Available online on 15.07.2022 at http://jddtonline.info

Journal of Drug Delivery and Therapeutics

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Research Article

In vivo assessment of acute and subchronic toxicity of hydroethanolic extract of *Ximenia americana L.* (Olacaceae) stem bark

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Article Info:

Article History:

Received 19 May 2022
Reviewed 29 June 2022
Accepted 06 July 2022
Published 15 July 2022

Cite this article as:

Togbossi LA, Lawson-Evi P, Atchou NK, Motto EA, Diallo A, Eklu-Gadegbeku K, Aklikokou K, *In vivo* assessment of acute and subchronic toxicity of hydroethanolic extract of *Ximenia americana L.* (Olacaceae) stem bark , Journal of Drug Delivery and Therapeutics. 2022; 12(4):81-86

DOI: http://dx.doi.org/10.22270/jddt.v12i4.5567

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Abstract

Many studies have confirmed the traditional use of *Ximenia americana* (Olacaceae). Its different parts, leaf, fruit, roots, stem bark and root bark have showed pharmacological potentials. *X. americana* is a plant which possessed antibacterial, antidiabetic, antiviral and anticancer properties. The present study was undertaken to evaluate the oral acute and subchronic toxicity of hydroethanolic extract of *X. americana* stem bark on Sprague Dawley rats. In acute test, female rats were administrated once 5000 mg/kg and observed during 14 days for determination of toxicity signs. For the subchronic toxicity the extract (500 mg/kg and 1000 mg/kg) was administrated daily to both sexes of rats during 28 days. Control rats were administrated distilled water. Animals were observed, food and water consumption, mortality were monitoring during experiment period. Body weight, organs relative weight, hematological and biochemical parameters and histopathological changes were conducted at the end of treatment. Acute toxicity test revealed no adverse effects and no mortality. Extract administration during 28 days does not cause any behavioral change, change in body and organs relative weight and mortality. No significant variation was observed in hematological, biochemical parameters between treatment and control groups. No remarkable structural change was found in organs. However, triglycerides show significant variation in both sexes but this variation was not dose dependent. Results suggested that the LD_{50} of hydroethanolic extract of *X. americana* stem bark is higher than 5000 mg/kg and extract administration at 1000 mg/kg during 28 days does not cause toxicity in Sprague Dawley rats.

Keywords: *Ximenia americana*; stem bark; acute toxicity; subacute toxicity; hematological parameters; biochemical analysis.

INTRODUCTION

Plants have been used in developing countries in treatment of many diseases for long time. Up to 80 % of the world's population uses medicinal plants for their health care according to the World Health Organization. This practice continues in African's countries because of lack of health facilities due to cultural, traditional, socio-economic fact[1,2]. Many secondary metabolites synthetized by plants have been shown to have therapeutic properties making them potential targets in drug discovery. Several studies in the literature have demonstrated the efficacy of medicinal plants extracts in counteracting many pathologies[3]. Despite their availability and effectiveness, these plants can possess adverse effects. Some studies demonstrated that some medicinal plants contain potentially toxic substances [4,5,6]. It is therefore necessary to conduct toxicological studies on plant extracts to identify its potential adverse effects and to ensure their safety before its use[7]. *X. americana* (Olacaceae) named sea lemon is one of plants widely used in some African's regions for medicinal purposes. Authors have proved its therapeutic effect such as antiulcer, analgesic, antibacterial, anticancer, anti-diabetic[8,9,10,11]. Acute toxicity has been studying on its aqueous extract[12], acute and subchronic toxicological studies were carry out on its methanolic extract[13] but extracted molecules depend on the solvent used, hence the need to explore toxicity of its hydroalcoholic extract. Molecule's toxicity is correlate to the route and duration of exposure, the amount of its used, the effects on major organs and its physiological actions.

In the present study, we evaluated the oral acute and subchronic toxicity properties of *X. americana* stem bark hydroalcoholic extract on Sprague Dawley rats. Toxicological effects were studied trough variation of food and water consumption, body and organ weights, biochemical, hematological parameters and organs histopathology.

MATERIAL AND METHODS

Plant material

Ximenia americana L. (Olacaceae) stem bark was collected at Oubiog, 20 km of Dapaong (Togo) in August 2020 and identified. A voucher specimen was deposited on number TG 15213 in herbarium of Botany Laboratory and Plant Ecology of Lome University. *X. americana* L. stem bark was pulverized after washing and dried at 20°C for one week.

Animals

Studies were carried out on female and male Sprague Dawley rats weighing 120-140g from Faculty of Sciences laboratory of

Lome University. Rats were fed with normal pellet diet and water ad libitum, same sex were housed in standard polypropylene cages and maintained under standard laboratory conditions: temperature 24°C, relative humidity with cycle of 12 h darkness/ligh Experimental protocols were approved by the institutional guidelines and ethics of Laboratory of Physiology/Pharmacology of University of Lome-Togo (ref: 001/2012/ CB-FDS-UL).

Methods

Extract preparation

X. americana L. stem bark was pulverized after washing and dried at 20°C for one week and 400 g of powder were macerated in 4l of an ethanol-distilled water mixture (5:5, V/V) for 72 h. The extract mixture was filtered and the filtrate was evaporated under 45°C with rotavapor Heidolph made in Germany. Extraction yield was 27,85%.

Acute oral toxicity

Acute oral toxicity was performed on Healthy female rats according to the instructions of the Organization for Economic Cooperation and Development (OECD) test guidelines, 420 (OECD 420)[14]. Three female rats were fasted overnight with free access to water and were administrated orally *X. americana* stem bark hydroethanolic extract at the dose of 5000 $mg.kg^{-1}$. Animals were then observed for mortality, signs of acute toxicity, behavioural changes like aggression, agitation, somnolence, asphyxia, convulsions and paralysis once daily over 14 days. For the first day rats were observed during one hour after every thirty minutes.

Subchronic oral toxicity

This experiment was conducted according to the Organization for Economic Cooperation and Development guideline 407 for testing of chemicals (OECD 407)[15].

Thirty rats of both sexes were divided in three groups, each group was composed of 5 males and 5 females. Group I, control group received distilled water while Groups 2 and 3 (tested animals) received respectively 500 $mg.kg^{-1}$ and 1000 $mg.kg^{-1}$ of *X.americana* hydroethanolic extract. Distilled water and extract were daily administered orally during 28 consecutive days. Animals were observed during the experiment for morbidity and mortality. Body weight, food intake and water consumption were recorded daily.

At 29th day, animals were anesthetized and blood samples were collected via retro-orbital puncture in two different tubes for each animal. In EDTA tubes for hematological parameters and in in anticoagulant-free tubes for biochemical parameters. Animals were sacrificed were sacrificed by cervical dislocation. Liver, kidneys, brain, spleen, lungs, heart, testes for males and ovaries for female rats were isolated washed in saline solution (9g/l), examined macroscopically and weighed. Organs relative weight was calculated as follows:

Organ relative weight = (Organ weight/animal body weight) × 100

Hematological parameters

Blood samples were collected in EDTA containing tubes for hematological analysis. Blood components were determined using an automatic hematology analyzer (AJ-2400 Auto). Hematological parameters such as Red Blood Cells (RBC), White Blood Cells (WBC), Platelets (Plt), Hemoglobin (Hb), Hematocrit (Ht), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin Concentration (MCHC), Procalcitonin (PCT), Red Blood Cell distribution unit (RDW), Platelet Distribution Width (PDW), Platelet Large Cell Ratio (P-LCR), and Mean Platelet Volume were estimated.

Biochemical parameters

For biochemical profile, blood samples were collected in anticoagulant-free tubes. After centrifugation at 3000 rpm over 10 min, serums were separated. Serum glucose, creatinine, urea, alanine transaminase (ALT), aspartate transaminase (AST), C-reactive protein (CRP), creatinine phosphokinase (CPK), triglyceride (TG), total cholesterol (T-Chol) and HDL were determined using an automatic analyzer (Rayto Chemray-120).

Histological examination

Liver, kidney and spleen were fixed in 10% phosphate-buffered formalin solution and examined microscopically.

Statistical analyses

Results were analyzed using graphpad prism 6.02 (Software, San Diego California USA) and the values were expressed as means ± standard error (n = 5 replicates). Comparisons between the different values were made using one way analysis of variance (ANOVA) test followed by the Tukey's test as a post hoc analysis. Differences between groups were considered significant at $p < 0.05$.

RESULTS

Acute oral toxicity of hydroethanolic extract of *X.americana*

No death, signs of toxicity and behavioral changes (aggression, agitation, somnolence, asphyxia) were observed 24 hours after administration of *X. americana* stem bark hydroethanolic extract at 5000mg/kg. It is concluded that *X. americana* stem bark hydroethanolic extract oral median lethal dose (LD_{50}) is higher than 5000 $mg.kg^{-1}$ in rats.

Subchronic oral toxicity

Effect on Bodyweight, food and water consumption of hydroethanolic extract of *X.americana*

Administration of *X. americana* extract at 500mg/kg and 1000mg/kg did not affect negatively animals (females and males) weight compared to control groups (Fig 1A and 1B). There were no significant differences in water and food intake between the control and treated groups.

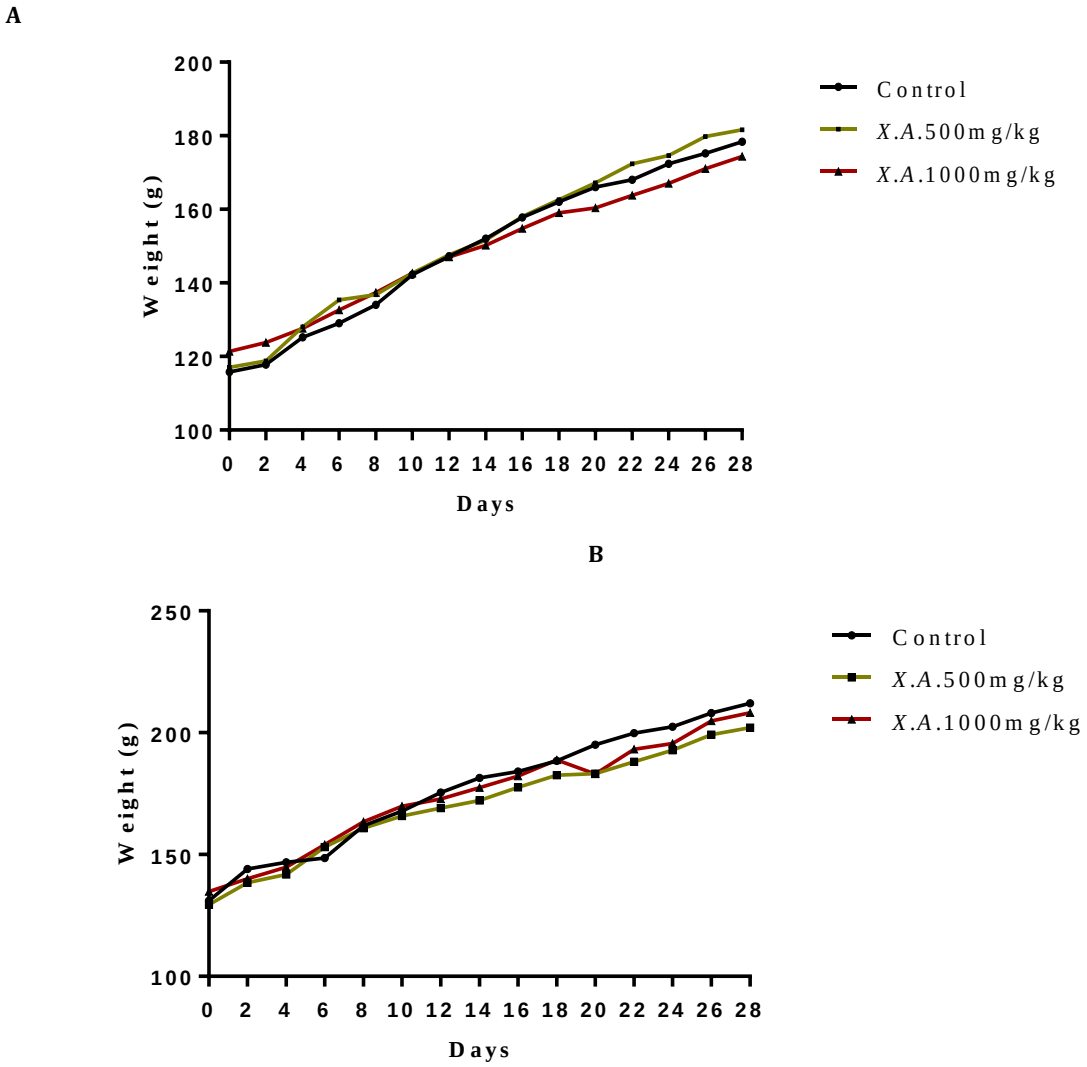

Figure 1: Effects of *X. americana* hydroethanolic extract on females (A) and males (B) rats body weight.

Extract effect on organs relative weights

Both sexes treated animals' organs did not show any change compared to control group in macroscopic examinations and organs relative weights didn't show significant variation compared to the control group (Table 1).

Table 1: Effects of *X. americana* hydroethanolic extract on relative weight of organs

Female rats	**Control**	***X.A.* 500 mg/kg**	***X.A.* 1000 mg/kg**
Liver	3.10 ± 0.14	3.27 ± 0.23	3.62 ± 0.21
Kidneys	0.68 ± 0.02	0.62 ± 0.01	0.67 ± 0.01
Brain	0.82 ± 0.05	0.88 ± 0.01	0.91 ± 0.03
Spleen	0.39 ± 0.03	0.45 ± 0.04	0.37 ± 0.02
Heart	0.37 ± 0.02	0.37 ± 0.01	0.34 ± 0.01
Lungs	0.67 ± 0.02	0.78 ± 0.07	0.72 ± 0.07
Ovaries	0.07 ± 0.01	0.06 ± 0.01	0.06 ± 0.01
Males rats	**Control**	***X.A.* 500 mg/kg**	***X.A.* 1000 mg/kg**
Liver	2.89 ± 0.14	3.30 ± 0.12	2.92 ± 0.02
Kidneys	0.61 ± 0.01	0.66 ± 0.03	0.65 ± 0.02
Brain	0.78 ± 0.01	0.82 ± 0.03	0.83 ± 0.03
Spleen	0.31 ± 0.01	0.30 ± 0.02	0.34 ± 0.02
Heart	0.38 ± 0.02	0.40 ± 0.01	0.40 ± 0.03
Lungs	0.68 ± 0.02	0.71 ± 0.01	0.69 ± 0.06
Testis	1.23 ± 0.05	1.19 ± 0.08	1.26 ± 0.03

Organs were removed and weighted on the last day. Data are expressed as Mean ±SEM (n =5). One-way ANOVA followed by Tukey's multiple comparisons test. Values not significant at $p<0.05$.

Extract effects on biochemical parameters

No significant changes were observed at *p<0,05* in serum glucose, hepatic enzymes (ALT, AST), kidneys markers (Creatinine, Urea), inflammation marker (CRP),Creatinine phosphokinase (CPK), Total Cholesterol (T-Chol) and HDL compared to control group. Nevertheless, significative variation in Triglyceride (TG) (*p<0,05)* were found in both sexes of animals (Table 2).

Table 2: Effects of *X. americana* hydroethanolic extract on biochemical parameters

Female rats	**Control**	***X.A*. 500 mg/kg**	***X.A*. 1000 mg/kg**
CRP(mg/dl)	20.48 ±1.42	21.96 ± 0.83	22.90 ± 1.13
Gly (g/l)	0.73 ± 0.04	0.74 ± 0.07	0.67 ± 0.05
Urea (g/l)	0.47 ± 0.01	0.49 ± 0.03	0.58 ± 0.04
Creat (mg/l)	6.60 ± 0.24	7.40 ± 0.60	6.80 ± 0.20
ASAT	179.40 ± 3.53	163.20 ± 15.21	177.00 ± 5.86
ALAT	75.80 ± 3.61	70.40 ± 7.07	81.40 ± 4.78
CPK (U/l)	229.94 ± 78.36	215.06 ± 36.44	268.88 ± 91.03
Chol T (g/l)	0.86 ± 0.03	0.92 ± 0.05	0.83 ± 0.01
HDL (g/l)	0.62 ± 0.02	0.72 ± 0.05	0.65 ± 0.01
TG (g/l)	0.602 ± 0.02	0.780 ± 0.04*	0.782± 0.03*
Males rats	**Control**	***X.A*. 500 mg/kg**	***X.A*. 1000 mg/kg**
CRP(mg/dl)	25.83 ± 5.23	20.48 ± 0.24	19.57 ± 1.85
Gly (g/l)	0.71 ± 0.05	0.65 ± 0.09	0.78 ± 0.06
Urea (g/l)	0.46 ± 0.01	0.56 ± 0.06	0.53 ± 0.04
Creat (mg/l)	7.40 ± 0.24	7.60 ± 0.68	7.20 ± 0.49
ASAT	177.80 ± 6.04	185.40 ± 10.37	185.80 ± 14.21
ALAT	74.60 ± 2.38	86.60 ± 5.69	86.00 ± 4.93
CPK (U/l)	383.51 ± 74.62	307.77 ± 67.51	436.20 ± 72.41
Chol T (g/l)	0.85 ± 0.06	0.76 ± 0.03	0.80 ± 0.03
HDL (g/l)	0.68 ± 0.05	0.59 ± 0.04	0.57 ± 0.05
TG (g/l)	0.634 ± 0.03	0.784 ± 0.05	0.841 ± 0.07*

Serums were collected for biochemical analyses. Data are expressed as Mean ±SEM (n =5). One-way ANOVA followed by Tukey's multiple comparisons test.

Extract effects on hematological parameters

The administration of *X. americana* did not cause any significant changes in blood components (red blood cells, white blood cells, platelets, hemoglobin) and parameters (hematocrit, mean corpuscular volume, mean corpuscular hemoglobin) in both sexes at the doses of 500 mg/kg and 1000 mg/kg(Table 3).

Table 3: Effects of *X. americana* hydroethanolic extract on hematological parameters

Females rats	**Control**	***X.A*. 500 mg/kg**	***X.A*. 1000 mg/kg**
WBC (10^3/µl)	11.53 ± 1.07	8.26 ± 0.19	9.81 ± 1.06
RBC(10^6/µl)	6.99 ± 0.26	7.25 ± 0.08	6.95 ± 0.14
PLT(10^3/µl)	732.80 ± 54.51	617.20 ± 73.93	758.20 ± 16.46
HGB(g/dl)	14.38 ± 0.24	14.78 ± 0.17	14.8 ± 0.09
HCT(%)	36.66 ± 0.94	38.02 ± 0.64	37.94 ± 0.26
MCV(fl)	52.62 ± 0.61	52.46 ± 0.35	54.60 ± 0.94
MCHC (g/dl)	39.18 ± 0.33	38.86 ± 0.26	38.94 ± 0.29
MCH (pg)	20.56 ± 0.39	20.32 ± 0.11	21.20 ± 0.51
PDW (fl)	8.96 ± 0.31	8.22 ± 0.2	8.68 ± 0.49
RDW (%)	15.62 ± 0.05	16.14 ± 0.22	15.66 ± 0.16
P-LCR (%)	18.25 ± 1.00	17.37 ± 1.06	17.14 ± 1.90
MPV (fl)	6.8 ± 0.12	6.44 ± 0.11	6.64 ± 0.20
PCT (%)	0.52 ± 0.03	0.47 ± 0.02	0.5 ± 0.02
Males rats	**Control**	***X.A*. 500 mg/kg**	***X.A*. 1000 mg/kg**
WBC (10^3/µl)	10.69 ± 2.01	9.34 ± 0.99	8.25 ± 0.74
RBC(10^6/µl)	7.35 ± 0.18	6.71 ± 0.38	7.02 ± 0.28
PLT(10^3/µl)	752.00 ± 30.86	750.40 ± 36.35	885.20 ± 43.31
HGB(g/dl)	14.82 ± 0.21	13.14 ± 0.75	14.2 ± 0.25
HCT(%)	38.1 ± 0.82	34.18 ± 2.06	37.38 ± 0.65
MCV(fl)	51.94 ± 0.50	50.98 ± 0.20	53.34 ± 1.52
MCHC (g/dl)	38.90 ± 0.29	38.42 ± 0.37	37.90 ± 0.05
MCH (pg)	20.12 ± 0.28	19.52 ± 0.17	20.16 ± 0.59
PDW (fl)	8.66 ± 0.44	8.26 ± 0.31	8.4 ± 0.21
RDW (%)	16.58 ± 0.46	17.24 ± 0.42	16.06 ± 0.24
P-LCR (%)	20.18 ± 2.61	19.59 ± 1.17	19.1 ± 1.43
MPV (fl)	6.52 ± 0.13	6.4 ± 0.13	6.6 ± 0.13
PCT (%)	0.47 ± 0.02	0.49 ± 0.01	0.53 ± 0.02

Blood samples were collected for hematological analysis. Data are expressed as Mean ±SEM (n =5). One-way ANOVA followed by Tukey's multiple comparisons test. Values not significant at p<.0.05).

Extract effect on organs histology

No histological changes were not observed between the control and treated groups. Liver of all groups showed hepatocyte spans forming lobules delimiting door spaces (Figure 2A), kidneys showed glomeruli and renal tubes of normal structure and morphology (Figure 2B), and spleen presented the lymphoid follicles formed by lymphocytes (Figure 2C).

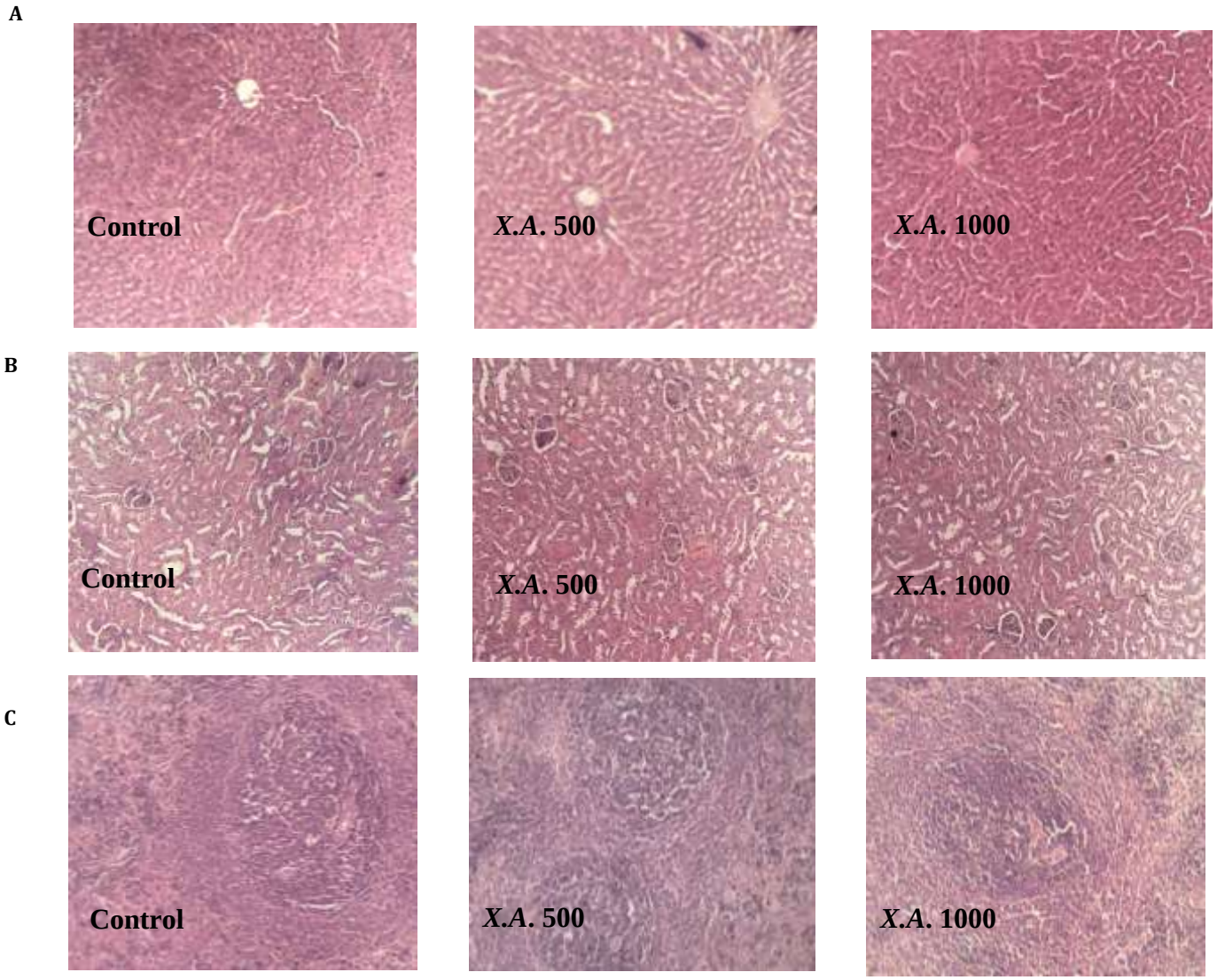

Figure 2: Histological study of liver (A), kidney (B) and spleen (C)

DISCUSSION

X. americana is one of important plants widely used traditionally. Our previous study showed it antioxidant activity *in vitro* and *in vivo* on ICR mice[16]. Other studies have proved therapeutics effects of its different parts (leaves, stem bark, roots, fruits)[8,10,17]. This species therefore constitutes a reserve of beneficial molecules to humans' health. According to its beneficial effects, it is necessary to explore the toxicological action on organism to ensure its use. Toxicological studies include acute and subchronique tests. Acute toxicity test gives the range of doses that could be toxic to the animal while the subchronic toxicity test is used to assess the health hazards which can result from long-term exposure to chemicals (drugs and xenobiotics). Both toxicity tests were used to estimate the adverse effects and target organ toxicity of molecules on animal's organisms. Toxicological studies are therefore conducted to ensure the safety of substances.

As mentioned earlier, toxicity studies have been conducted on aqueous and methanolic extracts of *X. americana* and results showed that methanol extract conduct to vascular congestion in kidney and distorted germinal centres in spleen[13]. Acute toxicity of aqueous extract on Swiss albino mice showed excitement, lack of appetite and later reduced activity during the first 24 h but no alteration in hematological and histopathological examination (Maikai *et al.,* 2008). In our study, we evaluated acute and subchronic toxicological effects of hydroethanolic extract of *X. americana* stem bark. No death was observed over 14 days after treatment with a single dose of extract at 5000mg/kg, in addition, no signs of gross toxicity and behavioral changes were noticed. Therefore, the LD_{50} of the extract is greater than 5000 mg/kg.

In 28-days oral toxicity study, rats were administrated with two doses of extract (500 and 1000 mg/kg/day) during 28 days with. No mortality and toxicity signs were observed throughout the experiment period. The findings didn't reveal treatment-related changes in organs morphological alterations, physiological toxic effects and tissue damages. Food and water consumption, body and target organs relative weight of animals did not show any significant change indicate that the extract did not affect negatively appetite, animals' growth and organs integrity contrary to previous study which showed significative increased in lungs weight with methanolic extract[13].

Hematopoietic system is one of targets of toxic molecules and change in this system is correlate to pathology statement, hematological parameters are therefore linked to toxicity studies. Blood components and red blood cell indices were not significantly *(p<0.05)* different from the control group in both sexes suggesting that extracts have no adverse effects on bone marrow activity and immune system of the treated animals[18,19].

Kidneys and liver are two major organs whose dysfunction leads to serious physiological disorders. Determination of molecules actions on those organs still crucial, so we evaluated extract effect on biochemical parameters including hepatic enzymes (AST and ALT) and kidneys function markers (urea and creatinine). Those parameters did not present any significant change in both sexes rats indicating that extract did not affect liver and kidney's function. CPK values were not significantly different from the control group in both male and female rats showing that *X. americana* did not disturb muscular and cardiac function[20]. Daily administration of the *extract* for 28 days increased triglycerides in both sexes but HDL content variation was not significant. Females rats showed important modifications because they are physiologically more sensitive than males[21,22]. This can be explained by the fact that triglyceride levels are very sensitive more than HDL cholesterol.

The absence of variations found in body weight, organs relative weight and biochemical parameters were confirmed by organs histopathology which not show any alteration compared to control animals. Liver, kidneys and spleen of treated animals showed normal architecture.

Results showed slight differences in our studies and previous studies. This may be due to differences in extraction methods, solvents use and the sampling sites.

CONCLUSION

This study didn't present mortality, gross pathological changes and treatment-related adverse effects in Sprague Dawley rats. The finding showed no toxic effect with a single oral administration of extract at dose of 5000 mg/kg. Administration of *X. americana* extract did not affect body weight, organ weight, food and water intake. No significant differences were found in hematological parameters, no abnormalities in liver enzymes, renal function markers and organs architectures after 28 days treatment with *X. americana* extract. Therefore, this species can be used safely by population in treatment of various pathologies. Nevertheless, there is need to explore other aspects of its toxicity to confirm the safety of its use.

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