Evaluation of the acute toxicity of the aqueous extract of *Annona squamosa* leaves on female Wistar rats

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Abstract :
Annona squamosa is a plant traditionally used in Ivory Coast to treat cases of female infertility. Excessive use of different parts of this plant can present risks of poisoning for users. The objective of this study is to evaluate the toxicity of the aqueous extract of *A. squamosa* leaves on female rats according to the European OECD guideline 423. The aqueous extract of the leaves was administered to the test rats and distilled water to the control rats on oral administration. The effects of this extract on the behavior and body weight of rats were observed. Biochemical and hematological parameters were analyzed according to standard methods. The histopathological study of the kidney and liver experimental animals was also carried out using the classic histology method. Regarding the behavior of rats, the acute toxicity test of the aqueous extract of A. squamosa at a single dose of 2000 mg/kg bw showed no signs apparent toxicity, mortality and morbidity via the oral route. At the level of the body weight of the animals, no difference was observed between the treated rats and the control rats. This teste also showed no difference between the weight of the vital organs (heart, liver and kidney) of the treated rats and those of the control rats. The lethal dose (LD50) would therefore be greater than 2000 mg/kg bw. The aqueous extract of A. squamosa did not cause any significant change at level of blood parameters and biochemical of treated rats compared to control rats. Microscopic examination of the liver and kidney of animals treated with the single dose of 2000 mg/kg bw of *A. squamosa* extract showed normal structural architecture compared to control animals.

Keywords : Toxic effect, *Annona squamosa*, female rats, behavior, vital organs.

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Résumé :
Annona squamosa est une plante utilisée traditionnellement en Côte d'Ivoire pour traiter des cas d'infertilité féminine. L'utilisation exagérée des différentes parties de cette plante peut présenter des risques d'intoxication pour les utilisateurs. L'objectif de cette étude est d'évaluer la toxicité de l'extrait aqueux des feuilles de *A. squamosa* sur les rats femelles selon ligne directrice 423 de L'OCDE. L'extrait aqueux des feuilles a été administré aux rattes tests et de l'eau distillée aux rattes témoins par voie orale. Les effets de cet extrait sur le comportement et le poids corporel des rattes ont été observés. Les paramètres biochimiques et hématologiques ont été analysés selon les méthodes classiques. L'étude histopathologique du rein et du foie des rattes tests et témoins a été également réalisée selon la méthode classique. Concernant le comportement des rattes, le test de toxicité aigüe de l'extrait aqueux de A. squamosa (EAAS) à la dose unique de 2000 mg/kg de poids corporel n'a montré aucun signe apparent de toxicité, mortalité et de morbidité par voie orale. Au niveau du poids corporel des animaux, aucune différence n'a été observée entre les rattes traitées et les rattes témoins. Cette toxicité n'a montré également aucune différence entre le poids des organes vitaux (Cœur, Foie et Rein) des rattes traitées et ceux des rattes témoins. La dose létale (DL50) serait donc supérieure à 2000mg/kg de poids corporel. L'EAAS n'a entrainé aucune modification significative des paramètres sanguins et des paramètres biochimiques des rattes traitées comparés aux rattes témoins. L'examen microscopique du foie et du rein des animaux traités avec la dose unique de 2000 mg/kg de poids corporel d'extrait de A. squamosa a montré une architecture structurale normale comparée aux animaux témoins.

Mots-clés : Effet toxique, *Annona squamosa*, rattes, comportement, organes vitaux.

Introduction

Infertility is the inability of a couple to procreate or carry a pregnancy to term after one year or more of regular, unprotected sexual intercourse (WHO, 2004). It deserves to be handled because it often constitutes a reason for the separation of couples and psychological disorders (Laborie, 2000). In African countries, given the low purchasing power of the population for modern medical consultations, this population resorts to traditional medicine through the use of plants to treat cases of female and male infertility. The World Health Organization (WHO) estimates that nearly 80% of people on the African continent mainly rely on traditional medicines (WHO, 2004). The strong use of plants for health needs gave rise to herbal medicine. This therapy shows that plants can be authentic medicines (Bouzouita, 2016). Phytotherapy research then became one of the greatest scientific concerns (Njike et al., 2005). WHO has developed a strategy for traditional medicine. The aim is to maximize the possibilities of using this

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traditional medicine as a source of health care and protection of the raw material (WHO, 2004).

Annona squamosa, also called cinnamon apple, is a fruit shrub native to Central America and the Caribbean reaching 3 m to 6 m high. It belongs to the Annonaceae family. This family contains approximately 130 genera and 2300 species of trees and shrubs (Shehata et al., 2021). Traditionally, all parts of the plant have medicinal properties (Logbo et al., 2022). Different parts of *A. Squamosa* such as bark, root, seeds, fruits, flowers and leaves have been used in traditional medicine to treat several diseases (Ma et al., 2017). According to Logbo et al. (2022), the leaves are used in various preparations to treat cancerous tumors. Logbo et al. (2022) also showed that *Annona squamosa* is effective against stored product insects. In Ivory Coast, *Annona squamosa* leaves are traditionally used to treat female infertility without prior assessment of toxicity. Except for the study by Saleh et al. (2021) that evaluated the acute toxicity of methanolic extract of *Annona squamosa* on male albino rats in Nigeria, there are not enough studies that have evaluated the acute toxicity of *Annona squamosa* leaves on female rats. The objective of this study is to evaluate the toxicity of the aqueous extract of *Annona squamosa* leaves in female rats.

1. Materials et Methods

1.1. Study site

The studies were conducted at the Endocrinology and Reproductive Biology Teaching and research unit of Biology and Health Laboratory of Félix Houphouet-Boigny University.

1.2. Plant material

The plant material consists of leaves of *Annona squamosa*. The leaves of A. Squamosa were harvested between October and November 2020 in the commune of Abobo (Abidjan-Côte d'Ivoire), north of Abidjan and identified by the National Floristic Center (CNF).

1.3. Animal material

Des The animal material consists of *Rattus norvegicus* rats of Wistar strain for the acute toxicity teste.

1.4. Methods

1.4.1. Preparation of the aqueous extract of the leaves of Annona squamosa

The leaves of the plant were collected in the commune of Abobo (Abidjan-Ivory Coast). The fresh leaves of *A. squamosa* are harvested early in the morning from 6 a.m., then dried in the open air away from direct sunlight, at a temperature of 26°C to 27°C for one (1) week. The dried leaves were crushed using a Moulinex machine type electric grinder; and the powder obtained was used for the preparation of the aqueous extract. This extract has been used for acute toxicity in rats. The aqueous extract of *A. squamosa* leaves was prepared following the method adopted by Yapo et al. (2016).

Fifty (50) grams of leaf powder were placed in 1 l of distilled water in a blender. The solution was mixed for 3 min, repeated three times at room temperature. The homogenate obtained was filtered twice (2) times on white poplin fabric cut into 900 cm² squares, then five (5) times on hydrophilic cotton. The obtained filtrate was evaporated at 55°C in a venticell® type oven to obtain the aqueous extract of *A. squamosa* leaves (EAAS).

1.4.2. Acute toxicity of the aqueous extract of Annona squamosa (EAAS) on female rats

The acute toxicity of the aqueous extract of *Annona squamosa* leaves (EAAS) was carried out in virgin and adult rats according to the European OECD guideline 423. Each animal received 1 mL/100 g of the 2000 mg dose /kg bw of aqueous extract of *A. squamosa* orally using an appropriate gastric tube. In accordance with the lethal dose 50 greater than 5000mg/kg body weight reported in the work of Saleh et al. (2016) on male rats orally with the methanolic extract of *A. squamosa* leaves, in this study, the initial limit dose of 2000 mg/kg bw was chosen from the following doses 5, 50, 300 and 2000 mg/kg bw. Animals were then observed individually for possible toxicological signs 4 hours after access to food. They were also observed the first 30 minutes, 24 hours after treatment and daily for 14 days. Signs such as tremor, convulsion, salivation, diarrhea, lethargy, sleep and coma were noted. The skin, hair, eyes and mucous membranes, as well as the respiratory system were explored. A new dose of 2000 mg/kg bw EAAS was administered to another group of three rats as recommended by OECD guideline 423. The animals underwent the same protocols and observations as preview group. At the end of 14 days of observation, the rats were sacrificed after ether anesthesia. Blood samples were collected in EDTA tubes for hematological studies and dry tubes for biochemical studies. The rats were then dissected. Vital organs such as the liver and kidney were removed and rinsed in 9‰ NaCl. These organs were then weighed to determine the relative weight.

RW (g/100 g bw) **:** Relative weight (g/100g of body weight) ;

OW : Organ weight ;

BW : Body weight) x 100.

1.4.3. Determination of biochemical parameters

Using a Rotofix 32 A type centrifuge (Germany), the blood collected in the dry tubes is centrifuged at 3000 rpm for 4 min. An aliquot of serum is taken into Eppendorf tubes and stored in the freezer in order to measure the various biochemical parameters. Various methods have been used to measure the desired parameters according to their specificities. Thus, urea, glucose and cholesterol were measured by colorimetric enzymatic methods. The determination of the enzymatic activities of transaminases (ALT and AST) and creatinine was carried out by the kinetic method. These dosages are carried out with a HITACHI 704 R type automaton (Japan).

1.4.4. Determination of hematological parameters

Blood collected in EDTA tubes is gently shaken to prevent the formation of micro clots. The numeration of blood count consisted of determining the number of white blood cells, red blood cells and platelets, the hematocrit and hemoglobin levels, the mean corpuscular volume, the mean corpuscular hemoglobin content and the mean blood concentration. Hemoglobin using a Sysmex XS-500i automated hematological analyzer (Germany).

1.4.5. Histopathological analysis of organs

The histology of the collected organs (liver, kidney) aims to highlight the toxic effects of the aqueous extract of *Annona squamosa* (EAAS) at microscopic dimensions. The different steps (fixation, dehydration, impregnation, embedding, sectioning, deparaffinization and staining) were performed according to the technique described by Djoudad-Kadji et al. (2011), Alturkistani et al. (2016).

1.4.6. Data analysis

Analysis of variance is a statistical tests used to compare means of multiple samples. The Mann-Whitney test was carried out with the Statistica software (version 6.0) to compare the means of the different parameters studied of rats treated with the aqueous extract of *Annona squamosa* (EAAS: 2000 mg/kg of bw) to those having received distilled water (control).

2. Résults

2.1. Effect of the aqueous extract of A. squamosa on the behavior of animals and determination of the lethal dose (LD50)

Oral administration of 2000 mg/kg bw of the aqueous extract of *Annona squamosa* (EAAS) to rats did not cause any deaths. No clinical signs of toxicity were recorded (Table I). Also, this dose of 2000 mg/kg bw did not have a significant effect ($p > 0.05$) on the body mass of the animals compared to control animals having received distilled water (Figure 1).

The treated animals showed signs of well-being (active movement, normal food and water intake) compared to the controls.

Thus, the leaves of *A. squamosa* would have an LD50 of between 2000 mg/kg bw and 5000 mg/kg bw or greater than 5000 mg/kg bw according to OECD guideline 423.

Table I: Results of clinical signs of the acute toxicity test after 14 days of observation

Clinical signs	Trials	
	EAAS (2000 mg/Kg bw)	Control (Distilled water)
Apathy	-	-
Excitement	-	-
Breathing problems	-	-
Refusal of food	-	-
Mouth bleeding	-	-
Abdominal pain	-	-
Convulsion	-	-
Tremor	-	-
Diarrhea	-	-
Coma	-	-

(-) : absence

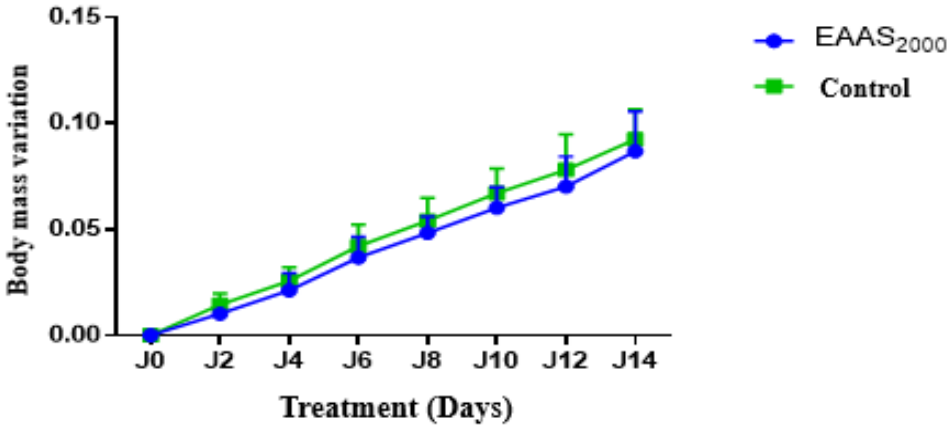

Figure 1: Variation of rat body mass by the time

2.2. *Effects of the aqueous extract of Annona squamosa (EAAS) on the relative weight of organs and on hematological parameters*

The relative weights of the kidneys, liver and heart of the treated rats compared to the control showed no significant difference (P > 0.05) (Table II). The hematological analysis also showed no significant modification (P > 0.05) in the blood parameters of the rats treated with the aqueous extract of A. squamosa compared to the control rats having received distilled water (Table III).

Table II : Effect of the aqueous extract of *A. squamosa* leaves on the relative weight of the organs collected

Treatements	Kidneys	Liver	Heart
Control (Distilled water)	0.573 ± 0.03 a	3.16 ± 0.24 a	0.343 ± 0.02 a
EAAS (2000 mg/Kg BW)	0.616 ± 0.06 a	3.897 ± 0.5 a	0.4267 ± 0.04 a

The Mann-Whitney test was used to make comparisons against Controls. The comparison of the table is made in the vertical direction. In the table, values followed by the same letter are not significantly different.

Table III : Effect of the aqueous extract of *A. squamosa* leaves on the hematological parameters of rats

Parameters	Control (Distilled water)	EAAS (2000 mg/Kg bw)
GR (10^6/µL)	7.77 ± 0.18 a	7.56 ± 0.33 a
HGB (g/dL)	13.15 ± 0.15 a	12.9 ± 0.0 a
HCT (%)	40.6 ± 0.6 a	40.9 ± 0.4 a
VGM (fL)	52.3 ± 2.00 a	54.2 ± 2.9 a
TCMH (pg)	16.9 ± 0.2 a	17.05± 0.75 a
CCMH (g/dL)	32.45 ± 0.85 a	31.55 ± 0.35 a
GB (10^3/µL)	12.84± 0.345 a	13.41 ± 2.345 a
NEUT (%)	15.95 ± 1.45 a	16.15 ± 2.75 a
PLQ (10^3/µL)	977.5 ± 2.50 a	981.5 ± 2.62 a

WBC : White Blood Cells; **GR :** Red Blood Cells; **HGB :** Hemoglobin; **HCT :** Hematocrit; **MCV :** Mean Globular Volume; **TCMH :** Corpuscular Hemoglobin Content; **CCMH :** Corpuscular Hemoglobin Concentration; **PLQ** : Platelets; **NEUT :** Neutrophil.

The Mann-Whitney test was used to make comparisons against Controls. The comparison of the table is made in the horizontal direction. In the table, values followed by the same letter are not significantly different.

2.3. *Effects of aqueous extract of leaves of A. squamosa on biochemical parameters*

The treatment of rats with the aqueous extract of *A. squamosa* (EAAS) at a single dose of 2000 mg/kg of body weight did not cause any disorders in the metabolism of the biochemical parameters (Urea, Creatinine, ALT and AST) studied in comparison with the control group **(**Table IV).

2.4. *Effects of the aqueous extract of A. squamosa leaves on histological parameters*

The histopathological study carried out on the kidney (Figure 2) and liver (Figure 3) revealed no structural abnormality, inflammation, hepatic cell necrosis and apoptosis in all rats treated with a single dose of 2000 mg/kg bw of the aqueous extract of A. squamosa leaves.

Table IV : Effects of the aqueous extract of *A. squamosa* on the biochemical parameters of rats

Parameters	Control (Distilled water)	EAAS (2000 mg/Kg Bw)
Urea	0.287±0.075 a	0.377± 0.015 a
Creatinine	5.000± 0.000 a	6.000± 0.000 a
ALAT	12±0.732 a	13.333±0.082 a
AST	139.66±0.506 a	140.667±0.371 a

ALT: Alanine-Amino transferase; **AST:** Aspartate amino transferase; **EAAS:** Aqueous extract of *Anonna squamosa*
The Mann-Whitney test was used to make comparisons against Controls. The comparison of the table is made in the horizontal direction. In the table, values followed by the same letter are not significantly different.

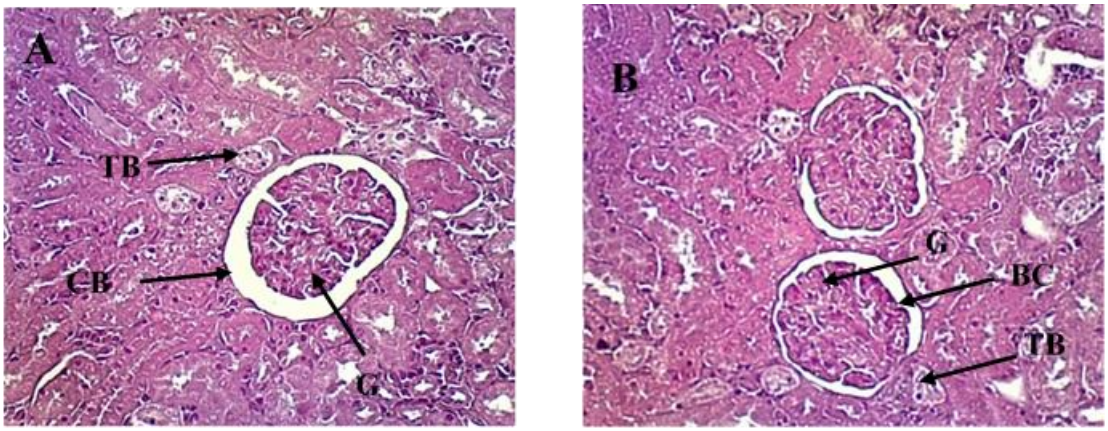

Figure 2: Histological section of the kidney (GX100)
A: Control (distilled water) ; **B:** EAAS (2000 mg/kg bw) ; **CB:** Bowman capsule ; **G:** Glomerulus ; **TB:** Tube Bypassed

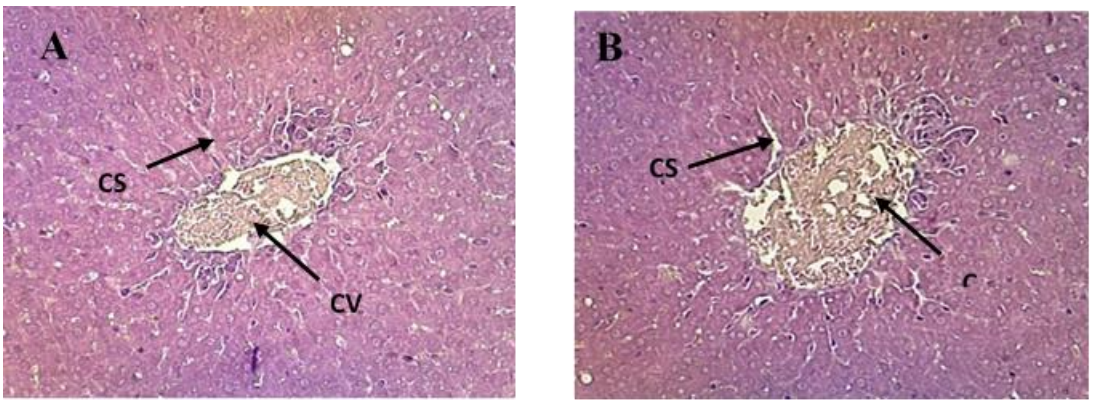

Figure 3: Histological section of the liver (GX100),
A: Control (distilled water) ; **B:** EAAS (2000 mg/kg bw) ; **CV:** Centrilobular Vein ; **CS :** Capillary Sinusoids.

3. Discussion

3.1.1. Identification des éleveurs

The evaluation of the acute toxicity of the aqueous extract of *A. squamosa* (EAAS) consisted of recording the various adverse effects which appeared after the administration of the single dose of 2000 mg/kg bw to adult rats. The toxicity of chemical substances was established according to the value of their LD50. Following the acute toxicity of the aqueous extract of the leaves of *A. squamosa* in female rats, administered orally, no deaths and no clinical signs were recorded at the single dose of 2000 mg/kg bw. If the administered dose is 2000 mg/kg bw, the LD50 would be greater than 2000 mg/kg bw orally.

Given the absence of results on the toxicity of the aqueous extract of *A. squamosa* leaves in female rats, the results of the present study were compared to that of Saleh et al. (2021) who showed that the LD50 of the methanolic extract of the leaves of *A. squamosa* is greater than 5000 mg/kg bw, administered orally, in male rats. According to the Globally Harmonized Classification System for Labeling of Chemicals OECD (2001), the aqueous extract of *A. squamosa* can be classified as category 5 or unclassified.

According to the toxicity scale of Hodge and Sterner (1943), the estimate of the LD50 at a value greater than 5000 mg/kg shows that the aqueous extract of the leaves of *A. squamosa* would be relatively harmless. The relative organ weights of treated rats showed no significant difference compared to the control. This result suggested that the single dose of 2000 mg/kg bw of the aqueous extract of *A. squamosa* would not influence the morphology of the organs.

The analysis of the hematological and biochemical parameters showed that the different parameters measured in the rats treated did not change significantly ($P > 0.05$) compared to the control. Saleh et al. (2021) also showed that biochemical analysis of aspartate amino transferase (AST), alanine amino transferase (ALT), albumin and globulin of animals administered with the methanolic extract of *A. squamosa* showed no significant differences compared to control groups.

The aqueous extract of the leaves of *A. squamosa* would therefore not be toxic at a single dose of 2000 mg/kg bw in adult rats. Microscopic examination of the liver and kidney of animals treated with the single dose of 2000 mg/kg bw of *A. squamosa* extract showed normal structural architecture compared to the control. This shows that oral administration of a single dose of 2000 mg/kg bw of *A. squamosa* extract does not cause any harmful changes and no morphological disorders. The absence of structural abnormality, inflammation, hepatic cell necrosis and apoptosis of the liver and kidney in rats treated with methanolic extracts of *A. squamosa* leaves confirms the results of the relative weight of organs and biochemical and hematological parameters. However, in terms of kidney histopathology, the results obtained in the present study were different from those of Saleh et al. (2021) who showed that kidney histopathology revealed some inflammation at 1000 mg/kg bw, 1600 mg/kg bw and 5000 mg/kg bw of the methanolic extract of *A. squamosa* leaves. This difference obtained in the results would be linked to the nature of the extract used which are not the same.

Conclusion

The acute toxicity of the aqueous extract of Annona squamosa at a single dose of 2000 mg/kg bw showed no toxicity on the behavior of female Wistar rats via the oral route. The analysis of the weight of vital organs (liver, kidney and heart) of treated rats did not differ from the control. The aqueous extract of Annona squamosa leaves (EAAS) did not modify the blood parameters and biochemical parameters of the treated rats. At a single dose of 2000 mg/kg bw, the substance tested is therefore not toxic in the short term to rats.

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