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Original Article

Xylopia aethiopica Fruit Macerate Inhibits Carrageenan-induced Pleurisy in Rats

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ABSTRACT

Background: Pleurisy is an inflammatory condition of the pleura that can lead to severe morbidity and mortality. Controlling excessive pleural inflammation is crucial. *Xylopia aethiopica*, known for its anti-inflammatory properties, may be a potential treatment. **Objectives:** This study evaluates its immunological mechanisms and toxicological impact. **Materials and Methods:** Wistar rats were pre-treated with either chloroquine or *X. aethiopica* fruit macerate before carrageenan-induced pleurisy. Interleukin-6 (IL-6) and Tumor Necrosis Factor-Alpha (TNF-α) levels were measured in blood and pleural exudate via sandwich ELISA. Leukocyte counts were also assessed. Acute and subacute toxicity profiles of the macerate were evaluated in rats. **Results:** Carrageenan injection induced an inflammatory condition, resulting in increased local and systemic TNF-α and IL-6 levels, with increased leukocyte influx in the pleural cavity. *X. aethiopica* macerate significantly reduced systemic (p=0.0026) and local (p=0.0004) TNF-α levels, comparable to chloroquine. No acute toxic effects were observed within 48 hours or 14 days. A 28-day oral administration did not induce behavioral changes, and biochemical analyses suggested safety at studied doses. However, histological analyses revealed vascular congestion and inflammation in the liver and kidneys. **Conclusion:** *X. aethiopica* macerate exhibits significant anti-inflammatory effects, supporting its potential as a pleurisy treatment. Further research is needed to confirm its clinical applications.

Keywords: Anti-inflammatory activity, Pleurisy, Toxicity, *Xylopia aethiopica* Fruit Macerate.

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INTRODUCTION

Pleurisy is an inflammation of the pleura, the membrane covering the lungs.[1] The pleura is a tissue made up of two layers that slide over each other thanks to a lubricating liquid called pleural fluid. The pleura effusion, due to the increase in the quantity of pleural liquid, can result from two mechanisms: an inflammatory, infectious, or tumorous condition of the pleura; or a disturbance in the secretion/reabsorption balance due to a mechanical anomaly.[2,3]

Carrageenan (Cg) is a seaweed-derived sulfated polysaccharide used to induce local inflammation and evaluate the anti-inflammatory effects of drugs.[4] Indeed, carrageenan injection into the pleural space leads to pleurisy, leucocyte infiltration, and lung injury.[5] Models of carrageenan-induced pleurisy have been widely employed to investigate the pathophysiology of acute inflammation and to evaluate the efficacy of drugs in inflammation.[6] Therefore, carrageenan-induced local pleurisy is a useful model to study mediators involved in inflammatroy processes. Inflammatory conditions induced by carrageenan are dependent on the activation of macrophages through the production of proinflammatory cytokines, especially TNF-α, and IL-6.[7,8]

Xylopia aethiopica (Annonaceae), a tree that grows in the forests of tropical and subtropical Africa, commonly referred to as "African guinea pepper" or "Ethiopian pepper", has been shown to have anti-inflammatory properties in the carrageenan-induced pleurisy model in mice.[9,10] Fruits of *X. aethiopica* are widely available on the markets of Togo as in other West African countries. They are used as traditional medicine as well as spices to flavor food and beverages.[11,12] Extracts, which can be made by decocting dried fruit, are frequently used to treat a variety of gastrointestinal, respiratory, and inflammatory diseases and

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infections, such as malaria and dysentery.[11] Due to their ability to imbalance between reactive oxygen species and antioxidant defenses, *X. aethiopica* extracts have historically been used to treat a variety of medical conditions, including diabetes, asthma, arthritis, and immunological diseases.[13,14] Previous research examined the antioxidant capacity of *X. aethiopica* extracts as food preservatives as well as the effects of these macerates on several animal models of diabetes, radiation, lipids, and cholesterol, as well as inflammation and discomfort.[15,16] In addition, our team recently demonstrated the anti-Th17 and anti-Th2 responses effects of hydro-ethanolic extracts of *X. aethiopica* in hyperreactive onchocerciasis individuals' peripheral blood mononuclear cells.[17] Using a murine model of pleurisy induced by carrageenan, the present study aimed to conduct an in-depth immunological analysis *in vivo* to assess the mechanisms of anti-inflammatory protection provided by *X. aethiopica* fruit macerate and its toxicological impact as an endogenous treatment for pleurisy and lung injuries.

MATERIALS AND METHODS

Study Framework

This experimental study was conducted at the « Unité de Recherche en Immunologie et Immunomodulation » (UR2IM) within the « Laboratoire de Microbiologie et de Contrôle de Qualité des Denrées Alimentaires » (LAMICODA) at the « Ecole Supérieure des Techniques Biologiques *et al*imentaires » (ESTBA), University of Lomé, Togo.

Ethical Consideration

All experiments involving rats were conducted in strict adherence to the guidelines established by the Organization for Economic Co-operation and Development (OECD) for the ethical care and use of laboratory animals.[18,19] The use of the minimum number of animals necessary to ensure the reliability of results was in accordance with the principles outlined in the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, 1985, revised 2011)[20] and followed the 3R (replacement, reduction and refinement) principles. This study was permitted and ethically approved by the institutional ethical board « Comité d'Ethique pour l'Expérimentation Animale (C2EA) » of the LAMICODA under the N° 001/2021/C2EA.

Animals, Samples and Compounds

Male and female Swiss albino (Wistar) rats, with a weight range of 150±250 g and an age of approximately 8-9 months, outbred at the LAMICODA/ESTBA Animal House in Lomé (Togo), were utilized. These animals were housed in standard polypropylene cages, maintained under natural light-dark cycles equivalent to 12±1 h of daylight and night, at a temperature of 30±2°C, and fed on commercial rodent food and water *ad libitum*.

Carrageenan and chloroquine were purchased from Sigma-Aldrich in Darmstadt, Germany, and Tongmei Laboratoire, Lomé-Togo, respectively. The *X. aethiopica* fruit were harvested from the Tchavadè forest in Sokodé, situated in the central region of Togo. All drugs, carrageenan, chloroquine, and the *Xylopia aethiopica* fruit macerate, were freshly prepared, sterilized through a millipore membrane, and administered as previously described.[21,22]

Preparation of *Xylopia aethiopica* Fruit Macerate

The harvested *Xylopia aethiopica* fruits were first washed, followed by air-drying under ambient laboratory conditions until they were adequately desiccated. The dried fruits were subsequently transformed into a fine powder. To produce the macerate, 50 g of this powdered material was mixed with 500 mL of distilled water, i.e 0.1 g/mL. The working concentration (1 mg/mL) was prepared from this stock concentration through dilution. The resulting mixture was then filtered using Whatman filter paper (Thermo Fisher, Waltham, Massachusetts, USA) and stored at 4°C.

Acute Oral Toxicity Profile Assessment of *Xylopia aethiopica* Fruit Macerate

The OECD guideline 403 was used to evaluate acute oral toxicity at a single dose.[18] In brief, after an acclimatization period, rats were subjected to an overnight fast with free access to water; then they were marked, weighed, and divided into two groups: a control group and a test group, each consisting of four male rats. The control group received a single oral dose of normal saline solution. In contrast, the test group received an oral administration of the *X. aethiopica* fruit macerate at a unique dose of 5,000 mg/kg of body weight. Animals were observed individually at 30-min intervals during the first 24 h following treatment, followed by daily observations throughout the 14-day experiment.

Throughout the observation period, toxicological symptoms and signs were carefully documented. This included monitoring alterations in the skin, fur, mucous membranes, eyes, respiratory and circulatory functions, central and autonomic nervous systems, as well as somatomotor activity and overall behavior. Special attention was also given to potential occurrences of seizures, excessive salivation, diarrhea, lethargy, drowsiness, coma and death.[22-24]

Pleurisy Induction in Rats

Pleurisy was induced as described by Vigil de Mello *et al.*, 2016.[25] Briefly, a single Intrapleural (i.pl.) injection of 0.2 mL of sterile saline solution (0.95% NaCl) containing 1% carrageenan (Sigma-Aldrich, Darmstadt, Germany) was performed.

Assessment of Anti-inflammatory Activity of *X. aethiopica* Fruit Macerate

To study anti-inflammatory activity of *X. aethiopica* fruit macerate, 12 Wistar rats were divided into 4 groups of 3 rats each and utilized as follow: i) group n°1 (negative control): rats received an intrapleural injection of 0.2 mL of sterile normal saline into the pleural cavity; ii) group n°2 (positive control): rats were administered an intrapleural injection of 0.2 mL of 1% carrageenan in sterile saline solution into the pleural cavity; iii) group n°3 (chloroquine treatment): rats were treated with chloroquine before pleurisy induction. Specifically, 30 min prior to the intrapleural injection of carrageenan, rats received an intraperitoneal injection of chloroquine at a concentration of 1 mg/mL, adjusted according to their weight (10 mg/kg); iv) group n°4 (*X. aethiopica* fruit macerate treatment): rats were treated with the *X. aethiopica* fruit macerate before pleurisy induction. Similar to group n°3, 30 min before the intrapleural injection of carrageenan, rats received an intraperitoneal injection of the *X. aethiopica* fruit macerate at a concentration of 1 mg/mL, adjusted based on their weight (10 mg/kg). 4 h later, the animals were anesthetized with ether and blood samples were collected from the retro-orbital sinus in EDTA tubes and anticoagulant-free tubes (dry tubes for sera collection after spinning). Subsequently, the animals were euthanized with an overdose of ether. The thoracic cavity was carefully opened, the abundance and aspect of pleural liquid noted, and the pleural cavity was flushed using push-pull movements with 1 mL of Phosphate-Buffered Saline (PBS) containing heparin (20 IU/mL). Finally, the lungs from each rat were harvested.

The extent of inflammation was evaluated through the visual inspection of the lungs to identify signs of edema and redness. Additionally, we measured the amounts of leukocytes and levels of cytokines in both peripheral blood samples and the exudate obtained from flushing the pleural cavity.[26]

Leukocyte Influx Determination

To determine leukocyte influx, 50 µL of the rat pleural cavity washing exudate and 50 µL of peripheral blood were assayed in an automated hematology analyzer (Sysmex KX-21N, Nanshan, Shenzhen, China).

Assessment of Cytokine Levels

Cytokine levels in pleural cavity washing exudate and sera from rats were analyzed by the sandwich ELISA technique using ELISA MAX™ Deluxe Set Mouse TNF-α and ELISA MAX™ Deluxe Set Mouse IL-6 kits (BioLegend, San Diego, CA, USA) following the manufacturer's instructions. Cytokines' concentrations were assessed using a HumaReader HS ELISA plate reader (Human, Wiesbaden, Germany) following the procedure previously described.[17]

Phytochemical Screening of *Xylopia aethiopica* Fruit Macerate

Qualitative phytochemical screening of the *X. aethiopica* fruit macerate was carried out as described by Shaikh and Patil (2020),[27] to determine the presence of some chemical groups such as coumarins, polyphenols, proteins, tannins, reducing sugars, alkaloids, saponins, triterpenes, sterols, and flavonoids.

Sub-acute Toxicity Profile Assessment of *Xylopia aethiopica* Fruit Macerate

The repeated oral dose toxicity study was conducted in accordance with OECD guidelines, method 407,[19] with slight modifications. Briefly, rats were weighed and randomly assigned to two groups, each consisting of three rats. Group 1: Each rat in this group received a daily oral dose of 10 mL/kg of body weight of normal saline solution. Group 2: Rats in this group were administered a daily oral dose of 1 mg/mL/kg of body weight of the *X. aethiopica* fruit macerate. Both normal saline solution and the *X. aethiopica* fruit macerate were administered daily for a duration of 28 days at the same time, while the rats were closely monitored twice a day throughout the experimental period to detect any signs of toxicity or mortality. Daily records of each rat's weight were maintained. Additionally, the rats' behavior, including their levels of aggressiveness, mobility, hunger, and response to sound stimulation, was assessed. After the 28-day treatment period, on the 29th day, following an overnight fast, the body weight of each rat was measured and documented. Subsequently, the rats were anesthetized with ether before blood samples were collected from the retro-orbital sinus for hematological analysis (using EDTA tubes) and biochemical analysis (using anticoagulant-free or dry tubes).

All the rats were sacrificed in accordance with OECD recommendations. The organs, including the heart, lungs, liver, kidneys, and testes, were examined for macroscopic visual characteristics, promptly excised, and weighed. The relative organ weight was calculated using the following formula:

$$\text{Relative organ weight} = \left(\frac{\textit{weight of the organ}}{\textit{bodyweight of the animal on sacrifice day}}\right) \times 100.$$

Organ samples were then fixed in a 10% formaldehyde solution (Central Drug House (Pvt) Ltd., Daryaganj, New Delhi, India) for subsequent histopathological analysis.

Hematological parameters, such as Red Blood Cell (RBC) count, White Blood Cell (WBC) count, Hemoglobin (HGB), Hematocrit (HCT), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC), and Platelet (PLT) count, were measured using the Sysmex KX-21N automated system (Nanshan, Shenzhen, China). Blood smear preparations were stained with May-Grünwald-Giemsa (Cypress Diagnostic,

Langdorp, Belgium) for a differential leukocyte count using optical microscopy, counting 100 cells in each case.

Biochemical parameters, such as glucose, urea, creatinine, Aspartate Aminotransferase (AST), And alanine Aminotransferase (ALT), were assessed using Labkit and Eu-gen kits as per the manufacturers' instructions. The level of each parameter was then determined using the HumaReader HS.

Organ preparation and histological examination

Rats were sacrificed 24 h after the final dose of the macerate. Immediately following dissection, the liver and kidneys were excised, rinsed with tap water, and fixed in 10% formaldehyde (Central Drug House (P) Ltd., Daryaganj, New Delhi, India) for at least 24 h. The organs were then sectioned transversely to create histological cassettes (Bio-Optica Milano Spa via San Faustino, Milano - Italy), which underwent a dehydration process using an ascending series of alcohol concentrations (Central Drug House (P) Ltd., Daryaganj, New Delhi, India). Subsequently, the tissues were embedded in paraffin (Central Drug House (P), Daryaganj, New Delhi, India).

Thin sections, 0.5 µm thick, were obtained using the HM 340 E semi-automatic rotary microtome. These sections were mounted on slides and stained with hematoxylin and eosin (HandE) (Bio-Optica Milano Spa via San Faustino, Milano-Italy). The slides were then covered with coverslips secured with a few drops of biological glue (Kaltek SRL, Via Liguria, Italy). Microscopic examination of the slides was conducted using a Euromex bscope trinocular optical microscope equipped with a Leica camera, at magnifications of X10 and X40.

Statistical Analysis

GraphPad Prism 5.02 (GraphPad Software, Inc., La Jolla, USA) was used for statistical analyses. Given that the variables exhibited a non-parametric distribution following testing with the Kolmogorov-Smirnov test, the Kruskal-Wallis was applied for the comparison of groups and when significant the Duns Post Hoc test was applied for the comparison between the different groups. A p-value ≤0.05 was considered statistically significant.

RESULTS

No Mortality or Adverse Effects were Observed in the Acute Oral Toxicity Test

In the acute oral toxicity test, the administration of a limit test dose of 5,000 mg/kg body weight did not result in any fatalities or acute toxic reactions in the three treated rats, both during a short observation period of 48 hrs and a longer 14-day observation period. Our assessment considered a range of indicators, including manifestations of tremors, convulsions, diarrhea, changes in skin and hair, salivation, sleep, coma, and death. Based on this test, the Maximum Tolerated Dose (MTD) for the *X. aethiopica* fruit macerate was determined to be 5,000 mg/kg body weight.

Xylopia aethiopica Fruit Macerate reduced Inflammation in rats' lungs

To evaluate observable indicators of inflammation, specifically edema and redness, the rats' lung tissues were examined through macroscopic observation. The results indicated that rats receiving an intrapleural injection of normal saline solution (used as a negative control) exhibited no signs of redness and edema, presenting as having smooth lungs (Figure 1A). In contrast, rats receiving only a 1% carrageenan intrapleural injection (used as a positive control) displayed reddish lungs with visible edema or ulcerations, indicating pleural inflammation (Figure 1B). As anticipated, rats that received intraperitoneal chloroquine before pleurisy induction by 1% carrageenan exhibited lungs devoid of redness and edema, suggesting a potential inhibition of pleura inflammation by chloroquine, which served as the reference drug (Figure 1C). This was in contrast to rats that received only the 1% carrageenan injection, where the aforementioned signs of inflammation were evident. Similarly, rats previously treated with intraperitoneal *Xylopia aethiopica* fruit macerate before pleurisy induction exhibited lung tissues showed no redness or edema, as depicted in Figure 1D and Table 1.

Low Leukocyte Counts in Rats Treated with *Xylopia aethiopica* Fruit Macerate

To investigate the local influx of leukocytes and systemic leukocyte variation, we performed a White Blood Cell (WBC) count on the peripheral blood and pleural cavity washing exudate in all groups of rats. In general, no significant differences were observed in leukocyte counts in peripheral blood between rats given 1% carrageenan (positive control) and those given normal saline (negative control). The same pattern was observed in rats previously treated with chloroquine or the *X. aethiopica* macerate before inducing pleurisy with 1% carrageenan. However, the number of white blood cells was lower compared to that observed in rats receiving only intrapleural 1% carrageenan (Figure 2A). On the other hand, a substantial increase in leukocyte influx was observed in the pleural cavity washing exudate across all groups of rats (Figure 2B). However, in rats that received combinations of chloroquine and 1% carrageenan or the *X. aethiopica* macerate and 1% carrageenan, there was a reduced influx of white blood cells, though this difference was not statistically significant compared to rats that received 1% carrageenan alone.

Decreased TNF-α but increased IL-6 levels in Rats treated with the Macerate

We assessed the anti-inflammatory properties of the *X. aethiopica* fruit macerate by measuring the levels of pro-inflammatory mediators, namely IL-6 and TNF-α, in both the peripheral blood a statiscally significant and pleural cavity washing exudate of rats. In general, there was statistically significant down-regulation of TNF-α in the group of rats that received the *X. aethiopica* fruit macerate before pleurisy induction with 1% carrageenan, both in

peripheral blood (p=0.0026) and pleural cavity washing exudate (p=0.0004), compared to rats treated with 1% carrageenan alone (Figures 3A and 3B). Conversely, the production of IL-6 showed a significant increase locally (p=0.020), but not systemically (p=0.6065), in rats treated with *X. aethiopica* macerate prior to pleurisy induction, compared to those receiving only intrapleural 1% carrageenan, as illustrated in Figures 3C and 3D. As expected, the combination of chloroquine and 1% carrageenan significantly abrogated IL-6 and TNF-α production in both peripheral blood and pleural cavity washing exudate, compared to rats treated with 1% carrageenan alone. Notably, the TNF-α inhibition in the chloroquine group was comparable to that of the *X. aethiopica* macerate (Figure 3).

Phytochemical Composition of the Macerate

An assessment of the qualitative phytochemical components in the *X. aethiopica* fruit macerate revealed the presence of alkaloids, tannins, flavonoids, triterpenes, sterols, reducing sugars, and coumarins. These findings are summarized in Table 2.

No adverse effects were detected in the Subacute Oral Toxicity Assessment

Throughout the 28-day period during which the rats were orally administered the *X. aethiopica* fruit macerate, no alterations in their behavior were observed. Importantly, there were no deaths or adverse reactions noted during the experiment. Moreover, the administration of the *X. aethiopica* fruit macerate had no significant impact on the average weight of the rats after the 28-day treatment period, as detailed in Table 3.

Furthermore, a comparison of the relative organ weights of the rats did not reveal significant differences between the test and control groups, as shown in Table 4.

With the exception of a significant increase in the Mean Corpuscular Hemoglobin (MCH) levels (p=0.01), there were no significant changes in hematological parameters following 28 days of administration of the *Xylopia aethiopica* fruit macerate, as detailed in Table 5.

In terms of biochemical parameters, although the group of rats treated with the *Xylopia aethiopica* fruit macerate showed elevated levels of Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST), these differences were not statistically significant compared to those treated with sterile normal saline solution. However, creatinine levels demonstrated a significant increase when compared to the saline-treated group (p=0.05), while urea and glucose concentrations remained consistent, as shown in Table 6.

Lastly, gross examination of the organs in both the test and control groups revealed no abnormalities. No signs of visible inflammation, internal bleeding, lesions, or deformities were evident in the liver, kidneys, testis, and heart of the treated rats in comparison to the control groups following 28 days of treatment with the *X. aethiopica* fruit macerate (data not shown).

Table 1: Score of observable signs of inflammation.

Parameter		Groups			
		Normal saline	Carrageenan 1%	Chloroquine+carrageenan 1%	*Xylopia aethiopica* Fruit Macerate+carrageenan 1%
Pleural cavity exudate	Volume	+	+++	+	+
	Aspect/Appearance	-	+++	+	+
Lung examination	Edema	0	3	0	0
	Redness	0	3	0	0
	Ulcerations	0	3	0	0
	Inflammation	0	3	0	0

(-)=not abundant/normal; (+)=mildly abundant/slightly abnormal; (++)=moderately abundant/moderately abnormal; (+++)=very abundant/severely abnormal. 0=No signs of inflammation, edema, redness, or ulcerations; 1=Mild inflammation, edema, redness, or ulcerations; 2=Moderate inflammation, edema, redness, or ulcerations; 3=Severe inflammation, edema, redness, or ulcerations.

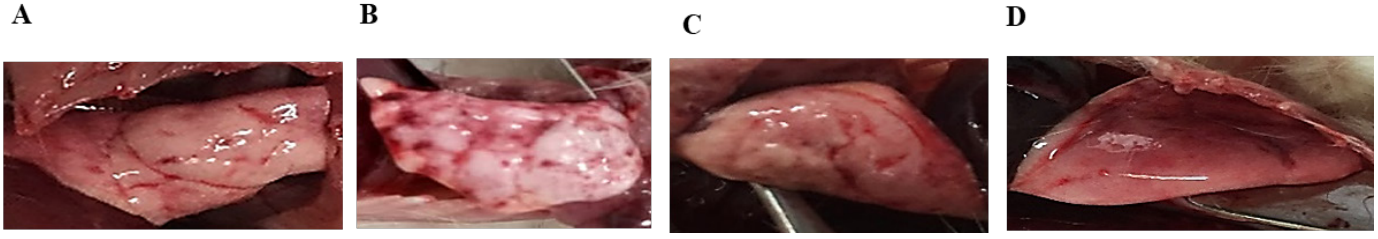

Figure 1: Photographs showing the effects of the fruit macerate of *Xylopia aethiopica* on rats' lungs: (A) normal saline solution; (B) carrageenan 1%; (C) chloroquine+carrageenan 1%; (D) *Xylopia aethiopica*+carrageenan 1%.

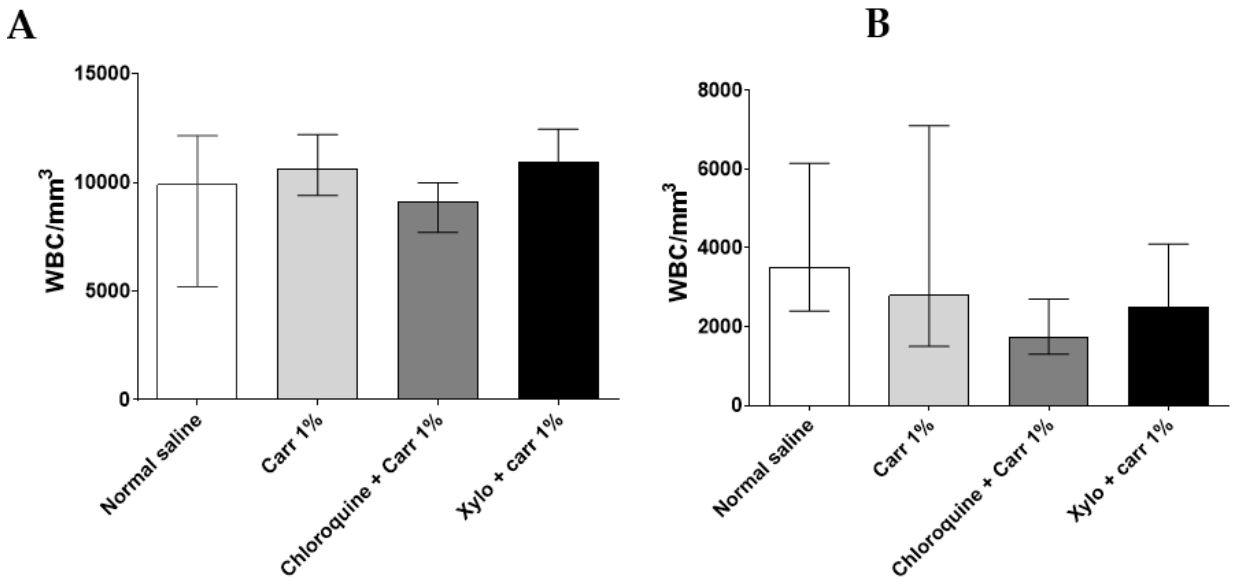

Figure 2: White Blood Cells (WBC) counts in peripheral blood and pleural cavity washing exudate. Systemic (A) and pleural (B) WBC counts are represented by boxes. Data are expressed as median with interquartile ranges. WBC=white blood cells; Carr 1%=carrageenan 1%; Xylo=*Xylopia aethiopica* Fruit Macerate.

Histological Evaluation of the Liver and Kidneys

Liver Histology

Representative pictures of liver sections from all treatment groups are presented in Figure 4. In the normal untreated control group and rats treated with normal saline, histological analysis revealed congestion in the portal vein located within the portal space (Figures 4A and 4D), as well as in the centrilobular vein and hepatocyte trabeculae separated by sinusoids (Figures 4B and 4E). The hepatocytes appeared normal and unremarkable (Figures 4C and 4F). In the group treated with *X. aethiopica* fruit macerate, similar congestion was observed in both the portal vein (Figure 4G) and the centrilobular vein. Additionally, periportal inflammatory infiltration by lymphoplasmacytes was evident (Figure 4H), though the hepatocytes remained normal (Figure 4I).

Kidney Histology

Figure 5 displays the histological observations in the kidney across different groups. Figures 5A and 5B show kidney sections from the untreated control group, displaying a normal glomerulus (Figure 5A) and renal tubules (Figure 5B), both separated by the interstitial space of the capsule with intact epithelial cells. Similarly, Figures 5C and 5D, representing kidneys from rats treated with normal saline, demonstrate a normal glomerulus (Figure 5C) and renal tubules (Figure 5D). In rats treated with *X. aethiopica* fruit macerate, Figure 5E shows a normal glomerulus, renal tubules, and epithelial cells, all separated by the interstitial space, but with evident vascular congestion (Figures 5E and 5F).

DISCUSSION

Pleurisy is characterized by an inflammation of the pleura, and excessive inflammation can lead to severe complications.[2,3] This study evaluated the in-depth immunological mechanisms of anti-inflammatory protection of *X. aethiopica* fruit macerate and its toxicological impact. Toxicity assessment of a therapeutic substance, particularly when used in traditional practices, is necessary to ensure its safe utilization without the risk of harm. This study began with an evaluation of the acute toxicity of *X. aethiopica* fruit macerates in Wistar albino rats. Interestingly, no abnormal behavior or mortality was observed over the 14-day period following the oral administration of a single dose of 5,000 mg/kg, indicating that the macerate's Lethal Dose (LD_{50}) exceeds this threshold. These findings are consistent with earlier reports by Ataba *et al.* (2020), which also demonstrated the safety of *X. aethiopica* extracts in acute toxicity models.[22] Furthermore, according to Diezi, substances with an LD_{50} ranging between 50 and 500 mg/kg body weight are considered toxic, whereas those with an LD_{50} above 5,000 mg/kg are deemed practically non-toxic.[28] Consequently, *Xylopia aethiopica* fruit macerate is classified as non-toxic when administered orally. Building on this foundational toxicity data, we proceeded to investigate the immunological mechanisms of anti-inflammatory protection of *X. aethiopica* using a murine model of pleurisy induced by carrageenan. The carrageenan-induced murine model of pleurisy is a standard and practical model, widely used for the evaluation of the anti-inflammatory properties of different new anti-inflammatory agents, particularly substances derived from natural plants. Carrageenan is a mucopolysaccharide that induces maximum edema from the 3rd h after injection.[29,30] It is already known how carrageenan triggers the inflammatory process at the

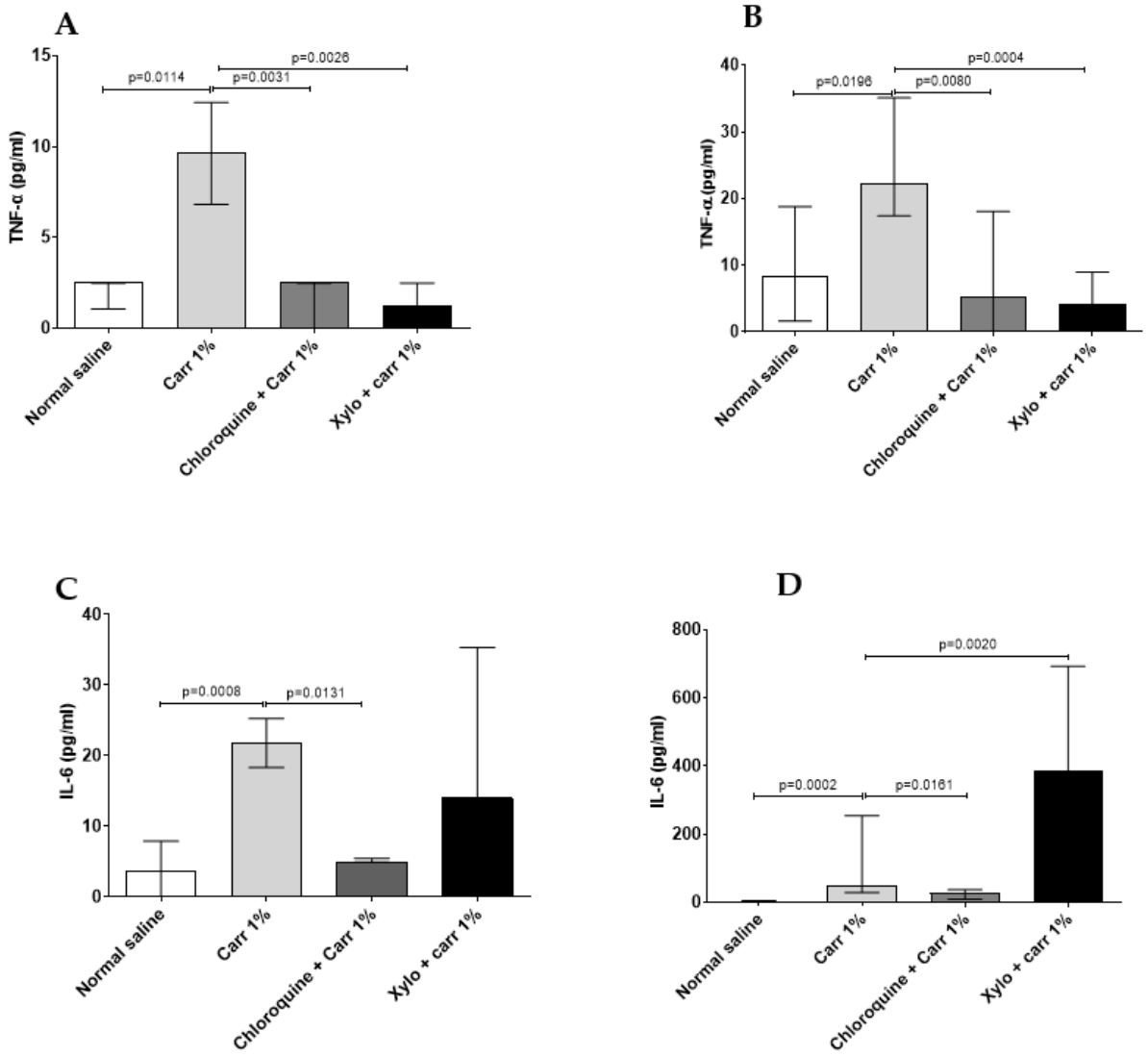

Figure 3: Levels of TNF-α and IL-6 in peripheral blood and pleural cavity washing exudate. Systemic (A) and pleural (B) concentrations of TNF-α and systemic (C) and pleural (D) concentrations of IL-6 are represented by boxes. Data are expressed as median with interquartile range. Statistical differences (Kruskal-Wallis test with Duns Post Hoc test) between 2 batches are shown in lines. *p*-value ≤ 0.05 is considered as significant. Carr 1%=carrageenan 1%, Xylo=*Xylopia aethiopica* Fruit Macerate.

cellular and molecular levels. In fact, carrageenan causes mast cells to release histamine and serotonin, which initiates a cascade of events that results in the production of other mediators that help to establish the acute inflammatory response.[29] This model has the distinct benefit of an early phase (4 h after carrageenan administration) that is characterized by a markedly increased leukocyte infiltration into the pleural cavity consequent to neutrophil influx.[25,26,30] Indeed, carrageenan induces during the early phase (1-2 hr) of the inflammatory reaction, the production of pro-inflammatory factors such as histamine, serotonin, leukotrienes, Platelet-Activating Factor (PAF) and prostanoids that cause vascular changes and result in plasma exudation. During the late phase of this inflammatory process (4-12 hr), these chemo-attractants induce neutrophil recruitment by chemotaxis to the inflammatory site, where they release their cytotoxic arsenal and other inflammatory mediators.[31-33]

Pleurisy has been identified as a significant risk factor for the severity and mortality of certain respiratory infections including COVID-19, as demonstrated by scientific findings.[34-37] Evidence indicates that during such infections, the excess production of early response pro-inflammatory cytokines such as Tumor Necrosis Factor Alpha (TNF-α), Interleukin (IL)-6, and IL-1β results in what has been dubbed a "cytokine storm". This condition leads to an increased risk of vascular hyperpermeability, multi-organ failure (especially in the lungs) and ultimately death when the high cytokine concentrations are unabated over time.[34-37] Therefore, effective and earlier regulation of the cytokine storm through therapies such as immune modulators, immune suppressants, and cytokine inhibitors is essential to reduce mortality rates in severely affected patients. However, these treatment approaches must be balanced with maintaining a sufficient inflammatory response for pathogen clearance.[37-40] Macroscopic observation of edema,

redness on lungs, levels of TNF-α and IL-6 pro-inflammatory cytokines in the pleural washing exudate and peripheral blood, and leukocytes infiltration into the pleural cavity of rats during the first 4 h after carrageenan injection were exploited in the present study. Our findings indicate that the intraperitoneal administration of *X. aethiopica* fruit macerate in rats significantly attenuated the development of pleurisy, evidenced by reduced exudate volume, lower systemic and pleural TNF-α levels, and diminished lung injury. Several investigations have looked at the anti-inflammatory activity of *X. aethiopica* fruit macerate and their main components, using carrageenan-induced paw oedema, xylene or croton oil-induced ear oedema, cotton wool-induced granuloma and air pocket-induced oedema. However, only a few studies have employed our model to assess the anti-inflammatory properties of *X. aethiopica*.[9,41,42] Notably, it has been demonstrated that *X. aethiopica* fruit macerates can reduce mast cell-dependent acute allergic reactions. This is achieved through anti-inflammatory effects, preventing mast cells from releasing histamine by stabilizing the cell membrane.[41] Previous studies from our group have also highlighted the traditional use of *X. aethiopica* in treating inflammation. For instance, Ataba *et al.*, conducted a study in 2020 illustrating the plant's anti-inflammatory properties by inhibiting the activity of Cyclooxygenase-2 (COX-2).[22] Similarly, in 2022, Katawa *et al.* demonstrated the anti-inflammatory activity, particularly the anti-Th17 and anti-Th2 response effects, of three traditionally used plants, including *X. aethiopica*, in hyperreactive onchocerciasis, a condition characterized by severe skin inflammation with elevated Th17-Th2 combined responses.[17]

The mode of action of SARS-CoV-2 includes hyper-inflammation, characterized by a fulminant and fatal hypokinemia leading to multi-organ failure, immunosuppression, reduction of ACE2 resulting in increased pulmonary vascular permeability and damage of the alveoli, and activation of ORF3a, ORF3b, and ORF7a via c-Jun N-Terminal Kinase (JNK) pathway, ultimately causing lung damage.[43-47] A recent study demonstrated that an aptamer targeting TNF-α can prevent TNF-α-mediated acute lung injury.[48] Given that acute lung injury is considered a hallmark feature of certain respiratory infections, our study then provides a potential therapeutic strategy for treating the cytokine storm accompanied by acute lung injury. These findings align with observations in rat lungs which showed inhibition of edema and redness, suggesting a potential abrogation of the carrageenan-induced pleurisy in rats.[49] In addition to inhibiting

Table 2: Phytochemical screening of *X. aethiopica* fruits macerate.

Phytochemical group	Result
Alkaloids	+
Tannins	+
Flavonoids	+
Triterpenes and sterols	+
Reducing sugars	+
Coumarins	+
Saponins	-
Proteins	-
Total polyphenols	-

(+) Present (-) absent.

Table 3: Effect of *X. aethiopica* fruit macerate on rats' bodyweight after twenty-eight days of experiment. Each value is a mean weight (g)±SD. The number of rats per group, *n*=3. The body weight of each animal was daily recorded. SD=Standard Deviation.

Days	Normal saline	*Xylopia aethiopica* Fruit Macerate (1 mg/mL)	*p*-value
0	177.67±50.05	155.67±24.00	0.53
1	175.67±52.59	153.33±21.57	0.53
7	175.00±46.00	147.33±20.55	0.40
14	175.33±47.01	155.00±19.00	0.53
21	180.00±47.57	156.67±19.85	0.47
28	185.00±50.11	153.33±25.01	0.38

Table 4: Effect of *X. aethiopica* fruit macerate on organs relative body weight after twenty-eight days of experiment. Each value is a mean weight (g)±SD. The number of rats per group, *n*=3. SD=standard deviation.

Organ	Normal saline	*Xylopia aethiopica* Fruit Macerate at 1 mg/mL	*p*-value
Liver	3.29±00.28	3.12±0.26	0.49
Lungs	1.00±0.20	1.25±0.16	0.17
Heart	0.55±0.14	0.45±0.16	0.50
Kidneys	0.69±0.10	0.75±0.13	0.52
Testis	1.33±0.27	1.20±0.12	0.50

Table 5: Effect of the *Xylopia aethiopica* fruit macerate on hematological parameters after twenty-eight days of experiment.

Parameters	Normal saline	*Xylopia aethiopica* Fruit Macerate (1 mg/mL)	*p*-value
WBC (x 10^3/µL)	13.83±3.23	9.50±2.22	0.13
RBC (x 10^6/µL)	9.10±0.46	9.60±0.50	0.28
HGB (g/dL)	13.86±0.80	13.90±0.52	0.96
HCT (%)	49.00±3.27	49.03±2.80	0.99
MCV (fL)	53.83±1.68	51.03±1.17	0.78
MCH (pg)	15.26±0.15	15.50±0.20	0.01*
MCHC (g/dL)	28.30±0.62	28.33±0.86	0.96
PLT (x 10^5/µL)	674.00±200.08	419.67±273.55	0.27

Each value is a mean±SD. The number of rats per group (*n*)=3. Hematological tests were performed at the end of the experiment. WBC, white blood cell; RBC, red blood cell; HCT, hematocrit; HGB, hemoglobin; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PLT, platelet. SD=standard deviation.

Table 6: Effect of *Xylopia aethiopica* fruit macerate on biochemical parameters after twenty-eight days of experiment.

Parameters	Normal saline	*Xylopia aethiopica* Fruit Macerate (1 mg/mL)	*p*-value
Urea (mg/dL)	66.67±14.43	58.33±14.43	0.52
Creatinine (mg/dL)	1.52±0.32	2.16±0.26	0.05*
Glucose (mmol/L)	5.63±1.55	5.74±1.42	0.93
AST (IU/L)	1520.18±299.58	2867.22±931.32	0.07
ALT (IU/L)	105.5±10.67	183.62±58.19	0.08

Each value is a mean±SD. The number of rats per group (*n*)=3. ALT, alanine aminotransferase; AST, aspartate aminotransferase. SD=standard deviation.

the production of the pro-inflammatory mediator TNF-α, the fruit macerate of *X. aethiopica* also inhibits the recruitment of leucocytes to the pleural cavity, albeit not significant. This inhibition may occur through the suppression of adhesion molecule expression on the vein wall of endothelial cells.[50] The lack of significant differences in leukocyte reduction in the pleural cavity of rats treated intraperitoneally with *X. aethiopica* fruit macerate before pleurisy induction could be attributed to the relatively short period (4 hr) of the post-carrageenan injection for the macerate to exert its anti-inflammatory activity. Taken together, these findings suggest that *X. aethiopica* fruit macerate may exert its anti-pleural effect by reducing the production of inflammatory mediator such as TNF-α involved in the stages of the acute inflammatory reaction induced by carrageenan and possibly inhibiting leucocyte recruitment to the pleural cavity through anti-chemoattractant effects.

We then argued that the observed biological characteristics might be influenced by specific chemical groups active in *X. aethiopica* fruit macerate. Our qualitative phytochemical analyses identified distinct chemical groups in the fruit macerate, encompassing coumarins, polyphenols, proteins, tannins, reducing sugars, alkaloids, saponins, triterpenes, sterols, and flavonoids. These findings align with previous studies demonstrating the anti-inflammatory properties of these chemical. For instance, polyphenols and coumarins have been reported for their direct antiviral action and their anti-inflammatory activity, inhibiting COX and Lipoxygenase (LOX), reducing oedema formation, stimulating phagocytosis and inhibiting Inducible Nitric Oxide Synthase (iNOS).[51-53] According to Kim *et al.*, gallic acid (a phenolic acid) and its derivatives are known to inhibit p38 Mitogen Activated Protein Kinases (MAPK) activation, and the inhibition of nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) binding, essential for the expression of pro-inflammatory cytokines such as histamine, TNF-α and IL-6.[54] Additionally, tannins, recognized for inhibiting phospholipase A2, play a role in prostaglandin and leukotriene inhibition.[55-57] Saponins, known for angiogenetic activities, exhibit anti-inflammatory and antiviral properties.[58] Triterpenes modify immune responses, inhibiting pro-inflammatory mediators such as nitric oxide, TNF-α, IL-6, and COX-2 and reducing edema in carrageenan-induced rats.[59] Similarly, plant alkaloids, a significant compound family, possess anti-inflammatory activity, decreasing the development of pro-inflammatory factors, including cytokines, lipid mediators, histamine, and inflammatory response enzymes.[60] The anti-inflammatory effect of plant sterols and flavonoids has also been documented. Indeed, flavonoids inhibit leukocytes migration by blocking their adhesion to the vascular wall.[61,62] This effect is thought to be due to the inhibition of the synthesis of IL-1 and TNF-α, the main inducers of the expression of adhesive molecules on the vascular wall.[63] TNF-α is known to direct the inflammatory response and serve as a signaling

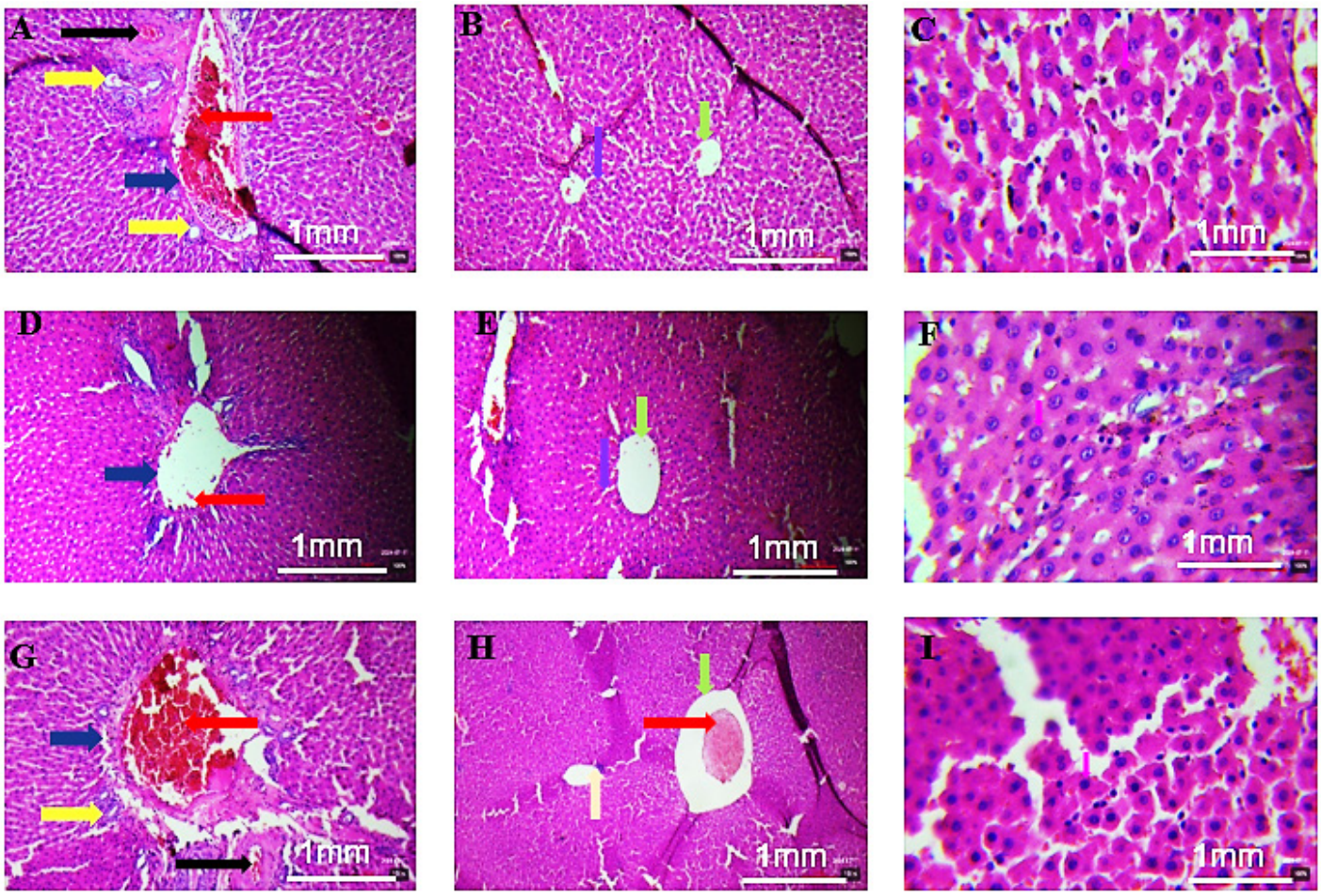

Figure 4: Representative pictures of liver sections from both untreated and treated rats are shown, highlighting key anatomical features: portal vein (dark blue arrow), hepatic artery (black arrow), bile ducts (yellow arrow), centrilobular vein (green arrow), sinusoids (light blue arrow), hepatocytes (pink arrow), congestion (bright red arrow), and infiltration of inflammatory cells (orange arrow). Batch 1: normal untreated control group (A-C). Batch 2: normal saline-treated control group (D-F). Batch 3: *X. aethiopica*-treated group (G-I). Liver sections were stained with hematoxylin and eosin (H and E). Images A, B, D, E, G, H, and I were captured at X100 magnification, while images C, F, and I were taken at X400 magnification.

molecule for immune cells. In this study, the identified chemical groups in *X. aethiopica* fruit macerate appear to contribute to its anti-inflammatory properties, potentially by strongly inhibiting TNF-α production. Nevertheless, further fractionation studies are essential to ascertain the precise fractions responsible for the pharmacological properties of the fruit macerate and elucidate their modes of action.

After evaluating both the acute toxicity and anti-inflammatory effects, we extended our investigation to a 28-day oral toxicity assessment to ensure the safety of prolonged administration of *X. aethiopica* fruit macerate. The sub-acute toxicity for 28 days was carried out with the selected dosage for rat administration based on the outcomes of the acute toxicity test and *in vivo* anti-inflammatory activity assessments. Administering *X. aethiopica* fruit macerate at 1 mg/mL body weight did not induce any visible harmful reactions leading to mortality or alterations in the behavior of the treated animals. Body weight loss serves as a sensitive and simple indicator of toxicity, and in this case, there was no significant difference in the body weight of the tested rats after 28 days of treatment compared to the control group. Similar results were reported by Ataba *et al.*, in 2020,[22] suggesting that the absence of body weight changes could be attributed to the animals' normal physiological adaptation to *X. aethiopica* fruit macerate. This also implies that the macerate at 1 mg/kg body weight does not exert stimulatory or inhibitory effects on the animals' appetite, thereby avoiding alterations in nutritional intake and subsequent weight gain or loss. In contrast, Pieme *et al.*, observed an increase in body weight in rats after 26 days of administering the aqueous macerate of *Senna alata*, indicating the appetite-stimulating effects of their macerate.[64] Importantly, at the end of our experiment, the relative weights of removed organs, including the liver, lungs, heart, kidneys, and testis, exhibited no significant differences between the treated and control groups suggesting that the 28-day oral treatment with *X. aethiopica* fruit macerate at 1 mg/kg body weight did not result in any organ hypertrophy.

Biochemical and hematological parameters serve as reliable indicators of various organ activities, and fluctuations in these

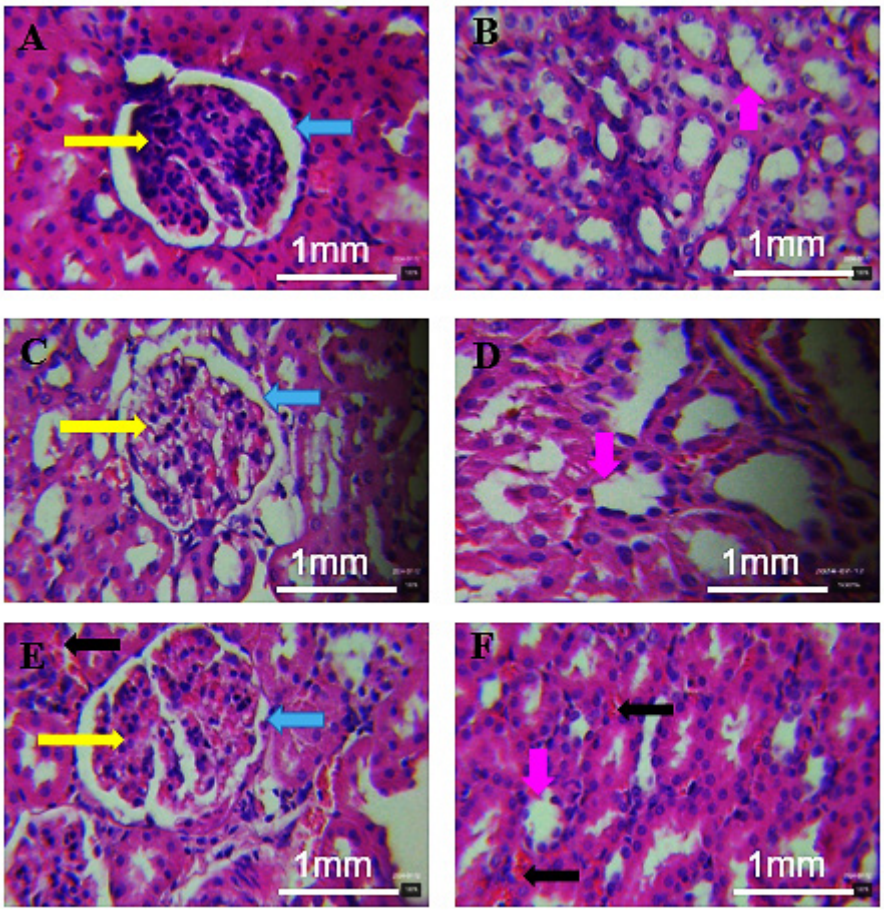

Figure 5: Representative pictures of cross-sections of kidneys from rats stained with HandE (magnification X100). Batch 1: normal untreated control group (A and B). Batch 2: normal saline-treated control group (C and D). Batch 3: *X. aethiopica*-treated group (E and F). Key structures such as glomerulus (yellow arrow), Bowman's capsule (blue arrow), vascular congestion (black arrow) and renal tubules (pink arrow) are highlighted.

factors can indicate oragan-related differences. In our current study, we investigated hepato-renal toxicity by assessing biochemical markers indicative of liver damage, including transaminases such as alanine Aminotransferase (ALT) and aspartate Aminotransferase (AST).[65-67] ALT is a cytosolic, liver-specific enzyme which is released into the bloodstream in cases of hepatocyte necrosis, making it a crucial and highly sensitive indicator of hepatotoxicity.[68,69] Additionally, AST, while also present in damaged liver cells, is found in the heart, kidney, lung, and skeletal muscle.[68] Given that AST and ALT are considered leakage enzymes located in hepatocytes, cytoplasm, and mitochondria (for ALT), an elevation in their levels in the blood serum may indicate alterations in hepatocellular or mitochondrial membranes resulting from liver injury due to conditions like hepatic cell necrosis, hepatitis, cirrhosis, or hepatotoxicity from specific drugs.[68,69] Our study showed no significant differences in AST and ALT levels between the *X. aethiopica*-treated group and the control group, suggesting normal liver function at the administered dose. This contradicts findings from Obodo *et al*, who reported hepatotoxic effects after 21 days of oral treatment of *X. aethiopica* leaf macerates.[70] We also evaluated urea and creatinine levels, excellent markers for evaluating kidney function. While there were no significant changes in urea levels, creatinine showed significant variations, indicating some degree of renal stress. Histological analysis further confirmed this, revealing kidney vascular congestion in the *X. aethiopica*-treated group though the capsular spaces, glomerulus, renal tubules, and epithelial cells remained intact.

Liver histology showed no significant pathological changes in control rats, whether untreated or treated with normal saline. However, rats treated with *X. aethiopica* macerate displayed periportal inflammatory infiltration. This inflammation, likely driven by bioactive compounds such as tannins, alkaloids, and flavonoids, suggests early signs of hepatic distress that could escalate with prolonged exposure.[71-73] The lack of significant changes in AST and ALT levels implies that liver function may remain intact at the dose tested, though the histological evidence points to potential early-stage liver stress. Similarly, the kidneys of *X. aethiopica*-treated rats exhibited vascular congestion in some renal corpuscles, despite preserved capsular

spaces, glomerulus, renal tubules, and epithelial cells. While the renal architecture was not entirely compromised, the observed changes indicate that the macerate may induce nephrotoxic effects with continued exposure. Although serum creatinine showed significant variations, its levels were not elevated to a degree that would indicate immediate renal failure. This suggests that kidney function was largely preserved despite the structural alterations. Nonetheless, the histological findings emphasize the need for caution in dosing and highlight the importance of long-term studies to fully understand the potential renal effects of *X. aethiopica*.

When a toxin reaches the systemic circulation, blood cells are the initial cells exposed to it, providing a reliable indication of the molecule's potential toxicity.[66] Our study revealed a significant increase in Mean Corpuscular Hemoglobin (MCH) levels (p=0.01) after 28 days of administering *X. aethiopica* fruit macerate at 1 mg/kg body weight, while other hematological parameters showed no significant changes. The most common reason for high MCH is macrocytic anemia, which is a blood disorder in which the body fails to produce enough red blood cells. In macrocytic anemia, red blood cells that are produced are larger than usual, each carrying more hemoglobin than normal-sized cells would. Since White Blood Cells (WBC) play a protective role against infections and platelets safeguard the vascular endothelium from free radical damage, the observed low levels of WBC and platelets induced by *Xylopia aethiopica* fruit macerate, though not statistically significant, suggest a lack of a stress response. This may imply that *Xylopia aethiopica* fruit macerate modulates the immune system through the regulation of various cytokines. A recent study by Ogbuagu *et al.*, demonstrated that an ethanolic extract of *Xylopia aethiopica* fruits induced oxidative stress in Wistar rats, with the potential to suppress the immune system.[74] Although the mechanism by which *Xylopia aethiopica* fruit macerate reduced white blood cell values is unclear, it may be linked to the presence of Xylopic acid.[75] The non-significant lower platelet counts in *Xylopia aethiopica* fruit-treated animals compared to the control group may suggest inhibition of Platelet-Activating Factor (PAF) activity and a potential effect on blood clotting or down-regulation of thrombopoietin production.[76,77] These findings collectively suggest that active ingredients in *Xylopia aethiopica* fruit macerate could potentially contribute to the observed induced oxidative stress, consequently impacting the immune system by reducing white blood cells and platelets.

While this study provides valuable insights, it has limitations and therefore suggests possible future approaches to be explored. One notable limitation is the relatively small number of rats used in the subacute toxicity assessment due to financial constraints. The sample size in toxicity studies is crucial for drawing robust conclusions about the safety profile of a substance. The use of a limited number of rats may impact the generalizability of the toxicity results and limits the ability to detect rare adverse effects. Another noteworthy constraint is the absence of female rats in the subacute toxicity study, also attributed to financial constraints. This introduces a potential gender bias, as responses to treatments can vary between male and female subjects due to hormonal differences. Moreover, the study utilized only one dose in the subacute toxicity assessment, whereas a more comprehensive understanding of dose-response relationships would require the evaluation of multiple doses. This single-dose approach may not capture potential dose-dependent effects and could impact the accuracy of risk assessment. Furthermore, histological examinations on lung sections following carrageenan challenge would have demonstrated if the fruit macerate of *X. aethiopica* is able to maintain normal alveolar architecture with decreased influx of neutrophils and edema formation. A broader histological examination of all organs could reveal structural anomalies, while an electrolytes panel evaluation would provide more information on renal function and provide a more comprehensive understanding of the plant's safety profile. Finally, further fractionation studies are necessary to determine and isolate the specific fractions involved in the pharmacological and toxicological properties of *X. aethiopica* fruit macerate.

CONCLUSION

The abrogation of carrageenan-induced pleurisy in Wistar rats by *X. aethiopica* fruit macerate demonstrates its pharmacological potential, supporting its traditional use for managing various inflammatory conditions including pleurisy. While *X. aethiopica* holds promise for various therapeutic applications, its use must be carefully regulated to avoid potential adverse effects on vital organs, particularly the liver and kidneys. This study reinforces the need for a balanced approach to the use of traditional remedies, ensuring that their benefits are harnessed without compromising safety. Therefore, additional investigations are imperative not only to elucidate the molecular and cellular mechanisms underpinning these therapeutic effects but also to provide a more comprehensive understanding of the plant's safety profile. Future research should also aim to identify the active compounds within *X. aethiopica* fruit macerate and assess their impact on the signaling pathways associated with the inflammatory process.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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ABBREVIATIONS

ACE2: Angiotensin-converting enzyme 2; **ALT:** Alanine aminotransferase; **AST:** Aspartate aminotransferase; **Carr 1%:** Carrageenan 1%; **COVID-19:** Coronavirus disease 2019; **COX-2:** Cyclooxygenase-2; **ELISA:** Enzyme linked immuno-sorbent assay; **HCT:** Hematocrit; **HGB:** Hemoglobin; **IL-6:** Interleukin-6; **IL-1β:** Interleukin-1 beta; **JNK:** c-Jun N-terminal kinase; **LD_{50}:** Lethal dose; **MAPK:** Mitogen activated protein kinases; **MCH:** Mean corpuscular hemoglobin; **MCHC:** Mean corpuscular hemoglobin concentration; **MCV:** Mean corpuscular volume; **NIH:** National institute of health; **NF-κB:** Nuclear factor kappa-light-chain-enhancer of activated B-cells; **OECD:** Organization for Economic Co-operation and Development; **PBS:** Phosphate-buffered saline; **PAF:** Platelet-activating factor; **PLT:** Platelet**; RBC:** Red blood cell; **SARS-CoV-2:** Severe acute respiratory syndrome coronavirus-2; **SD:** Standard deviation**; TNF-α:** Tumor necrosis factor-alpha; **WBC:** White blood cells; **Xylo:** *Xylopia aethiopica.*

SUMMARY

Pleurisy, characterized by excessive pleural inflammation, remains a significant clinical concern. This study investigates the anti-inflammatory potential of *Xylopia aethiopica*, a plant widely used in African traditional medicine, and assesses its immunological mechanisms and toxicological impact in a carrageenan-induced pleurisy model in Wistar rats. The findings demonstrate that *X. aethiopica* fruit macerate significantly reduces inflammatory markers, particularly TNF-α levels, with effects comparable to chloroquine. Moreover, toxicity evaluations suggest relative safety at the studied doses, although histological analyses highlight the need for further investigation.

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