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Anti-Inflammatory Potentials of Aqueous –Methanolic Extract of Soursop (Annona Muricata) Leaves on Liver Function Parameters in Formalin-Induced Inflammation and Oedema in Male Rats

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Abstracts
This study investigated the anti-inflammatory and hepatoprotective effects of aqueous-methanolic extract of soursop (Annona muricata) leaves in formalin-induced inflammation and edema in male rats. Inflammation and oxidative stress are major contributors to liver damage, and soursop leaves have traditional medicinal applications. Thirty male rats were assigned to six groups: normal saline control, formalin-induced control, two treatment groups receiving 100 mg/kg and 300 mg/kg of soursop leaf extract, and two groups treated with standard drugs Diclofenac and Aspirin. Liver function markers, including Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), Alkaline Phosphatase (ALP), and total protein, were evaluated. Formalin induction significantly increased AST, ALT, and ALP levels in untreated rats. Treatment with soursop extract at both doses ameliorated ALT

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and AST elevations, with greater efficacy observed at 300 mg/kg. Diclofenac-treated rats also exhibited significant reductions in these enzymes. Notably, ALP levels were markedly reduced in rats receiving 300 mg/kg extract, indicating restoration of hepatocyte function, while Diclofenac produced a moderate effect. These findings demonstrate that soursop leaf extract effectively mitigates formalin-induced liver enzyme alterations and exhibits significant anti-inflammatory and hepatoprotective activity. The study highlights the therapeutic potential of soursop leaves as a natural intervention for protecting liver function in inflammatory conditions.
Keywords: Annona muricata, anti-inflammatory, hepatoprotection, formalin-induced oedema, liver enzymes.

1. Introduction

A complicated physiological reaction, inflammation frequently leads to oedema, which is defined as the buildup of extra fluid in tissues as a result of increased vascular permeability. Swelling and decreased organ function might result from this illness. Since the liver is essential for detoxification and metabolic control, inflammatory processes are intimately linked to liver dysfunction (Van Damme *et al.,* 2020). Hepatocellular damage, oxidative stress, and changed liver function parameters like elevated bilirubin and liver enzyme levels can all be caused by persistent inflammation. Thus, controlling inflammation is essential to preserving liver health (Idowu *et al.,* 2023).

The potential of natural plant extracts, especially those high in bioactive chemicals, to reduce inflammation and enhance liver function has drawn interest. Soursop, or *Annona muricata*, has been utilized in traditional medicine due to its many medicinal qualities, which include hepatoprotective, anti-inflammatory, and antioxidant benefits. Recent research on the effectiveness of antioxidant-rich medicinal plants in regulating liver function during inflammatory circumstances has shown encouraging outcomes in lowering liver enzyme levels and enhancing liver health in general (Owolabi *et al.,* 2022).

A tropical plant species in the Annonaceae family, soursop (*Annona muricata*) is well-known for its several ethnomedical applications (Agu *et al*., 2017). In tropical regions, all parts of Annona are utilized in natural medicine. It is thought to be an excellent natural antioxidant source for a number of illnesses. The leaves have historically been used to treat liver issues, headaches, sleeplessness, cystitis, anticancer, and inflammation (Agu *et al.,* 2017). This plant's distinct phytochemical makeup has been linked to its health advantages (Ekpo *et al.,* 2013). The importance of plant active components in agriculture and medicine has sparked scientists' interest in the biological activities of plants in recent years (Adebowale-Tellez *et al.,* 2019; Kumari *et al*., 2021).

In order to create inflammation and simulate clinical situations like liver damage, formalin is frequently employed in experimental models. Formalin exposure causes notable elevations in liver enzymes in male rats, such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT), which are markers of liver damage. These models have demonstrated a decrease in these enzymes when medicinal herbs with antioxidant qualities are administered, indicating their potential to shield the liver against formalin-induced toxicity and inflammation (Adebayo *et al.,* 2021).

2. Literature Review.

According to Oladeji *et al*. (2020), plants possessing therapeutic properties are categorized as one of the agents utilized in the past to fight illnesses. Numerous experts have noted and documented the roles these medicinal plants play in treating a variety of human ailments. Herbal medicines are regarded as an alternative treatment to achieve wholeness against a number of illnesses, including cancer, fever, liver, gonorrhea, heart, malaria, analgesics, calcium channel blockers, kidney, etc. (Chaudhari *et al*., 2020).

Plants having medicinal potential are widely accepted natural resources that were thought to have less adverse effects. According to Oladeji and Oyebamiji (2020), the widespread acceptance of medications derived from herbs may be attributed to their affordability and ease of access. The entire plant or specific plant parts, such as roots, leaves, bark, flowers, seeds, etc., may be used as medications (Alamgir, 2017). The entire medicinal plant or any portion of it can be applied directly to human skin, inhaled through the nasal canal, or taken orally (Bassey *et al.,* 2022).

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Numerous scholars have focused on the importance of medicinal plants for both individual and societal health care. Drugs made from natural products are also seen as aiding the healthcare system for treatments that are widely accepted. Phytochemicals found in medicinal plants have been shown to be crucial in drug discovery in a number of publications, and many scientists have been interested in their biological efficiency, which is deemed essential (Anand *et al*., 2019).

Due to its potential for medicinal use, *Annona muricata* L. (*A. muricata*), a member of the Annonaceae family, has been extensively researched in recent decades. Due to its bioactivity and traditional usage, this species has garnered attention since the Annonaceae family's therapeutic benefits were first documented a long time ago (Gavamukulya *et al.,* 2021). Worldwide, medicinal plants are regarded as the cornerstone of health treatment and preservation. Treatments for chronic degenerative diseases are crucial since they have reached epidemic proportions and are regarded as major health issues (Sun and Li, 2023).
According to ethnobotanical research, A. muricata has been used as a parasiticide and insecticide. Fruit juice and leaf or branch infusions have also been used to treat fever, malaria, gastrointestinal issues, liver, heart, and kidney affections, and sedative respiratory illnesses (Oyebamiji and Semire, 2022). It has gained popularity recently as a treatment for cancer, hypoglycemia, and hypotension. The available scientific research on A. muricata has been integrated into a variety of papers and reviews, with a focus on acetogenins as the primary bioactive components. Along with a few documented toxicities, further bioactive chemicals have been discovered, more bioactivities have been assessed, and pharmaceutical uses have been expanded (Dar *et al.*, 2023).

3.0 Materials and Methods

3.1 Plant materials and Experimental Animals

Fresh leaves of soursop (*Annona muricata*) (1000 kg) were collected from Heipang, Barkin Ladi Local Government Area, Plateau State. The leaves were authenticated by Dr. Noel Dawam of Biology Unit, Science Department, Plateau State Polythenic, Barkin Ladi. Forty (40) male albino rats weighed (134-185g) were purchased at the College of Health Sciences, Animal House Unit of the College of Medicine, Benue State University, Makurdi, Nigeria. The rats were allowed to acclimatize to the laboratory for 14 days before the study. The animals were given vital feeds and tap water.

3.2 Apparatus and Chemical Drugs Used for the Analysis

3.2.1 Equipment and Apparatus used for the Analysis: The equipment used include; weighing balance, hot plate (Gallenkamp), vacuum pump (CIT-Alcatel Annely, France), rotary evaporator (Büchi), hot air-drying oven (Gallenkamp), heating mantle (Gallenkamp), UV/Vis cabinet (254 and 366 nm) (Lamag UV-Cabinet II, Refrigerator (Thermocool). The apparatus includes; measuring cylinders (1L, 500 ml, 100 ml, 10 ml), glass Pasteur pipettes, micropipette, test tube (15 ml), microcapillary tube, conical flasks, beakers (250, 500 ml), round bottom flasks (various sizes), volumetric flask (100 mL). All glass wares were washed, solvent rinsed and oven dried prior to use.

3.2.2 Chemicals and Drugs used for the Analysis: Methanol, normal saline, distilled water, chloroform, diclofenac sodium, aspirin were purchased from VINCAL Pharmacy, Wadata market, Benue State. All the chemicals and solvents were of standard and analytical grade.

3.3 Location of the Study

This study was carried out at Biochemistry Laboratory, Department of Biochemistry, College of Biological Sciences, Morton Sarwuan Tarka University, Makurdi, Benue State.

3.4 Preparation of Aqueous methanolic Extract of Soursop (*Annona muricata*)

The leaves of soursop (*Annona muricata*) were air dried at room temperature for 30 days. The dried leaves were later subjected to mechanical crushing using mortar and pestle and then to a blender to obtain a fine powdery homogenous mixture which was sieved using sieving machine. 100g 0f the powdered soursop was dissolved in 700 ml of 99.8 % of absolute methanol and 300 ml distilled water (500 g of dried soursop was dissolved in 3500 ml of

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99.8 % of methanol and 1500 ml of distilled water) for 72 hours. The resulting mixture were sieved using a muslin cloth and to a whatman filter paper. The filtrate was evaporated on a water bath at 45°C to get the slurry residue which was used as drug in the experiment.

3.5 Experimental Design for Induction of Inflammation using Rat Model of Formalin-Induced Paw Oedema

Wistar albino rats weighing approximately (160–250 g body weight) were used. Animals were housed and used at least one week after their arrival. Five rats were housed per cage; animals were fed a standard laboratory diet and tap water ad libitum, and kept at 23 ± 1°C with a 12 h light/dark cycle. The animals were used according to standards guidelines of the Committee on Care and Use of Experimental Animal Resources.

Anti-inflammatory potentials of aqueous - methanolic extract of soursop (*Annona muricata*) leaves were measured in rat model of formalin-induced paw oedema. Albino rats fasted overnight were divided into 6 groups of five animals each, the dosage of the drugs administered to the different groups was as follows:

Group I - Normal Control (normal saline)

Group II - Formalin induced/ Test Control (0.1ml/kg b.wt.)

Group III - Formalin + lower dosage 100 mg/kg b.wt. of plant extract

Group IV - Formalin + higher dosage 300 mg/kg b.wt. of plant extract

Group V - Formalin + Standard drug Diclofenac sodium (10 mg/kg b.wt.)

Group VI - Formalin + Standard drug Aspirin (10 mg/kg b.wt.)

Thirty minutes pre oral treatment with extract/drug, following injection of formalin (0.1ml of 10% v/v) into the right hind paw of the tested rats. No injection of formalin into the normal control group animals. The paw thickness was measured before and after induction of inflammation by using vernier calliper. The increase in paw oedema was measured by vernier calliper according to method described by Chan *et al*. 2021; Hunter *et al*. (2000) and Morton *et al.* (2005) with some modifications.

The difference in paw thickness after and before induction of inflammation was calculated and presented as mean increase in paw thickness (cm). The ability of aqueous - methanolic extract of soursop (*Annona muricata*) leaves and the standard drugs (ibuprofen and aspirin) used as anti-inflammatory drugs to suppress paw inflammation was expressed as a percentage of inhibition of paw oedema (Chan *et al*. 2021; Hunter *et al*., 2000 and Morton *et al.*, 2005).

3.6 Collection and Preparation of Serum Sample for Liver Function Test

At the end of 3 days experimental period, rats were sacrificed by cervical dislocation. Blood samples were collected by cardiac puncture into plain serum tubes and allowed to stay for 1 hr. The clotted blood was centrifuged for 10 min at 3000 rpm. The serum was transferred into clean tubes and stored at 4°C until needed for use.

3.6.1 Assay of Aspartate Aminotransferase (AST) Activity

Aspartate aminotransferase (AST) activity was determined based on the method of Li and Frankel (1957) using commercially available kits (Randox laboratories, UK).

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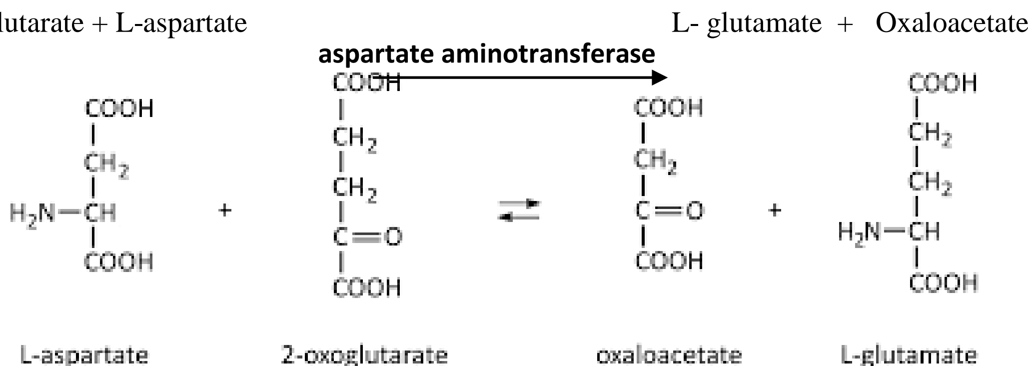
Principle

α-Oxoglutarate + L-aspartate L- glutamate + Oxaloacetate



Aspartate aminotransferase activity was estimated by monitoring the concentration of oxaloacetate hydrazone formed with 2,4-dinitrophenylhydrazine.

Procedure

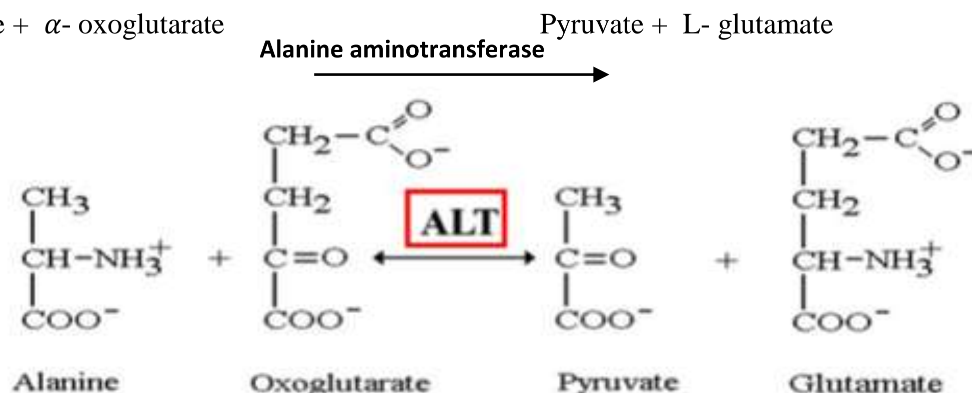
Briefly, 0.1 ml of diluted serum (2 fold dilution with normal saline) was mixed with 0.5 ml of reagent 1, R1 containing 100 mM, phosphate buffer pH 7.4, 100 mM, L-aspartate, 2mM α-oxoglutarate. The reaction mixture was incubated for exactly 30 min at 37°C. Thereafter, 0.5 ml of reagent 2, R2 (2 mM 2, 4-dinitrphenylhydrazine) was added to the reaction mixture and allowed to stand for exactly 20 min at 37°C. Finally, 5.0 ml of NaOH (0.4 M) was added, mixed gently and the absorbance was read against the reagent blank after 5 minutes at 546 nm against reagent blank. Values for AST activity were extrapolated from the standard curve. See appendix.

3.6.2 Assay of Alanine Aminotransferase (ALT) Activity

Alanine aminotransferase (ALT) activity was determined in serum and hepatic homogenate based on the method of Li and Frankel (1957) using commercially available kits (Randox laboratories, UK).

Principle

L- alanine + α- oxoglutarate Pyruvate + L- glutamate



Alanine aminotransferase is measured by monitoring the concentration of pyruvate hydrazone formed with 2,4-dinitrophenylhydrazine (Li and Frankel, 1957). The enzyme alanine aminotransferase catalyzes the transfer of amino group from L-alanine to α- oxoglutarate to form L-glutamate and oxaloacetate. The oxaloacetate formed is unstable and is quantitatively decarboxylated to pyruvate which is then complexed with 2,4-

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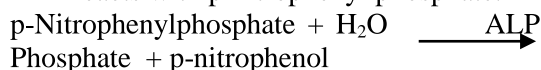
dinitrophenylhydrazine (DNPH) to produce an intensely coloured hydrazone on the addition of NaOH. This coloured complex absorbs radiation at 530-550nm.

Procedure
Briefly, 0.1 ml of diluted serum (2 fold dilution with normal saline) was mixed with 0.5 ml of reagent 1, R1 (100 mM, phosphate buffer pH 7.4, 200 mM, L-alanine, 2 mM α-oxoglutarate). The mixture was incubated for exactly 30 min at 37°C. 0.5 ml of reagent 2, R2 (2 mM 2, 4-dinitrphenylhydrazine) was added to the reaction mixture and allowed to stand for exactly 20 min at 25°C. Thereafter, 5.0 ml of NaOH (0.4 M) was added, mixed gently and the absorbance was read against the reagent blank after 5 minutes at 546 nm. Reagent blank was prepared as described above replacing sample with 0.1 ml of distilled water. Values for AST activity were extrapolated from the standard curve.

3.6.3 Assay of Alkaline Phosphatase (ALP) Activity
Alkaline phosphatase (ALP) activity was determined based on the method of Newman *et al.* (1970) using commercially available kits (Randox laboratories, UK).

Principle
ALP activity was measured by monitoring the concentration of p-nitrophenol formed when ALP reacts with p-nitrophenyl phosphate.



p-Nitrophenylphosphate + H_2O $\xrightarrow{ALP}$ Phosphate + p-nitrophenol

Procedure
Serum (0.02 ml) was added to 1.0 ml of the ALP reagent (buffer and substrate) and mixed. Initial reading was taken at 405nm and the timer started simultaneously. Subsequent readings were taken at 1 minute interval for 3 minutes. The ALP activity was calculated by taking into consideration the change in absorbance per minute
Calculation:
ALP Activity = 2760 x Δ Absorbance 405 nm/min

3.6.4 Determination of

4.1 Anti-inflammatory Activity of Aqueous - Methanolic Extract of Soursop (*Annona*

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Protein Concentration
This was carried out using the Biuret method as described by Weichselbaumin (1946) using a Randox total protein kit (Randox Ltd, UK).

Principle
Proteins form a coloured complex with cupric ions in an alkaline solution.
Reagent composition

1. Biuret reagent (R1): sodium hydroxide (100 mmol/l), sodium-potassium tartrate (16 mmol/l), potassium iodide (15 mmol/l) and copper II sulphate (6 mmol/l).
2. Blank reagent (R2)
3. Standard: a ready for use protein

Procedure
Exactly 1 ml of reagent R1 was added to 0.02 ml of the test sample, the mixture was incubated at 25℃ and the absorbance was read against reagent blank at 546 nm. The protein content of sample was calculated as follows:

$$\text{Protein (mg/ml)} = \frac{\text{Absorbance of sample x concentration of standard}}{\text{Absorbance of standard}}$$

3.7 Statistical analysis
Data were presented as a mean ± standard error of mean of five determinations. Statistical analysis was carried out using one way analysis of variance (ANOVA). Difference were statistically significant at p<0.000.

4.0 Results
4.1 Anti-inflammatory Activity of Aqueous - Methanolic Extract of Soursop (*Annona muricata)* Leaves on Liver Function ParametersinFormalin-Induced Inflammation and Oedema in Male Rats
The anti-inflammatory activity of aqueous methanolic extract of soursop (*Annona muricata*) leaves on liver function parameters in formalin induced inflammation and oedema is presented in Table 4.1. There is a significant difference in the parameters in comparison with the normal and aqueous methanolic extract of soursop (*Annona muricata*) leaves treatedgroups.

***muricata)* Leaves on Liver Function Parameters in Formalin-Induced Inflammation and Oedema in Male Rat**

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Groups (mg/kg)	ALT (IU/L) Mean ± SEM	AST (IU/L) Mean ± SEM	ALP (IU/L) Mean ± SEM	Total Protein (g/dL) Mean ± SEM
Control (Normal saline)	62.07 ± 1.92^{a}	55.20 ± 0.64^{a}	53.50 ± 2.60^{a}	76.87 ± 0.55^{a}
Induced/Formalin	$98.57 \pm 0.88^{e,f}$	103.87 ± 1.66^{f}	139.70 ± 0.91^{f}	52.67 ± 1.29^{e}
I + *Annona muricata* (100 mg/kg)	93.50 ± 1.44^{e}	81.13 ± 1.13^{e}	123.75 ± 1.59^{e}	59.67 ± 0.83^{d}
I + *Annona muricata* (300 mg/kg)	74.70 ± 2.66^{b}	71.63 ± 0.96^{b}	68.90 ± 2.89^{b}	73.43 ± 0.84^{b}
I + Diclofenac sodium (10 mg/kg)	82.87 ± 2.17^{c}	75.53 ± 0.23^{c}	85.30 ± 2.14^{c}	71.37 ± 0.32^{b}
I +Aspirin (10 mg/kg)	$89.65 \pm 0.14^{c,d}$	$78.77 \pm 0.23^{c,d}$	108.50 ± 1.32^{d}	62.03 ± 0.94^{c}

Values are expressed as mean ± standard error of mean (n=5). Values with different superscript(s) in a column are significantly different ($p<0.001$)
Keywords:ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase; ALP: Alkaline Phosphatase; T. Protein- Total Protein

5.0 Discussion and Conclusion

5.1 Discussion

Annona muricata is a medicinal plant that has high therapeutic efficacy index as an antimalarial, antipyretic, analgesic, anti-inflammatory and hypotensive properties. This study was carried out to investigate the anti-inflammatory activity of aqueous - methanolic extract of soursop (*Annona muricata*) leaves on the serum levels of ALP, AST, ALT and Total protein, in formalin-induced inflammation and oedema in right hind paw of wistar rats. Serum or plasma liver enzyme levels are considered as markers for monitoring the degree of chemically induced liver damage (Marghoob *et al*., 2013).

From the present study, significant decrease $p<0.001$ in serum concentration of total protein (52.67 ± 1.29^{e}) (Table 4.1) revealed increased proteinuria observed in the formalin-induced not treated rats compared with normal control (76.87 ± 0.55^{a}). Treatment with aqueous - methanolic extract of soursop (*Annona muricata*) leaves plant extract at the doses of 100 mg/kg (59.67 ± 0.83^{d}) and 300 mg/kg body weight (73.43 ± 0.84^{b}) respectively protected the observed alterations though 300 mg/kg was more effective than 100 mg/kg. Lower dosage of serum concentration of total protein (71.37 ± 0.32^{b}) was also recorded for diclofenac sodium, the standard drug.

The result of the liver function test presented in Table 4.1 below indicates that formalin induction into the right hind paw of the rats led to significant increase at ($p<0.001$) in serum levels of Aspartate aminotransferase (AST) 103.87 ± 1.66^{f}), Alanine aminotransferase (ALT) ($98.57 \pm 0.88^{e,f}$), and Alkaline phosphatase (ALP) (139.70 ± 0.91^{f}) in formalin –induced not treated rats compared to the normal control and treated group rats. These results therefore imply that formalin adversely affected liver function enzymes. The report of Aguwa *et al*. (2016) indicates that rise in serum levels of AST, ALP but especially ALT is a valid indicator of liver damage. Liver enzymes ALT and AST are normally present in normal hepatocytes. These enzymes however, leak out into the circulation when hepatocytes or their cell membranes are damaged as reported by Goldring (2012).

Treatment with aqueous - methanolic extract of soursop (*Annona muricata*) leaves plant extract at the doses of 100 mg/kg and 300 mg/kg body weight respectively has ameliorate the ALT (93.50 ± 1.44^{e} at the dose of 100 mg/kg; 74.70 ± 2.66^{b} at the dose of 300 mg/kg) and AST (81.13 ± 1.13^{e} at dose of 100

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mg/kg; 71.63 ± 0.96^{b} at dose of 300 mg/kg) hepatotoxicity associated with the formalin at these doses though more effective at 300 mg/kg) This result is in accordance with result reported by Eseoghene *et al*. (2022). Also, there was a highly significant reduction in serum AST (75.53 ± 0.23^{c}) and ALT (82.87 ± 2.17^{c}) concentrations ($p<0.001$) in diclofenac sodium treated rats when compared to the formalin-induced not treated rats.

Serum alkaline phosphatase (ALP) is a sensitive detector in diseases characterized by inflammation, regeneration, intrahepatic and extrahepatic bile obstruction (Marghoob *et al*., 2013). The highly significant dramastic reduction of ALP (68.90 ± 2.89^{b}) seen at 300 mg/kg dose aqueous - methanolic extract of soursop (*Annona muricata*) leaves plant extract treated rats showed that the plant extract has the potential to resuscitate the hepatocytes from damage (Eseoghene *et al*., 2022). Lower dosage of ALP (85.30 ± 2.14^{c}) was also recorded for diclofenac sodium, the standard drug.

5.2 Conclusion

Results from the study support the fact that aqueous - methanolic extract of soursop (*Annona muricata*) leaves plant extract have excellent anti-inflammatory activity and compared favourably with the standard drug (diclofenac sodium) in several instances.

5.0 Recommendation

The results from this study support the traditional use of aqueous -methanolic extract of soursop (*Annona muricata)* leaves for treatment inflammation and improved health for liver function enzymes.

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