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Acute and subacute toxicity of the aqueous extract of the leaves of *Annona senegalensis* Pers. (Annonacee) in rats and mice

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Abstract

Annona senegalensis pers. is used in Africa for therapeutic and dietary purposes. However, few studies have been carried out in Burkina Faso on the toxicity of this plant's leaves. The aim of the study was to assess the acute and subacute toxicity of the aqueous extract of the leaves of *Annona senegalensis* on female rats and mice. The acute toxicity study was carried out on six female mice. The control group, received distilled water and the the test group received the extract at a single dose of 2000 mg/ kg bw. After administration, animals were observed for 14 days. For subacute toxicity, thirty female rats were used. The control group received distilled water. The test groups received *Annona senegalensis* extract at doses of 40, 100 and 200 mg/kg bw respectively for 28 consecutive days. Satellite groups were used. In the acute test, no sign of toxicity or mortality were recorded. The LD50 of the extract is therefore greater than 2000 mg/kg. In the sub-acute test, the body weight of treated rats increased ($p > 0.05$) compared to the control. Hematological analyses of white and red blood cell and platelet counts showed no significant variations ($p > 0.05$) compared with the control. Biochemical analyses showed no significant variations ($p > 0.05$) compared with the control. Histological sections showed no tissue damage in the liver and kidneys of rats treated with extract compared with the control. Our results show that the leaves of *Annona senegalensis* were non-toxic.

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Introduction

The use of herbal medicine, which is linked to the social, cultural and economic context of developing countries, plays a key role on human well-being. In Africa, over 80% of the population use it for primary health care (Duchene-Marullaz, 1989 ; WHO, 2002). The ongoing quest for natural remedies may stem from patients' disappointment with the efficacy of standard treatments. Moreover, according to some beliefs, the use of plants guarantees a healthier lifestyle and that medicinal plants, being natural, seem to be « harmless » (Ekor, 2014).

Annona senegalensis pers. is a species of the Annonaceae family native to Senegal, and then widely distributed throughout the semi-arid and sub-humid regions of Africa. All parts of the plant - leaves, flowers, fruit, stem and roots - have several medicinal uses (Arbonnier, 2009). Fruits are eaten directly when ripe and are a real source of food for the local population in Burkina Faso (Traoré *et al.*, 2011). The roots are eaten to relieve pulmonary disorders (Sofowora, 1993). The leaves possess antioxidant, antimicrobial, anticonvulsant, antimalarial and anti-inflammatory properties (Johnson *et al.*, 2002 ; Ajaiyeoba *et al.*, 2006 ; Ajboye *et al.*, 2010 ; Yeo *et al.*, 2011 ; Konate *et al.*, 2012).

Plant metabolites, although perceived as harmless, often contain phytochemical compounds responsible for toxic effects (Babu *et al.*, 2016 ; Poivre *et al.*, 2017 ; Hudson *et al.*, 2018).

The aim of this study was to assess the acute and subacute toxicity of the aqueous extract of the leaves of *Annona senegalensis* on female rats and mice.

Material and methods

Plant

The leaves of *Annona senegalensis* were collected in the Hauts Bassins, a region of western part of Burkina Faso. They were washed and dried under ventilation without sunlight and finely powdered. The plant was identified at the Department of Plant Biology and Ecology, Joseph Ki-ZERBO University, Ouagadougou, where a sample was kept under identification number 18049.

Animals

Naval Medical Research Institute (NMRI) mice and Wistar rats were used. These animals weighed an average of 22.5 g for the mice and 162.5 g for the rats. They were provided by the Joseph KI-ZERBO University, where they were bred at an average temperature of 22±3°C, a relative humidity of 50 ± 10% and subjected to a 12-hour light cycle. They had free access to food and water.

Preparation of extract

One hundred grams (100 g) of the leaves' powder was macerated in 1000 mL of distilled water for 24 hours. The filtrate was centrifuged at 2000 rpm for 10 minutes. The supernatant was frozen at -23°C and freeze-dried. The aqueous extract of *Annona senegalensis* leaves (EAAS) was thus obtained. The extraction yield was 24.23%.

Acute toxicity

The acute toxicity study was carried out in accordance with OECD (2001) guideline 423. Six (6) female mice aged between 8 and 12 weeks and weighing an average of 22.5g were divided into 2 groups. They were fasted for 4 hours before the start of the test. The control group received distilled water. The test group received the aqueous extract of *Annona senegalensis* at a single dose of 2000 mg/kg body weight (bw). The volume of water and extract administered was 1 ml/100 g body weight (bw). After treatment, all mice were carefully observed for the first 4 h, 24 h, 48 h and 72 h after extract administration, then daily for 14 days. Observations included behavioral changes such as tremor, convulsion, salivation, lethargy, somnolence, coma and mortality.

Subacute toxicity

The subacute toxicity study was carried out in accordance with the method described in OECD (2008) guideline 407. It was performed on 30 female rats divided into 6 groups of 5. The control group (group 1) received distilled water. The 3 test groups (2, 3, 4) received *Annona senegalensis* extract at doses of 40, 100 and 200 mg/kg bw respectively.

The satellite control group (group 5) received distilled water and the satellite group (group 6) received the extract at 200 mg/kg bw. Treatments lasted 18 days and the volume of water and extract administered was 1 ml/100 g bw. Satellite groups were observed for a further 14 days to detect any reversibility or delayed onset of toxic effects. During treatment, rat body weights were recorded every 4 days. At the end of treatment, rats were anesthetized with ketamine and necropsied. Organs such as heart, liver, kidneys, lungs and spleen were removed, and weighed.

Biochemical and haematological analysis

At the end of the various treatments, the rats were fasted for 12 h. They were anaesthetized, then by decapitation the blood was collected in EDTA tubes for blood counts. The remaining blood was collected in dry tubes and centrifuged at 3,000 rpm for 15 minutes. The serum obtained was stored at -20°C for biochemical analysis. Biochemical parameters such as AST, ALT, total cholesterol, creatinine, triglycerides, urea and alkaline phosphatase were assayed according to the Atlas Medical kit protocol.

Histological analysis

The liver and kidneys of rats exposed to 200 mg/kg and control rats were removed and fixed in formalin (10%) at the end of autopsy. After immersion in alcohol and xylene baths, the organs were embedded in kerosene. Histological sections of these organs were cut and spread on glass slides. After drying, the prepared slides were stained with hematoxylin and eosin (H&E) for morphological observation under the light microscope.

Statistical analysis

Data were expressed as means ± standard error. Graph Pad Prism version 5.03 was used to generate graphs and perform statistical tests. A one-factor analysis of variance (ANOVA) followed by Tukey's post-test was used to compare test groups with control groups. Means were compared at a significance level of 5% ($P<0.05$).

Results

Acute toxicity tests

At a single dose of 2000 mg/kg bw, *Annona senegalensis* extract showed no sign of toxicity in mice. No mouse mortality was either recorded. The lethal dose 50 (LD_{50}) is therefore assumed to be higher than 2000 mg/kg body weight.

Subacute toxicity study

Effects of aqueous extract of Annona senegalensis leaves on weight gain in rats

Aqueous extract of *Annona senegalensis* leaves at all dose levels produced a non-significant ($P>0.05$) increase in the body weight of treated rats compared with the control (Fig. 1).

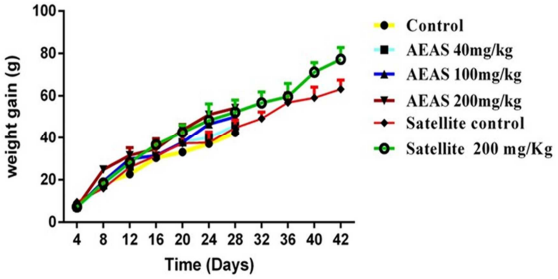

Fig. 1. Effects of aqueous extract of the leaves of *Annona senegalensis* on weight gain in rats

Effects of aqueous extract of the leaves of Annona senegalensis on the relative weight of rat organs

Table 1 show the results of relative organ weights exposed to different doses of aqueous extract of *Annona senegalensis*. All doses of the extract resulted in a non-significant decrease ($p> 0.05$) in the relative weights of liver and lung compared with the control. On the other hand, a non-significant ($p> 0.05$) increase in the relative weight of the spleen was recorded compared with the control. The doses of 40 and 100 mg/kg bw of extract produced a non-significant ($p> 0.05$) increase in relative weight of the kidney compared with the control. For the heart, extract at the dose of 40 mg/kg produced a non-significant ($p> 0.05$) increase in relative weight, while extract at the doses of 100 mg/kg and 200 mg/kg produced a non-significant ($p> 0.05$) decrease in relative weight when compared with the control. For satellite groups, the extract produced a non-significant increase in relative liver and lung weights.

Table 1. Effects of aqueous extract of the leaves of *Annona senegalensis* on the relative weight of organs

Organs	Treatment (mg/kg)					
	Control	EAAS 40	EAAS 100	EAAS 200	Control satellite	Satellite
Liver	2,88±0,02	2,84±0,02	2,87±0,02	2,81±0,01	2,38±0,01	2,80±0,02
Kidneys	0,59±0,04	0,60±0,06	0,60±0,03	0,59±0,02	0,59±0,03	0,61±0,04
Spleen	0,27±0,07	0,30±0,03	0,32±0,02	0,29±0,02	0,29±0,06	0,30±0,02
Lungs	0,97±0,01	0,76±0,01	0,84±0,02	0,78±0,02	0,71±0,02	0,82±0,01
Heart	0,43±0,03	0,44±0,03	0,42±0,04	0,41±0,03	0,42±0,03	0,41±0,04

Values are expressed as mean ± standard error to the mean (SEM) ; n = 5

Table 2. Effect of extract on haematological parameters

Parametres	Témoin	EAAS 40mg/kg	EAAS 100mg/kg	EAAS 200mg/kg	Control satellite	Satellite
WBC x 10^3/µl	05,845±2,614	05,61±0,385	05,610±0,385	05,96±0,284	05,684±0,435	05,491±0,573
LYM (%)	75,62±33,818	68,64±4,208	68,64±4,208	68,86±4,339	76,12±3,862	78,136±0,226
RBC x 10^6/µl	08,144±3,642	7,919±0,310	07,919±0,310	06,971±0,381	07,892±0,204	07,336±0,202
HB (g/dl)	14,41±6,444	14,207±0,602	14,207±0,602	12,546±0,613	13,552±0,148	13,102±0,135
HCT (%)	50,35±22,517	49,256±1,977	49,256±1,977	43,313±2,054	44,686±0,896	42,958±0,209
PLT (10^3/µL)	788,4±352,58	774,578±39,498	738,55±63,346	727,124±26,228	803,866±55,581	762,435±29,724

Values are expressed as mean ± standard error to the mean (SEM) ; n = 5, WBC : White blood cells, LYM : Lymphocytes, RBC : Red blood cells, HB : Hemoglobin, HCT : Hematocrits, PLT : Platelets

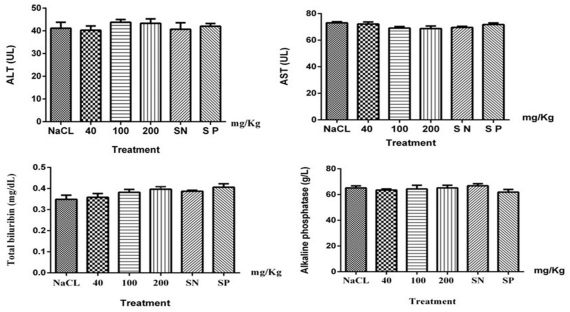

Fig. 2. Effects of aqueous extract of the leaves of *Annona senegalensis* on liver markers in rats

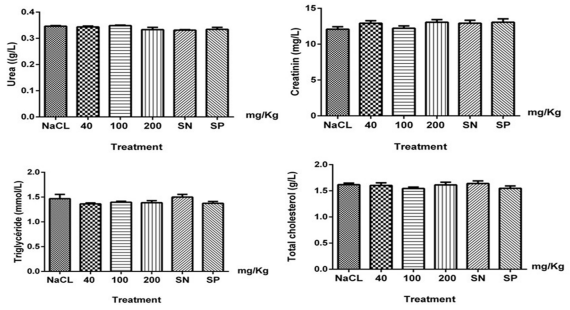

Fig. 3. Effects of aqueous extract of the leaves of *Annona senegalensis* on renal markers and lipid peroxidation in rats

Effects of aqueous extract of the leaves of Annona senegalensis on haematological parameters

Table 2 shows haemogram results of rats exposed to aqueous extract of *Annona senegalensis*. The extract at all doses caused a non-significant (p> 0.05) decrease in white and red blood cell count, platelet count, hematocrit and lymphocyte count compared with the control. The dose of 200 mg/kg extract caused a non-significant (p> 0.05) decrease in hemoglobin level compared with the control. For satellite groups, the extract induced a non-significant increase in lymphocyte count.

Effects of Annona senegalensis extract on biochemical parameters

At doses of 100 and 200 mg/kg, the extract produced a non-significant (p> 0.05) increase in ALT and total bilirubin levels compared with the control. On the other hand, at the same doses, the extract caused a non-significant (p> 0.05) decrease in AST levels compared with the control. Satellite groups showed a non-significant variation (p> 0.05) in ALT, AST, BT and PAL (Fig. 2). The doses of 40 and 200 mg/kg bw of extracts produced a non-significant (p> 0.05) increase in creatinine levels compared with the control. For all the doses, the extract caused a non-significant (p> 0.05) decrease in triglyceride levels compared with the control. Satellite groups showed a non-significant variation (p> 0.05) in creatinine and triglyceride levels (Fig. 3).

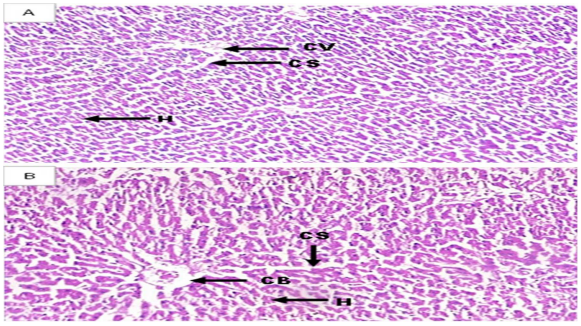

Fig. 4. Effects of the extract of the leaves of *Annona senegalensis* on rat liver sections (H&E x 400). A : control rat liver, B : rat liver treated with EAAS 200 mg/kg. CB : biliary canaliculus, CV : central vein, H : hepatocytes, CS : sinusoidal canaliculus

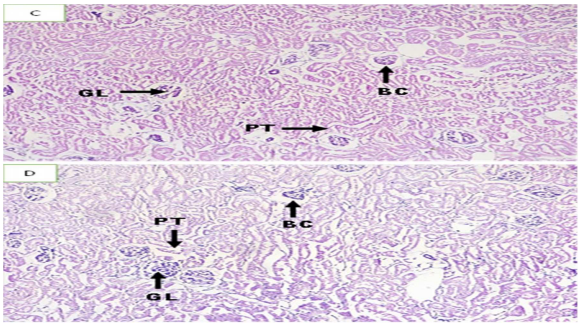

Fig. 5. Effects of the extract of the leaves of *Annona senegalensis* on rat kidney sections (H&E x 400). C : control rat kidney, D : rat kidney treated with EAAS 200 mg/kg. PT : proximal tubule, CB : Bowman's capsule, GL : glomerulus

Effects of Annona senegalensis extract on the histological structure of rat liver and kidney

Fig. 4 and 5 illustrate histological sections of liver and kidney from rats exposed to maximum doses of 200 mg/kg. The liver shows hepatic parenchyma with a centrilobular vein and distinct hepatocytes. The kidneys show normal proximal tubules and a glomerulus surrounded by Bowman's capsule. These sections show normal liver and kidney architecture in control and treated rats. No significant changes in liver and kidney parenchyma were observed when rats were exposed to 200 mg/kg *Annona senegalensis*.

Discussion

Oral administration of the aqueous extract of the leaves of *Annona senegalensis* at a single dose of 2000 mg/kg body weight did not cause behavioural changes or death in mice. Aqueous extract of the leaves of *Annona senegalensis* is therefore not toxic at this dose. The lethal dose 50 (LD50) is therefore assumed to be greater than 2000 mg/kg body weight. Onwusonye *et al.* (2014) showed that the methanoic extract of *Annona senegalensis* is not toxic at a dose of 5000 mg/kg bw. As a result, the extract of the leaves of *Annona senegalensis* could be classified in category 5, considered non-toxic by the oral route according to the OECD global harmonized classification system.

Weight changes in rats are regularly assessed in toxicology studies and are essential for interpreting compound-related effects (Hoffman *et al.*, 2002). An increase in body weight after drug administration generally indicates the absence of toxicity (Agrawal *et al.,* 2007). Aqueous extract of the leaves of *Annona senegalensis* at all doses resulted in a non-significant increase in body weight in rats compared with the control group. Yeo *et al.* (2011) demonstrated the presence of phenolics, tannins, coumarins, terpenes and flavonoids in the leaves of *Annona senegalensis*. These compounds could provoke reflex stimulation of gastric and biliary secretions in rats (Duarte *et al.*, 2005). They could induce an increase in lipase and amylase activities in the intestinal mucosa (Daferera *et al.*, 2003 ; Iscan *et al.*, 2002). These compounds may improve intestinal transit by stimulating intestinal peristalsis (Baba Aissa, 1991). The combined action of these bioavailable metabolites could induce an increase in the weight of rats treated with the aqueous extract of *Annona senegalensis*.

Relative organ weight is considered a sensitive indicator in toxicity studies (Lullmann-Rauch, 2008). It is often used for toxicological evaluation (Michael *et al.*, 2007). The extract of *Annona senegalensis* did not cause a significant change in the relative weight of certain organs (liver, lung, kidney, heart and spleen) in test animals compared with controls. Therefore, the aqueous extract of the leaves of *Annona senegalensis* does not contain -toxic substances.

One of the targets of therapeutic substances in the body is the hematopoietic system (Kplé *et al.,* 2022). Impaired erythropoiesis after administration of a therapeutic substance is characterized by anemia following lysis of blood cells by the product's active agents (Gandhare *et al.*, 2013). The extract of *Annona senegalensis* at all doses did not cause any abnormal variation in haematological parameters. Flavonoids are known for their ability to prevent various hematological disorders (Sadzuka *et al.* 1997). In fact, flavonoids provide protection against free radicals by preventing their binding to cell membrane lipids, thus protecting hematological parameters by enabling proper erythrocyte regeneration and preventing the leukopenia and thrombocytopenia observed in the presence of free radicals (Chaudhuri *et al.*, 2007, Chandana Venkateswara and Vijayakuma, 2008). *Annona senegalensis* contains secondary metabolites, including coumarins, lignans, sterols and triterpenes, anthracene derivatives, tannins, flavonoids and alkaloids (Roland *et al.*, 2022). The aqueous extract of the leaves of *annona senegalensis* may possess hematoprotective properties that may be attributed to the flavonoids. Kitadi *et al.* (2020) also demonstrated that the aqueous extract of the leaves of *Annona senegalensis* significantly prevents hemolysis of red blood cells. Furthermore, our results showed a non-significant increase in white blood cell count in satellite group treated with the extract at a dose of 200 mg/kg body weight, suggesting a later immunostimulatory effect of our extract (Alberts, 2005). *Annona senegalensis* could therefore be used as a complementary therapeutic agent in the treatment of immunodeficiency.

Transaminases (ALT and AST) and alkaline phosphatase are the main enzymes used to assess the state of liver function (Wallace *et al.*, 2010). They are widely used to assess liver damage caused by drugs or any other hepatotoxin (Ramaiah *et al.*, 2011). Indeed, any cell necrosis, destruction of liver parenchyma or increase in hepatocyte membrane permeability leads to the release of these enzymes into the bloodstream, thereby increasing their serum levels (Adeneye, 2006). The extract of *Annona senegalensis* at different doses did not significantly increase serum levels of these enzymes. These results show that our extract preserved liver function. Flavonoids and tannins are known to be hepatoprotective molecules (Narayana *et al.*, 2001 ; Da *et al.*, 2023). We can therefore assume that the flavonoids in our extract provided hepatocyte protection. Our results are comparable to those of Da *et al.*, (2023) who demonstrated that the aqueous extract of fruits of *Sarcocephalus latifolius* exerted a hepatoprotective effect in the case of paracetamol-induced hepatotoxicity in rats. This hepatoprotective effect was attributed to the flavonoids and tannins contained in the extract. Nevertheless more investigation is need in order to explain these findings. Histopathological examinations of the liver showed no structural alterations, confirming the absence of toxicity of the extract of *Annona senegalensis*.

The kidney, the main excretory organ for xenobiotics and their metabolites, is particularly sensitive to toxic effects (Gueguen *et al.*, 2006). Creatinine and urea are excellent markers of renal function. Their increase or decrease reflects renal dysfunction (Sirwal *et al.*, 2004). A decrease of at least 50% in glomerular filtration rate can lead to hypercreatinemia (Adler *et al.*, 2003). No significant changes in creatinine and urea values were observed when comparing test groups and control groups. Moreover, histological sections showed no structural alteration in the kidneys of test animals compared with the control. Therefore, the extract of the leaves of *Annona senegalensis* had no adverse effect on renal function. Various phenol and flavonoid derivatives have a potential nephroprotective effect (Hasan *et al.*, 2022). Through these secondary metabolites, our extract could have provided renal cytoprotection. Our results are in line with those of Neelima et *al* (2020), who also demonstrated the nephroprotective capacity of ethanolic extract of the leaves of *Annona squamosa* against paracetamol-induced nephrotoxicity. This nephroprotective effect was attributed to the flavonoids, glycosides, terpenes, tannins, polyphenols and alkaloids present in the extract.

There was no significant variation in lipid parameters (triglyceridemia, cholesteridemia). We noted a non-significant ($p > 0.05$) decrease in cholesterol and triglyceride levels at all extract doses. Many saponins have the ability to lower serum cholesterol levels (Malinow *et al.* 1980). Ikeda *et al.* (1998) have also demonstrated that phytosterols, with their greater affinity for micelles than cholesterol, reduce the incorporation of cholesterol into micelles, thereby increasing cholesterol elimination via the faeces. The extract of *Annona senegalensis*, through its secondary metabolites, would therefore have a beneficial effect on the cardiovascular system. A comparison of the different results in animals from satellite groups showed that the extract has no has no reverse and/or.late toxic effects.

Conclusion

The extract of the leaves of *Annona senegalensis* does not induce mortality and has no toxic effect. The overall results on haematological and biochemical parameters support the leaves of *Annona senegalensis* can be safe for medicinal use. These overall conclusions support the use of the leaves of *Annona senegalensis* for medicinal and dietary purposes for many years to come. More studies are needed to isolate the phytocompounds of the leaves *of Annona senegalensis* and determine their biological activities.

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