Pharmacogn. Res., 2025; 17(4):1348-1361.
https://www.phcogres.com

Original Article

Medical Plants *Aframomum melegueta* and *Xylopia aethiopica* Show Low Toxicity *in vitro* and *in vivo* Highlighting their Potential for Therapeutic Applications

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ABSTRACT

Background: We previously demonstrated that crude Hydro-Ethanolic Extracts (HEE) of Aframomum melegueta (AM) and Xylopia aethiopica (XA) exhibited anti-helminthic and anti-inflammatory properties with low toxicity. **Objectives:** This study aimed to investigate the toxicological effects of HEE-derived Petroleum Ether (EF), Dichloromethane (DCMF) and Aqueous (AF) fractions of AM and XA. **Materials and Methods:** The HEE of each plant underwent bioguided fractionation between petroleum ether, dichloromethane and water, yielding three fractions. For the cytotoxicity test, PBMCs were co-cultured with these fractions for 72 hr and stained with Propidium Iodide. Oral acute and sub-acute toxicities were tested using Sprague-Dawley rats after 14 and 28 days, respectively. Histological analysis was performed on the kidneys and livers after staining with hematoxylin and eosin. **Results:** HEE-derived fraction showed low cytotoxicity with cell death less than 5%. No signs of toxicity or death were recorded at concentrations of 5,000 mg/Kg and 100 µl/Kg for acute toxicity. The sub-acute toxicity studies showed that fractions do not affect the weight of the rats and no significant changes in whole blood cell composition and biochemical parameters were observed. Histological analysis reveals the presence of inflammatory cells infiltrating with DCMF and EF of AM and XA in liver and kidney sections. **Conclusion:** These findings highlighted the low toxicity of these fractions, indicating their safety for potential therapeutic use.

Keywords: *A. melegueta*, Fractions, Togo, Toxicity, *X. aethiopica*.

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Received: 07-05-2025;
Revised: 24-07-2025;
Accepted: 19-09-2025.

INTRODUCTION

Herbal remedies for immune disorders, such as allergies and chronic inflammatory diseases, are increasing.[1] Plants used for health care are a matter of culture and tradition in Africa.[2] It should be noted that for primary health needs, a large part of the African population uses traditional medicine, whose make-up is mainly based on plants.[3,4] Medicinal plants have the advantage of being easy to obtain, less expensive to produce and are often associated with a lower incidence of adverse effects than current pharmaceutical treatments.[5] Given the ongoing threat of filariae developing resistance to current drug therapy, there is a need to search for potential new plant molecules with anti-filarial activities. Consequently, researchers are trying to identify new molecules that treat or provide benefits to people who do not respond well to current therapies.[6] So, biological screening in the laboratory on animal models is performed to find novel drugs.[4,7] Ouadja and colleagues showed that the essential oil of *Chenopodium ambrosioides* leaf was toxic, but the plant could be a source of antioxidant and anti-inflammatory drugs at very low doses.[8] Our previous studies revealed that traditional healers use *Aframomum melegueta* (AM) and *Xylopia aethiopica* (XA) to treat helminthiasis. Furthermore, our studies have shown

DOI: 10.5530/pres.20250030

that hydro-ethanolic crude extracts of AM and XA possess anti-helminthic and antioxidant activities without cytotoxicity *in vitro* and no toxic effects on female and male Wistar albino rats *in vivo*.[9,10] We have recently demonstrated that AM and XA fractions have anthelmintic activity against Taenia worms.[11] Moreover, Adeyemo and colleagues discovered that at doses above 500 mg/kg of the petroleum ether extract of AM caused enzymatic leakages from the organs (kidney and liver) into the serum, compared to the control group with especially high levels of bilirubin and creatinine in the serum.[12] The methanolic extract of AM seeds and its fractions from successive partition in n-hexane, dichloromethane and ethyl acetate showed significant cytotoxic properties on many cancer cell lines, but not for the normal cell lines.[13] On the other hand, the dichloromethane fraction of XA fruit methanolic extract induced mitochondrial-mediated apoptosis in a study and may be useful for drug development in diseases where apoptosis is compromised.[14] It was reported that XA fruit extracts can have an impact on the body weight, glucose concentration and lipid profile of animals.[15,16] Ethanolic extract of XA fruit showed toxic effects on the liver with kidney at doses above 389 mg/kg.[17] But, the results of Imo and colleagues suggested that XA fruit extracts have no apparent toxic effect on the liver and kidneys of experimental rats and showed a correlation with the results of biochemical analysis.[18]

The diverse biological properties of plant extracts may be due to the variability of their chemical composition, and the choice of solvent used in an extraction defines the chemical profile of the extract and potentially influences its biological effects.[19] But fraction activity does not depend on solvent polarity.[20] On the other hand, the relative solubility of phytochemicals in different solvents depends on the nature of the polarity of the solvents and their chemical bonding.[21] Various solvents have been used in studies, including methanol, water, ethanol, hexane and dichloromethane, with mixed results.[19] In this study, hydro-ethanolic crude extracts of AM and XA underwent successive fractionation in petroleum ether, dichloromethane and water, yielding three fractions per plant. Less is known about these hydro-ethanolic extract-derived fractions. Thus, the present study aims to investigate the toxicity of the petroleum Ether (EF), Dichloromethane (DCMF), and Water (AF) fractions derived from the hydro-ethanolic crude extracts of AM and XA.

MATERIALS AND METHODS

Preparation of Plant Material

Fresh fruits of AM and XA were collected and subjected to hydro-ethanolic extraction following the method previously described.[9] The fresh fruits were purchased from the market in Danyi (Kloto prefecture, Plateaux region), rinsed, and dried at laboratory temperatures under permanent ventilation (Figure 1). The dried fruits were then ground into powder for the extraction process.

Hydro-ethanolic Extraction and Liquid-liquid Separation

Extraction was performed by maceration of 500 g of dried fruit powder in 2 L of 70% ethanol (ethanol-water mixture, 70/30, v/v) for 72 hr, repeated twice. The solution was first filtered through cotton and then through No. 1 Whatman filter paper (Whatman Labware Products, Maidstone, Kent, England). Collected solutions were then concentrated using a Rotavapor (Heidolph Scientific Products GmbH, Schwabach, Germany) at 50°C under reduced pressure. One-third of the crude extract underwent total evaporation to yield a paste, constituting the Hydro-Ethanolic Extract (HEE).

The remaining two-thirds of the crude extract was partially evaporated to remove ethanol, resulting in a crude aqueous Fraction (F1). This fraction was further subject to liquid-liquid partitioning between petroleum ether (Park Scientific Limited, Moulton Park, Northampton, UK), dichloromethane (Scharlab S.L., Barcelona, España) and water as described by Dosso *et al.*,[22] The process resulted in two organic fractions, namely the Ether Fraction (EF) and Dichloromethane Fraction (DCMF) and the Aqueous Fraction (AF). Solvents were evaporated from the organic fractions in an oven at 44°C for at least two weeks. The aqueous fractions were lyophilised. After all, fractions were stored at +4°C until use.

Plant Fraction Working Concentration Determination

To assess toxicity, fractions were dissolved in distilled water and/or Dimethyl Sulfoxide (DMSO) (Sigma-Aldrich, Saint-Quentin-Fallavier, France) and diluted in distilled water to the desired concentrations. A preparation method was adopted depending on the nature of the fraction. For HEE, DCMF and AF from AM, as well as HEE and AF from XA, 1 g of each fraction was dissolved in 10 mL of distilled water to obtain a stock concentration of 100 mg/mL. For DCMF and EF of XA, 1 g of the mass obtained was first dissolved in 2 mL 100% DMSO and then 8 mL distilled water was added to obtain a stock concentration of 100 mg/mL. For EF of AM with an oily appearance, it has been diluted to 2% (EF2%) with pure DMSO, then to 1% with distilled water from EF2%. EF1% was the base concentration. The highest residual concentration of DMSO for the working concentrations was 0.245%. A control was prepared with this concentration of DMSO and the culture medium without the extract to ensure the effect of DMSO on the cells. All dilutions were filtered through a 0.45 µm Millipore membrane (VWR International, North America, Puerto Rico) before being used in various *in vitro* assays.

To determine the working concentration, a successive dilution to half was carried out. We started with 11 decreasing concentrations from 1 mg/mL to 0.87 µg/mL for all fractions except EF of AM, which had 5 concentrations from 0.2% to 0.0025%. In parallel, five control concentrations of DMSO were prepared from 1.96% to 0.1225%. The concentrations that demonstrated optimal

activity with minimal toxicity were selected for the following experiments.

In vitro Cytotoxicity on White Blood Cells using Flow Cytometry

The cytotoxicity of the fractions of the hydro-ethanolic extracts of AM and XA was evaluated according to the standard NF EN ISO 10993-5 recommendations from 2009. PBMCs from healthy volunteers were isolated using the Ficoll gradient centrifugation technique as previously descirbed[23] Six (6) healthy voluntary blood donors were recruited at the "Centre National de Transfusion Sanguine" (CNTS) of Lomé during blood donation. PBMCs (2×10^5 cells/well) were cultured alone in RPMI 1640 medium supplemented with gentamycin (50 pg/mL), penicillin/streptomycin (50 pg/mL) and L-glutamine (292.3 pg/mL)) (Gibco by Life Technologies Corporation, Carlsbad, USA) supplemented with 10% fetal bovine serum (PAN Biotech, Aidenbach, Germany) or co-cultured with decreasing concentrations (1 mg/mL to 0.87 µg/mL) of HEE, DCMF, AF, and EF from XA, and HEE, DCMF and AF from AM. Additionally, cells were activated using microbeads (ratio microbeads/cells: 1/10) coated with monoclonal αCD3/CD28 (Invitrogen, Carlsbad, California, USA) in the presence or absence of EF from AM at concentrations from 0.2% to 0.0025%. Pure DMSO was used as a toxicity control. After 72 hr of incubation at 37°C in a humidified atmosphere with 5% CO_2, cell pellets were collected and stained with 0.2% of Propidium Iodide (PI) (Biotium, *Inc.* Fremont, USA). Thereafter, cells were acquired using a Cytoflex flow cytometer (Beckman Coulter, Brea, California, USA). Based on compensation beads and unstained controls, a gating strategy (Figure S1) was applied to analyse the percentage of cells expressing PI (dead cells).

In vivo Toxicological Assessment on Sprague-Dawley (SD) Rats

Experimental Animals and Housing Conditions

The study was conducted using SD rats provided by the Faculty of Science at the University of Lomé. The rats were housed in Tecniplast plastic cages (Tecniplast S.p.a., Buguggiate, Italy) and fed with standard food pellets, with water *ad libitum*. During the experiments, the animals were maintained under controlled temperature conditions (20±2°C) and a 12 hr light/dark cycle.

Acute Toxicity Test

The acute toxicity was conducted on 30 female SD rats aged 12 weeks, with a weight between 100 g and 120 g. A dose of 5000 mg/kg for HEE of XA and its Derived Fractions (DCMF, AF, and EF), as well as for the HEE of AM and its Derived Fractions (DCMF and AF), along with 100 µL/kg for EF of AM, was administered orally by force-feeding to each strain following the Organization for Economic Cooperation and Development (OECD) guidelines, method 423.[24] In total, 10 groups of 3 female SD rats per group were involved. Eight (8) groups were treated with the hydro-ethanolic extracts of AM and XA and with their derived fractions. One group served as a negative control, receiving only the vehicle (distilled water), while another group that received no treatment was considered the normal control. All animals were individually monitored approximately 30 min after administration, followed by hourly observations for the first 5 hr, and then daily for the subsequent 14 days of the study, to identify any signs and symptoms of toxicity. Observations focused on alterations in the skin, coat, mucous membranes, and eyes, as well as assessments of the respiratory and circulatory systems, central and autonomic nervous systems, somatic activity and behaviour, and the potential onset of seizures, salivation, diarrhoea, lethargy, drowsiness, coma, mortality, and the timing of any adverse effects.

Sub-acute Toxicity Assessment

The safety of fractions derived from crude Hydro-Ethanolic Extracts (HEE) of AM and XA was assessed in SD rats over a 28-day period using *in vivo* sub-chronic toxicity methods. This study followed the promising results obtained from *in vitro* cytotoxicity assays with the working concentrations used in this project. The sub-acute oral toxicity study adhered to the OECD guideline 407 for chemical testing.[25]

A total of 40 SD rats (20 females and 20 males) were divided into 10 groups of 4 rats each, with 2 rats per sex. Nine groups, comprising 36 rats, were force-fed the extracts for 28 days, while 1 group, consisting of 4 rats, remained untreated for the same duration. The fractions and Distilled Water (DW) were administered daily at a consistent time, and the rats were monitored closely twice daily for any signs of toxicity or mortality. Rat weights

Figure 1: Investigated plant organs. A) Fresh fruits of *A. melegueta* K. Schum; B) Dried grains of *A. melegueta* K. Schum; C) Fresh fruits of *X. aethiopica* (Dunal) A. Rich. and D) Dried fruits of *X. aethiopica* (Dunal) A. Rich.

were recorded daily throughout the experiment. Fractions were prepared at a concentration of 12 mg/Kg of body weight for HEE from XA and its Derived Fractions (DCMF, AF, and EF), as well as from AM and its Derived Fractions (DCMF and AF) on distilled water. Additionally, a concentration of 0.3%/Kg of body weight was prepared for EF of AM, with rats receiving 1 mL of extract per 100 g body weight.

Euthanasia and Sample Collection

After 28 days of treatment, rats from both control and treated groups were weighed, euthanized using Carbon Dioxide (CO_2) inhalation after fasting for at least 12 hr. The euthanasia was performed in the animal house of « Laboratoire de Microbiologie et de Controle de Qualité des Denrées Alimentaires (LAMICODA) » of the University of Lomé. The rat was placed in the euthanasia chamber filled at a rate of 30% of the total chamber volume/min until the chamber reached 100%. The euthanasia cycle lasted 7-8 min, including a continued flow of CO_2 into the chamber for at least 1 min after the rat stopped breathing. In addition, gases were exchanged between consecutive euthanasia procedures.

Following euthanasia, the rats were dissected, and blood was collected in EDTA tubes and anticoagulant-free tubes, respectively. Blood samples were used for the measurement of haematological parameters (e.g., red blood cells, white blood cells, platelets, and haemoglobin levels) on the Sysmex automated system SN A4201 (Sysmex, Kobe, Japan). The samples in anticoagulant-free tubes were centrifuged at 2,500 x g for 10 min to collect sera for the measurement of the biochemical parameters (e.g., glycaemia, uraemia, creatininaemia, transaminases, Alanine Aminotransferase (ALT), and Aspartate Aminotransferase (AST)) using LabKit reagents (Labkit, Eu-gen) and the 'HumaReader HS' plate reader according to the instructions of the manufacturer. Organs such as the liver, kidneys, heart, spleen, lungs, ovaries, and testes were removed, weighed, and preserved in 10% formalin for histopathological study.

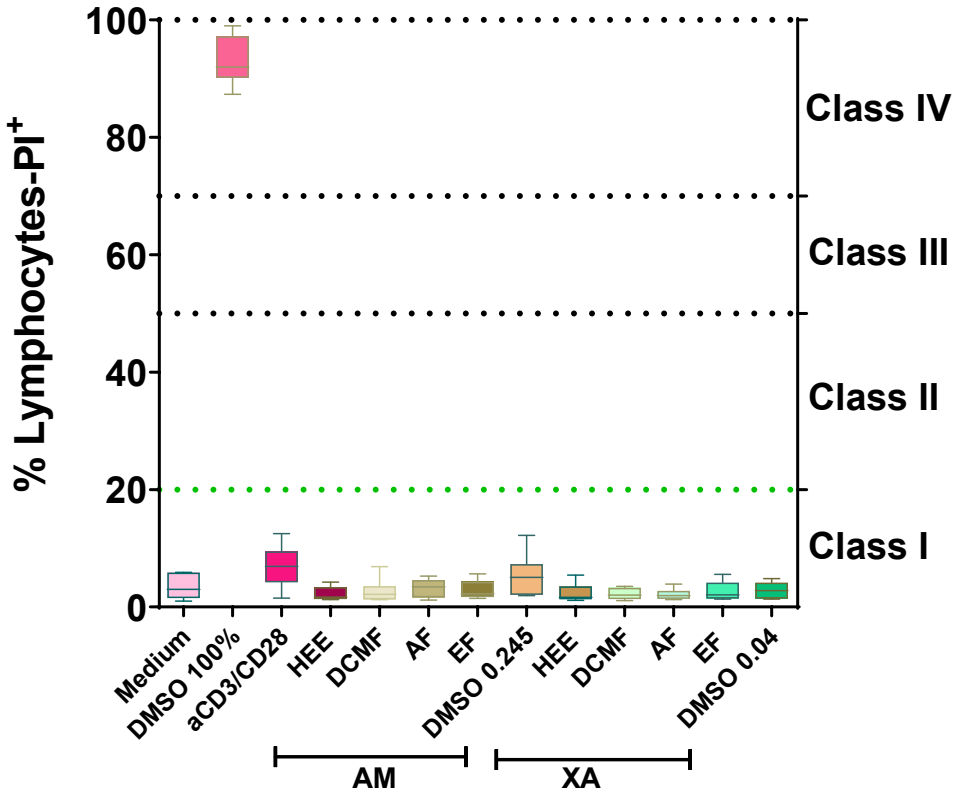

Figure 2: Cytotoxicity of hydro-ethanolic crude extracts and fractions of AM and XA. PBMCs (2×10^5 cells/well) from six (6) healthy donors were either left unstimulated (Medium) or stimulated with 200 µg/mL of HEE, DCMF, AF, EF of XA and AM and 0.005% EF of AM, as well as 100% of DMSO, 0.245% and 0.04% residual DMSO concentrations, respectively, in EF of AM and DCMF, EF of XA for 72 hr. Cells were then stained with propidium iodide (PI) dye and analysed by flow cytometry. Box whiskers (Tukey) represent the percentage of lymphocytes expressing PI. The NF EN ISO 10993-5: 2009 standards classifications are indicated by class I (not cytotoxic), class II (moderate cytotoxicity), class III (mild cytotoxicity) and class IV (severe cytotoxicity). AM: *A. melegueta*, XA: *X. aethiopica*, HEE: Hydro-Ethanolic Extract, DCMF: Dichloromethane fraction, AF: Aqueous Fraction, EF: Ether Fraction, DMSO: Dimethyl Sulfoxide.

Relative Organ Weights

The relative weights of the weighed organs were calculated using the formula:

Relative organ weight=(OW/AW)×100

Where OW represents organ weight and AW represents animal weight on the day of sacrifice.

Organ Preparation and Histological Examination

Tissue Collection and Fixation

To facilitate a histological comparison between the organs of control and treated rats, organs such as the liver, kidneys, heart, spleen, lungs, ovaries, and testes were removed, rinsed with tap water, weighed, and fixed in 10% formaldehyde (Central Drug House Pvt. Ltd., CDH, New Delhi, India). Only the liver and kidneys, which play a crucial role in drug metabolism, were subjected to this histopathological study.

Processing and Sectioning

After fixation in 10% formaldehyde for a minimum of 24 hr, the organs were cut transversely to create histological cassettes (Bio-Optica Milano Spa via San Faustino, Milano, Italy), which underwent dehydration in increasing concentrations of alcohol (CDH, New Delhi, India), followed by embedding in paraffin (CDH, New Delhi, India).

Staining and Microscopic Examination

After embedding, 0.5 µm sections of the organs were cut using the HM 340 E semi-automatic rotary microtome. The prepared slides were stained with haematoxylin and eosin (Bio-Optica Milano Spa via San Faustino, Milano - Italy). The stained slides were then covered with coverslips containing a few drops of biological glue. Microscopic observation of the slides was performed using a 'bscope' trinocular optical microscope (Euromex), equipped with a Leica camera, employing magnifications of X 10 and X 40.

Statistical Analysis

GraphPad Prism version 5.02 (GraphPad Software, San Diego, California, USA) was used to create histograms. The data were expressed as Median±IQR.[26]

Ethical Consideration

This study obtained bioethical approval (Opinion No. 029/2021/CBRS) from the "Comité de Bioéthique pour la Recherche en Santé (CBRS)" under the Ministry of Health, Public Hygiene, and Universal Access to Care. Participants were informed of the voluntary nature of the study and gave their informed consent by signing the consent form.

This study obtained also animal bioethical approval (Opinion No. 003/2024/C2EA) from "Comité d'éthique pour l'Expérimentation Animale" of Laboratoire de Microbiologie et de Controle de Qualité des Denrées Alimentaires (LAMICODA).

All aspects of the rearing and experimentation on SD rats were in accordance with the OECD ethical guidelines.

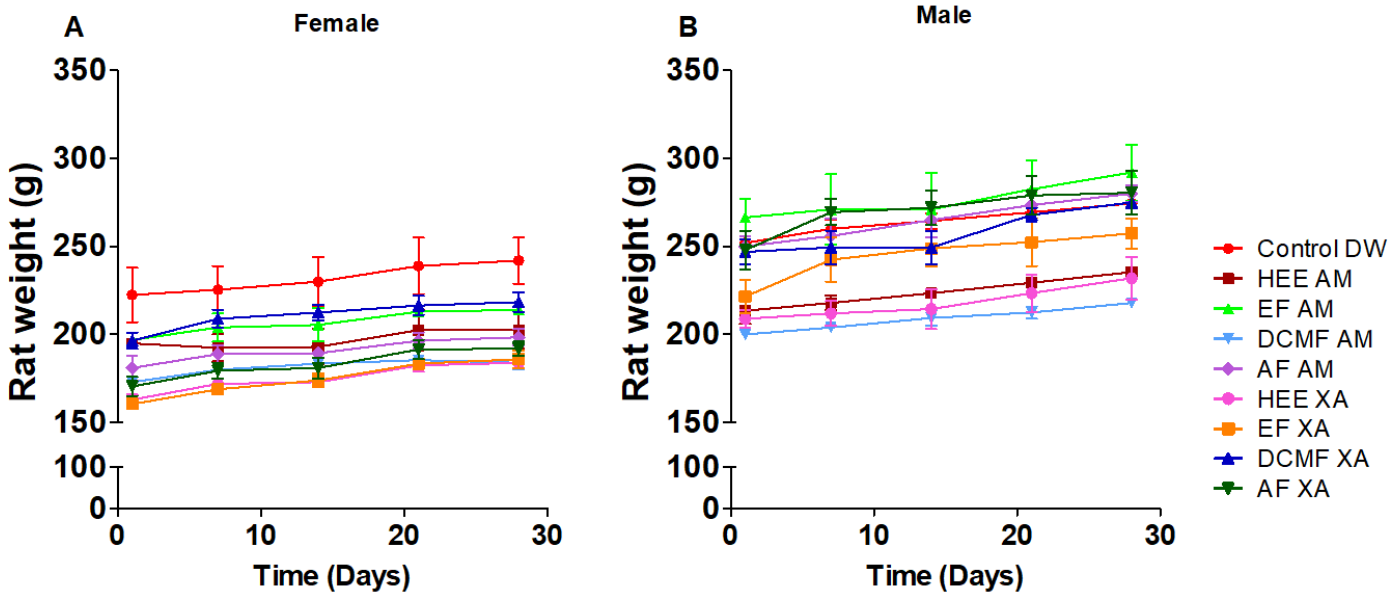

Figure 3: Variation of body weight of female (A) and male (B) rats after 28 days treatment with fractions derived from AM and XA hydro-ethanolic extracts. Values are median±range (*n*=2 / sex / group), AM: *A. melegueta*, XA: *X. aethiopica*, HEE: Hydro-Ethanolic Extract, DCMF: Dichloromethane Fraction, AF: Aqueous Fraction, EF: Ether Fraction, DW: Distilled Water.

RESULTS

Low Cytotoxicity of *A. melegueta* and *X. aethiopica* Hydro-ethanolic Extract Derived Fractions

To determine the cytotoxicity of AM and XA hydro-ethanolic fractions. PBMCs (2×10^5 cells/well) from healthy donors were left alone (Medium) or stimulated with αCD3/CD28 in the presence or not of 200 µg/mL of HEE, DCMF, AF, EF of XA and AM and 0.005% EF of AM, as well as 100% of DMSO, 0.245% and 0.04% residual DMSO concentrations, respectively, in EF of AM and DCMF, EF of XA for 72 hr. Cells were then stained with Propidium Iodide (PI) dye and analysed by flow cytometry

Analysis of the flow cytometry results using the gating strategy depicted in Figure S1 showed that pure DMSO (positive control) induced over 90% cell death (Figure 2) and stimulation of cells with αCD3/CD28 (negative control) showed a mean cell death rate of 6.89%. After 3 days of co-culture with fractions, we could observe an average cell death of 2.70%, 3.23%, and 2.86%, respectively, for DCMF, AF, EF of AM and 2.19%, 2.14%, 2.70% for DCMF, AF, EF of XA (Figure 2). These cell death rates were all less than 20%. According to the French standard NF EN ISO 10993-5 "Biological evaluation of medical devices - Part 5: tests for *in vitro* cytotoxicity" that describes test methods for measuring the *in vitro* cytotoxicity of medical devices with mammalian cells, using appropriate biological parameters of 2009, last revised and confirmed in 2022, these fractions are classified as Class I (non-cytotoxic) products at concentrations of 200 µg/mL for DCMF, EF and AF fractions of AM and XA and at 0.005% for EF of AM.

Acute Toxicity Assessment Reveals no Mortality in Rats Treated with *A. melegueta* and *X. aethiopica* Hydro-ethanolic Extract-derived Fractions

The *in vitro* cytotoxicity using PBMCs demonstrated that our fractions were not toxic (Class I). Next, we investigated the *in vivo* toxicity using Sprague-Dawley (SD) rats and we could indeed show that after administration of a single dose of 5,000 mg/Kg body weight (bw) of AM and XA extracts and fractions during 14 days of monitoring, no changes in rat behaviour and no sign of toxicity such as alterations in skin, coat, mucous membranes and eyes, as well as assessments of respiratory and circulatory systems, central and autonomic nervous systems, somatic activity and behaviour, and the potential appearance of seizures, salivation, diarrhoea, lethargy, somnolence, coma, mortality and neither possible adverse effects were recorded. Interestingly, no deaths occurred in any of the treated rat groups.

The limit dose of 5,000 mg/kg of fractions derived from the HEE of AM and XA, followed by 100 µl/Kg of FE AM, did not cause mortality or acute toxic effects in treated rats. Thus, the LD_{50} would then be greater than 5,000 mg/kg bw and 100 µl/Kg bw.

No Body Weight Loss after 28 Days of Treatment with Hydro-ethanolic Extracts-derived Fractions

Evaluation of the sub-acute toxicity of fractions derived from the fractionation of crude HEE of AM and XA showed that treatment with these fractions had no impact on the animals' weight gain. In general, an increase in body weight over time was observed in both treated and control rats (Figure 3).

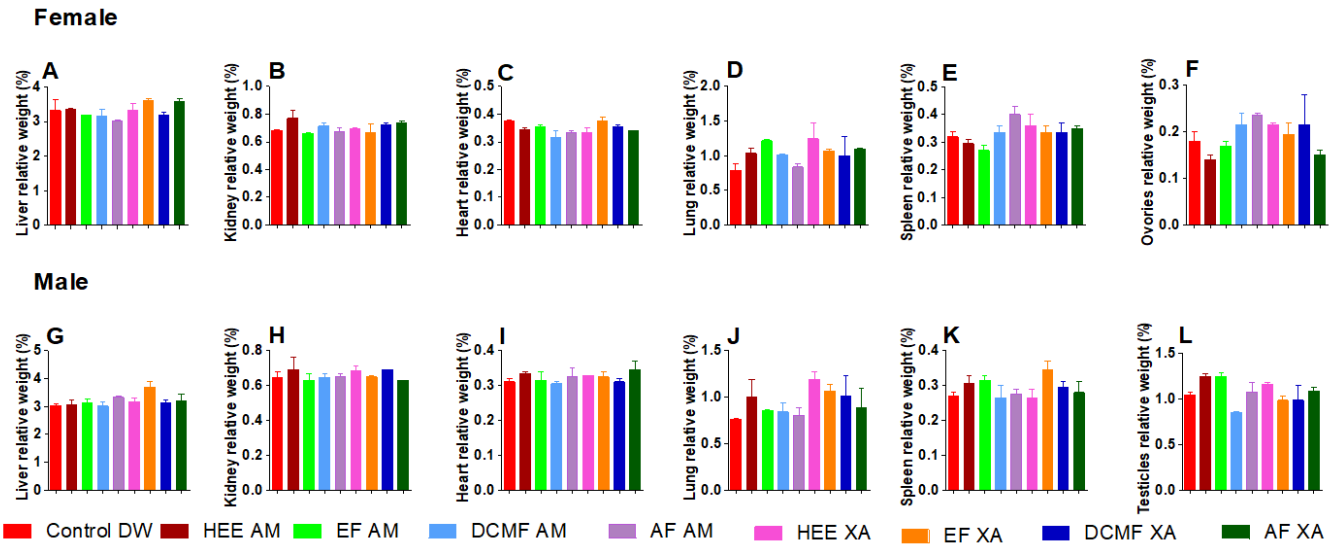

Figure 4: Relative organs weight variation of female (A-F) and male (G-L) rats after 28 days treatment with fractions derived from hydro-ethanolic extracts of AM and XA. Values are Median±IQR (*n*=2 / sex / group). AM: *A. melegueta*, XA: *X. aethiopica*, HEE: Hydro-Ethanolic Extract, DCMF: Dichloromethane Fraction, AF: aqueous fraction, EF: Ether Fraction, DW: Distilled Water.

No Alterations of Body and Organ Weight in Treated Female and Male rats

Evaluation of the sub-acute toxicity of fractions derived from the fractionation of crude HEE of AM and XA showed that treatment with these fractions had no impact on the animals' weight gain. In general, an increase in body weight over time was observed in both treated and control rats (Figure 3).

Also, no variation in the relative weight of liver, kidneys, heart, lungs, spleen, ovaries and testicles was observed in the different test groups with AM and XA fractions compared with the control as depicted in Figure 4.

Administration of Dichloromethane and Ether Fractions of *A. melegueta* Results in Periportal Plasma Cell Infiltrates in the Liver

As we had not observed any interference of treatment with fractions on body weight and relative organ weights of treated rats, we turned to the study of histological sections of the liver and kidney of these animals.

Microscopic examination of histological sections of the liver revealed varying degrees of congestion of the centrilobular vein and portal vein in all groups of treated rats, as well as in the control groups (Figures 5 and 6). Periportal lympho-plasmacytic inflammatory infiltrates (yellow arrow) were noted in groups of rats with HEE, DCMF and EF of AM (Figure 5). Hepatocytes had a normal histological appearance, characterized by their polygonal shape and tight clustering, with rounded basophilic central nuclei separated by sinusoids, observable in both treated and control groups (Figure 7).

Dichloromethane and Ether Fractions from *A. melegueta* and *X. aethiopica* Induce Glomerular Atrophy of the Kidneys in Treated Rats

The kidney, microscopic analysis of histological sections from treated rats revealed glomerular congestion in all fractions and glomerular atrophy associated with HEE, DCMF, EF of AM and

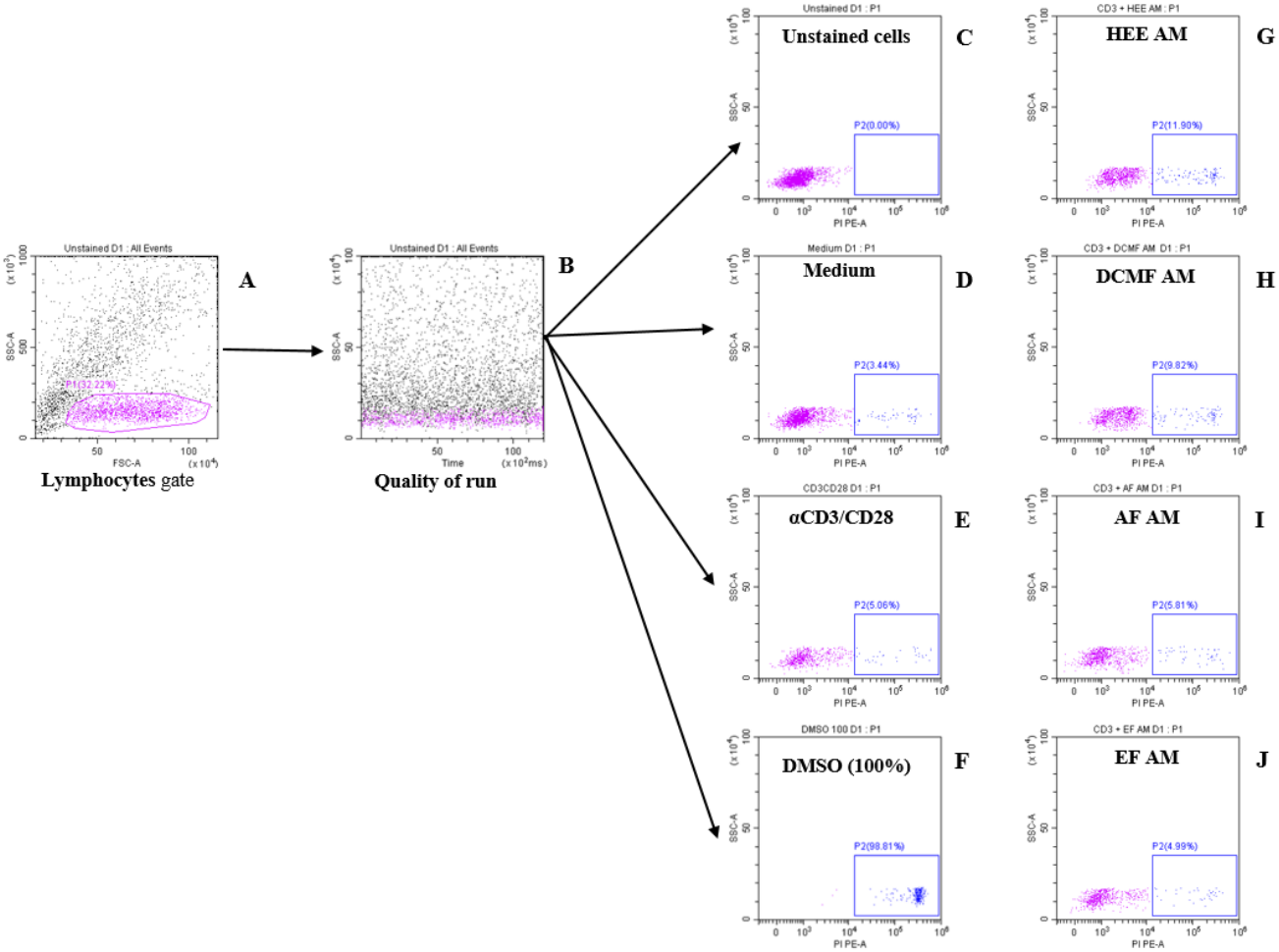

Figure S1: Gating strategy showing cytotoxicity of the fractions derived from *A. melegueta* and *X. aehiopica* hydro-ethanolic extracts. Human PBMCs (2 × 105 cells/well) were left alone (Medium) or stimulated with 200 µg/mL and 0.005% of *A. melegueta* and *X. aehiopica* fractions, DMSO 100% and anti-CD3/CD28 microbeads for 72h. Cells were stained with propidium iodide dye (PI) and analyzed by flow cytometry. A: lymphocytes gate, B: quality of the run, C to F: controls and G to J: AM fractions are PI+ cells in presence of differentes stimulations. AM: *A. melegueta*, HEE: Hydro-Ethanolic Extract, DCMF: Dichloromethane Fraction, AF: Aqueous Fraction, EF: Ether Fraction, DMSO: Dimethyl Sulfoxide.

XA as well as an inflammatory cell infiltrate noted in DCMF of AM (Figure 8). In contrast, kidneys from untreated rats showed normal renal parenchyma, with glomeruli surrounded by healthy interstitial tissue (Figure 8). In addition, Figure 9 shows photomicrographs of rat renal tubules, which exhibit regular architecture with well-defined epithelial cells and appropriate interstitial space between tubules in both treated and untreated rats.

Increased Leukocyte and Neutrophil Counts in Dichloromethane and Aqueous Fractions of *A. melegueta-treated* Rats

Since we have observed some inflammation in our histological sections, we wanted to know whether this was also noticeable in the peripheral blood.

Analysis of the complete blood count of rats in the treated groups compared to the vehicle (distilled water) treated ones showed no significant variation in the number of lymphocytes, monocytes, eosinophils and basophils (Figure S2), as well as in the number of erythrocytes, haemoglobin and haematocrit levels (Figure S3) and mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration (Figure S4) levels in both female and male rats. However, a peak of the number of white blood cells and neutrophils was observed in the groups treated with DCMF and AF from AM in both female and male rat groups. Similarly, this peak of the number of white blood cells and neutrophils was also observed in male rats with EF from XA and in the female rat group with DCMF from XA (Figure S2). A peak of platelet count was noted in the groups of male rats treated with DCMF from AM and HEE from XA (Figure S3A). However, the biological relevance of the significance needs to be confirmed using more animals per treatment group. In summary, the fractions do not change whole blood parameters in rats.

Treatment does not Change Biochemical Parameters in Treated Rats

Damage to vital organs can be detected by biochemical tests. Therefore, even if the histological sections of the livers and kidneys did not show any worrying changes, we checked some biochemical parameters.

Comparison of the biochemical data of female and male rats from treated groups versus vehicle showed no significant variation in blood glucose, creatinine, urea, ASAT and ALAT levels with AM and XA hydro-ethanolic derived fractions (Figure S5). However, XA fractions caused an increase in urea, ALT and AST levels in female rats (Figures S5, C-E), which needs to be statistically proven with a larger number of treated rats.

In summary, treatment of rats with the different fractions of AM and XA did not interfere with the weight gain, whole blood cell composition, and biochemical profile of the animals.

DISCUSSION

The anthelmintic and anti-inflammatory properties of crude hydro-ethanolic extracts of AM and XA have been demonstrated in our previous studies.[9,27] These extracts had shown no signs of toxicity either *in vitro* on PBMCs or *in vivo* in Wistar albino rats.[10] Bioguided fractionation of these crude extracts was carried to test the toxicity of derived fractions.

The cytotoxicity test showed a rate of cells positive for PI staining of less than 20% for all fractions of these two medicinal plants. According to the standard classification NF EN ISO 10993-5: 2009, a PI-positive cell count of less than 20% means that the product is not cytotoxic and these results confirm our previous observation. In a recent study, ethanolic and methanolic extracts of AM at doses of 20 µg/mL to 100 µg/mL did not show cytotoxicity or toxicity on peripheral mononuclear cells using the MTT test.[28] Also, no cytotoxicity was found on the human

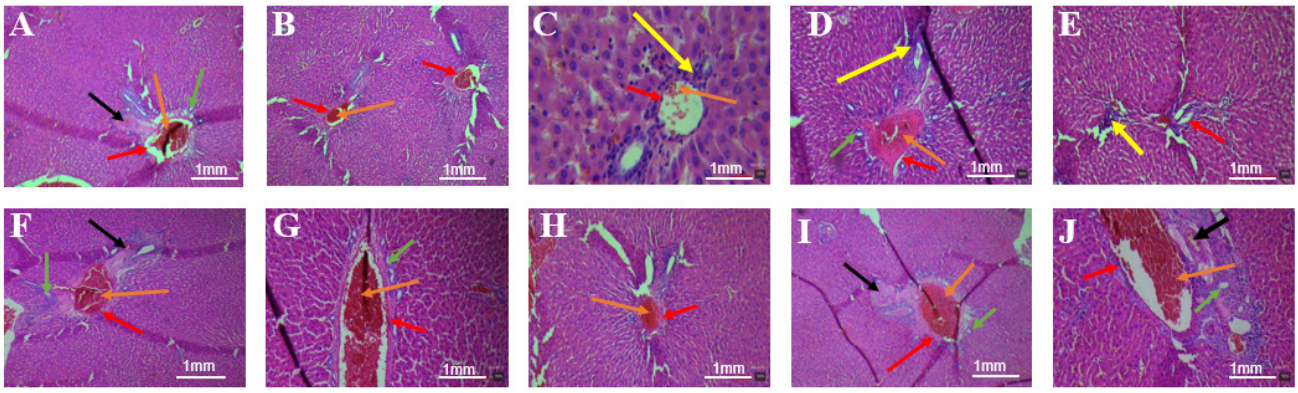

Figure 5: Microscopic images of transverse sections of rat liver showing portal spaces composed with portal vein (red arrow), hepatic artery (black arrow), bile ducts (green arrow), congest portal vein (orange arrow) and congestion (yellow arrow): A- Group 1: Normal untreated control, B- Group 2: DW; C- Group 3: HEE AM. D- Group 4: DCMF AM; E- Group 5: EF of AM; F- Group 6: AF of AM; G- Group 7: HEE of XA; H- Group 8: DCMF of XA; I- Group 9: AF of XA; J- Group 10: EF of XA. H&E staining, photos A, B, D, E, F, G, H, I, J (magnification X100) and photo C (magnification X400). AM: *A. melegueta*, XA: *X. aethiopica*, HEE: Hydro-Ethanolic Extract, DCMF: Dichloromethane Fraction, AF: Aqueous Fraction, EF: Ether Fraction, DW: Distilled Water.

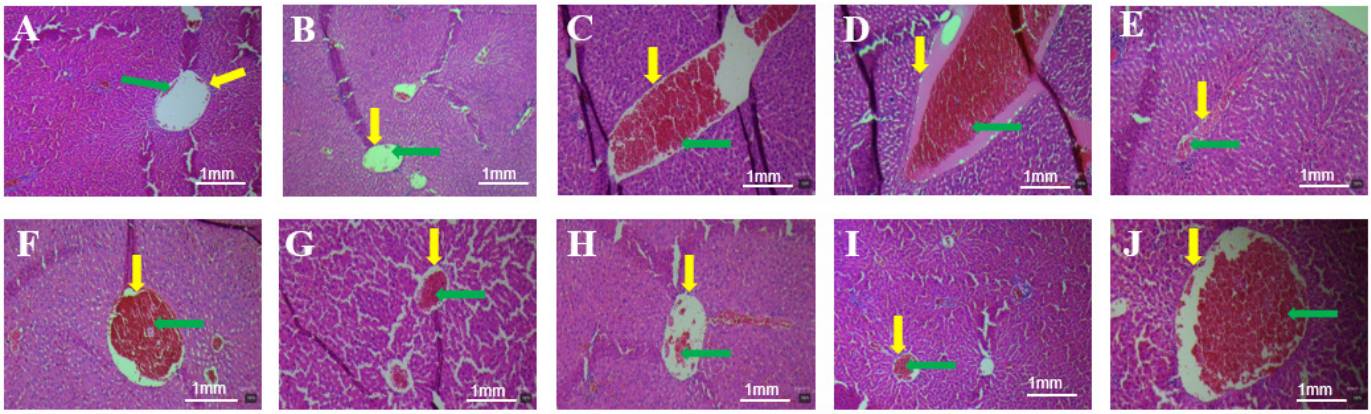

Figure 6: Microscopic images of rat liver cross-sections showing the centrolobular veins (yellow arrow) with congestion (green arrow). The micrographs of the control (Photographs A and B) and treated groups (Photos C to J) show a normal histological structure of the Centrolobular Vein (CLV) with trabeculae of hepatocytes separated by sinusoids and show minimal to intense congestion of the CVL. A- Group 1: Normal untreated control, B- Group 2: DW; C- Group 3: HEE AM. D- Group 4: DCMF AM; E- Group 5: EF of AM; F- Group 6: AF of AM; G- Group 7: HEE of XA; H- Group 8: DCMF of XA; I- Group 9: AF of XA; J- Group 10: EF of XA. (H&E staining, magnification X100). AM: *A. melegueta*, XA: *X. aethiopica*, HEE: Hydro-Ethanolic Extract, DCMF: Dichloromethane Fraction, AF: Aqueous Fraction, EF: Ether Fraction, DW: Distilled Water.

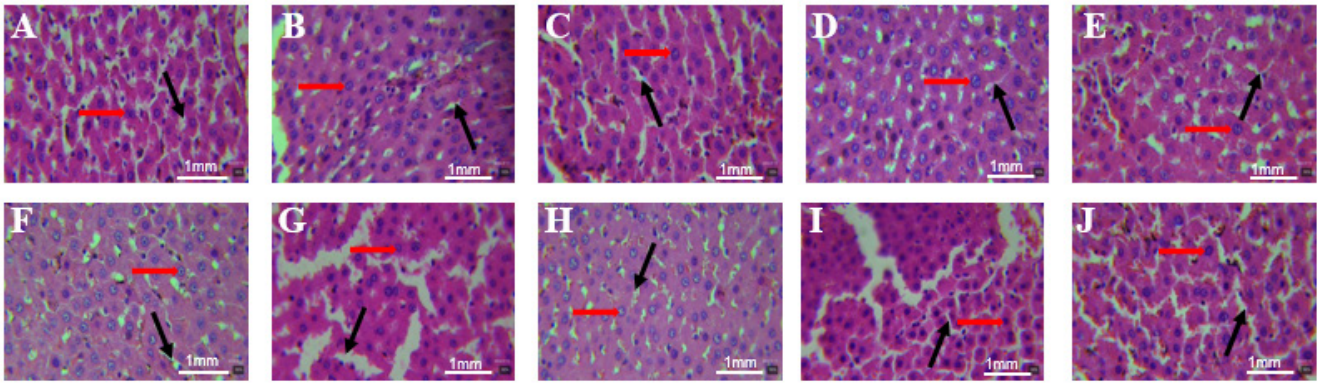

Figure 7: Microscopic images of rat liver cross-sections showing hepatocytes (red arrow) and sinusoids (black arrow). (H&E staining, magnification X400). Overall, the photomicrographs show a normal histological structure of the hepatocytes, which are polygonal in shape, tightly clustered, containing rounded basophilic central nuclei separated by sinusoids. A- Group 1: Normal untreated control, B- Group 2: DW; C- Group 3: HEE AM. D- Group 4: DCMF AM; E- Group 5: EF of AM; F- Group 6: AF of AM; G- Group 7: HEE of XA; H- Group 8: DCMF of XA; I- Group 9: AF of XA; J- Group 10: EF of XA. AM: *A. melegueta*, XA: *X. aethiopica*, HEE: Hydro-Ethanolic Extract, DCMF: Dichloromethane Fraction, AF: Aqueous Fraction, EF: Ether Fraction, DW: Distilled Water.

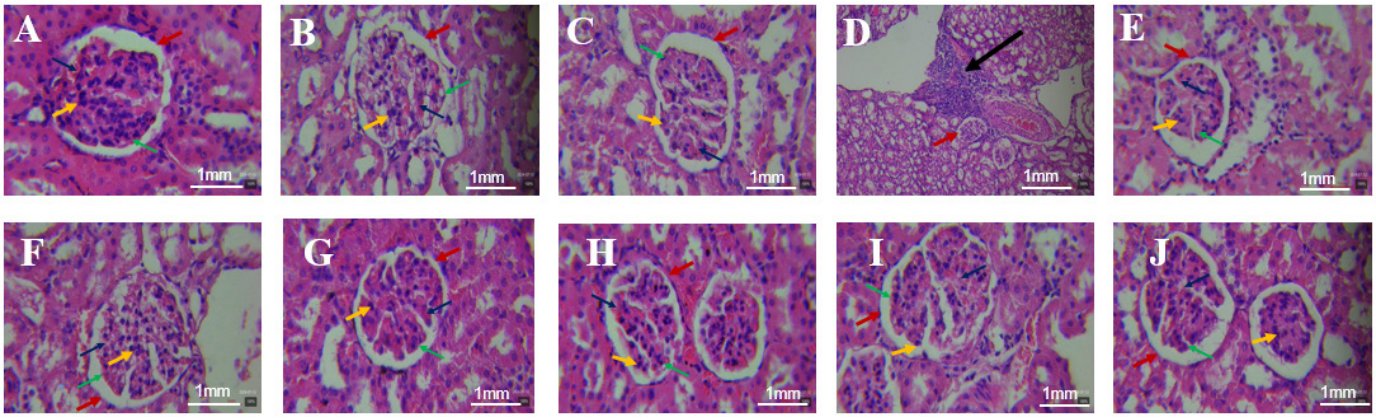

Figure 8: Microscopic images of cross-sections of kidneys from SD rats (H&E staining, magnification ×400). The micrographs of the kidney showing a regular structure of renal architecture with podocytes (yellow arrow), Bowman's capsule (red arrow), regular structure of glomeruli (green arrow), congestion (dark blue arrow) and infiltrate of inflammatory cells (black arrow). A- Group 1: Normal untreated control, B- Group 2: DW; C- Group 3: HEE AM. D- Group 4: DCMF AM; E- Group 5: EF of AM; F- Group 6: AF of AM; G- Group 7: HEE of XA; H- Group 8: DCMF of XA; I- Group 9: AF of XA; J- Group 10: EF of XA. AM: *A. melegueta*, XA: *X. aethiopica*, HEE: Hydro-Ethanolic Extract, DCMF: Dichloromethane Fraction, AF: Aqueous Fraction, EF: Ether Fraction, CF: Chloroform Fraction, DW: Distilled Water.

epidermal cell line HaCaT *in vitro* with the volatile of XA fruits harvested in Togo, with IC_{50}>3000 µg/mL.[29] The methanolic fraction showed high cytotoxic activity in the brine shrimp lethality bioassay with an LC_{50} value of 3.4 µg/mL, followed by the n-hexane fraction 12.4 µg/mL, crude extract 19.7 µg/mL, interface 25.0 µg/mL and chloroform fraction 185.5 µg/mL from partitioning of the ethanolic extracts of XA fruits. This showed polarity-independent activity, which is inconsistent with the polarity of the extracts tested in the order: hexane<chloroform<ethanol<methanol<interface.[20]

Also, no sign of toxicity was found during the acute toxicity test in the case of the *in vivo* toxicity test at 5000 mg/kg for all fractions except EF from AM, which was at 100 µL/kg of pure fraction. In the same line, sub-chronic toxicity on females and males SD rats with AM and XA fractions during 28 days of oral treatment showed no visible or clinical signs of toxicity and no deaths occurred during the experimental procedure.

The subacute toxicity study for 28 days showed that administration of AM and XA fractions did not interfere with body weight or relative organ weights in treated rats. This assumes that these fractions were non-toxic and therefore the treatment had no impact on the general health of treated rats. Similarly, Ilic and colleagues noted no difference in body weight gain between treated and untreated rats with 120, 450 and 1500 mg/ kg/day of AM HEE in male and female SD rats.[30] confirming results from XA seed extracts on albino rats.[31] In contrast, Woode and colleagues found an increase in body weight as well as sexual organ weights with XA fruit ethanolic extract administered orally to male SD rats at doses of 30 to 300 mg/kg for 60 days.[32] Also, in another study, the untreated Wistar rats had greater weight gain than the treated groups with XA fruit extract and the higher the dose of the extract, the lower the weight gain.[33] Thus, our results can be explained by the very low dose used, which was 12 mg/kg bw. However, the low number of rats is a limitation in our results and needs to be confirmed with increased number of treated rats.

In addition, in the *in vivo* toxicity studies, the anatomical and pathological aspects of the liver and kidneys are crucial. So, histological sections of the liver showed congestion in both the centrolobular vein and portal vein across all groups of treated rats, as well as the control groups and periportal inflammatory infiltrate cells in rats' groups treated with hydro-ethanolic extract, dichloromethane and ether fractions from AM. This result shows that congestion is not due to the treatment. Previously, oral administration of 100 mg/kg to 300 mg/kg of XA extract to Wistar albino rats for 21 days showed in the liver of treated rats normal hepatocytes, filled with a periportal inflammatory infiltrate and a congested central vein.[34] Also, histological changes such as a slightly cholestatic central vein and congestion in liver tissue were observed respectively at doses of 40 mg/kg and 80 mg/kg of methanolic extract of AM administered for 30 days.[35] Moreover, kidney sections from treated rats revealed glomerular congestion in all our fractions and glomerular atrophy associated with HEE, DCMF, EF from AM and XA, as well as an inflammatory cell infiltrate noted in DCMF of AM. Our recent studies showed vascular congestion and inflammation in the liver and kidneys of Wistar rats treated with *X. aethiopica* macerate, but this did not affect its pharmacological properties.[36] Instead, a study showed that AM seed extract at a dose of 100 mg/kg attenuated acute kidney injury induced by diclofenac at a dose of 50 mg/kg for 5 days.[37] In the same vein, aqueous extract of XA was also pre-treated with 250 mg/kg to 1000 mg/kg and

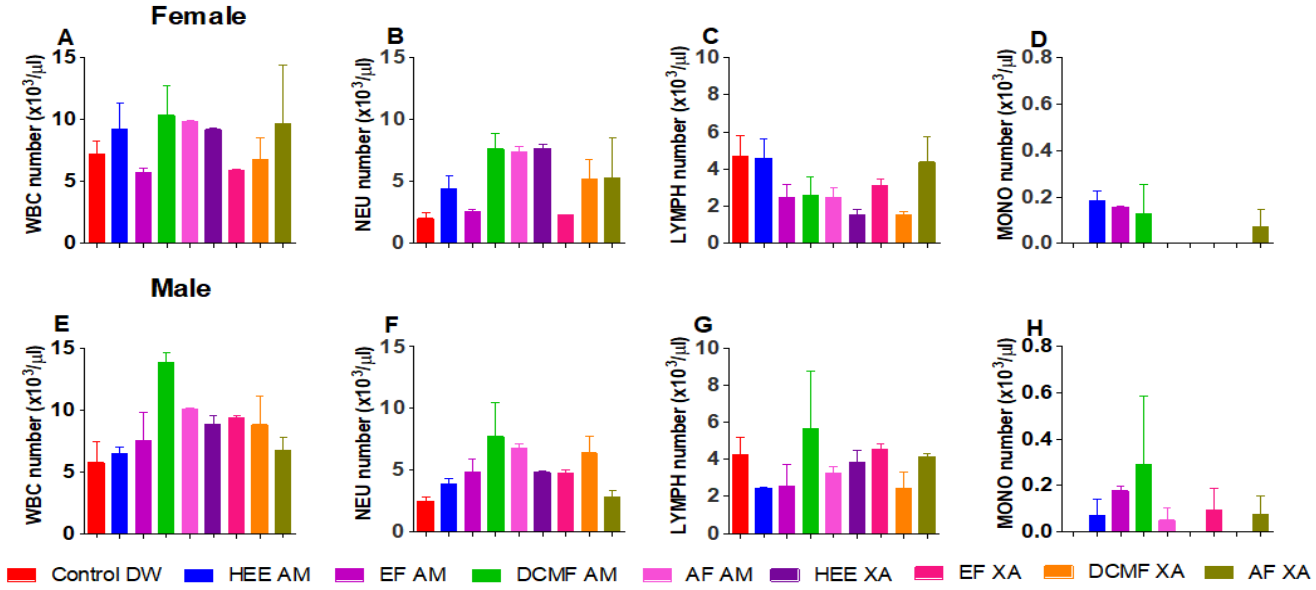

Figure S2: White blood cells profile in female (A-D) and male (E-H) rat groups after 28 days of treatment with the fractions derived from AM and XA. Values are Median±IQR (n=2 / sex / group). AM: *A. melegueta*, XA: *X. aethiopica*, HEE: hydro-ethanolic extract, DCMF: dichloromethane fraction, AF: aqueous fraction, EF: Ether Fraction, DW: Distilled Water, WBC: White blood cells; NEUT: Neutrophils; LYMPH: Lymphocytes; MONO: Monocytes.

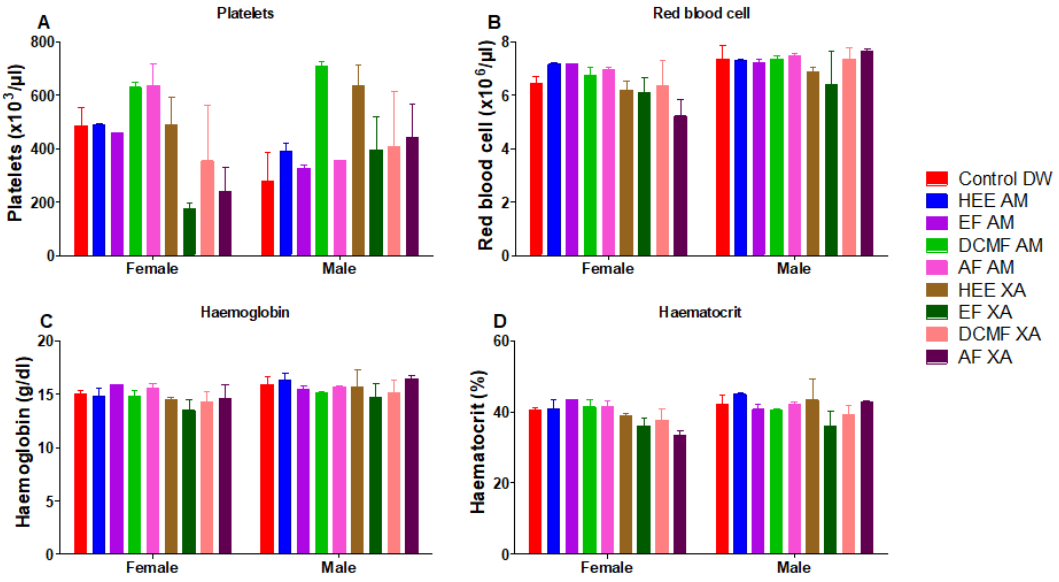

Figure S3: Platelets (A), erythrocytes (B), haemoglobin (C) and haematocrit (D) level profile in female and male rat groups after 28 days of treatment with the fractions derived from AM and XA. Values are Median±IQR, (*n*=2 / sex / group). AM: *A. melegueta*, XA: *X. aethiopica*, HEE: Hydro-Ethanolic Extract, DCMF: Dichloromethane Fraction, AF: Aqueous Fraction, EF: Ether Fraction, DW: Distilled Water.

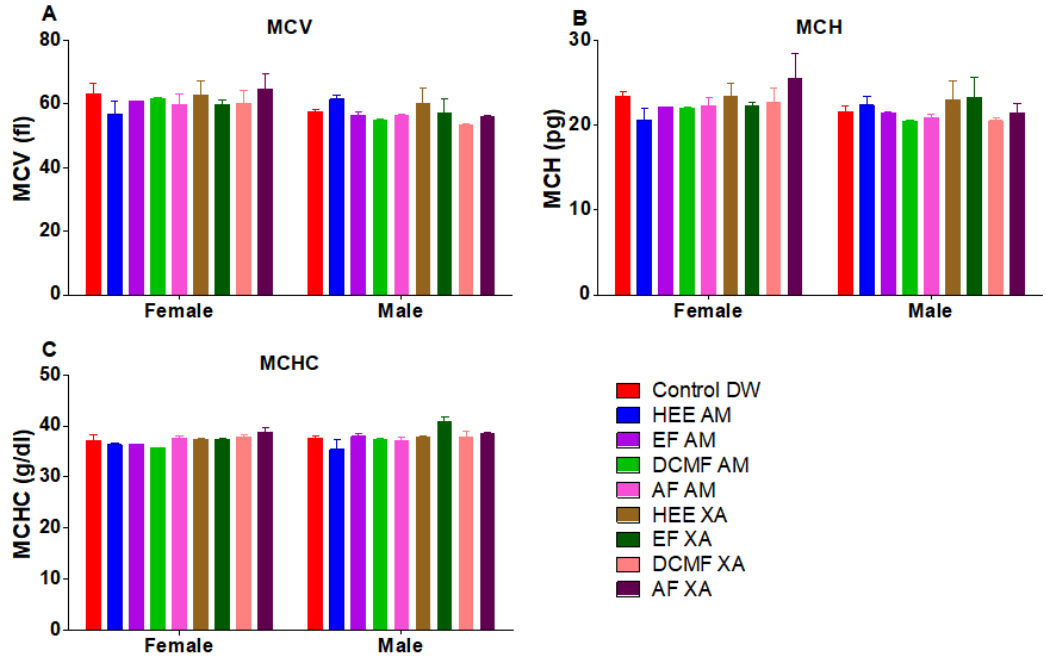

Figure S4: MCV (A), MCH (B) and MCHC (C) level profile in female and male rat groups after 28 days of treatment with the fractions derived from AM and XA. Values are Median±IQR, (*n*=2 / sex / group). AM: *A. melegueta*, XA: *X. aethiopica*, HEE: Hydro-Ethanolic Extract, DCMF: Dichloromethane Fraction, AF: Aqueous Fraction, EF: Ether Fraction, DW: Distilled Water, MCV: Mean Corpuscular Volume; MCH: Mean Corpuscular Haemoglobin; MCHC: Mean Corpuscular Haemoglobin Concentration.

showed a dose-dependent improvement in CCL4-induced renal and hepatic toxicity.[38] Also, AM extract attenuated epithelial cell degeneration and promoted regeneration of the renal tubular epithelium in lambda-Cyhalothrin-induced kidney injury rat model,[39] and protected liver injury from ethanol-induced toxicity in male Wistar rats.[40] Previously, Obike and colleagues, found no histological disorders in the kidney tissue compared to the control and they associated it with the antioxidant and hepatoprotective effects of AM aqueous extract.[41] So, AM and XA extracts can attenuate the toxic effects of conventional drugs.

Moreover, investigation on haematological parameters in this study revealed no significant variation in both treated and control rat groups. However, the inflammatory infiltrate observed in liver and kidney sections with the DCMF AM fraction was

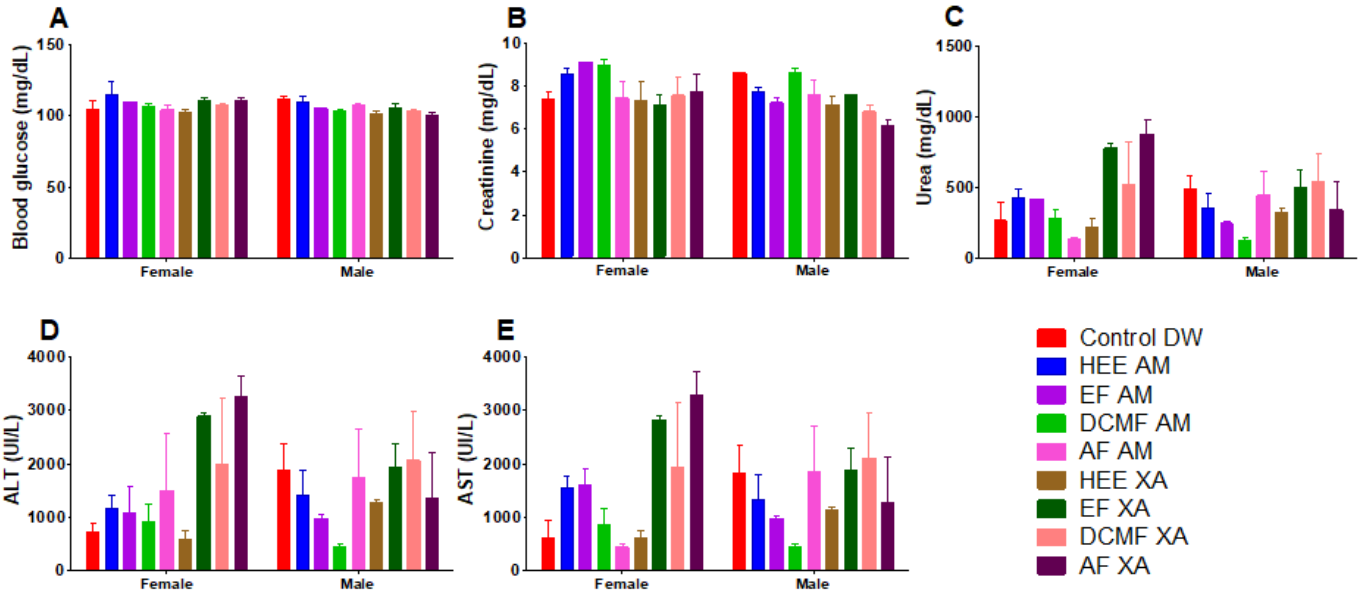

Figure S5: Biochemical data from female and male rats treated with AM and XA fractions. Values are Median±IQR, (*n*=2 / sex / group). AM: *A. melegueta*, XA: *X. aethiopica*, HEE: Hydro-Ethanolic Extract, DCMF: Dichloromethane Fraction, AF: Aqueous Fraction, EF: Ether Fraction, DW: Distilled Water. ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase.

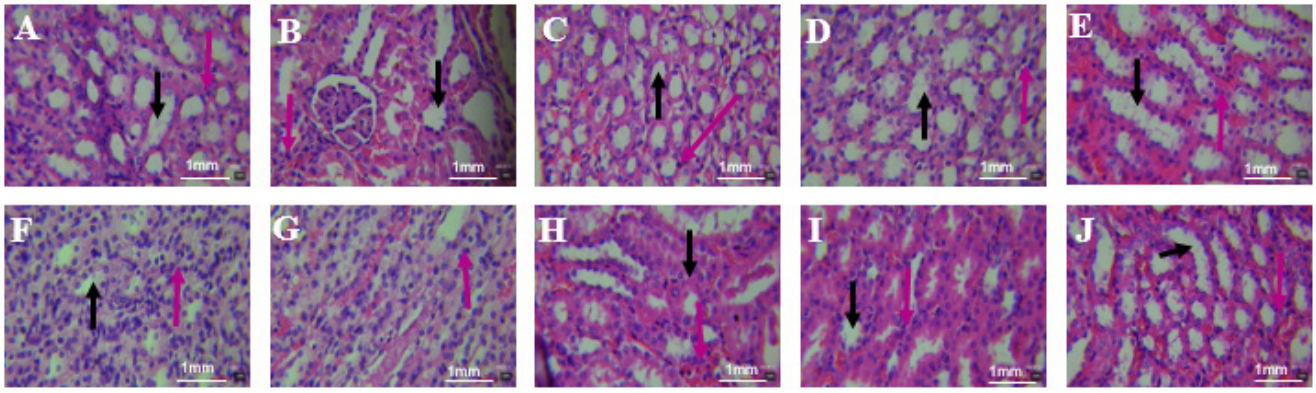

Figure 9: Microscopic images of cross-sections of kidneys showing the renal tubules (black arrow) and epithelial cells (pink arrow) of SD rats (H&E stain, magnification ×400). Overall, the micrographs show regular structures of the architecture of the renal tubules, including the epithelial cells, the regular structure of the tubules separated by the interstitial space, both in the rats of the control groups and in the treated groups. A- Group 1: Normal untreated control, B- Group 2: DW; C- Group 3: HEE AM. D- Group 4: DCMF AM; E- Group 5: EF of AM; F- Group 6: AF of AM; G- Group 7: HEE of XA; H- Group 8: DCMF of XA; I- Group 9: AF of XA; J- Group 10: EF of XA. AM: *A. melegueta*, XA: *X. aethiopica*, HEE: Hydro-Ethanolic Extract, DCMF: Dichloromethane Fraction, AF: Aqueous Fraction, EF: Ether Fraction, DW: Distilled Water.

supported by haematological data showing elevated leukocytes and neutrophils with this fraction. Also, an isolated increase in the number of white blood cells and neutrophils was observed in the groups treated with AF from AM in both sexes and, respectively, in male rats with EF from XA and the female rat groups with DCMF from XA. An increase in platelet count was noted in the male rats treated with DCMF from AM and HEE from XA. Our results suggest that these fractions can stimulate the immune system in the management of pathologies. These properties are the result of the bioactive molecules present in AM and XA hydro-ethanolic extracts-derived fractions. Thus, a previous study showed that prolonged period administration of AM seed extracts can accumulate active phytochemicals such as flavonoids, tannins, alkaloids and trace components (iron, copper and vitamin B) to reach a stable state.[42] In the same lines, a study noted an increase in blood cells with 40 mg/kg and 80 mg/kg of AM extract and associated it with the presence of trace elements such as copper, iron and other metabolites at haematopoietic sites.[35] Furthermore, it has been revealed that XA fruit extract increased red blood cells and decreased white blood cells and their sub-population at 389 mg/kg after 28 days of oral administration.[43] Also, the XA aqueous extract administration over 200 mg/kg during four weeks in female Wistar rats significantly increases platelet count.[44] Thus, all these results on AM and XA extracts suggest that these plants can promote immune cell recruitment.

Our biochemical data showed no variation in blood glucose, creatinine, urea, ASAT and ALAT levels in treated groups with no histological impact on the organs concerned. This result

shows that our fractions are neither nephrotoxic nor hepatotoxic at the dose used. Adeyemo and colleagues observed that the petroleum ether extract of AM caused renal and hepatic toxicity only at doses above 500 mg/kg, with a significant increase in serum proteins like total protein, AST, ALT, urea and creatinine in male Albino Wistar rats.[12] However, in their study, they did not perform histological investigations. Indeed, increases in ALT and/or AST are common symptoms of hepatotoxicity.[45] The liver releases ALT and AST, while the kidneys release creatinine and urea. A significant rise in ALT is often a sign of liver damage and biliary tract problems. A very high ALT level (more than 10 times normal) is generally due to acute hepatitis or to a viral infection.[12,46] A recent study on the effect of the n-hexane, aqueous and ethanolic fractions derived from the fractionation of XA crude extracts on the biochemical parameters of male Wistar rats, treated daily with oral doses of 100 mg/kg bw and 200 mg/kg bw for 14 days, revealed in plasma a significant decrease in urea and creatinine levels and an increase in AST and ALT levels in all treated animals compared with the control group.[47] However, the biological relevance of the significance in this study needs to be confirmed using more animals per treatment group.

CONCLUSION

This study investigated the toxicity of AM and XA hydro-ethanolic extracts-derived fractions through bioguided fractionation. The fractions *in vitro* were not cytotoxic and the *in vivo* assessments revealed that treatment of rats with the different fractions of AM and XA did not interfere with the body weight. In addition, the overall health status of the treated rats showed slight inflammation in the organs which does not affect biochemical and immunological parameters. These findings highlighted the low toxicity of these fractions, indicating their safety for potential therapeutic use.

ACKNOWLEDGEMENT

We thank healthy donors from the National Blood Transfusion Center of Lomé, for participating in this study.

ABBREVIATIONS

AF: Aqueous Fraction; **ALT:** Alanine Aminotransferase; **AM:** *Aframomum melegueta*; **AST:** Aspartate Aminotransferase; **bw:** Body Weight; **DCMF:** Dichloromethane; **DMSO:** Dimethyl Sulfoxide; **DW:** Distilled Water; **EF:** Petroleum Ether; **H&E:** Hematoxylin and Eosin; **HEE:** Hydro-Ethanolic Extract; **LD_{50}:** Lethal Dose 50; **OECD:** Organization for Economic Cooperation and Development; **PBMCs:** Peripheral Blood Mononuclear Cells; **SD:** Sprague-Dawley; **XA:** *Xylopia aethiopica*.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FUNDING

This research was funded through a Grant awarded to GK from the German Research Foundation (DFG); Grant LA2746/1-2 within the "German African Cooperation Projects in Infectiology".

SUMMARY

This study investigates the toxicological effects of Hydro-Ethanolic Extract (HEE)-derived Petroleum Ether (EF), Dichloromethane (DCMF) and Aqueous (AF) fractions of *Aframomum melegueta* and *Xylopia aethiopica*. HEE-derived fraction showed low cytotoxicity and no signs of toxicity or death during acute toxicity. The sub-acute toxicity studies showed that fractions do not affect the weight of the rats. Histological analysis reveals slight inflammation in the organs which does not affect biochemical and immunological parameters. These findings support their potential therapeutic applications.

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Cite this article: Awesso ER, Arndts K, Katawa G, Douti FV, Bara FD, Karou SD, *et al*. Medical Plants *Aframomum melegueta* and *Xylopia aethiopica* Show Low Toxicity *in vitro* and *in vivo* Highlighting their Potential for Therapeutic Applications. Pharmacog Res. 2025;17(4):1348-61.