

Original Research Article

Cyclophosphamide Induced Toxicity and Oxidative Stress in Liver of Male Wistar Rat: Protection by Ethanolic Soursop (*Annona muricata*) Leaves Extract

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Abstract: The aim of this study is to determine the protection of ethanolic *annona muricata* (soursop) leaves extract on cyclophosphamide induced toxicity and oxidative stress in liver of male wistar rat. The healthy male albino rats of wistar strain, after acclimatization for a period of two weeks were randomly distributed to three (3) groups with four (4) rats in each group, group 1 served as control and was feed on pelletized growers feed and distilled water for 14 days. Group 2 served as positive control and was feed on pelletized growers feed and distilled water throughout the experiment (14days), then received 100mg/kg per body weight (b.w.) of cyclophosphamide via injection, and were sacrificed after one day. While group 3 served as test and was feed on pelletized growers feed and distilled water, and also 100mg/100kg (b.w.) of ethanolic leaves extract of soursop for 14 days, then received 100mg/kg per body weight (b.w.) of cyclophosphamide via injection, and were sacrificed after one day. For the methodology, hepatic anti-oxidant enzymes, ALT, AST, and histopathology of the liver were the parameters assayed using biochemical methods. The results showed that there was a significant decrease ($P<0.05$) in SOD activities of wistar albino rats following the administration of cyclophosphamide. However, treatment of rats with cyclophosphamide was observed to have caused a significant increase in MDA levels. Administration of *Annona muricata* extract was observed to have reversed the adverse effects caused by cyclophosphamide. The results showed that there was a significant increase ($P<0.05$) in the enzyme levels compared to the control in albino male rat liver after the administration of the cyclophosphamide. However, administration of extract was observed to have ameliorated the effects of cyclophosphamide induced damage thereby causing the decrease in ALT and AST concentrations. In conclusion, *Annona muricata* leaves possess hepatoprotective properties and can therefore be recommended for treatment of hepatotoxicity and oxidative stress.

Keywords: Cyclophosphamide, Toxicity, Ethanolic, *Annona Muricata*.

INTRODUCTION

Natural products, especially those derived from plants, are used to help mankind sustain its health since the dawn of medicine. Over the past century, the phytochemicals in plants are a pivotal pipeline for pharmaceutical discovery. The importance of the active ingredients of plants in agriculture and medicine has stimulated significant scientific interest within the biological activities of these substances [1]. Although these studies have been done, a restricted range of plant species has experienced detailed scientific inspection, and our knowledge is comparatively insufficient concerning their potential role in nature. Hence, the attainment of a cheap perception of natural products necessitates comprehensive investigations on the biological activities of these plants and their key phytochemicals [2]. During a pharmaceutical landscape, plants with an extended history of use in ethno medicine are a rich source of active phytoconstituents that provide medicinal or health benefits against various ailments and diseases. One such plant with extensive traditional use is soursop. *Annona muricata* could also be a member of the Annonaceae family and is an evergreen tree. It's cultivated in tropical and subtropical regions and thought of as a typical medicine [3]. Its leaves contain several groups of medicine collectively called annonaceous acetogenins that include murihexocin, annocuricin [4] annopentocin A, B and C, (2, 4-cis)-annomuricin-D-one, murihexocin A and B, (2,4-trans)annomuricin-D-one, 4-

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acetyl gigantetrocin, cis-gigantrionin [5] muricatocin A, B and C [6] and annohexocin [7]. These compounds are highly potent and selective against microbial resistance [8]. They showed anti-tumor effects in vivo and in vitro [9-11]. The essential oils of *A. muricata* leave have parasitocidal, anti-diarrheal, rheumatological, and anti-neuralgic properties [12, 13]. The leaves extracts are gastroprotective [15], anti-diabetic, hepatoprotective [14] anti-bacterial [17], anti-arthritis, anti-inflammatory [16] and are modulators of the innate system [18]. Cyclophosphamide (CP) is an alkylating agent widely used for treating many human malignant tumors and as immunosuppressant drug [19; Mahmoud *et al.*, [20]; Omole *et al.*, [21]. However, its clinical application is typically limited thanks to its adverse side effects, including hepatotoxicity [19-21]. Exposure to high doses of CP can induce acute hepatotoxic effects provoked by oxidative stress and activation of inflammatory cascade reaction [22, 23]. The deleterious effects of CP are attributed to its metabolites phosphoramidate mustard and acrolein produced through the action of hepatic microsomal cytochrome P450 (CYP450) [24]. The highly reactive metabolite acrolein features a brief biological half-life and triggers the assembly of reactive oxygen species (ROS) [25]. In turn, ROS provoke lipid peroxidation (LPO), protein carbonylation and oxidative DNA damage, and activate multiple signalling molecules, including nuclear factor-kappaB (NF- κ B), eventually resulting in necrobiosis. Oxidative stress and inflammation can activate the apoptotic signaling pathways in hepatocytes and are therefore implicated in CP hepatotoxicity [26, 20, 22, 23]. Medicinal plants are identified and used throughout human history. Plants have the facility to synthesize an honest kind of chemical compounds that are used to perform important biological functions and to defend against attack from predators like insects, fungi and herbivorous mammals [27]. Chemical compounds in plants mediate their effects on the human body through processes a bit like those already well understood for the chemical compounds in conventional drugs; thus herbal medicines don't differ greatly from conventional drugs in terms of how they work [28]. This allows herbal medicines to be as effective as conventional medicines, but also gives them the same potential to cause harmful side effects. The use of plants as medicines predates written human history. Ethno-botany is recognized as an efficient because of discover future medicines. Many of the pharmaceuticals currently available to physicians have an extended history of use as herbal remedies including aspirin, digitalis, quinine, and opium. The use of herbs to treat diseases is almost universal among non-industrialized societies and is typically cheaper than purchasing expensive modern pharmaceuticals [29]. Herbal medicine is that the utilization of medicinal plants for prevention and treatment of diseases: it ranges from traditional and popular medicines of every country to the use of standardized and titrated herbal extracts [30]. Traditional medical system may indicate safety, but not efficacy of treatments, especially in herbal medicine where tradition is almost completely supported remedies containing active principles at low and ultra-low concentrations, or depending on magical-energetic principles [31].

MATERIALS AND METHODS

Plant Source

The leaves of *Annona muricata* were obtained from a farm in Amassoma, Wilberforce Island.

Preparation of plant leaves extract

Fresh leaves of *Annona muricata* was rinsed in a clean water and shade dried at room temperature. The dried leaves were grounded into fine powder with a mechanical grinder. 500g of the powdered leaf was dissolved with 1900ml of 75% ethanol. The resulting mixture was filtered with the aid of a sterile cheese cloth and the filtrate was subjected to a low but complete solvent evaporation using a water bath at a temperature of 60°C. The extract was stored in an air tight container, labelled and stored at room temperature.

Chemicals/Reagents

All chemicals used were of analytical grade. Ethanol, 30mM Hydrogen peroxide, 6M hydrogen tetraoxosulphate (H₂SO₄), Tris amino methane buffer (hydroxyl methyl), Phosphate buffer, Carbonate buffer, KMNO₄, NaCl, NaOH, Na₂HPO₄, HCl, Na₂CO₃, NaHCO₃, EDTA (Ethylenediaminetetraacetic acid), Adrenaline solution.

Experimental animals

Twelve (12) healthy adult male wistar albino rats with an average weight of 124g to 186g were used for this study. They were obtained from the animal house of the Department of Pharmacology, Niger Delta University, Wilberforce Island, Bayelsa State, Nigeria and were maintained under standard housing conditions (photoperiod: 12h natural light and 12h dark). The animals were acclimatized for three weeks and were fed pelletized growers feed and were exposed to clean tap water throughout the period of the study.

Experiment design

The healthy male albino rats of wistar strain, after acclimatization for a period of two weeks were randomly distributed to three (3) groups, with four (4) rats in each group. Group 1 (control) was feed on pelletized growers feed and distilled water throughout the experiment (14days). Group 2 (positive control) was feed on pelletized growers feed and distilled water for 14 days, then received 100mg/kg per body weight (b.w.) of cyclophosphamide via injection, and was left for a day for the cyclophosphamide to take effect. Group 3 was feed on pelletized growers feed and distilled water for 14 days, received 100mg/kg per body weight (b.w.) of ethanoic extract of *Annona muricata* for 14 days, then

received 100mg/kg per body weight (b.w.) of cyclophosphamide via injection, and was left for a day for the cyclophosphamide to take effect.

Method of collection and handling sample (liver)

Annona muricata leaf extract was administered orally with gavage for 14 days after which the animals were sacrificed via chloroform anaesthetization. The whole body was dissected and the liver tissue excised and washed in normal saline and part of it was placed in a sample bottle containing 10% formal saline. This was subjected to histological analysis. Then 10% homogenate was prepared. This was subjected to biochemical analysis. The blood was centrifuged for 10 minutes at 3000rpm and the serum was collected.

Histopathological studies of the liver

The liver tissue was removed, weighed and immediately fixed in 10% formalin for histological studies. The tissue samples were cleared in xylene and embedded in paraffin wax and sections were cut using 5-micron in a rotary microtome. The sections were then examined using the light microscope after staining with haematoxylin and eosin dyes and interpreted by an expert histologist.

Biochemical assay

The level of superoxide dismutase (SOD) activities was determined by the method of Misra and Fridovich [32]. Lipid peroxidation was determined by measuring the thiobarbituric acid (TBA) and reactive substrates (RS) produced during lipid peroxidation. Catalase assay was carried out according to the method described by Aebi [33].

STATISTICAL ANALYSIS

Results obtained were expressed as mean \pm standard deviation (SD). Analysis of variance (ANOVA) was carried out on the result data at 95% confidence level using SPSS statistical software package, version 17.

RESULTS

Effect of *Annona muricata* on liver MDA and SOD

Table-1.0: The effect of administration of *Annona muricata* on liver MDA and SOD.

Treatment	SOD (U/ml)	MDA (mM/l)
Control (feed and water)	5.25 \pm 1.16 ^a	401.09 \pm 1 ^a
Cyclophosphamide	2.48 \pm 0.29 ^b	1207.98 \pm 1.39 ^b
Cyclophosphamide + ethanolic soursop leave extract	4.58 \pm 0.17 ^a	385.95 \pm 31.14 ^a

Data are Mean \pm SD (n = 4). Mean in the same column with different superscript letter(s) are significantly different, (P<0.05).

The results showed that there was a significant decrease (P<0.05) SOD activity of wistar albino rats following the administration of cyclophosphamide. However, treatment of rats with cyclophosphamide was observed to have caused a significant increase (P<0.05) in MDA levels. Administration of *Annona muricata* extract was observed to have reversed the adverse effects caused by cyclophosphamide. Administration of *Annona muricata* caused an increase (P<0.05) in SOD activity bringing it close to the original level of activity. The administration of *Annona muricata* also caused a significant decrease (P<0.05) in the levels of MDA bringing it close to the original MDA levels.

Effect of *Annona muricata* on liver ALT and AST level

Table-2.0: The effect of administration of *Annona muricata* extract on the ALT and AST level of liver of albino male wistar rat

Treatment	AST (IU/L)	ALT (IU/L)
Control (feed and water)	67.83 \pm 1.39 ^a	43.73 \pm 1 ^a
Cyclophosphamide	173.4 \pm 0.81 ^b	128.25 \pm 0.68 ^b
Cyclophosphamide + ethanolic soursop leave extract	67.06 \pm 1.28 ^a	42.75 \pm 1.42 ^a

Data are Mean \pm SD (n = 4). Mean in the same column with different superscript letter(s) are significantly different, (P<0.05).

The results showed that there was a significant increase (P<0.05) in the enzyme levels compared to the control in albino male rat liver after the administration of the cyclophosphamide. However, administration of *Annona muricata* extract was observed to have ameliorated the effects of cyclophosphamide induced damage thereby causing the decrease in ALT and AST concentrations.

Effects of *Annona muricata* on histopathology of liver

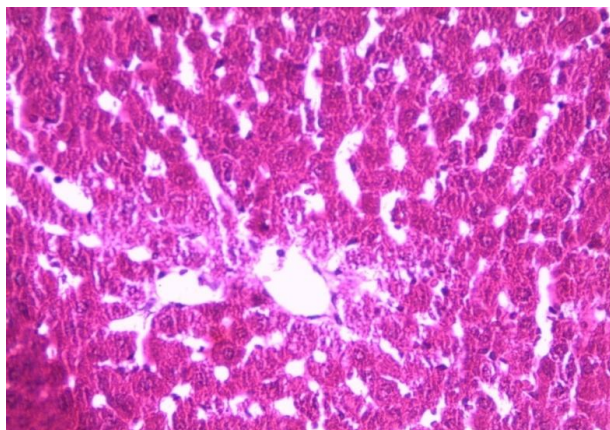


Fig-1

Fig-1: (Control group) Liver with cords of hepatocytes well preserved, cytoplasm not vacuolated, sinusoids well demarcated, no area of necrosis, no fatty change, and no fatty degeneration. The result shows the control group only fed with feed and water. There are no signs of damage

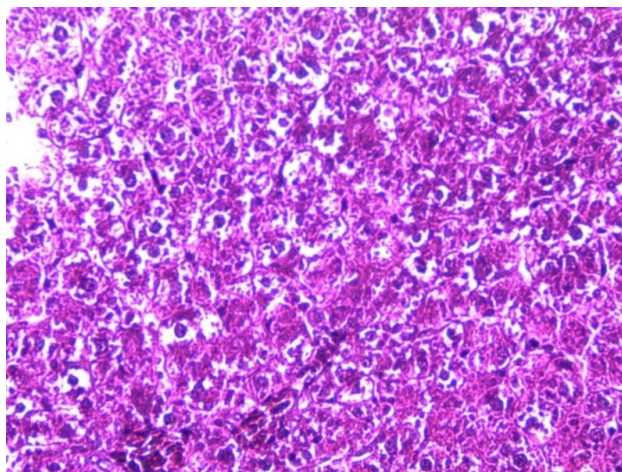


Fig-2

Fig-2: (Positive control group treated with cyclophosphamide 100kg/mg b. wt) Liver showing enlarged hepatocytes with vacuolation of cytoplasm. There is compression of the sinusoid. The result shows the positive control group showed signs of liver damage caused by the administration of cyclophosphamide.

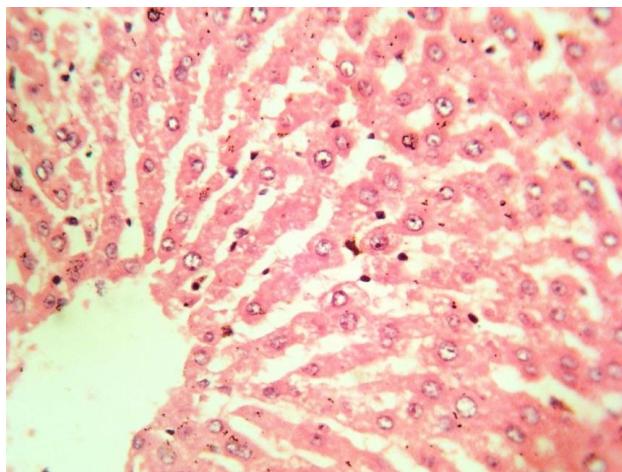


Fig-3

Fig 3: Liver (treated with cyclophosphamide 100mg/kg bwt + ethanolic soursop leave extract 100mg/kg bwt) with normal architecture. Showing central vein .Cords of hepatocytes well preserved cytoplasm not vacuolated. Sinusoids well demarcated no area of necrosis, no fatty change, no fatty degeneration. The result shows the test group after administration of cyclophosmide and then the leaf extract. The liver architecture is normal, showing central vein, cords of hepatocytes well preserved and cytoplasm not vacuolated. Sinusoids are well demarcated with no area of necrosis, no fatty change, and no fatty degeneration.

DISCUSSION

Liver disease and toxicity is common, especially with many drug treatments. Serum activities of AST and ALT are the foremost commonly used biochemical markers of liver injuries. SOD and MDA levels can also function biomarkers of oxidative stress in liver. The results of this study showed increase within the levels of ALT and AST within the test group injected with cyclophosphamide as compared to the control group. The rise in serum level of AST and ALT has been attributed to the damaged structural integrity of the liver. This is often because they're cytoplasmic in their location and are released into circulation after cellular damage [34]. Enzymes are proteins found within the body that increase the speed of chemical reactions. Liver has enzymes which perform these actions within the liver. the foremost common liver enzymes are Aspartate aminotransferase (AST), Alanine aminotransferase(ALT) and Alkaline Phosphatase (ALP) which are useful biomarkers of liver injury during a patient with a point of intact liver function [35]. Tests are performed on patient's blood sample, a number of which are related to functionality of the liver (e.g. albumin) and cellular integrity (e.g. transaminase) while some are related to conditions linked to the biliary tract (gammaglutamyl transferase and alkaline phosphatase) [36]. Several biochemical tests are useful within the evaluation and management of patients with hepatic dysfunction. These tests are often wont to detect the presence of liver diseases, distinguish among differing types of liver disorders, gauge the extent of known liver damage, and follow]liver injury [35]. An initial step in detecting liver damage may be a simple biopsy to work out the extent of certain liver enzymes (proteins) within the blood. Under normal circumstances, these enzymes mostly reside within the cells of the liver. But when the liver is injured for any reason, these enzymes are spilled into the bloodstream [37]. Oxidative stress (OS) may be a condition produced by the imbalance between oxidants and antioxidants during a biological system. The imbalance occurs as results of the surplus level of reactive oxygen species (ROS) or improper functioning of the antioxidant system [38]. SOD (SOD) may be a liver antioxidant, that is, SOD is an enzyme that helps break down potentially harmful oxygen molecules within the liver. In event of a big change within the normal enzyme activity of SOD, it can cause oxidative stress within the liver as there would be more free radicals and fewer SOD to assist break them down. Malondialdehyde (MDA) within the liver is one among the ultimate products of polyunsaturated fatty acids peroxidation within the cells. a rise in free radicals causes overproduction of MDA, so MDA is usually referred to as a marker of oxidative stress and antioxidant status. The present study was conducted to guage the protective effect of the extract of soursop leaves on cyclophosphamide induced oxidative stress and toxicity in liver of male rats of wistar strain. Soursop was chosen because it's widely used and readily available in Nigeria also as in many parts of Africa and therefore the world. Soursop is understood as graviola, soursop and guanabana. This plant is distributed in tropical areas of Central and South America, Africa and south-east Asia as alittle evergreen tree that grows around 4 to eight m tall and produces green spiny heart-shaped fruit. The bark, fruit, seed and leaves are traditionally used for the management of a good sort of diseases like malaria, diabetes and parasitosis [39].The observed effect of soursop on liver enzymes has been attributed to its leaves containing several groups of drugs collectively called annonaceous acetogenins [40] and also other phytochemicals of the plant including alkaloids (ALKs) [41, 42], megastigmanes (MGs) [43], flavonol triglycosides (FTGs) [44], phenolics (PLs) [45], cyclopeptides (CPs) and essential oils [46, 47]. Flavonoids are known to be antioxidants, radical scavengers and antilipoperoxidant resulting in hepatoprotection. The mechanism by which soursop exerts protection against cyclophosphamide induced alterations within the liver could also be thanks to the antioxidative and acetogeninic effect of the plant extract. The pharmacological effects of *A. muricata* leaf extracts and acetogenins isolated from *A. muricata* on major body systems suggests a favourable safety profile [39].

The observed effect of *Annona muricata* on liver enzymes has been attributed to its leaves containing several groups of substances collectively called annonaceous acetogenins [40] and also other phytochemicals of the plant including alkaloids (ALKs) [41, 42], megastigmanes (MGs) [43], flavonol triglycosides (FTGs) [44], phenolics (PLs) [45], cyclopeptides (CPs) and essential oils [46, 47]. Flavonoids are known to be antioxidants, free radical scavengers and antilipoperoxidant leading to hepatoprotection. The mechanism by which *Annona muricata* exerts protection against cyclophosphamide induced alterations in the liver may be due to the antioxidative and acetogeninic effect of the plant extract. The pharmacological effects of *A. muricata* leaf extracts and acetogenins isolated from *A. muricata* on major body systems suggests a favourable safety profile [39].

The activity of SOD when compared to the control decreased significantly ($P < 0.05$) from 5.25 ± 1.16 to 2.48 ± 0.29 (U/ml) after treatment with cyclophosphamide. On the other hand, administration of *Annona muricata* increased the level of SOD from 2.48 ± 0.29 to 4.58 ± 0.17 (Table 1).

The level of MDA when compared to the control increased significantly ($P < 0.05$) from 401.09 ± 1 to 1207.98 ± 1.39 (mM/l) after treatment with cyclophosphamide. On the other hand, administration of *Annona muricata* decreased the level of MDA from 1207.98 ± 1.39 to 385.95 ± 31.14 (Table 1).

Histopathological study showed that the control sample showed no signs of injury, that is, no area of necrosis, no fatty change, no fatty degeneration and cytoplasm not vacuolated (Fig 1). The results show the group administered cyclophosphamide feature enlarged hepatocytes with vacuolation of cytoplasm and there's compression of the sinusoid (Fig 2). The results also show the group treated with soursop leaves extract after administration of cyclophosphamide with normal architecture, showing central vein, cords of hepatocytes, well preserved cytoplasm not vacuolated, sinusoids well demarcated no area of necrosis, no fatty change, and no fatty degeneration (Fig 3). The level of AST and ALT in comparison to the control increased significantly ($P < 0.05$) from 67.83 ± 1.39 to 173.4 ± 0.81 for AST and 43.72 ± 1 to 128.25 ± 0.68 for ALT, after treatment with cyclophosphamide (Table 2). On the other hand, administration of *Annona muricata* increased the level of AST and ALT from 173.4 ± 0.81 to 67.06 ± 1.28 for AST and 128.25 ± 0.68 to 42.75 ± 1.42 for ALT (Table 2).

The results of this study however demonstrated that pre-treatment with ethanolic leaf extract of soursop significantly caused a decrease in serum ALT and AST as compared to treatment with cyclophosphamide alone. Thus the extract protected the hepatocytes from cyclophosphamide induced injuries. The stabilization of transaminases denotes the renewal of the normal hepatic activity. The efficacy of any hepatoprotective drug depends on its capacity of either reducing the harmful effect or restoring the traditional hepatic physiology that has been distributed by a hepatotoxin. Soursop ethanolic leaf extract decreased ($p < 0.05$) cyclophosphamide induced high ALT and AST levels in tested groups, indicating the protection of structural integrity of hepatocytic cell wall or regeneration of damaged liver cells [48].

The results of this study however demonstrated that pre-treatment with ethanolic leaf extract of *Annona muricata* significantly caused a decrease in serum ALT and AST as compared to treatment with cyclophosphamide alone. Thus the extract protected the hepatocytes from cyclophosphamide induced injuries. The stabilization of transaminases denotes the renewal of the original hepatic activity. The efficacy of any hepatoprotective drug is dependent on its capacity of either reducing the harmful effect or restoring the normal hepatic physiology that has been distributed by a hepatotoxin. *Annona muricata* ethanolic leaf extract decreased ($p < 0.05$) cyclophosphamide induced high ALT and AST levels in tested groups, indicating the protection of structural integrity of hepatocytic cell membrane or regeneration of damaged liver cells [48].

These results accept as true with the study of Offor *et al.*, [49] on "Effect of Ethanol Leaf-Extract of soursop on Liver Enzymes of Albino Rats" which reported that soursop might be used to treat hepatic toxicity and restore liver enzyme level. This result agrees with the study of Arhoghro *et al.* [50] on "Effect Of *Costus Afer* On Fertility Parameters In Cyclophosphamide-Induced Reproductive Toxicity In Male Albino Rats" which reported that cyclophosphamide induced toxicity in male albino wistar rats. These results also accept as true with the study of Usunobun *et al.*, [51] on "Attenuation of N, N-Dimethylnitrosamine-Induced Liver Fibrosis in Rats by Ethanolic Leaf Extract of *Annona muricata*" which reported that soursop might be used to treat oxidative stress of the liver because the extract restored the hepatic antioxidants to healthy levels when administered after induction of oxidative stress. These results also accept as true with the study of Adewole and Ojewole, [15] on "Protective Effects Of soursop Linn. (Annonaceae) Leaf Aqueous Extract on Serum Lipid Profiles and Oxidative Stress in Hepatocytes of Streptozotocin-Treated Diabetic Rats" which reported that *A. muricata* leaves extract possess antioxidant properties which is in a position to inhibit and/or prevent hepatic oxidative damage.

The study of Wai-Jo *et al.*, [39] on "The Safety and Tolerability of *Annona muricata* Leaf Extract: A Systematic Review" reported and show that the pharmacological effects of *A. muricata* leaf extracts and acetogenins isolated from *A. muricata* on major body systems suggests a favourable safety profile.

CONCLUSION

From the results of this study, the *Annona muricata* had a significant effect on the liver enzymes and antioxidants. Treatment with *Annona muricata* leaf extract restored the level of enzymes and antioxidant function in the liver, and Histopathological study showed liver damage repair. This justifies the conclusion that the extract might help with alleviation of liver damage as such might be recommended for treatment of hepatotoxicity.

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