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PROTECTIVE EFFECT OF ETHANOLIC LEAF EXTRACT OF *ANNONA MURICATA* LINN ON SOME EARLY EVENTS IN CYCAS-INDUCED COLORECTAL CARCINOGENESIS IN RATS

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ABSTRACT
The study was carried out to investigate the effect of ethanolic leaf extract of *Annona muricata*, ELAM on some early changes in cycas-induced colorectal carcinogenesis in rats. 7 groups of male Wistar rats (10 rats /group) were used. Groups were fed either mash alone or mash mixed with 5 % (w/w) cycas leaf powder. ELAM (100 mg/kg body weight) was administered before, simultaneously with, or after exposure to 5 % (w/w) cycas, for 30 days. After sacrifice, colon sections were dissected out for histological and some biochemical analysis. Results showed that cycas alone caused significant decreases in colon total protein ($p < 0.05$), but administration of cycas with ELAM (irrespective of which came first) led to reversal of these decreases, with colon protein restored to levels higher than values for controls. Cycas-induced increases in LDH were significantly ($p < 0.05$) reduced by ELAM. Colon SOD, catalase and GPx activities were significantly decreased in the group fed cycas, while these enzymes were significantly increased above control values in the groups fed both cycas and ELAM ($p < 0.05$). In addition, the cycas-treated group had significant elevation of colon MDA, but MDA levels were significantly lowered in the groups fed cycas and ELAM. Histology results revealed early cancer-related morphological changes in the cycas group, including mucosal erosion, oedema and glandular hyperplasia. These lesions were absent in the groups given ELAM and cycas, irrespective of the duration of ELAM exposure. These results demonstrate evidence for protective role of ELAM in early biochemical events associated with colorectal carcinogenesis in rats.
Keywords: Cycas, Colorectal Cancer, *Annona muricata,* Protection.

INTRODUCTION

Cancer is recognized as a leading cause of mortality in the world[1]. The disease affects practically all tissues, but the most prominent forms include breast, prostate, liver and colon (colorectal) cancers. These neoplasms constitute over 50 % of all newly-diagnosed cancers in the United Kingdom[2]. Colorectal cancer is the most prevalent malignancy in the US, ranking third behind prostate and lung cancer in men, and behind breast and lung cancer in women[3]. Studies have shown that colon cancer accounts for 10-50 % of gastro-intestinal malignancies in Nigeria[4-6]. Colon cancer, which begins as benign polyps, has been associated with several pre-disposing factors including age and diet[7]; inactivity and obesity[8]; as well as sedentary life style[9]. Conventional treatments for colorectal cancer are the same as in other cancers and include surgery, chemotherapy and radiotherapy. However the toxicity and side effects commonly associated with the chemotherapy and radiotherapy, which target all dividing cells, have tended to decrease survival rates of cancer patients[10]. This has led to increasing attention on alternative remedies with more precision and minimum side effects. Herbal remedies have been used for centuries as alternative to chemotherapy and radiotherapy[11]. Indeed the search for plant-based cancer treatment strategies has continued to engage the attention of researchers[12]. Many effective anti-neoplastic agents have been isolated from plants[13]. Intensive research has focused on the cancer-killing properties of the leaves and stem of *A. muricata* since their anti-carcinogenic properties became known[14]. The anti-neoplastic agents in *Annona muricata* have been identified as acetogenins, a group of polyketide-derived fatty acids present in plants of the family *Annonaceae*[15,16]. Researchers at Purdue University, Indiana, have reported that acetogenins selectively suppress proliferation of cancer cells, especially those resistant to the most potent chemotherapy drug adriamycin, without apparent toxicity to normal cells[10]. Acetogenins exert their selective anti-neoplastic effects by inhibiting complex 1 of the mitochondrial respiratory chain, thereby blocking ATP supply to the cancer cells[17]. Several *in vitro* and *in vivo* studies have consistently demonstrated strong anti-carcinogenic effects of acetogenins on various cancers in experimental animals and cancer cell lines[18-22]. However these studies were based on pure acetogenins which may not be readily available and affordable as alternative cancer remedies, especially for the vast majority of poor dwellers in rural settings in the tropics, for whom the leaves and stem of *Annona muricata* offer much cheaper and more readily-available options. For these people, results from studies based on the crude plant extract would be of more practical significance. The present study is aimed at assessing the protective effect of ethanolic leaf extract of *Annona muricata* against some early events in cycas-induced colorectal cancer in rats.

MATERIALS AND METHODS

Preparation of ethanolic leaf extract of *Annona muricata* (ELAM)

A large quantity of fresh, green leaves of *Annona muricata* was collected from trees in house-hold gardens in Benin City, Edo State, Nigeria. The plant was identified by Dr Bamidele of Department of Plant Biology and Biotechnology, University of Benin and authenticated by Professor Idu of the same Department. A voucher specimen, number UBHa 0205 was deposited at the Herbarium of Department of Plant

Biology and Biotechnology, University of Benin. The leaves were rinsed in tap water and dried at room temperature (about 25°C) for about 4 weeks. Thereafter the leaves were ground to fine powder in an electric blender. For extraction, 402 g of the powder was soaked in 2.6 liters of absolute ethanol (99 % w/v) for 72 hours with regular stirring. The extract was filtered through sintered funnel and Whatman No.1 filter paper and the filtrate was concentrated *in vacuo* in a rotary evaporator. The resultant dense, greenish emulsion was dried in a beaker under ambient air to yield 16.3 g of powdery extract. This was kept refrigerated in a clean plastic jar prior to use.

Collection and preparation of cycas (*Cycas circinalis)* leaf powder

Fresh cycas leaves were collected from the Ugbowo Campus of University of Benin. The leaves were dried at room temperature (about 25°C), and subsequently ground to powder. The powder was kept in air-tight glass jar until used. Strict safety procedures were adopted during the grinding and subsequent handling of the cycas powder, by regular use of nasal masks and thorough washing of hands afterwards.

Experimental Animals, grouping and treatment

70 male Wistar rats (mean weight =199 g) were randomly assigned to 7 groups of 10 rats each and members of each group housed separately in clean, disinfected cages in a room with a 12-hour light/dark cycle. The rats were acclimatized to growers mash (Bendel Feed and Flour Mills Ltd, Ewu, Nigeria) for 2 weeks prior to commencement of the experiment. Each group received either pure mash, or mash with 5 % (w/w) cycas with or without *Annona muricata* (100 mg/kg body weight) given either prior to or post cycas exposure. All groups were fed their respective diets for 4 weeks. The groupings and treatments are summarized in Table 1.

Table1: Animal groups and their treatments

Group	Treatment
I (Control)	Mash
II	Mash + 5 % (w/w) cycas
III	Mash + 5 % (w/w) cycas for 3 weeks, then 100 mg ELAM/kg body wt. for 1 week
IV	Mash + 5 % (w/w) cycas for 1 week, then 100 mg ELAM/kg body wt. for 3 weeks
V	Mash + 100 mg ELAM/kg body wt. for 1 week, then 5 % (w/w) cycas for 3 weeks
VI	Mash + 100 mg ELAM/kg body wt for 4 weeks
VII	Mash + 5 % (w/w) cycas + 100 mg ELAM/kg body wt for 4 weeks

The study was carried out in strict compliance with the ethics in Guidelines and Specifications on Experimental Animal Care[23]. All groups had unrestricted access to clean drinking water. The experiment was conducted at an average room temperature of 25°C and 12-hour day light cycle. After 30 days, the rats were sacrificed by cervical dislocation and sections of the colon were dissected out, rinsed severally in saline and used for preparation of tissue homogenates for some biochemical assays. Sections for histology were immediately fixed in 10 % formol-saline prior to processing. The tissue homogenate was prepared by grinding 0.5 g of colon in a mortar with acid-washed sand and 5 ml of normal saline for 10 minutes at room temperature (25°C). The homogenate was centrifuged at 10,000 g for 5 minutes and the resultant supernatant was used for the biochemical assays.

Biochemical Assays

LDH was assayed spectrophotometrically using Randox kits, by monitoring NAD^+ reduction at 340 nm. SOD was assayed by following auto-oxidation of adrenaline at 420 nm[24]. Catalase assay was as described by Cohen *et al*[25], in which the rate of decomposition of hydrogen peroxide is monitored at 480 nm. GPx activity was determined by measuring the production of purpurogallen from pyrogallol at 420 nm[26]. Total protein was estimated colorimetrically using Randox Biuret test kits. MDA levels were measured in a colorimetric reaction with thiobarbituric acid as described by Varshney and Kale[27].

Histology

Colon sections fixed in formol-saline were processed for light microscopy at the Department of Anatomy, Faculty of Basic Medical Sciences, College of Medicine, University of Benin. The slides were read and interpreted by one of us, G.I.E, a Consultant Pathologist.

Statistics

Results from biochemical assays were expressed as Mean ± S.E.M. Data were analyzed by paired sample Student's t-test using SPSS package (version 15). Values of $p < 0.05$ were taken as significant.

RESULTS

Results for colon total protein and LDH activities for rats in the various groups are shown on Table 2. Group II given cycas alone had significantly higher colon LDH activity ($p<0.01$) and significantly lower colon total protein ($p< 0.05$) than in any other group. 1 week treatment with ELAM following prior exposure of the rats to cycas for three weeks significantly reduced colon LDH below control levels and increased total protein significantly above control values ($p<0.05$). A similar pattern was seen in rats given cycas for 1 week before exposure to ELAM for 3 weeks and also in those exposed simultaneously to both cycas and ELAM.

Table 2: Changes in colon LDH (U/100g fresh weight) and total protein (mg/g fresh weight) of rats in the various groups

Groups	LDH	Total protein
I	12.30 ± 1.80^{a}	0.443 ± 0.01^{a}
II	94.81 ± 10.20^{b}	0.416 ± 0.10^{b}
III	18.83 ± 3.10^{ac}	0.433 ± 0.10^{ab}
IV	21.69 ± 0.60^{c}	0.535 ± 0.20^{c}
V	4.92 ± 0.01^{d}	0.544 ± 0.10^{c}
VI	4.43 ± 1.40^{de}	0.551 ± 0.01^{cd}
VII	$3.44 \pm 0.80d^{ef}$	0.515 ± 0.12^{ce}

Values are Mean ± S.E.M. (n=10). Values having different superscripts between groups differ significantly ($p< 0.05$).

Table 3: Colon MDA levels, and activities of SOD, Catalase and GPx of rats in the various groups

Group	MDA×10^{-3} (mmoles/g fresh weight)	SOD (U/g fresh weight)	Catalase (U/g fresh weight)	GPx (U/mg fresh weight)
I	14.73 ± 0.30^{a}	2.11 ± 0.010^{a}	321.00 ± 6.00^{a}	56.90 ± 4.00^{a}
II	74.22 ± 0.30^{b}	1.71 ± 0.003^{b}	256.00 ± 6.00^{b}	37.60 ± 2.00^{b}
III	53.54 ± 0.90^{c}	3.13 ± 0.030^{c}	363.00 ± 7.00^{c}	59.50 ± 7.00^{a}
IV	33.26 ± 1.70^{d}	3.14 ± 0.004^{cd}	350.00 ± 9.00^{acf}	184.00 ± 2.00^{c}
V	48.16 ± 1.40^{e}	3.00 ± 0.001^{e}	341.00 ± 10.00^{acfg}	131.00 ± 2.00^{d}
VI	48.99 ± 0.60^{ef}	3.14 ± 0.040^{cdf}	367.00 ± 4.00^{acfh}	119.00 ± 7.00^{de}
VII	40.12 ± 0.22^{g}	3.00 ± 0.001^{eg}	363.00 ± 10.00^{acfgh}	85.00 ± 4.00^{ef}

Values are represented as Mean ±S .E.M. (n=10). For each parameter, values having different superscripts between groups differ significantly ($p < 0.05$).

Figures 1 to 7 are photomicrographs of colon sections taken from rats in most of the groups investigated, depicting either pathological changes or normal colon architecture, as indicated.

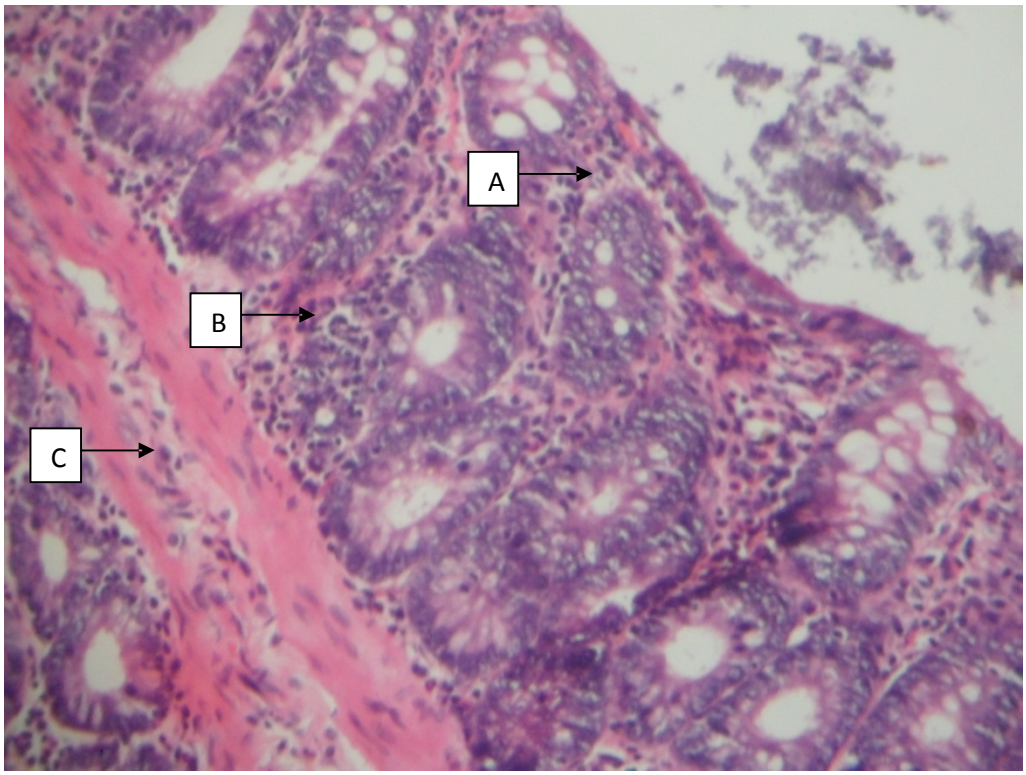

Figure 1: Rat colon from control group showing normal mucosal lining A, glands B and muscularis mucosa C (H and E; x 10)

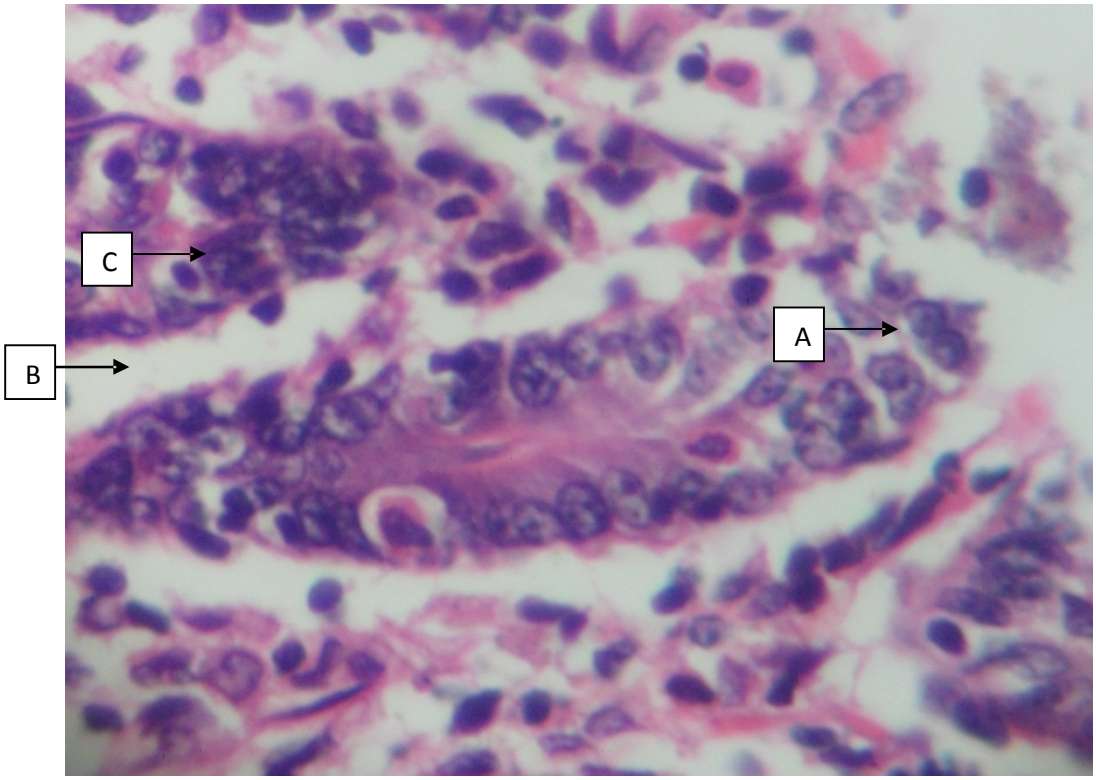

Figure 2 : Colon section of rats treated with cycas for 4 weeks, showing mucosal erosion (A); oedema (B) and glandular hyperplasia (C). (H and E; x 10)

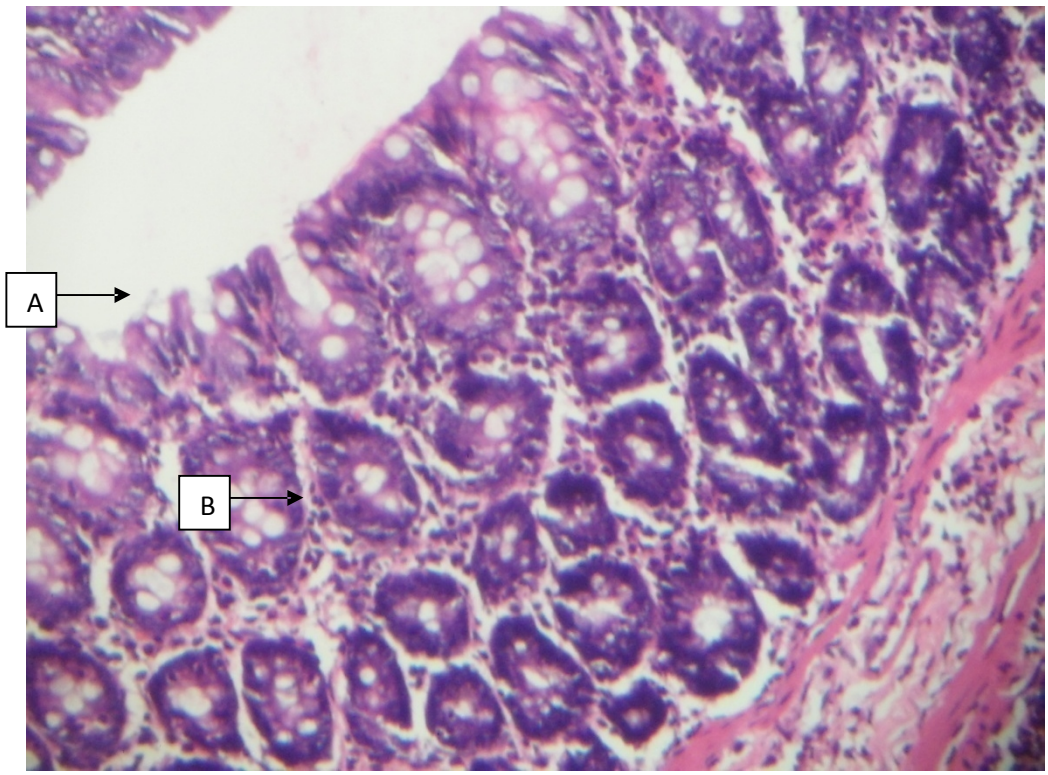

Figure 3: Rat colon treated with cycas for 3 weeks and *Annona muricata* for 1 week showing reduced mucosal damage A and reduced glandular abnormality B (H and E; x 10)

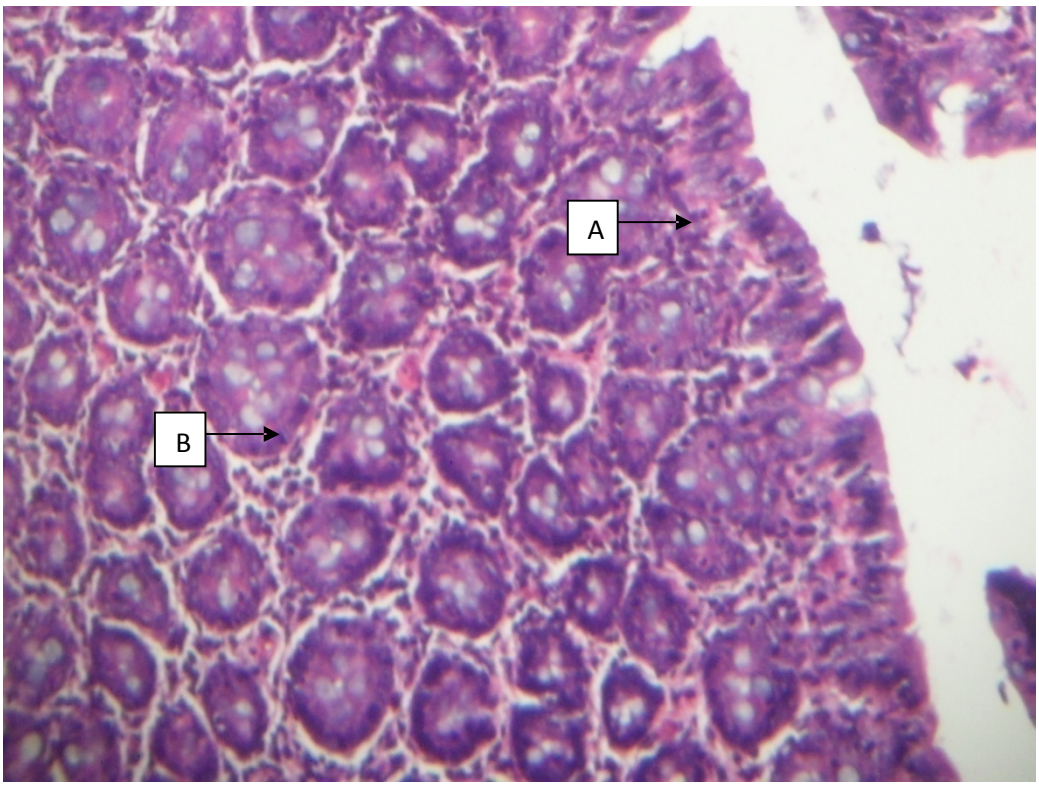

Figure 4: Rat colon treated with cycas for 1 week and *Annona muricata* for 3 weeks showing marked reduction in the mucosal damage A and in the glandular abnormality B (H and E; x 10)

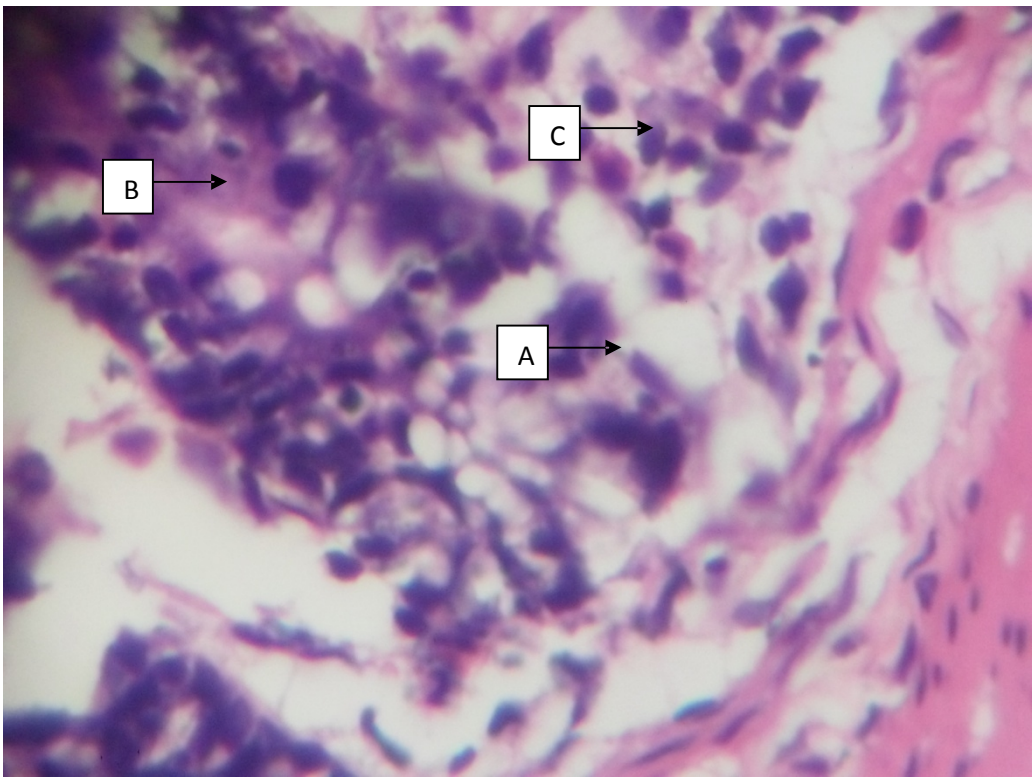

Figure 5: Colon section from rats treated with *Annona muricata* for 1 week followed by cycas for 3 weeks. Mucosal oedema A, abnormal mitosis B and mild infiltrates of chronic inflammatory cells C are shown (H and E; x 40)

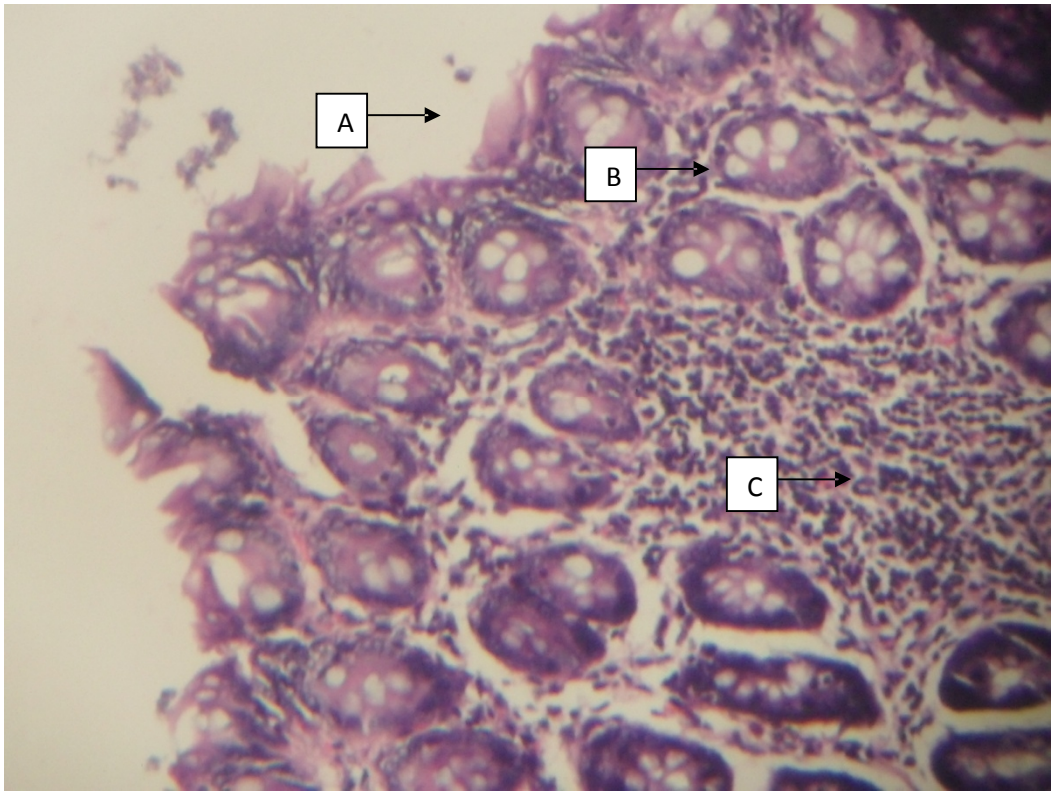

Figure 6: Section of colon from rats treated with *Annona muricata* for 4 weeks showing fairly unremarkable mucosal lining A, glands B and mildly activated lymphoid aggregates C (H and E; x 10)

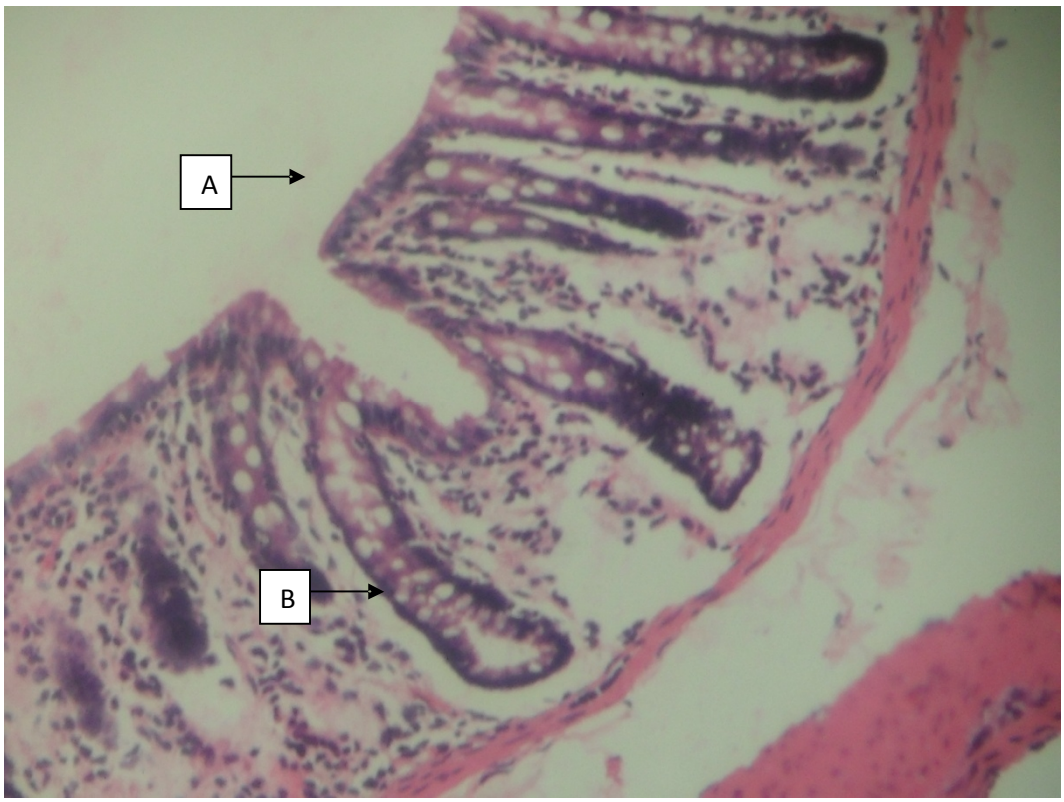

Figure 7: Colon section of rats treated simultaneously with *Annona muricata* and cycas for 4 weeks. Fairly unremarkable mucosal lining A and glands B are seen (H and E; x 10)

DISCUSSION

Extracts of medicinal plants have been employed for centuries in the treatment of a variety of diseases. Indeed cancer is one of the diseases for which intensive research towards a plant-based treatment is on-going[12]. The quest for herbal remedy for cancer is informed by the toxic side effects, lack of specificity and resistance associated with conventional chemotherapeutic strategies[10]. In the present study, the abnormal changes seen in the colon photomicrographs of the rats given cycas alone (i.e. mucosal erosion, oedema and glandular hyperplasia) are consistent with early events in cycas-induced carcinogenesis. Cycas

(*Cycas circinalis*) contains cycasin and its aglycone methyl azoxymethanol, which are known to induce colon cancer in lower animals[28]. On ingestion of a cycas meal, intestinal bacteria hydrolyse the ß-glucoside bond of cycasin to release the aglycone, methyl azoxymethanol[28]. Since researchers became aware of the potent carcinogenic properties of cycasin and methyl azoxymethanol, these agents have been employed as reliable animal models for inducing a range of intestinal cancers, including colorectal cancer[29,30]. Due to increased energy demand and mitochondrial dysfunction, cancer cells are known to exhibit high rates of glycolysis, even under aerobic conditions[31,32]. It is now known that succinate, an intermediate of tricarboxylic acid cycle, serves as the link between mitochondrial dysfunction and oncogenesis via hypoxia-inducible factor, HIF-1α[33]. Mitochondrial dysfunction in turn leads to accumulation of the glycolytic intermediate pyruvate and elevated activities of LDH through substrate-activation of LDH gene[31]. This is in agreement with the significant increases in colon LDH seen in the rats given cycas alone, which is a manifestation of some early events in carcinogenesis. These very high increases in colon LDH were significantly reduced to control values by administration of ELAM after 3 weeks of cycas exposure, with significant reduction in the accompanying pathological changes i.e. reduced mucosal damage and reduced glandular abnormality. This demonstrates a protective role of *Annona muricata* in cycas-induced carcinogenesis. Further evidence is seen in the *Annona muricata*-mediated reversal of decreases in colon tissue protein, anti-oxidant enzymes (catalase, GPx and SOD); and the reduction in MDA levels (lipid peroxidation). Lipid peroxidation is an important pre-disposing factor in carcinogenesis[34]. The pattern of changes in the levels of the antioxidant enzymes, as well as LDH and MDA also indicate that co-administration of ELAM and cycas neutralizes the deleterious effect of cycas. This is corroborated by histological evidence of near-normal or completely normal colon architecture in the groups given cycas and *Annona muricata* extract simultaneously and in the group given cycas for one week prior to a 3-week exposure to cycas. The cycas-induced decrease in colon protein is in agreement with tissue protein loss usually associated with most cancers and the ability of *Annona muricata* to reverse it is a further indication of its protective effect. Weight loss, which is an important prognostic factor in cancer[36]; has been attributed to increased protein degradation and/or decreased protein synthesis[37,38]. The observed protective effect of *Annona muricata* in this study can be attributed to annonaceous acetogenins present in the plant[15,16]. Acetogenins destroy cancer cells by blocking NADH-CoQ oxidoreductase (complex 1), thereby starving the cancer cells of much-needed ATP[17]. Annonaceous acetogenins also possess potent anti-oxidant properties[39]. This may in part account for the observed significant decreases in lipid peroxidation in the rats exposed to *Annona muricata* and the significantly enhanced anti-oxidant potential of these groups relative to those exposed to cycas alone. Although data from several *in vitro* and *in vivo* studies have confirmed the potent anti-neoplastic properties of annonaceous acetogenins, the absence of direct information from human studies have tended to play down the therapeutic import of these findings.

CONCLUSION

The results obtained in this study demonstrate evidence for a protective role of ethanolic extract of *Annona muricata* on some early events associated with colorectal carcinogenesis in rats. Since the properties of intact biological systems are often more than the sum of properties of purified individual components, the use of crude leaf extracts of *Annona muricata* may indeed be more beneficial than the application of purified acetogenins. If animal-to-human extrapolation is permitted, our results indicate a promising potential for the use of leaves of *Annona muricata* in the management of colorectal cancer. This is considered beneficial and of more practical significance where commercial preparations of acetogenins are not readily available or affordable, especially in poor, rural communities with unrestricted access to the plant in its natural form.

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