



Protective Effect of Ethanol Extract of Soursop (*Annona Muricata* Linn) Leaves on Cycad Induced Oxidative Stress in Male Albino Wistar Rats

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ABSTRACT

This study was carried out to investigate the protective effect of *Annona muricata* ethanol extract (AME) on oxidative stress induced by oral administration of Cycads. Seventy male albino Wistar rats were divided into seven groups; Group A rats served as normal control, they were given normal diet (growers marsh) group B received normal diet + 5% cycad (w/w) only for 4 weeks, group C received normal diet + 5% cycad for 3 weeks +100 mg/kg b.w of AME for 1 week, group D received normal diet + 5% cycad for 1 week before treating with 100mg/kg for 3 weeks, group E received normal diet + 100 mg/kg AME for 1 week and then 5% cycad for the remaining 3 weeks group F received normal diet + 100 mg/kg AME for 4 weeks and group G received normal diet + 5% cycad + 100 mg/kg AME for 4weeks. At the end of the feeding experiment, the animals were sacrificed and their heart and liver were excised for: total protein, LDH, MDA, SOD, Catalase and GPx assays respectively. The result showed a significant increase ($P < 0.05$) in liver total protein and LDH in group B compared to group A, liver and heart MDA levels were highest in group B; 45.05 and 60.29 Units/mL respectively and significantly decreased in groups C, D, E, F G treated with AME, also there was a marked decrease in Liver and Heart's SOD and Catalase activity in group B (2.52 Units/mg and 0.42 Units/mg) compared to group A (5.29 and 1.92Units/mg), but increased in other group C, D, E, F and G that were treated with AME. There was no significant difference ($P < 0.05$) in GPx activities between groups. These observations shows that *Annona muricata* ethanolic extract could have conferred a protective effect on the liver and heart tissues, preventing oxidative attack as a result of its antioxidant potential.

1. Introduction

Annona muricata linn is a member of the family of custard apples tree called *Annonaceae* and of the species of the genus *Annona* known for its edible fruit called Soursop. This is a native of the Caribbean and Central America but now cultivated in other parts of the world and even in Nigeria with its fruit locally known as "Abo" in Yoruba, "uhuru ocha" in igbo and "Gwandardaji" in Hausa [1]. Soursop are highly aromatic fruits with white juicy flesh containing carbohydrate,

protein, folic acid, calcium, phosphorus, iron, vitamin C, large amount of Vitamin B₁ and B₂ [2]. *A. muricata* L. is also known to contain acids such as ellagic acid, *p*-coumaric acids, stearic acid, myristic acid, Gama-amino butyric acid (GABA) as well as some inorganic acids it also contain lactones, coumarins, procyanidins, flavonoids, pentacyclic terpenoid saponins, tannins sugar, alcohol aldehydes phenolic compounds and so on [3, 4]. Annonaceous acetogenins are common to the *annonaceae* family with *A. muricata* found to contain annonacin (the major acetogenin) and 81 other acetogenin [5, 6]. These chemical compounds have been reported to kill cancerous cell by blocking ATP production [7]. It is effective against multi-drug resistant cancer (MDR-Cancer) that can survive chemotherapy. It does this by inhibiting ATP transfer into the cancer cell, retarding their functions and eventually leading to the cancer cell death. Healthy cell are not affected in this process because they do not require infusion of ATP [8]. Ethno-medically, *Annona muricata* plant has been reported to be effective in the treatment of Diabetes, Hyperlipidemia and also exhibit anti-microbial and antifungal activities [9, 10, 11]. The efficacy of this plant in the management of cardiovascular diseases, intestinal flora diseases have also been reported [12, 13]. Extract of *A. muricata* possess potent *in vitro* anti-oxidant activity [14].

Cycad cirncinalis is an exotic ornamental tree belonging to the family of *Cycadaceae* [15, 16]. Research has established the carcinogenic properties of the cycad plant and that when ingested orally, it exclusively induces tumor in the large intestine (colon) [17, 18]. This study was aimed at evaluating the protective effect of ethanol extract of *Annona muricata* (leaves) on liver and heart tissue oxidative stress during the early stages of cycad induced colorectal cancer.

2. Materials and Method

2.1 Animals and Diet

Seventy (70) male adult albino Wister rats of weight range 181.80g to 216.70g were obtained from the animal house of the Department of Medical Biochemistry, School of Basic Medical Sciences, University of Benin, Benin City. The animals were housed in wooden cages and in a room with controlled lighting (12hours light: dark cycle) and at constant temperature of 24⁰C. They were handled in accordance with the guidelines and specifications of the Council of European communities 1986.

2.2 Plant Leaves and Extracts Preparation

2.2.1 Preparation of Ethanolic Extract of *Annona muricata* (Linn) Leaves.

Annona muricata leaves was collected from the botanical garden in the Department of Botany, Faculty of Life Science University of Benin, Benin City. The leaves were properly identified and authenticated by Prof. Anoliefo and Prof M.I Idu of the Department of Botany, Faculty of Life Science, University of Benin. After which the leaves were sorted out and air dried under room temperature until it was free of moisture and the dried leaves were pulverized using an electronic blender (Saisho S-423 blender). Extraction was carried out using 2.6 L 99% ethanol in a ratio of 2:3 for 72hours after which the filtration was done using a sintered filter and then Whatman filter paper. The extract was concentrated using a rotary evaporator and further dried in a wash glass under ambient temperature. The extract was reconstituted with water for use at 5 g% (w/v). A gavage syringe was used in the oral administration of the extract to rats.

2.2.2. Collection of Cycad Leaves

The *Cycad cirncinalis* leaves were collected from the university of Benin environment and also identified and authenticated by Prof E. Anoliefo of the Department of Plants and Biotechnology

Faculty of Life Sciences University of Benin. Benin City. The leaves were dried, grinded in powdered form and mixed with the animals feeds at 5% (w/w) for the duration of the experiment.

2.3 Experimental Design

The animals were allowed access to water and standard diet *ad libitum* and they were randomly divided into seven groups of ten rats each.

Table 1: Animal Groupings and Duration of Administration of Cycad and *A. muricata* Leave

Groups	Treatment	Duration Of Treatment With Cycad	Duration Of Treatment With <i>Annona muricata</i> Extract
Group A	Normal feed only	-	-
Group B	Normal feed and 5% Cycad only	4 weeks	-
Group C	Normal feed + 5% Cycad and AME (100mg/kg b.w)	3weeks	1 week
Group D	Normal feed + 5% Cycad and AME	1 week	3 weeks
Group E	Normal feed + AME (100mg/kg b. w) and 5%Cycad	1 week	3 weeks
Group F	Normal feed + AME (100mg/kg b. w) for 4weeks	-	4 weeks
Group G	Normal feed + 5%Cycad + AME (100mg/kg b. w)		4 weeks

Key: AME= *Annona muricata* Extract

2.4 Animals Tissues Collection

At the end of the experiments, animals were sacrificed by cervical dislocation. The target organs were harvested and homogenized in normal saline using a mortar and a pestle (0.5g of tissue per 5ml of normal saline). The homogenized tissues were centrifuged at 10,000g for 5minutes and the supernatant collected for biochemical assay.

2.5 Biochemical Assays

Liver and heart tissues total proteins were determined by Biuret Kit methods as described by Tietz, [19]. While SOD levels were assayed for in both tissues by Misra and Fridovich methods [20], catalase activity were determined as described by Cohen *et al.* [21]. Lipid peroxidation in the form of MDA-TBA was assayed for by method described by Varshney and Kale [22]. Glutathione Peroxidase (GPx) was determined by Flohe and Gunzler [23]. Energy metabolism level in these tissue were ascertained as LDH by UV-kit method.

2.6 Statistical Analysis

Values were expressed as Mean \pm Standard Error of Mean (SEM). Experimental data were analyzed using paired sample student t-test (2 way t-test), using SPSS package (version 15.0) with confidence interval at 95% ($p < 0.05$).

2.7 Results and Discussions

Results of experimental values of Lactate dehydrogenase (LDH) activities, malodialdehyde levels, catalase, superoxide dismutase and glutathione peroxidase activities evaluated in the liver and heart tissues of the rats are shown in Tables 2,3,4,5 and 6. This study showed that liver and heart LDH activities and MDA levels was significantly higher ($p < 0.05$) in group B (Cycad fed rats)

compared to group A (normal control) in both Group C (Cycad fed rats treated with 100mg/mL of *A. muricata* extract) however had the least LDH values in both liver and heart but the highest MDA levels in both tissues compared to Cycad fed and non-Cycad fed groups treated with *A. muricata* leaves (groups D-G). Although, Groups D and E had the lowest heart tissue MDA levels compared to the Cycad fed rats (group B) but no significant difference was observed even when compared to groups D, E, F and G. Cycad fed rat treated with *A. muricata* extract (groups C and G) and *A. muricata* extract fed extracts (group G). Values are expressed in **Table 3**. Liver superoxide dismutase activities were significantly decreased ($p<0.05$) in group B (Cycad fed rats) compared to group A (normal control) and other groups (*A. muricata* fed rats and Cycad fed groups treated with *A. muricata* extract). Group G (Cycad co-administered with *A. muricata* extract rats) gave the highest SOD activity in liver tissues compared to all groups. For hearts superoxide dismutase activities it showed the same trend with that of liver; an initial decrease in group B (Cycad fed rats) compared to group A (control) to increase in other groups C, D, E, F and G which were also significantly different ($p<0.05$) except for group C. Also, catalase activities (**Table 5**) in liver tissues showed a significant difference ($p<0.05$) between group B (Cycad fed rats) and *Annona muricata* ethanol extract treated rats as there was an initial decrease in value in group B which began to increase from groups C to D and then a decrease in group E (though higher than group B) and finally peaking at group G which has the highest value. Heart catalase activities was however, reduced in all groups. Group B had the least and group C had the highest CAT activities compared to other groups. Finally **Table 6** shows value for glutathione peroxidase activities of the liver and heart tissues respectively. Statistically, there was no significant difference ($p<0.05$) between group B (Cycad fed rats), normal control (group A) and other groups treated with *Annona muricata* extract (groups C to G). Group C (Cycad fed rats treated with 100mg/mL of *A. muricata* extract) showed a significantly raised ($p<0.05$) glutathione peroxidase activity in the heart tissues compared to the control and other groups. Also group B (Cycad fed rats) had a significantly reduced ($p<0.05$) glutathione peroxidase activity compared to the *A. muricata* treated Cycad and non-Cycad fed rats (groups C-G). When compared to the normal control, although the glutathione peroxidase activity for Cycad fed rats was lower but this was not statistically significant.

Table 2: Lactate Dehydrogenase (LDH) Levels in Liver and Heart Tissues of Cycad Fed Rats Treated with *A. muricata* Leave Extracts After Four Weeks

Groups	Liver LDH (units/l)	Heart LDH (units/l)
Group A	1.97 ±0.80 ^a	1.96±0.80 ^a
Group B	4.92±0.00 ^b	4.43±0.50 ^b
Group C	3.44±0.80 ^{abg}	3.44±0.80 ^{ab}
Group D	4.43±0.50 ^{bcd}	3.94±0.70 ^{ab}
Group E	4.92±0.00 ^c	4.43±0.50 ^{acd}
Group F	4.92±0.00 ^c	4.43±0.50 ^{ac}
Group G	4.92±0.00 ^c	3.94±0.70 ^{abdef}

*Values are expressed as Mean±S.E.M. **Values with different superscripts are significantly difference ($p<0.05$) for comparison down the groups.

Table 3: Lipid peroxidation level in Liver and Heart Tissues of Cycad fed rats treated with *A. muricata* leave extracts after four weeks

Groups	Liver MDA (10^{-3} units/mL)	Heart MDA (10^{-3} units/mL)
Group A	40.73±0.30 ^a	56.30±1.40 ^a
Group B	45.05±1.40 ^b	60.29±3.00 ^a
Group C	47.04±1.40 ^{bc}	59.39±1.00 ^a
Group D	44.04±1.00 ^{bdh}	46.02±1.30 ^b
Group E	45.75±1.00 ^{bceh}	48.44±1.30 ^{bc}
Group F	45.06±1.10 ^{bcefn}	56.75±0.05 ^{ade}
Group G	44.96±1.00 ^{bcfrgh}	57.61±2.00 ^{ae}

*Values are Mean±S.E.M of assay for a particular tissue. **Values with different superscripts are significantly difference ($p<0.05$) for comparison down the groups

Table 4: Superoxide Dismutase in Liver and Heart Tissues of Cycad Fed Rats Treated with *A. muricata* Leave Extracts After Four Weeks

Groups	Liver SOD (units/mg)	Hearts SOD (units/mg)
Group A	5.29±0.002 ^a	1.92±0.10 ^a
Group B	2.52±0.20 ^b	0.42±0.06 ^b
Group C	6.12±0.08 ^c	1.34±0.40 ^{abc}
Group D	6.39±0.20 ^{cd}	1.16±0.20 ^{cd}
Group E	6.69±0.50 ^e	1.68±0.20 ^{acd}
Group F	5.48±0.10 ^{cdf}	1.58±0.10 ^{cd}
Group G	9.77±0.003 ^{eg}	1.89±0.20 ^{ace}

*Values are Mean±S.E.M. **Values with different superscripts are significantly difference ($p<0.05$) for comparison down the groups

Table 5: Catalase Activity in Liver and Heart Tissues of Cycad Fed Rats Treated with *A. muricata* Leave Extracts After Four Weeks

Groups	Liver Catalase (mg/mL)	Hearts Catalase (mg/mL)
Group A	161.19±9.00 ^a	0.061±0.003 ^a
Group B	73.71±7.00 ^b	0.053±0.004 ^b
Group C	166.73±11.00 ^a	0.117±0.008 ^c
Group D	333.18±18.00 ^c	0.055±0.004 ^b
Group E	241.27±10.00 ^d	0.075±0.01 ^{abcde}
Group F	299.03±6.00 ^{ce}	0.083±0.01 ^e
Group G	337.37±12.30 ^{cf}	0.064±0.004 ^{abde}

*Values are Mean ± S.E.M. **Values with different superscripts are significantly difference ($p<0.05$) for comparison down the groups

Table 6: Glutathione Peroxidase level in Liver and Heart Tissues of Cycad Fed Rats treated Treated with *A. muricata* leave extracts after four weeks

Groups	Liver GPx (mg/mL)	Hearts GPx (mg/mL)
Group A	207.96±1.00 ^a	213.86±9.60 ^a
Group B	206.65±1.40 ^a	212.75±11.0 ^a
Group C	208.96±1.00 ^a	252.25±1.00 ^b
Group D	208.94±1.00 ^a	200.65±5.00 ^{ac}
Group E	209.74±0.20 ^a	224.21±5.00 ^{ad}
Group F	207.86±1.30 ^a	222.16±6.00 ^{ac}
Group G	209.27±2.00 ^a	217.29±6.00 ^{ace}

*Values are Mean ± S.E.M. **Values with different superscripts are significantly difference ($p < 0.05$) for comparison down the groups

Reports from various research has suggested that disruption of oxidative balance is one of the major biochemical features of colorectal carcinogenesis [25]. Oral exposure to Cycasin can lead to colorectal cancer [17, 26]. Cycasin is the primary procarcinogen in Cycad plant, metabolized to the ideal carcinogen by the microbial flora of the gastrointestinal tract [27]. In most animal cells, oxidative stress induced by a predominance of oxidants cause a particular type of cellular injury to biological molecules. This damage by oxidants and other genotoxic agents play important roles in the development and generation of cancer. Earlier studies have demonstrated the production of extra cellular superoxide (O_2) by the intestinal micro flora as a source for generating these oxidants that can cause chromosomal instability and colon cancer [28]. In spite of the destructive activity of oxidants, they can act as second messengers in a various cellular processes. For instance, reactive oxygen species (ROS) could serve as signaling molecules or could cause oxidative damage to body tissues. This is dependent on the delicate equilibrium between ROS production, and their scavenging. Efficient scavenging of ROS produced during various biological processes requires the action of several enzymatic and non-enzymatic antioxidants present in the tissues. The extent of damage caused by these oxidants is dependent on the activities of detoxifying enzymes.

In recent times, there is increasing interest on the evaluation of antioxidant properties of medicinal plants for scientific research as well as for therapeutic purposes [29]. This is primarily based on their strong biological activity which overwhelms those of commonly used synthetic drugs with many deleterious side effects [30]. The efficacy of medicinal plants has been attributed to their ability to scavenge free radicals and alleviate diseases associated with oxidative stress such as Cancer, Cardiovascular diseases, Diabetes, Hypertension, Alzheimer's disease. Antioxidant compounds protect cells against the damaging effects of reactive oxygen species (ROS) such as singlet oxygen, superoxide, peroxy radicals, hydroxyl radicals and peroxynitrite which results in oxidative stress leading to cellular damage [31]. *A. muricata* is a plant that has been well known for its anticancer properties due to its ability to kill cancer cells. The leaves of this plant have been reported to possess antioxidant potentials and play a role as an effective radical scavenger which augments its therapeutic effect [32].

Lactate dehydrogenase is a cytoplasmic enzyme found in almost all tissues. The extracellular appearance of this enzyme is used as an indicator of cell damage and cell death [33]. This was observed in group B, D, E, F and G in the liver and heart tissues. The observed elevation of LDH in the respective groups compared to the normal control as stated above is due to the tissue (liver

and heart) damage caused by exposure of the rats to Cycad a renowned toxin. Eventhough the *A. muricata* extract was unable to reverse the existing damage in groups other groups except groups C, D and E (heart tissues only). This could be attributed to the duration of exposure to the Cycas toxin and treatment with the *A. muricata* extract. Malondialdehyde (MDA) is an index of peroxidation of polyunsaturated lipids resulting from reactive oxygen species (ROS). These lipids forms an integral component of the structures of biological membranes. Free radicals primarily attacks membrane lipids leading to their peroxidation [33]. This results in membrane instability, destruction of membrane proteins as well as other macromolecules and cellular damage [34]. The elevated MDA levels observed in the liver and heart tissues of the Cycad fed rats can be explained by the presence of Cycasin, a component of the Cycad plant which forms agents with elevated free radicals activities [27]. These free radicals attacks the membrane lipids leading to their peroxidation. The higher levels of MDA produced in other groups C-G (Cycad fed rats and non-Cycad fed rats treated with *A. muricata* extract) could be as a result of an irreversible damage to the membrane lipids. This results in decreased membrane fluidity and reduction of catalyst and receptor activity as well as injury to the macromolecules of the membrane, finally triggers cell inactivation and death [35].

Tissues of the liver and heart contains some antioxidants enzymes such as SOD, CAT and GPx which protects them from oxidative attack from free radicals [36, 37]. However when oxidative stress is induced, cells responds to combat the activities of free radicals by doubling the activities of these antioxidant enzymes to counter the effect [38]. Results from this study suggests that the leaves of *A. muricata* could have ameliorated the oxidative damage on the liver and heart tissues of the Cycad fed rats. This was indicated by the increase in SOD and CAT activities in the organs of Cycad fed rats treated with *A. muricata* extract compared to the Cycad fed rats that didn't receive the extract. Also, the reduced SOD and CAT activities in the Cycad fed rats could be attributed to the elevated levels of oxidative stress induced by the free radical activities of Cycasin present in the Cycad plant. These findings corroborates with those of Lolodi and Eriyamremu [39] that observed a decrease in SOD and CAT activities in Cycads fed rats. Glutathione peroxidase (GPx) activity was significantly elevated in group C rats (rats fed with Cycad and treated with *A. muricata* extract) an indication that GPx protected the tissues membrane from oxidative attack by decomposition of hydrogen peroxide radical from other organic peroxides. This reduction is indicative that the scavenging abilities of this antioxidant enzyme have been overwhelmed by free radicals produced by the toxicant present in the Cycad plant. This observation, therefore, suggests that the Cycad plant altered the expression and activities of antioxidant enzymes as a result of toxic metabolites generated during their biotransformation.

4. Conclusion

Results from this study showed that *A. muricata* extract reduced the deleterious effect of the Cycad induced oxidative damage on the liver and heart tissues. This means that *A. muricata* extract could have conferred a protective effect on the liver and heart tissues, preventing oxidative attack as a result of its antioxidant potential.

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