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# ANNONA MURICATA ETHANOLIC LEAF EXTRACT POTENTIATES CYCLOPHOSPHAMIDE-INDUCED JEJUNAL TOXICITY

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## ABSTRACT

**Objective:** The practice of combination therapy of herbal concoctions with conventional medications is evident in Nigeria, however with numerous undesired effects. *Annona muricata* leaves alcohol-based concoctions have been deployed for the treatment of cancer and some other ailments, sometimes in combination with other cytotoxic drugs such as cyclophosphamide, thus, a heightened interest in understanding their possible synergism or mitigating effects in recent studies. Cyclophosphamide has been deemed cytotoxic and therefore in high concentrations can result in devastating effects on normal tissues. The same can be said for *Annona muricata* leaves, hence the need to establish the relationship between oral intake of ethanolic extract of *Annona muricata* leaves and cyclophosphamide in jejunal toxicity. **Methods:** A total of 24 male Wistar rats weighing between 180-250g were randomly grouped into six (A-F; n=4). Group A served as the normal control and received normal saline treatment only for 14 days. Group B received 50mg/kg BW of cyclophosphamide only on days 1 and 8 by intravenous route. Group C received oral administration of ethanolic extract of *Annona muricata* leaves at a dose of 50mg/kg BW considered to be a low-dose daily for 14 days. Groups D, E, and F were administered a weekly dose of 50mg/kg of cyclophosphamide on days 1 and 8 only via intravenous route, followed by daily oral administration of ethanolic extract of *Annona muricata* leaves for 14 days at 50mg/kg BW, 100mg/kg BW, and 150mg/kg BW respectively. **Results:** Malondialdehyde and Superoxide Dismutase levels were measured to determine the extent of oxidative stress in each group. Groups B and C demonstrated significant *($p<0.05$)* differences compared to the normal control in the malondialdehyde analysis. The level of significance *($p<0.05$)* in the reduced levels of superoxide dismutase seen in groups D-F compared to the rest of the groups was remarkable. This corresponded to the histological lesions characterized by severe depletion of the apical villi in groups E and F, suggesting ulcerative enteritis of the jejunum. **Conclusion:** Co-administration of *Annona muricata* leaf extract and cyclophosphamide should be discouraged as a means to prevent ulcerative enteritis during cancer therapies.

**KEYWORDS:** *Annona muricata* leaves, Cyclophosphamide, Oxidative stress, Jejunal toxicity.

## 1. INTRODUCTION

Cyclophosphamide is an alkylating agent deployed as a cytotoxic and immunosuppressive agent due to its potential to inhibit protein synthesis.[1,2] In high doses, cyclophosphamide is considered one of the many anticancer agents used in the eradication therapy of different kinds of cancers.[3] However, in lower dosages, it could serve as a selective immunomodulatory agent of T cells thereby promoting its use in the management of post-transplant alloreactivity, autoimmune disorders, and vasculitis.[4-6] Cyclophosphamide is known to have some undesired effects,[2] interactions with other drugs, and toxicities[1,3,6] to the individuals on its therapy, therefore care should be taken when used in combination with other medications.

In Nigeria, trado-medical practices currently involve an astonishing range of 43 to 88% percentage of herbal concoctions co-administered with conventional medications.[7-9] This has incited research interest in understanding the mechanism of action, interactions, undesired effects, and the effectiveness of some of these concoctions. Some of the most common herbs used to prepare these concoctions include Soursop leaves (*Annona muricata* Lin.) which comprises more than 2300 species.[10] The plant itself grows in the tropics and subtropics, produces an edible fruit–

soursop, and has been attributed to various pharmacological activities.[10] Almost every part of the plant is said to be medicinal which includes its seeds combating parasitic infections, the fruit combating musculoskeletal disorders, and its leaves combating cystitis, headaches, and cancer.[11] The main active components of the leaves of *Annona muricata* include secondary metabolites such as phytosterols, saponins, tannins, terpenoids, alkaloids, flavonoids, coumarins, lactones, glycosides, and anthraquinones.[12,13] Some of the bioactive compounds have been shown to have varying degrees of pharmacologic and toxic effects on the body.[14] However, the leaves of *Annona muricata* are claimed to have gastroprotective activities and thus prevent gastric ulceration by way of protecting against reactive oxygen species (ROS) scavenging effects and gastric wall damage through upregulating Superoxide Dismutase (SOD) expression while downregulating Malondialdehyde (MDA) expression, amongst other biomarkers. Therefore, it can serve both functions as a cytotoxic or an antioxidative agent when deployed in the management of cancers.[14] However, nothing concrete has been studied concerning its protective or delirious effect on the small bowel.

Various kinds of toxicity have been associated with cyclophosphamide use, some of which include hemorrhagic cystitis, immunosuppression, hepatotoxicity, and gastrointestinal toxicity. Gastrointestinal toxicity is characterized by nausea, vomiting, abdominal discomfort or pain, and diarrhea. Ulceration of the oral mucosa and hemorrhagic colitis has also been reported occasionally.[15] The effect of the combination of cyclophosphamide and *Annona muricata* in the gastrointestinal system is yet to be reported although *Annona muricata* mitigating effects have been reported on cyclophosphamide-induced oxidative stress.[16,17]

The aim is to establish the relationship between oral intake of *Annona muricata* ethanolic leaf extract and cyclophosphamide-induced jejunal toxicity. Bioactive agents can alter the metabolism of cyclophosphamide by the hepatic microsomal enzymes and may lead to increased toxicity of the drug when co-administered as inferred by Conney.[18] It also seeks to discover any histomorphological changes in the jejunum of animals on combination therapy of cyclophosphamide and *Annona muricata*.

## 2. MATERIALS AND METHODS

### 2.1 Experimental Animals

A total number of 24 adult male Wistar rats weighing between 180 and 250 grams, and of similar age were obtained and housed in conventional wired mesh cross-ventilated cages in the animal house of the Department of Anatomy, University of Nigeria Enugu Campus. The conditions of the facility were optimal with 12-hour light/dark cycles. The animals were grouped into six (Table 1) and acclimated for two weeks before initiating the experimental manipulations while allowing them unrestricted access to food and clean water. The standard operating procedures and guidelines (SOPGs) of Animal Care and Use were followed after gaining ethical clearance for the study from the Ethics and Research Committee of the Faculty of Basic Medical Sciences, Enugu State University of Science and Technology with ethical right permission number: ESUCOM/ FBMS/ETR/2022/051.

### 2.2 Preparation of Plant Material and Extract

*Annona muricata* leaves were obtained from a farm settlement in Thinker's Corner, Emene, Enugu-East local government area in Enugu State, Nigeria between June 2022 and September 2022. They were authenticated by the Department of Plant Science and Biotechnology, University of Nigeria before the leaves were air-dried at room temperature for two months and then pulverized into a fine powder with the use of a mechanical grinder. About 3000 grams of the leaf powder was cold-macerated and extracted in 3 liters of absolute alcohol at room temperature for over 3 weeks.[19] The mixture was filtered using a mesh cloth and a grade 4 Whatman's filter paper and then left open in a jar to evaporate the ethanol in the extract.[20] A paste of approximately 2100 grams was yielded and stored in a sample bottle at -4°c until ready for use. Phytochemical analysis of the extract was done and it demonstrated positive tests for phytates, phenols, terpenoids, flavonoids, saponins, and tannins. The protocol outlined by Fofana *et al.*[21] was used for the ethanolic extraction of *Annona muricata* leaves.

### 2.3 Drugs

Cyclophosphamide (500mg) was purchased from the Alpha pharmaceutical store in Enugu metropolis, Nigeria. Batch Number: BUX 1061 with manufacturing date as 05/2021 and expiry date as 04/2023. Manufacturing license number: 27/MDCHL/TS/2017/F/G(L) by Cadila Healthcare Limited.

### 2.4 Experimental design

**Table 1: Experimental animal grouping and experimental design.**

| | Groups | Treatment |
|---|---|---|
| A | Normal Control | 0.2mls of Normal Saline orally for 14 days. |
| B | CP only | Intravenous cyclophosphamide at a weekly dose of 40mg/kg BW for 14 days. (Days 1 and 8) |
| C | Low dose AM only | Oral AM at a daily dose of 50mg/kg BW for 21 days. |

| | | |
|---|---|---|
| D | Low dose AM + CP | Oral AM at a daily dose of 50mg/kg BW and intravenous cyclophosphamide at a weekly dose of 40mg/kg BW for 14 days. |
| E | Medium dose AM + CP | Oral AM at a daily dose of 100mg/kg BW and intravenous cyclophosphamide at a weekly dose of 40mg/kg BW for 14 days. |
| F | High dose AM + CP | Oral AM at a daily dose of 150mg/kg BW and Intravenous cyclophosphamide at a weekly dose of 40mg/kg BW for 14 days. |

### 2.5 Biochemical analysis

#### 2.5.1 Malondialdehyde (MDA) Analysis

According to methods used by Bevan *et al*,[22] the index of lipid peroxidation was measured in nmol/ml spectrophotometrically at 540nm wavelength. At the expense of NADPH, glutathione reductase regenerates glutathione from glutathione disulfide in an enzymatic cycle. This reduces the concentration of NADPH while maintaining the concentration of GSH through the breakdown of hydrogen peroxide which converts reduced glutathione to oxidized glutathione/glutathione disulfide thereby maintaining the enzymatic cycle. The enzymatic activity is termed as nmol NADPH/g protein/min.

#### 2.5.2 Superoxide Dismutase (SOD) analysis

The technique by Marklund & Marklund[23] was used in the analysis of superoxide dismutase (SOD). It is based on the quantity of the enzyme (2.8ml Tris-EDTA) that reduces the rate of pyrogallol auto-oxidation by 50% in units/mg protein/min while measuring the absorbance at 420nm wavelength for 3 minutes using a spectrophotometer. This is termed as one unit of SOD activity.

### 2.6 Histopathological analysis

On Day 22, all experimental animals were sacrificed under proper anesthesia by inhalation of chloroform. Careful dissection of the abdomen was done with the small intestine rapidly harvested and fixed in 10% formal saline within 3 minutes according to the GUT bundling technique. The jejunum represents the major part of the small intestine in rodents[24] and it was trimmed into bundles of 1cm in length, stacked on top of each other, and tightened together with a 3M Micropore tape loop, after flushing the small intestine with ice-cold phosphate-buffered saline under pressure. These bundles were placed into tissue cassettes and labeled indelibly for each group. The cassettes were transferred into a tissue processor for standard tissue processing and sectioning at 4µm thickness for hematoxylin and eosin (H&E) staining.[25]

### 2.7 Statistical analysis

All quantitative data obtained were presented as mean and standard deviation (Mean ± SD). The obtained quantitative data were analyzed using SPSS Version 23 (IBM Corp., Armonk, NY, USA) software, using one-way ANOVA followed by Tukey's comparison test. Significance was set at $P < 0.05$. The results were demonstrated in tables.

## 3. RESULTS AND DISCUSSION

### 3.1 Biochemical analysis result

**Table 2: Result of the Mean ± Standard Deviation of MDA and SOD**

| Groups | MDA (µmol NADPH/g protein) | SOD (units/mg protein/min) |
|---|---|---|
| A | 106.04±3.19 | 22.07±0.36 [a] |
| B | 147.91±1.11[a] | 28.92±0.96 |
| C | 139.28±1.28 [a] | 21.94±0.32 [a] |
| D | 208.46±2.33 [c] | 10.67±1.21[b] |
| E | 211.25±1.14 [b c] | 9.10±1.48 [c] |
| F | 219.81±2.12 [b c] | 7.56±0.91 [c] |

Values were expressed as Mean ± SD; [a]$P<0.05$ showed a significant difference in groups B and C compared to other groups for MDA while in SOD similar result was seen in groups A-C when compared to the combination therapy groups (D-F)**;** [b]$P<0.05$ showed a significant difference compared to groups A and B. [c]$P<0.05$ showed a significant difference in groups E and F when compared to the normal control group, and included group D in the MDA analysis.

*MDA=MALONDIALDEHYDE

*SOD = SUPEROXIDE DISMUTASE

### *3.2* Histomorphology of jejunum of experimental animals

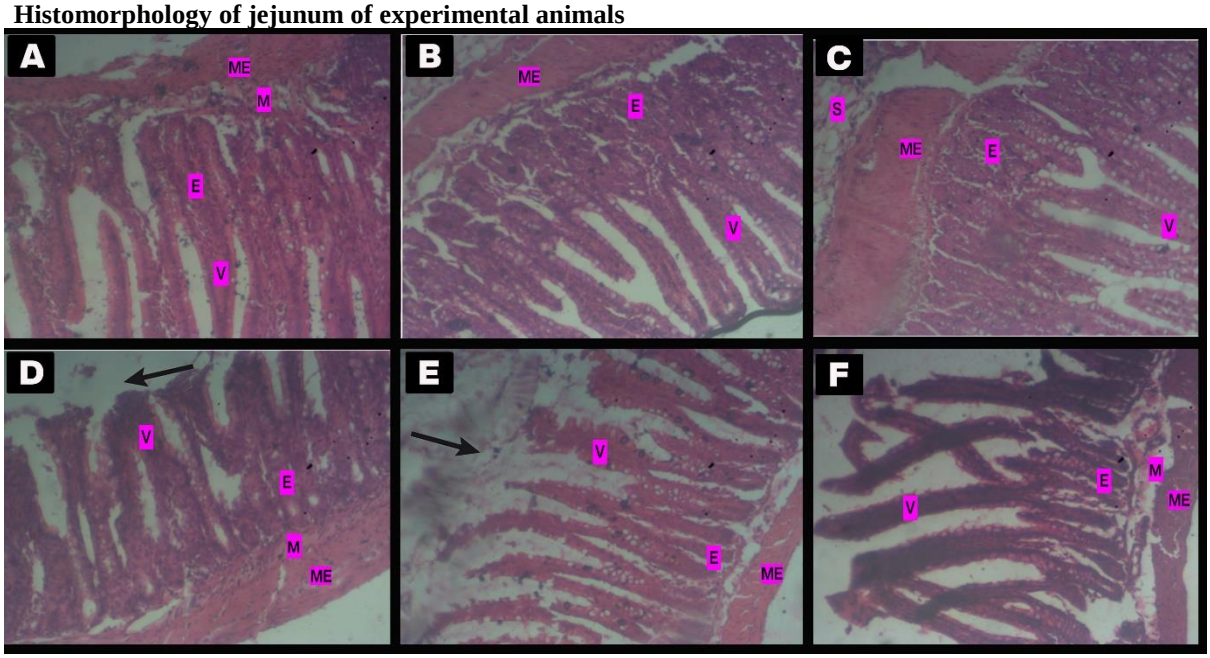


**Figure 1: Photomicrographs of the jejunum (A-F) demonstrating the various groups.** A (Normal control) shows several intestinal villi (V) in the epithelium (E) with a thin muscularis mucosae (M) and the muscularis externa (ME). The general architecture appears normal. B which received cyclophosphamide only also shows a normal distribution of several intestinal villi (V) in the epithelium (E) with a thin muscularis mucosae (M) and the muscularis externa (ME). The serous layer (S) was also demonstrated on photomicrograph B. Group 3 as seen in photomicrograph C which received a high dose of Annona muricata leaf extract only demonstrated several intestinal villi (V) in the epithelium (E) with a thin muscularis mucosae. The muscularis externa (ME) is elaborately demonstrated. The combination of cyclophosphamide and ethanolic extract of Annona muricata demonstrated remarkable changes in the epithelium of the jejunum as seen in photomicrographs D, E, and F. There is mild apical villous atrophy (V) in photomicrograph D as shown by the black arrow. Photomicrograph E shows moderate basal villous atrophy but severe apical villous atrophy. In photomicrograph F, there is marked severe apical atrophy of the villous with loss of basal villi within the epithelium (E) due to atrophy of muscularis mucosae (M) **[H&E x 200].**

### 3.3 DISCUSSION

The use of herbal concoctions in managing a variety of ailments and maintaining good health is common in Nigeria. This could be attributed to poverty, ignorance, and the desperation to get well. Also, a good number of recent studies have proven the efficacy of herbal medication, triggering a heightened interest in scientific research.[26,27] *Annona muricata* appears to be one of these compelling candidates to study due to its numerous phytochemical and antioxidative properties, and also due to its routine usage in the preparation of herbal concoctions for the traditional management of arthritis, diarrhea, liver, and heart ailments, and cancer.[28] Therefore, the implication of such practices is a major concern when considering the interaction with cyclophosphamide.

According to Yang *et al*,[29] cyclophosphamide can cause gastrointestinal toxicity such as hemorrhagic ulcerations of the small bowel amongst other undesired effects seen in cyclophosphamide administration. It is worth studying, the effects *Annona muricata* has on cyclophosphamide presumed toxicity in the gastrointestinal tract.

The malondialdehyde (MDA) level and superoxide dismutase (SOD) activity of each jejunal sample were evaluated. Analysis of lipid peroxidation assesses the oxidative degradation of lipids which predisposes the cellular membranes to discontinuity.[22] The generation of MDA in oxidative stress is a result of the oxidizing action of free radicals on unsaturated fatty acids with more than one double bond located within the cellular lipids.[30] The presence of MDA in elevated levels is related to oxidative stress due to the accumulation of free radicals,[22] hence its use as a biomarker for the assessment of lipid peroxidation. The MDA analysis in this study revealed the absence of lipid peroxidation in the normal control group. However, MDA levels in groups B and C were slightly elevated and were significant *($p<0.05$)* when compared to the normal control. This suggests oxidative activities of the free radicals on lipid peroxidation were present in the single therapy of cyclophosphamide or *Annona muricata*. Groups D-F demonstrated further increase and significantly *($p<0.05$)* elevated levels of MDA which is in agreement with reported MDA elevations during *Annona muricata* administration in the management of oxidative stress.[16,17]

SOD is charged with the conversion of harmful superoxides to hydrogen peroxides, which are then converted to water molecules by catalase. Levels of SOD are drastically reduced in disease conditions such as ulceration. In this study, the levels of SOD in the groups D, E, and F were also observed to be significantly *($p<0.05$)* reduced in a dose-dependent manner. The combination of 40mg/kg BW of cyclophosphamide and *Annona muricata* suggests a synergistic activity in jejunal toxicity induction which worsened with an increase in the dosage of *Annona muricata*. This suggests the overutilization of SOD in the breakdown of superoxide anions generated by lipid peroxidation.[23] However, there was no significant reduction in the levels of SOD in the groups treated with cyclophosphamide alone and those treated with *Annona muricata* alone (Table 2). This suggests that lone therapy of cyclophosphamide or *Annona muricata* may not predispose the gastrointestinal mucosa to ulcerations that may eventually lead to enteritis. However, this does not agree with Yang *et al.*[29] which claims severe multifocal ulcerative enterocolitis could affect the small bowel and colon during treatment with cyclophosphamide.

The photomicrographs in Figure 1 of the jejunum in A-C which represented groups A to C demonstrated normal cytoarchitecture of intestinal villi in the epithelium, muscularis mucosae, and muscularis externa. This suggests that both cyclophosphamide and *Annona muricata* when used separately as a single therapy do not lead to jejunal toxicity unlike seen in the combination therapy groups (D-F). This does not agree with the report from Marcellinus *et al.* which suggests that combination therapy involving cyclophosphamide and *Annona muricata* restores destroyed epithelium and prevents ulcerations due to mitigation of oxidative stress. Additionally, the extent of damage to the epithelium and intestinal villi observed in groups D and E suggests that the ulceration is dose-dependent on *Annona muricata*. This buttresses the toxicity of *Annona muricata* which is a result of the potentiating effect of cyclophosphamide perhaps enhancing its cytotoxic properties.[14]

## 4 CONCLUSION

The study discovered that oral administration of ethanolic extract of *Annona muricata* demonstrated a potentiating effect towards cyclophosphamide-induced jejunal toxicity in male Wistar rats. Therefore, oral administration of *Annona muricata* leaf extract should be avoided in individuals on cyclophosphamide treatment. Single therapy may not predispose to ulcerative enteritis as demonstrated in the study.

## ACKNOWLEDGMENTS

Not applicable.

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