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

Okolie Ngozi Paulinus 1,*, Agu Kinsley 2 and Eze Gerald Ikechi 3

1Department of Biochemistry, Faculty of Life Sciences, University of Benin, Edo State, Nigeria
2Department of Medical Biochemistry, School of Basic Medical Sciences, College of Medicine, University of Benin, Edo State, Nigeria
3Department of Anatomy, School of Basic Medical Sciences, College of Medicine, University of Benin, Benin City, Edo State, Nigeria

*Corresponding Author Email: okolie_ngozi@yahoo.co.uk
DOI: 10.7897/2272.02444

Published by Moksha Publishing House. Website www.mokshaph.com
All rights reserved.

Received on: 09/06/13 Revised on: 18/07/13 Accepted on: 26/07/13

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 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. 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. 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. Studies have shown that colon cancer accounts for 10-50% of gastro-intestinal malignancies in Nigeria. Colon cancer, which begins as benign polyps, has been associated with several pre-disposing factors including age and diet; inactivity and obesity; as well as sedentary life style. 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. 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. Indeed the search for plant-based cancer treatment strategies has continued to engage the attention of researchers. Many effective anti-neoplastic agents have been isolated from plants. Intensive research has focused on the cancer-killing properties of the leaves and stem of A. muricata since their anti-carcinogenic properties became known. 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. 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. Acetogenins exert their selective anti-neoplastic effects by inhibiting complex 1 of the mitochondrial respiratory chain, thereby blocking ATP supply to the cancer cells. 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. 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
The study was carried out in strict compliance with the ethics in Guidelines and Specifications on Experimental Animal Care\(^2\). 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% formal 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\(^2\). Catalase assay was as described by Cohen et al\(^2\), in which the rate of decomposition of hydrogen peroxide is monitored at 480 nm. GPx activity was determined by measuring the production of purpuragallen from pyrogallol at 420 nm\(^2\). 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\(^7\).

### Histology
Colon sections fixed in formal 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 1: Animal groups and their treatments

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

The study was carried out in strict compliance with the ethics in Guidelines and Specifications on Experimental Animal Care\(^2\). 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% formal 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. Catalase assay was as described by Cohen et al\(^2\), in which the rate of decomposition of hydrogen peroxide is monitored at 480 nm. GPx activity was determined by measuring the production of purpuragallen from pyrogallol at 420 nm. 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\(^7\).

### Histology
Colon sections fixed in formal 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

<table>
<thead>
<tr>
<th>Groups</th>
<th>LDH</th>
<th>Total protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>12.30 ± 1.80(^a)</td>
<td>0.443 ± 0.01(^a)</td>
</tr>
<tr>
<td>II</td>
<td>94.81 ± 10.20(^b)</td>
<td>0.416 ± 0.10(^b)</td>
</tr>
<tr>
<td>III</td>
<td>18.83 ± 3.10(^a)</td>
<td>0.433 ± 0.10(^a)</td>
</tr>
<tr>
<td>IV</td>
<td>21.69 ± 0.60(^a)</td>
<td>0.535 ± 0.20(^a)</td>
</tr>
<tr>
<td>V</td>
<td>4.92 ± 0.01(^b)</td>
<td>0.544 ± 0.10(^b)</td>
</tr>
<tr>
<td>VI</td>
<td>4.43 ± 1.40(^b)</td>
<td>0.535 ± 0.01(^b)</td>
</tr>
<tr>
<td>VII</td>
<td>3.44 ± 0.80(^d)</td>
<td>0.515 ± 0.12(^d)</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Group</th>
<th>MDA x 10^-3 (mmoles/g fresh weight)</th>
<th>SOD (U/g fresh weight)</th>
<th>Catalase (U/g fresh weight)</th>
<th>GPx (U/mg fresh weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>14.73 ± 0.30a</td>
<td>2.11 ± 0.010a</td>
<td>321.00 ± 6.000a</td>
<td>56.90 ± 4.000a</td>
</tr>
<tr>
<td>II</td>
<td>74.22 ± 0.30b</td>
<td>1.71 ± 0.003b</td>
<td>256.00 ± 6.000b</td>
<td>37.60 ± 2.000b</td>
</tr>
<tr>
<td>III</td>
<td>53.54 ± 0.90c</td>
<td>3.13 ± 0.030c</td>
<td>363.00 ± 7.000c</td>
<td>59.50 ± 7.000c</td>
</tr>
<tr>
<td>IV</td>
<td>33.26 ± 1.70d</td>
<td>3.14 ± 0.004d</td>
<td>350.00 ± 9.000d</td>
<td>184.00 ± 2.000d</td>
</tr>
<tr>
<td>V</td>
<td>48.16 ± 1.40e</td>
<td>3.00 ± 0.001e</td>
<td>341.00 ± 10.000e</td>
<td>131.00 ± 2.000e</td>
</tr>
<tr>
<td>VI</td>
<td>48.99 ± 0.60f</td>
<td>3.14 ± 0.040f</td>
<td>367.00 ± 4.000f</td>
<td>119.00 ± 7.000f</td>
</tr>
<tr>
<td>VII</td>
<td>40.12 ± 0.22g</td>
<td>3.00 ± 0.001g</td>
<td>363.00 ± 10.000g</td>
<td>85.00 ± 4.000g</td>
</tr>
</tbody>
</table>

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)
**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. The quest for herbal remedy for cancer is informed by the toxic side effects, lack of specificity and resistance associated with conventional chemotherapeutic strategies. 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...
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.

REFERENCES

Source of support: Nil, Conflict of interest: None Declared