received DMH for once a week for 12 weeks followed by daily EEAML administration for 4 weeks.

### Animal Sacrifice

After the 16-week study, the animals were fasted overnight (12 hours) prior to sacrifice, and then sacrificed using a pair of scissors dissecting the animals by cervical dislocation in order for the

colon, kidney to be intact. The kidney and colon were collected for biochemical tests.

#### **Preparation of Tissue Homogenate.**

The colon and kidneys were harvested from each rat, weighed, and then homogenized in a cold normal saline solution. The homogenates were then centrifuged at 4000 rpm for 20 minutes, and the supernatant was transferred into labeled containers. These supernatants were immediately used for biochemical analysis of some enzymatic antioxidants, which included the Superoxide dismutase assay (SOD), Total protein assay (TP), catalase (CAT), Glutathione peroxidase (GPx), lipid peroxidation assay (malondialdehyde).

#### **Biochemical Assays**

The level of superoxide dismutase activity was determined according to the method of Misra and Fridovich (1972). Adrenaline auto-oxidizes rapidly in the presence of superoxide anions, and SOD inhibits the reaction by catalyzing superoxide anions. The activity of the enzyme is reflected in its degree of inhibition. The concentration of adrenochrome released was read at 420nm. Malondialdehyde (MDA) levels were estimated by the method of Burge and Aust (1978). MDA is a product of lipid peroxidation. The absorbance of the pink-coloured complex produced as a result of the reaction mixture with 2-thiobarbituric acid was read at 535nm. The Cohen *et al.*, (1970) method was used to measure the catalase activity. Hydrogen peroxide is broken down by catalase into water and oxygen. At 480 nm, absorbance was measured. Glutathione peroxidase activity was carried out according to the method described by Nyman (1959).

#### **Statistical Analysis**

The values were presented as mean  $\pm$  SEM of the different groups. Differences between the mean values were estimated using one-way analysis of variance (ANOVA) using SPSS

version 23. The results were considered significant statistically at  $p < 0.05$ .

## **RESULTS**

### **Effects of Ethanol Extract of *Annona muricata* (EEAML) on Relative Colon and Kidney Body Weight in 1,2 – dimethylhydrazine Induced Wistar Rats**

The result presented in figure 1. showed that rats treated with EEAML only revealed an insignificant increase in colon body ratio relative to that of control. DMH control group showed an increase in colon body ratio that is significant to that of the control group ( $p < 0.05$ ). Co-treated, pre-treated and post treated groups colon body weight were not significantly affected ( $p > 0.05$ ) when compared with controls. EEAML control and post treated group revealed an insignificant increase in kidney body ratio relative to that of controls ( $p > 0.05$ ). Rats treated with DMH group only showed an increase in kidney body ratio that is significant to that of the control and EEAML group ( $p < 0.05$ ). Co-treated (DMH + EEA group) and pre-treated group differ significantly when compared to DMH control group ( $p < 0.05$ ).

### **Effects of the Extract on Anti-oxidant Enzymes in 1,2 – dimethylhydrazine Induced Rats**

SOD and GPx activity were significantly lower in DMH-treated rats compared to control and EEAML control rats ( $p < 0.05$ ). Co-treated DMH and EEAML group showed significantly lower activity in SOD and GPx in relative to control and EEAML control but a significant increase compared to DMH control. Enzymatic antioxidants activities in pre-treated and post treated groups diverge sharply ( $p < 0.05$ ) relative to control, DMH control and EEAML control ( $p > 0.05$ ).

### **Effects of Ethanol Extract of *Annona muricata* on Anti-oxidant enzymes in 1,2 – dimethylhydrazine Induced Kidney Toxicity in Wistar Rats**

Rats treated with DMH only showed a lower level in SOD, and GPx activities that is significant to that of the control and EEAML control groups ( $p < 0.05$ ).

These enzymatic antioxidants activities in pre-treated rats differ significantly to control, DMH control and EEAML control but the co-treated and post treated were not significantly affected by the treatments. The catalase activity of the different groups shows no statistical difference.

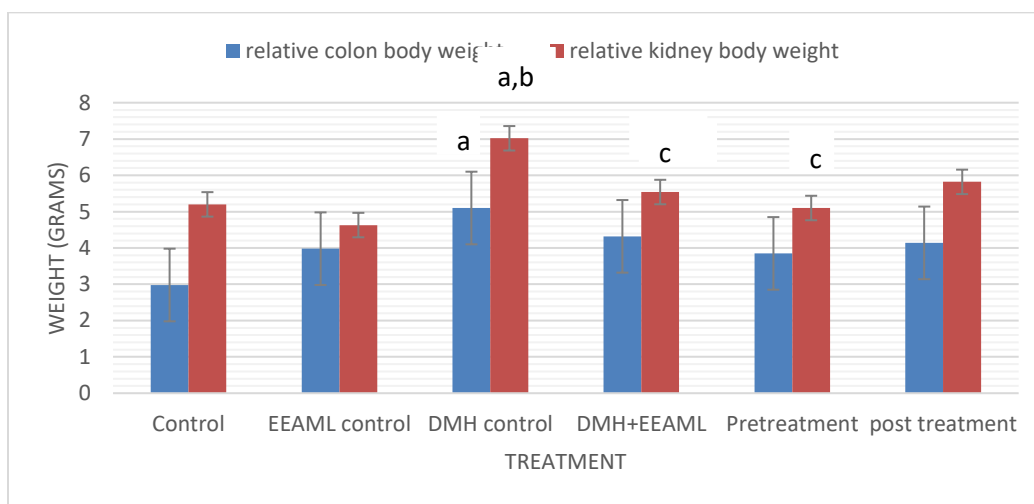


Figure 1: Effect of EEAML on relative colon and kidney body weight in 1,2 – dimethylhydrazine induced rats.

Data were presented as mean  $\pm$  SEM,  $n = 5$ , DMH = 1,2-dimethylhydrazine, EEAML = ethanol extract of *Annona muricata* leaves. Data were statistically significant at  $P < 0.05$ . Values with letter (a) differ significantly from normal control. Values with the letter (b) differ significantly from EEAML control. Values with the letter (c) differ significantly from DMH control.

### Effects of Ethanol Extract of *Annona muricata* on Malondialdehyde Level in 1,2 – dimethylhydrazine Induced Colon Toxicity In Wistar Rats

Result represented below showed that rats treated with DMH have increased MDA level significant to that of the controls group ( $p < 0.05$ ). Co-treated (DMH + EEAML) and pre-treated groups also revealed significant difference in MDA level relative to controls ( $p < 0.05$ ). However, post treated group differ significantly from DMH control, co-treated and pre-treated groups ( $p < 0.05$ ).

### Effects of Ethanol Extract of *Annona muricata* on Total Protein and Malondialdehyde in DMH Induced Kidney Toxicity in Rats.

The bar chart below displays the results, which suggest that rats fed with the leave extract alone did not exhibit a significant reduction in MDA level as compared to the control group. DMH group showed a significant and vast increase in MDA amount compared to that of the control and EEAML groups. Co-treated (DMH + EEA) group revealed a pronounced increase in MDA activity in relative to control and EEAML control but a decrease when compared to DMH control. MDA activity in pre-treated rats differ significantly from

the controls ( $p < 0.05$ ) while post-treated rats differ significantly from DMH control and pre-treated rats.

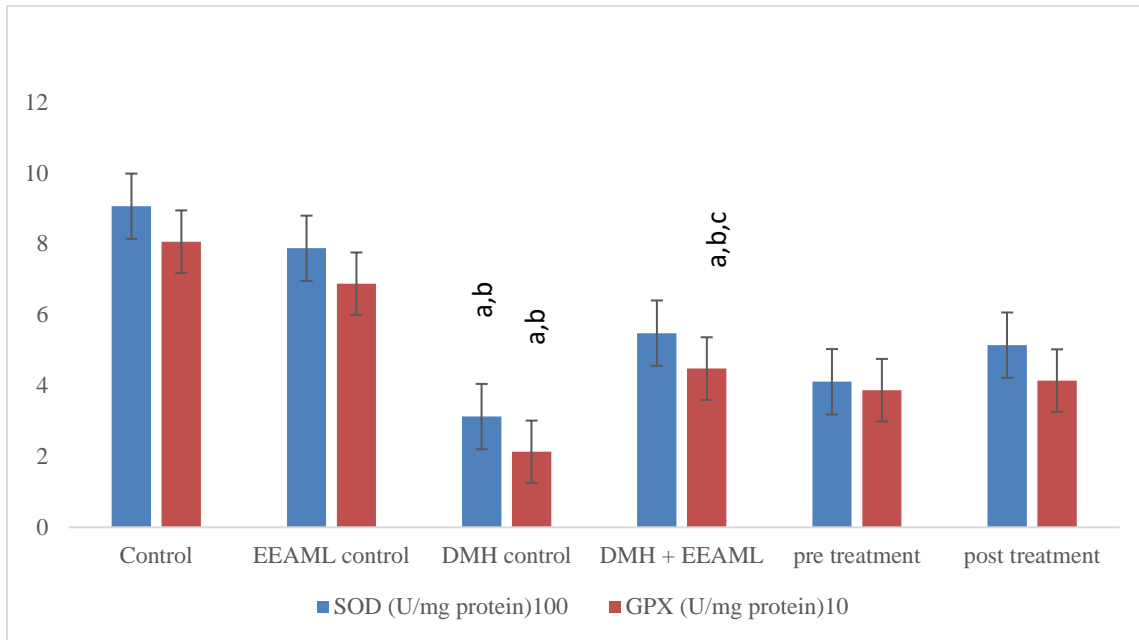


Figure 2: Effect of EEAML on GPx and SOD activities in 1,2 – dimethylhydrazine induced colon toxicity in rats. Values were presented as mean  $\pm$  SEM,  $n = 5$ , DMH =1,2-dimethylhydrazine, EEAML = ethanol extract of *Annona muricata* leaves. Data were statistically significant at  $P < 0.05$ . Lower case letter 'a' denotes a vast difference from normal control. Lower case letter 'b' implies there was a significant change compared to EEAML control. Lower case letter 'c' denotes sharp diverge from DMH control.

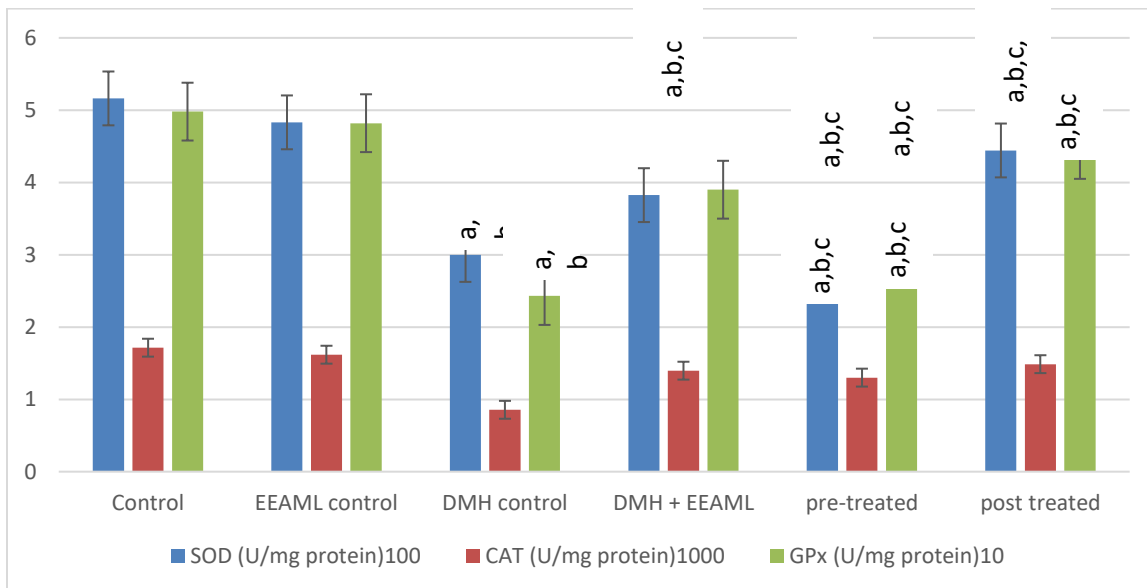


Figure 3: Effects of EEAML on anti-oxidant enzymes in 1,2 – dimethylhydrazine induced kidney toxicity in wistar rats. Values were presented as mean  $\pm$  SEM,  $n = 5$ , DMH =1,2-dimethylhydrazine, EEAML = ethanol extract of *Annona muricata* leaves. At  $P < 0.05$ , the statistics are statistically significant. The values with lower case 'a' are considerably different from the regular control. The values with the lower case 'b' are notably different from the EEAML control. The values with lower case 'c' vary from the DMH control.

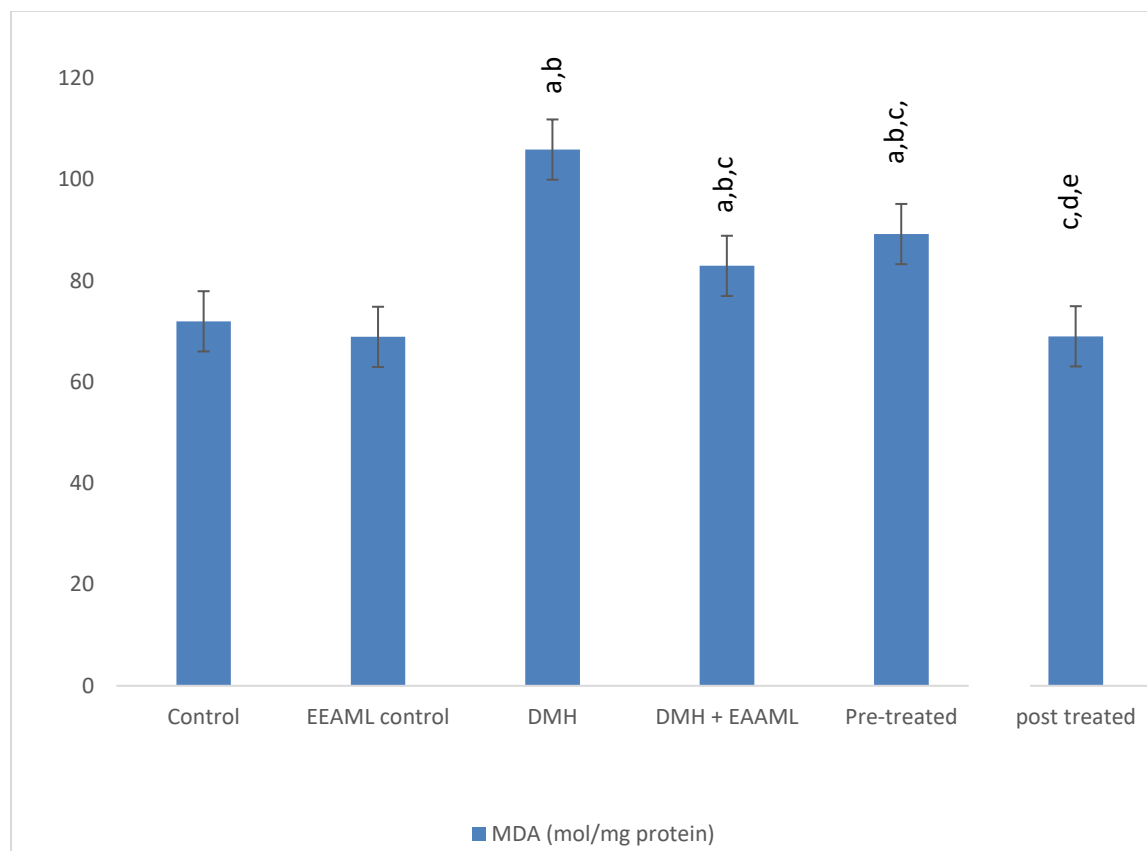


Figure 4: Effects of EEAML on malondialdehyde in 1,2 – dimethylhydrazine induced colon toxicity in wistar rats. Values are provided as mean  $\pm$  SEM, n= 5, DMH =1,2-dimethylhydrazine, EEAML = ethanol extract of *Annona muricata* leaves. At P < 0.05, the data were statistically significant. Values with a lower case "a" denote a significant departure from standard control. It was statically distinct from EEAML control, as indicated by the local case letter "b". Values that begin with the lowercase letter "c" denote large departures from the DMH control. The lowercase letter "d" stands for a significant departure from co-treatment (DMH+ EEAML). A statistically significant divergence from pre-treated is denoted by the lowercase letter "e".

## DISCUSSION

The kidney and colon of the rat's biochemical results and findings unambiguously show that administering the carcinogen DMH along with the ethanol extract of *Annona muricata* leaves (EEAML) causes a significant change in tissue lipid peroxidation and antioxidant state. Cancer tissues are known to have antioxidant and oxidant levels that are distorted and imbalanced (Jebakkan *et al.*, 2009; Manju and Nalini, 2010). The causes of oxidative stress and injury in cells have been linked to changes in cellular redox equilibrium and increased oxygen radical generation, associated with gene mutation, tumour development and promotion of

carcinogenesis (Ray *et al.*, 2010; Salima *et al.*, 2020). The liver is renowned for playing a significant part in metabolism. An electrophile is generated as a result of the metabolic activation of DMH by this organ from its procarcinogen state to its active form, carbonium. Oxidative stress is also caused by this aggressive electrophile (Salima *et al.*, 2020).

MDA, a lipid peroxidation marker, was measured in the kidney and colon tissues of the rats. MDA levels of DMH control were significantly higher when compared to control and EEAML control. The generation of free radicals and their functions in physiological cellular development were discussed in Loizzo and Tundis's (2019) report.

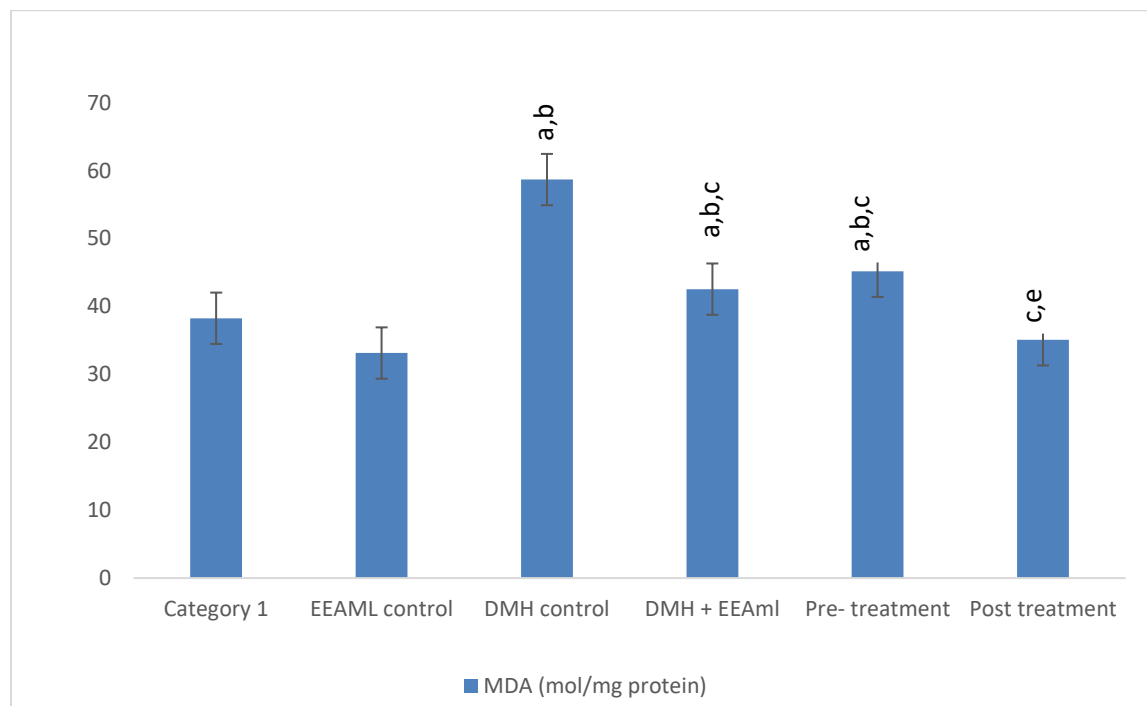


Figure 5: Leaf extract effect on malondialdehyde in 1,2 – dimethylhydrazine induced kidney toxicity in rats. Mean  $\pm$  SEM was the mode of data presentation, n= 5, DMH =1,2-dimethylhydrazine, EEAML = ethanol extract of *Annona muricata* leaves. At  $P < 0.05$ , the data were statistically significant. Values with the lower-case letter "a" were considerably significantly from the normal control ( $p < 0.05$ ). Values with the lower-case letter "b" were considerably different from the EEAML control ( $p < 0.05$ ). Values with the lower-case letter "c" significantly deviated from the DMH control ( $p < 0.05$ ). Values with the lower-case letter "e" deviate much from pre-treated values ( $p < 0.05$ ).

Their reactivity and the harmful and deleterious effects of the overproduction of free radicals on macromolecules have also been reported (Qingling *et al.*, 2020). DMH causes abnormal changes in colon tissues as a result of the active electrophile released by this chemical, which exacerbates lipid peroxidation. MDA is a strong marker of lipid peroxidation; it gives a clear picture of the extent to which a cell has been affected or damaged. High lipid peroxidation in tissues is directly proportional to the extensive damage perpetrated by DMH (Amirhasan *et al.*, 2022). In this study, significantly higher MDA (oxidative stress biomarker) concentration were seen in the DMH group compared to the control groups ( $P < 0.05$ ). Increased cell death and abnormal and excessive colon tissue growth are factors that contribute to the increased level of malondialdehyde. Malondialdehyde's increased level in DMH-induced oxidative stress occurs due to the imbalance between free radicals' production and the ability of the antioxidant

system to remove them before they mutate macromolecules, DNA and RNA proteins. This reactive radical's overproduction overwhelms the cells, resulting in cell damage (Amerizadeh *et al.*, 2018; Lokeshkumar *et al.*, 2015; López-Mejía *et al.*, 2021). Co-treated group (DMH + EEAML) expressed a decreased MDA level as well as the pre-treated and post treated group. This implies the potency of the EEAML to scavenge free radicals and reduce lipid peroxidation. Antioxidants inhibit the oxidation of other molecules, thereby maintaining free radical homeostasis. There are both enzymatic and non-enzymatic antioxidants. GPx, SOD, and catalase are examples of enzymatic antioxidants. Enzymatic antioxidants scavenge free radicals, thus preventing oxidative damage in cells. They are considered to be the first line of cellular defense against oxidative damage. The activity of these enzymes decreases as cancer progresses from stage 0 to stage IV. This reduction leads to less scavenging of free radicals and increased peroxidation of membranes (Nadeem *et al.*,



2021). SOD and CAT activities, like those in other tissues, define the antioxidant defense system in colorectal cancer tissue. SOD converts superoxide radicals into hydrogen peroxide and a molecule of oxygen. Catalase splits the hydrogen peroxide produced as a product of the SOD reaction and other hydrogen peroxides produced by the cells into oxygen and water (Hamiza *et al.*, 2012; Kopetz *et al.*, 2007). Glutathione is a tripeptide that detoxify many environmental carcinogens and protect the cells against free radicals (Jebakkan *et al.*, 2009). The reduced form of glutathione removes hydrogen peroxide in a reaction catalyzed by glutathione peroxidase. This enzyme contains four selenium cofactors that catalyzes the breakdown of hydrogen peroxide and organic hydroperoxides. Carcinogens are electrophilic in nature while GSH are nucleophilic in nature. The reduced form is oxidized due to the acceptance of the electron. Glutathione reductase helps in the regeneration of the reduced form. The three aforementioned enzymes work unanimously to scavenge free radicals. If not scavenged, it could combine with DNA, RNA, or cell proteins and lead to severe cell damage (Ray *et al.*, 2000; Woo *et al.*, 2012).

The enzymatic antioxidants assayed for in this study followed the report of Yu *et al* (2020) that showed that carcinogen administration is accompanied with a sharp drop in the activities of antioxidant enzymes. The decline in the antioxidant enzymes levels of DMH administered rats reflects increased detoxification mechanism exerted by these enzymes and an adaptive mechanism by which tumor cells achieves self-sufficient growth signals, insensitive to anti-growth signals, angiogenesis stimulation and evasion of apoptosis.

EEAML administration to DMH treated rats (post treatment) significantly normalized the SOD, CAT and GPx activities by suppressing the action of reactive oxygen species. This may be due to the free radical scavenging and antioxidant property of EEAML which is supposed to be achieved by its trapping O<sub>2</sub> to form stable radicals of itself. The cotreated group also displayed the ability of the EEAML to shield the effect of the carcinogen. Administration of EEAML alongside with DMH (Group 4), Pre-treatment (Group 5) and post treatment (Group 6) significantly decreased lipid peroxidation and enhanced enzymatic antioxidant concentrations.

This current study shows that ethanol extract of *Annona muricata* leaves have both combined anticarcinogenic and antioxidant activities that inhibited colon carcinogenesis and simultaneously suppress oxidative stress. The efficacy of EEAML on DMH induced colon carcinogenesis inhibition is likely boosted by its antioxidative action and presence of some compounds like acetogenins, flavonoids, tannins, proanthocyanidin, phenols, ferric acid reducing antioxidant potential of EEAML and DPPH radical scavenging activity of EEAML (Olude *et al.*, 2020; Usunobun *et al.*, 2014).

## CONCLUSION

*Annona muricata* has huge pharmacological properties. The enzymatic antioxidants assayed for in this study were reduced in DMH-administered rats but higher in the groups treated with plant extracts. The lipid peroxidation marker was more pronounced in the DMH-treated group but reduced in the groups treated with the plant extract. The results obtained in this study confirmed the antioxidant potential of an ethanol extract of *Annona muricata* leaves and its inhibitory effect on lipid peroxidation in 1,2-dimethylhydrazine-induced colon carcinogenesis. Further investigations are required to study the effect of the extract on colon molecular biomarkers, apoptotic genes, and inflammation.

## Conflict of interest

The authors declare no conflicting interest

## Author contribution

OOM and OFO prepared the materials, collected data, and analyzed the results. OOM wrote the initial draft of the manuscript, while OFO edited it. Both authors conceived and designed the study. The final paper was reviewed and approved by both authors.

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