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ACUTE AND SUBACUTE TOXICITIESOF THE AQUEOUS EXTRACT OF *SIMAROUBA AMARA* AUBLET STEM BARK

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Keywords:

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ABSTRACT: The decoction of *Simarouba amara* stem bark has been used to treat malaria, inflammation, diarrhea and as tonic. The aim of this study was to evaluate acute and subacute toxicities of the aqueous extract of *S. amara* stem bark (SAAE) in rats and mice, respectively. For the acute toxicity test, the SAAE was administered in a single oral dose of 2.0 g/kg (n = 5/group/sex) and in the subacute, the following doses were used: 0.25, 0.5 and 1.0 g/kg/day (n = 12/group/sex, p.o.), for 30 consecutive days. In the acute toxicity, SAAE did not produce mortality nor clinical signs of toxicity. The subacute treatment did not alter water intake, however, in the last week there were reductions body mass gain of males and females treated with the highest dose. The males showed increased levels of total protein and albumin (all doses), of alkaline phosphatase and total bilirubin (doses of 0.5 and 1.0 g/kg) and leukocytosis (1.0 g/kg). The females showed hyperuricemia (0.5 and 1.0 g/kg), hypertriglyceridemia (1.0 g/kg) and increased levels of RDW (1.0 g/kg).The histological analysis showed discrete lymphocytic infiltrate in the heart of males (1.0 g/kg), discrete hepatic steatosis in the females (0.5 and 1.0 g/kg) and increased renal capsular space and hypertrophy of adrenal gland espongiocytes (both sexes treated with 1.0 g/kg). In conclusion, the subacute administration of SAAE in the highest dose suggests some toxicity in the long term, but further studies should be conducted for confirmation.

INTRODUCTION: Since the advent of human begins, it is very much possible that they were afflicted with diseases and in course of time started using various ingredients including plants[1].

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Notably, the ethnomedicinal knowledge is usually passed from one generation to the next through members of the family or persons serving as apprentices to the practitioner.

Thus such indigenous communities' knowledge and particularly the smaller ones (i.e. communities whose population is below 500 persons) reflect knowledge acquired and accumulated over centuries and even possibly millennia[2]. Many studies prove that medicinal plants have high

therapeutic value and recognized properties of healing, prevention, diagnosis or treatment of disease symptoms. Thus, itsuse is beneficial and recommended. However, some researches show that many of these plants have harmful substances and, therefore, should be used with caution, respecting their toxicological risks[3].

Simarouba amara Aublet, popularly known as "simaruba", “praíba”, “marupá” and “pau-paraíba”, is a large tree that reaches up to 40 m height and 0.5 to 0.9 m diameter. In traditional medicine, the decoction of *S. amara* stem bark has been used to treat malaria, inflammation, diarrhea and as tonic[4]. Pharmacological assays showed that its fruits showed antimalarial action[5], its stem bark showed ambicide and bactericidal activities (against *Shigella flexineri* and *Salmonella typhosa*) and its root bark showed moisturizing action in the human epidermis[4].

Our laboratory has demonstrated anti-inflammatory, antiulcer and hepatoprotective activities of the aqueous extract of *Simarouba amara* stem bark at dose of 100 mg/kg of Wistar rats’ body weight (data under consideration for publication).

Simarouba amara is inserted only in the first volume of the Brazilian Pharmacopoeia (1926) and, recently, the National Health Surveillance Agency (Anvisa) through of the Pharmacopoeia Commission, has sought to develop, review and update the monographs for editing a consolidated Brazilian Pharmacopoeia. *Simarouba amara* monograph is one that should be reviewed and updated in accordance with this project[6].

However, there is a lack of toxicological information about *S. amara* in the literature. The aim of the present study was assess the acute and subacute toxicities of the aqueous extract of *S. amara* stem bark (SAAE) in Wistar rats and Swiss mice, respectively, in order to verify the safety of its use in the traditional medicine.

MATERIALS AND METHODS:

PLANT MATERIAL:

The stem barks of *Simarouba amara* Aublet were collected in Sao Joao, Pernambuco, Brazil (08° 52' 33" S and 36° 22' 01" The Gr) and identified at the Agronomic Institute of Pernambuco. A voucher specimen was deposited at the Dardano de Andrade Lima Herbarium under the number 85268.

Extract Preparation

The stem barks of *Simarouba amara* Aublet were collected in December, 2010. The samples were dried for 48 hours in the shade and then placed in a circulating air oven at a temperature of 45 ± 2 °C to stabilize the residual moisture. The barks were then ground in a Knives mill. The aqueous extract of *Simarouba amara* stem bark (SAAE) was obtained from the decoction of the powder (10:100 w/v) using distilled water as extractor solvent for a period of 10 minutes. The aqueous extract was concentrated in lyophilizer. The yield of *Simarouba amara* dried extract was 12.15 g per liter of the aqueous extract of *Simarouba amara* stem bark.

PHYTOCHEMICAL SCREENING

Thin Layer Chromatography

The dry extract of *Simarouba amara* stem bark were analyzed for the presence of hydrolyzable tannins (gallic and ellagic acids), condensed tannins (catechins), flavonoids, saponins, coumarins, phenylpropanoids, cinnamic acid derivatives, alkaloids, triterpenes/steroids, monoterpenes, sesquiterpenes, quassinoids, iridoids and sugars. The phytochemical profile was assessed in silica gel chromatographic plates (Merck® art. 105553, UV 250-366nm) using appropriate mobile phases, reagents and standards.

High Performance Liquid Chromatography (HPLC)

HPLC conditions: chromatographic analysis to quantify tannins and chlorogenic acid in the extractive solution were conducted in liquid chromatograph Shimadzu® (UFLC, Japan) controlled by the software LC Solution 1.0 and consisting of LC-20 AT pump, degasser DGU - 20A5, Sil-20A autosampler and detector diode array (DAD) SPD - M20A. It was used Restek® C18 column (250 mm x 4 mm, 5µm) maintained at 30 °C.

The standards and samples were eluted using a mobile phase gradient consisting of methanol (A) and 0.5% acetic acid (pH 3.0) (B). The conditions

were: 0-50 min - 10-90% A and 90-10% B, 50-55 min - 90-10% A and 10-90% B, 55-60 min - 10% A and 90% B. Flow rate of 0.8mL/min and injection volume of 20μL.

Samples preparation: for extractive solution, it was prepared an aqueous solution of the dried extract from *Simarouba amara* stem bark in a concentration of 1.6mg/mL. For standards, were prepared aqueous solutions of catechin (1.6 mg/mL), gallic and ellagic acids (0.16mg/mL), epicatechin (0.1mg/mL) and methanolic solution of chlorogenic acid (0.05mg/mL). The standard concentrations were used to construct their analytical curves. All samples and standards were filtered through membranes of 0.22μm (Millipore®) and injected in triplicate. The chromatograms were obtained at 290 nm.

Animals:

Male and female Wistar rats (*Rattus norvegicus* var. *albinus*) (aged 2 months, weighing 250-280 g and 210-240 g, respectively) were obtained from the Department of Physiology and Pharmacology at the Federal University of Pernambuco (UFPE), Pernambuco, Brazil. Male and female Swiss mice (aged 2 months, weighing 35-40 g and 30-35 g, respectively) were obtained from the Keizo Asami Immunopathology Laboratory (LIKA/UFPE), Pernambuco, Brazil. The animals were kept under standard environmental conditions (22 ± 2 °C); 12:12 h dark/light cycle. Water and industrialized dry food (Presence®, Purina, Brazil) were made available *ad libitum*. The experimental protocol was approved by the Animal Experimentation Ethics Committee of the UFPE (Process nº. 26449), in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals.

Acute Toxicity:

"Up and down" acute toxicity studies were performed on Wistarrats of both sexes as described by Organization for Economic Co-Operation and Development (OECD) [7], with slight modifications. The animals were randomly divided into four groups (n = 5/group/sex) and deprived of feed for 12 h with access to water *ad libitum*. The treated groups received SAAE in a single oral dose of 2.0g/kg and the control groups received water (1mL/100 g). The observations were performed at 30, 60, 120, 180 and 240 minutes after the oral treatments and daily for 14 days. Behavioral changes, weight, food and water intakes, toxicity clinical signs and mortality were recorded daily[8].

Subacute Toxicity

Male and female Swiss mice were randomly divided into 8 groups (n= 12/group/sex). Animals received water-vehicle orally (control group) or SAAE at the doses of 0.25, 0.5 and 1.0 g/kg/day for 30 consecutive days. Body weight was recorded weekly and food and water intakes were monitored daily.

Animals were observed for signs of abnormalities during the treatment period. At the end of the treatment, animals were fasted overnight, but allowed access to water *ad libitum*. They were then anesthetized using Nembutal® (0.035g/kg, i.p.), and blood samples were obtained by retro-orbital puncture[8], using capillary tubes for hematological and biochemical studies, with and without ethylenediamine tetra acetic acid (EDTA) anticoagulant, respectively.

Hematological and Biochemical Analysis

Hematological evaluation was performed using an automatic hematological analyzer (Coulter STKS, Beckman). Parameters included: red blood cell (RBC) count, white blood cell (WBC) count, hemoglobin (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), platelets count and mean platelet volume (MPV).

The differential leukocyte count was performed with an optical microscopy after staining and, in each case, 100 cells were counted.

For biochemical analysis, blood was centrifuged at 1500×g for 10 min to obtain serum, which was stored at -20 °C until the following parameters were determined: glucose; blood urea nitrogen (BUN); creatinine; uric acid; aspartate aminotransferase (AST); alanine aminotransferase (ALT); alkaline phosphatase (AF); total, direct and indirect bilirubin, lactate dehydrogenase (LDH),

total protein, albumin, total cholesterol and triglycerides. Dosages were made using Architect (Abbott®) automation with BoehringerIngelheim® biochemical kits[9].

Morphological Study

After euthanasia of the animals with an excess of Nembutal® (0.140g/kg, i.p.), necropsy was performed to analyze the macroscopic external features of the heart, lungs, stomach, liver, spleen, kidneys, adrenal gland, testicle, epididymis, seminal vesicle, uterus, ovaries. These organs were carefully removed and weighed individually. Organ weights were expressed in absolute and relative terms (g/100g of body weight). Histological evaluation was performed in six animals randomly selected from each group. Organ tissue sections were fixed in 10% buffered formalin. After setting, the samples were washed with water, immersed in 70% ethyl alcohol for 3-4 days and embedded in paraffin. Paraffin sections of 5 µm were obtained from rotational microtome and stained with hematoxylin and eosin (HE). Histopathological analyzes were carried out using an automatic microscopy system MICRO DIP® KacilInc [10].

Statistical Analysis

The results were expressed as mean ± standard error of mean (S.E.M). Statistical analysis was performed using Graph PadPrism5.0® software. The difference between groups was assessed by analysis of variance (ANOVA), followed, when necessary, by Newman – Keuls test. The significance level for rejection of the null hypothesis was always ≥5% ($p<0.05$)[8].

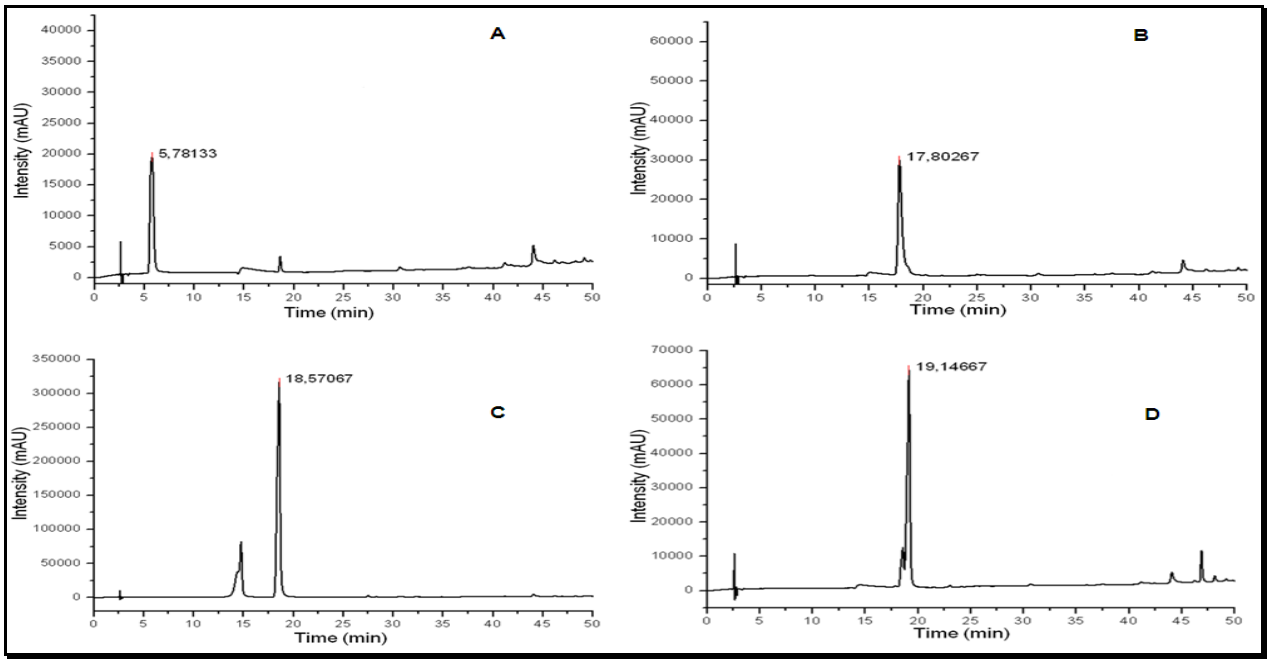
RESULTS:

Thin Layer Chromatography

Phytochemical analysis of dry extract from *S. amara* stem bark demonstrated the presence of hydrolyzable tannins (gallic and ellagic acids) and tannins condensed (proanthocyanidins and leucoanthocyanidins). Leucoanthocyanidins are precursors of procyanidins, class in which is inserted condensed tannins (catechin and epicatechin). Phenylpropanoids and cinnamic acid derivatives (cafeic acid) also were identified as well as traces of saponins, steroids and quassinoids.

High Performance Liquid Chromatography (HPLC)

It was observed the presence of chromatographic peaks consistent with the standards. Standard retention times were 5.78, 17.80, 18.57, 19.15 and 32.6 min for gallic acid (A), chlorogenic acid (B), catechin (C), epicatechin (D) and ellagic acid (E) **(Figure 1)**, respectively. The concentration of the main metabolites present in *S. amara* extractive solution were 5.76, 17.9, 18.57, 19.14 and 32.6 min (Figure 1E), respectively. Their concentrations were gallic acid (16.47µg/mL), chlorogenic acid (3.83µg/mL), catechin (349.96µg/mL), epicatechin (16.43µg/mL) and ellagic acid (1.29µg/mL). Linear regression analyzes indicated linearity of the method.



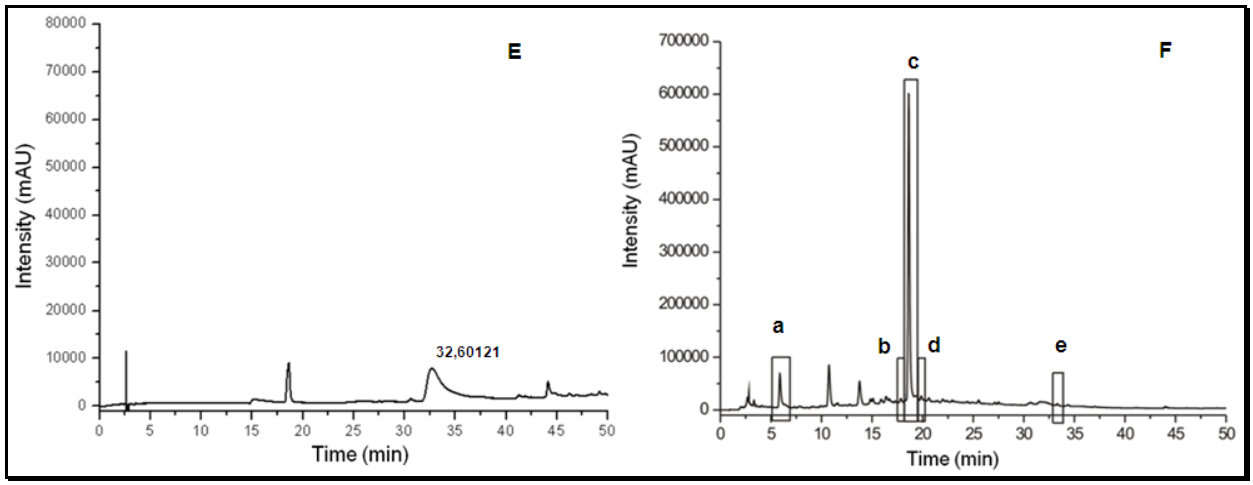


FIGURE 1: HPLC OF STANDARDS AND EXTRACTIVE SOLUTION OF *SIMAROUBA AMARA* STEM BARK. A – GALLIC ACID; B – CHLOROGENIC ACID; C – CATECHIN; D – EPICATECHIN; E – ELLAGIC ACID, F – EXTRACTIVE SOLUTION OF *S. AMARA* (A – GALLIC ACID; B – CHLOROGENIC ACID; C – CATECHIN; D – EPICATECHIN; E – ELLAGIC ACID).

Acute Toxicity

Acute oral treatment with SAAE in a single oral dose of 2.0g/kg did not produce any clinical signs of toxicity or mortality in male or female rats. Likewise, the food and water intakes and body mass gain of the animals treated with SAAE was similar to those of the control group during the 14 days of observation (data not shown).These results suggest a LD_{50} higher than 2.0 g/kg indicating low toxicity of SAAE.

Subacute Toxicity

No signs of toxicity (such as piloerection, alteration in the locomotor activity or diarrhea) or mortalities were recorded during the 30 consecutive days of treatment by oral route with SAAE at doses of 0.25, 0.5 or 1.0g/kg. The treatment of male mice did not change the water intake during the whole period compared to control **(Figure 2)**.

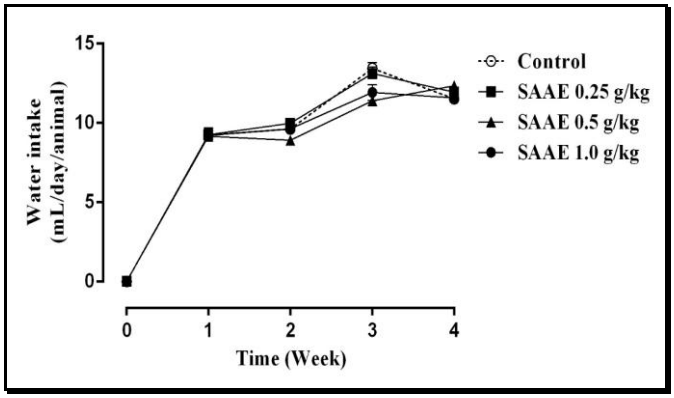


FIGURE 2: WATER INTAKE (ML/DAY/ANIMAL) OF MALE MICE TREATED WITH SAAE (0.25, 0.5 AND 1.0 G/KG, *P.O*) DURING 30 CONSECUTIVE DAYS. THE VALUES REPRESENT MEAN ± S.E.M. (N = 12/GROUP).

In the last week of treatment, there were significant reductions in food intake of the males treated with SAAE at doses of 0.5and 1.0g/kg compared to control **(Figure 3)**, but the reduction in the body mass gain only was reflected in the animals treated with the highest dose **(Figure 4)**.

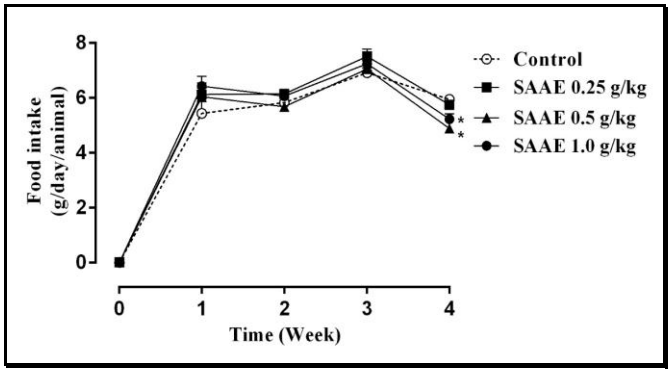


FIGURE 3: FOOD INTAKE (G/DAY/ANIMAL) OF MALE MICE TREATED WITH SAAE (0.25, 0.5 AND 1.0 G/KG, *P.O*) DURING 30 CONSECUTIVE DAYS. THE VALUES REPRESENT MEAN ± S.E.M. (N = 12/GROUP).*STATISTICALLY DIFFERENT FROM CONTROL (ANOVA FOLLOWED BY NEWMAN-KEULS, P < 0.05).

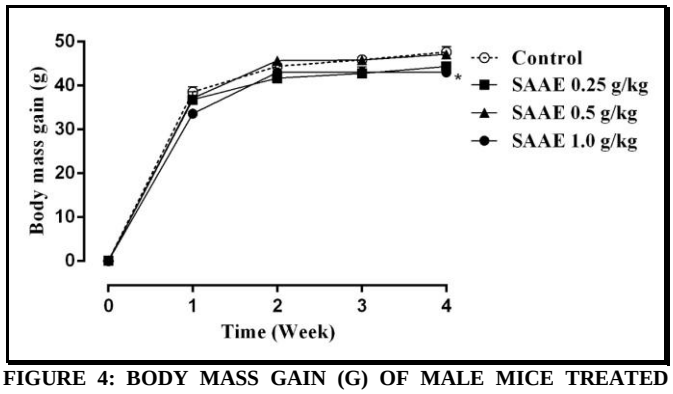


FIGURE 4: BODY MASS GAIN (G) OF MALE MICE TREATED WITH SAAE (0.25, 0.5 AND 1.0 G/KG, *P.O*) DURING 30 CONSECUTIVE DAYS. THE VALUES REPRESENT MEAN ± S.E.M. (N = 12/GROUP).*STATISTICALLY DIFFERENT FROM CONTROL (ANOVA FOLLOWED BY NEWMAN-KEULS, P < 0.05).

The treatment of the females did not compromise the water and food intakes when compared to control group **(Figures 5** and **6,** respectively).

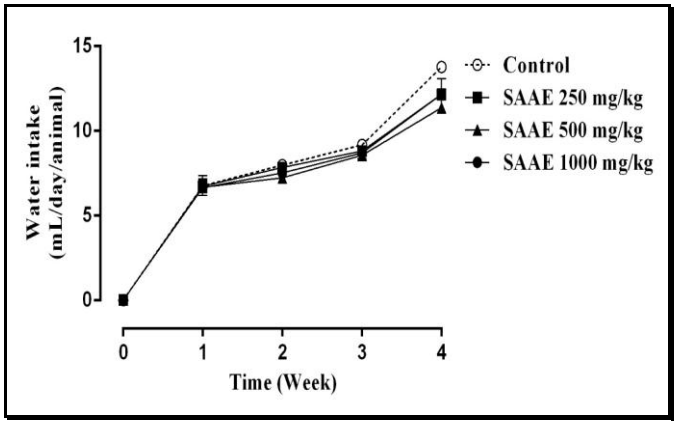


FIGURE 5: WATER INTAKE (ML/DAY/ANIMAL) OF FEMALE MICE TREATED WITH SAAE (0.25, 0.5 AND 1.0 G/KG, *P.O*) DURING 30 CONSECUTIVE DAYS. THE VALUES REPRESENT MEAN ± S.E.M. (N = 12/GROUP).

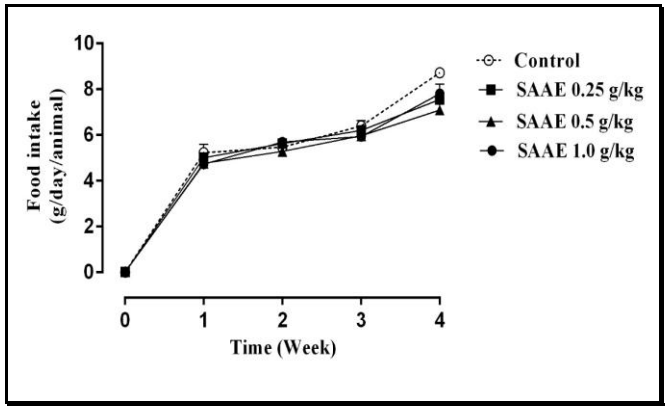


FIGURE 6: FOOD INTAKE (G/DAY/ANIMAL) OF FEMALE MICE TREATED WITH SAAE (0.25, 0.5 AND 1.0 G/KG, *P.O*) DURING 30 CONSECUTIVE DAYS. THE VALUES REPRESENT MEAN ± S.E.M. (N = 12/GROUP).

The animals treated with the SAAE at dose 1.0g/kg showed a decrease in body mass gain since the second week until the end of the treatment **(Figure7)**.

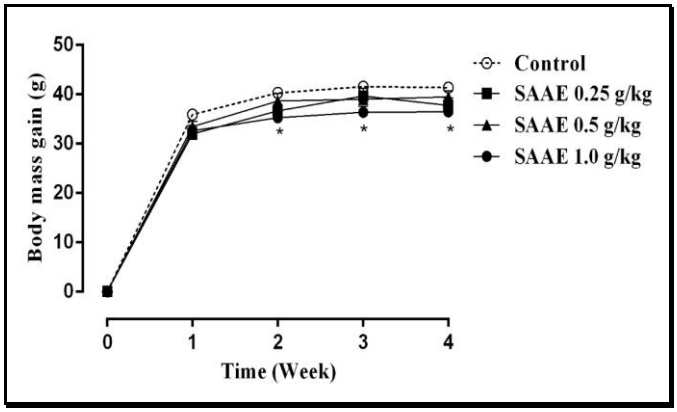


FIGURE 7: BODY MASS GAIN (G) OF FEMALE MICE TREATED WITH SAAE (0.25, 0.5 AND 1.0 G/Kg, *P.O*) DURING 30 CONSECUTIVE DAYS. THE VALUES REPRESENT MEAN ± S.E.M. (N = 12/GROUP).*STATISTICALLY DIFFERENT FROM CONTROL (ANOVA FOLLOWED BY NEWMAN-KEULS, P < 0.05).

Hematological and Biochemical Parameters

There were not significant changes in the most of hematological parameters for both sexes. The male and female mice when treated with the highest dose showed leukocytosis **(Table 1)** and increased levels of RDW **(Table 2)**, respectively, when compared to control.

In relation to biochemical parameters, the males showed increased levels of total protein and albumin when treated with all doses of SAAE, of alkaline phosphatase and total bilirubin when treated with the doses of 0.5 and 1.0 g/kg **(Table 3)**. The females treated with doses of 0.5 and 1.0 g/kg showed hyperuricemia and those treated only with the highest dose showed hypertriglyceridemia **(Table 4)**.

TABLE 1. EFFECTS OF SAAE (0.25, 0.5 AND 0.1G/Kg) BY ORAL ROUTE ON HEMATOLOGICAL PARAMETERS OF MALE SWISS MICE TREATED FOR 30 CONSECUTIVE DAYS.

Parameters	Control	SAAE 0.25 g/kg	SAAE 0.5 g/kg	SAAE 1.0 g/kg
Erythrocytes (10^6/µL)	9.32 ± 0.21	8.66 ± 0.47	9.87 ± 0.17	9.82 ± 0.26
Hemoglobin (g/dL)	15.39 ± 0.34	16.01 ± 0.62	16.23 ± 0.25	15.98 ± 0.47
Hematocrit (%)	44.65 ± 1.07	41.33 ± 1.96	47.75 ± 0.68	46.14 ± 1.13
MCV (fL)	47.67 ± 0.33	47.83 ± 1.22	48.33 ± 0.33	47.00 ± 0.32
MCH (pg)	16.50 ± 0.13	16.13 ± 0.36	16.43 ± 0.05	16.27 ± 0.09
MCHC (g/dL)	34.46 ± 0.15	33.69 ± 0.29	33.97 ± 0.21	34.63 ± 0.22
RDW (%)	17.68 ± 0.36	18.17 ± 0.76	16.25 ± 0.28	17.12 ± 0.23
Platelets (10^3/µL)	882.30 ± 32.65	913.30 ± 89.43	833.00 ± 39.05	787.40 ± 75.45
MPV (µm^3)	5.60 ± 0.07	5.43 ± 0.05	5.65 ± 0.08	5.74 ± 0.21
WBC (10^3/µL)	5.83 ± 0.10	5.00 ± 0.78	7.95 ± 0.96	10.54 ± 1.16*
Neutrophils (%)	7.68 ± 0.89	12.32 ± 1.89	9.98 ± 1.99	7.36 ± 1.20
Eosinophils (%)	0.02 ± 0.02	0.06 ± 0.05	0.03 ± 0.02	0.06 ± 0.02
Basophils (%)	0.22 ± 0.04	0.16 ± 0.02	0.12 ± 0.03	0.18 ± 0.06
Lymphocytes (%)	90.50 ± 1.25	85.53 ± 1.61	88.88 ± 2.07	91.54 ± 1.17
Monocytes (%)	1.58 ± 0.52	1.92 ± 0.55	0.98 ± 0.11	0.86 ± 0.02

Values represent the mean ± S.E.M. (n = 12/group). MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width; MPV, mean platelet volume; WBC, white blood cell. *Statistically different from control (ANOVA followed by Newman-Keuls, p < 0.05).

TABLE 2. EFFECTS OF SAAE (0.25, 0.5 AND 0.1G/KG) BY ORAL ROUTE ON HEMATOLOGICAL PARAMETERS OF FEMALE SWISS MICE TREATED FOR 30 CONSECUTIVE DAYS.

Parameters	Control	SAAE 0.25 g/kg	SAAE 0.5 g/kg	SAAE 1.0 g/kg
Erythrocytes (10^6/µL)	10.51 ± 0.86	8.66 ± 0.47	11.70 ± 1.61	11.00 ± 0.98
Hemoglobin (g/dL)	16.77 ± 0.91	16.58 ± 0.14	18.46 ± 2.20	18.64 ± 1.65
Hematocrit (%)	44.65 ± 1.07	41.33 ± 1.96	47.75 ± 0.68	46.14 ± 1.13
MCV (fL)	46.67 ± 0.33	47.80 ± 0.37	47.20 ± 0.49	47.50 ± 0.22
MCH (pg)	16.45 ± 0.21	16.55 ± 0.47	16.05 ± 0.58	16.93 ± 0.10
MCHC (g/dL)	34.56 ± 0.79	35.65 ± 0.16	34.00 ± 1.05	35.61 ± 0.20
RDW (%)	14.67 ± 0.16	14.54 ± 0.32	15.20 ± 0.39	16.38 ± 0.23*
Platelets (10^3/µL)	780.20 ± 43.59	754.40 ± 15.89	882.20 ± 104.40	781.30 ± 31.15
MPV (µm^3)	6.10 ± 0.10	6.04 ± 0.10	6.30 ± 0.24	6.25 ± 0.21
WBC (10^3/µL)	9867 ± 1636	12020 ± 1201	9920 ± 1941	12067 ± 1599
Neutrophils (%)	4.90 ± 0.72	3.96 ± 1.04	5.66 ± 1.04	3.53 ± 0.98
Eosinophils (%)	0.05 ± 0.03	0.02 ± 0.02	0.00 ± 0.00	0.00 ± 0.00
Basophils (%)	0.20 ± 0.05	0.14 ± 0.05	0.14 ± 0.02	0.15 ± 0.02
Lymphocytes (%)	93.98 ± 0.92	95.10 ± 1.25	93.40 ± 1.71	95.50 ± 1.21
Monocytes (%)	0.88 ± 0.36	0.78 ± 0.25	0.80 ± 0.33	0.82 ± 0.27

Values represent the mean ± S.E.M. (n = 12/group). MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width; MPV, mean platelet volume; WBC, white blood cell. *Statistically different from control (ANOVA followed by Newman-Keuls, $p < 0.05$).

TABLE 3. EFFECTS OF SAAE (0.25, 0.5 AND 0.1G/KG) BY ORAL ROUTE ON BIOCHEMICAL PARAMETERS OF MALE SWISS MICE TREATED FOR 30 CONSECUTIVE DAYS.

Parameters	Control	SAAE 0.25 g/kg	SAAE 0.5 g/kg	SAAE 1.0 g/kg
Glucose (mg/dL)	121.20 ± 10.71	142.00 ± 14.02	144.00 ± 17.22	146.20 ± 14.79
BUN (mg/dL)	34.42 ± 3.36	44.45 ± 1.59	27.58 ± 2.92	42.54 ± 2.64
Creatinine (mg/dL)	0.08 ± 0.01	0.11 ± 0.02	0.10 ± 0.00	0.10 ± 0.00
Uric acid (mg/dL)	1.83 ± 0.17	2.16 ± 0.12	2.15 ± 0.18	2.12 ± 0.05
AST(U/L)	116.70 ± 10.05	89.20 ± 12.74	116.80 ± 20.84	215.20 ± 54.31
ALT (U/L)	55.50 ± 2.77	51.20 ± 3.27	50.50 ± 2.66	72.17 ± 10.64
Alkalinephosphatase (U/L)	68.83 ± 6.07	90.80 ± 9.53	127.70 ± 28.43*	114.70 ± 9.20*
Total bilirubin (mg/dL)	0.25 ± 0.04	0.34 ± 0.02	0.45 ± 0.03*	0.42 ± 0.02*
Directbilirubin (mg/dL)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Indirectbilirubin (mg/dL)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
LDH (U/L)	1142.00 ± 147.20	870.80 ± 112.20	1067.00 ± 174.70	1228.00± 212.90
Total cholesterol (mg/dL)	102.70 ± 6.07	94.20 ± 3.07	96.25 ± 3.75	106.20 ± 2.70
Triglycerides (mg/dL)	115.00 ± 10.01	138.80 ± 14.97	110.80 ± 10.76	101.60 ± 7.05
Total protein (g/dL)	4.25 ± 0.16	4.84 ± 0.08*	4.97 ± 0.22*	5.54 ± 0.13*
Albumin (g/dL)	1.85 ± 0.09	2.12 ± 0.09*	2.37 ± 0.12*	2.66 ± 0.07*

Values represent the mean ± S.E.M. (n = 12/group). BUN, blood urea nitrogen; AST, aspartate aminotransferase; ALT, alanine aminotransferase, LDH, lactate dehydrogenase. *Statistically different from control (ANOVA followed by Newman-Keuls, $p < 0.05$).

TABLE 4. EFFECTS OF SAAE (0.25, 0.5 AND 0.1G/KG) BY ORAL ROUTE ON BIOCHEMICAL PARAMETERS OF FEMALE SWISS MICE TREATED FOR 30 CONSECUTIVE DAYS.

Parameters	Control	SAAE 0.25 g/kg	SAAE 0.5 g/kg	SAAE 1.0 g/kg
Glucose (mg/dL)	168.50 ± 6.89	157.70 ± 4.17	146.80 ± 11.59	144.70 ± 13.47
BUN (mg/dL)	45.13 ± 1.81	40.92 ± 1.90	40.68 ± 0.66	40.32 ± 4.72
Creatinine (mg/dL)	0.10 ± 0.00	0.10 ± 0.00	0.11 ± 0.02	0.10 ± 0.00
Uric acid (mg/dL)	0.82 ± 0.14	1.05 ± 0.14	1.30 ± 0.11*	1.57 ± 0.08*
AST(U/L)	97.00 ± 7.21	94.67 ± 16.22	184.80 ± 60.58	70.20 ± 5.46
ALT (U/L)	48.67 ± 0.66	48.67 ± 7.86	58.60 ± 6.29	45.50 ± 2.32
Alkalinephosphatase (U/L)	127.70 ± 6.88	155.00 ± 11.85	133.30 ± 13.69	113.40 ± 2.62
Total bilirubin (mg/dL)	0.25 ± 0.07	0.30 ± 0.06	0.32 ± 0.05	0.44 ± 0.02
Directbilirubin (mg/dL)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Indirectbilirubin (mg/dL)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
LDH (U/L)	854.00 ± 89.64	974.00 ± 149.30	1401.00 ± 339.40	643.00 ± 30.01
Total cholesterol (mg/dL)	83.00 ± 4.55	79.50 ± 11.50	101.00 ± 9.00	75.50 ± 5.04
Triglycerides (mg/dL)	103.80 ± 13.54	163.00 ± 3.00	110.00 ± 10.00	181.50 ± 13.88*
Total protein (g/dL)	5.66 ± 0.09	5.76 ± 0.08	5.46 ± 0.07	5.33 ± 0.13
Albumin (g/dL)	2.90 ± 0.06	2.87 ± 0.06	2.70 ± 0.07	2.85 ± 0.08

Values represent the mean ± S.E.M. (n = 12/group). BUN, blood urea nitrogen; AST, aspartate aminotransferase; ALT, alanine aminotransferase, LDH, lactate dehydrogenase. *Statistically different from control (ANOVA followed by Newman-Keuls, $p < 0.05$).

Morphological Parameters

The absolute and relative masses of the male and female mice organs were not altered by treatment with all doses of SAAE when compared to control (**Tables 5** and **6**, respectively). Macroscopic analysis of these target organs did not show significant changes in color or texture when compared to control group.

TABLE 5. EFFECTS OF THE SAAE (0.25, 0.5AND1.0G/KG) ON ABSOLUTE AND RELATIVE MASSES OF MALE SWISS MICE ORGANS TREATED FOR 30 CONSECUTIVE DAYS.

Organs	Control	SAAE 0.25 g/kg	SAAE 0.5 g/kg	SAAE 1.0 g/kg
Heart (g)	0.210± 0.006	0.220± 0.010	0.240± 0.020	0.230± 0.020
(g/100 g)	0.080± 0.004	0.040± 0.009	0.100± 0.008	0.070± 0.004
Lungs(g)	0.220± 0.006	0.240± 0.020	0.280± 0.030	0.230± 0.020
(g/100 g)	0.090± 0.006	0.050± 0.050	0.120± 0.020	0.090± 0.010
Liver (g)	1.88 ± 0.06	1.74 ± 0.07	1.79 ± 0.08	1.70 ± 0.08
(g/100g)	0.77 ± 0.06	0.59 ± 0.06	0.79 ± 0.06	0.75 ± 0.06
Stomach (g)	0.26 ± 0.01	0.24 ± 0.01	0.26 ± 0.01	0.25 ± 0.02
(g/100 g)	0.10 ± 0.008	0.10 ± 0.01	0.13 ± 0.01	0.10 ± 0.01
Kidneys (g)	0.49 ± 0.01	0.48 ± 0.02	0.47 ± 0.02	0.48 ± 0.03
(g/100 g)	0.190± 0.005	0.110± 0.010	0.110± 0.010	0.180± 0.020
Adrenal glands (g)	0.005 ± 0.001	0.005 ± 0.002	0.006 ± 0.002	0.005 ± 0.001
(g/100 g)	0.0020 ± 0.0003	0.0016 ± 0.0005	0.0020 ± 0.0003	0.0025 ± 0.0003
Spleen (g)	0.15 ± 0.02	0.16 ± 0.01	0.15 ± 0.01	0.16 ± 0.02
(g/100 g)	0.058 ± 0.007	0.057 ± 0.009	0.056 ± 0.005	0.047 ± 0.007
Testicle (g)	0.26 ± 0.01	0.28 ± 0.01	0.26 ± 0.02	0.27 ± 0.02
(g/100 g)	0.10 ± 0.01	0.07 ± 0.01	0.11 ± 0.01	0.07 ± 0.01
Epididymis (g)	0.18 ± 0.02	0.20 ± 0.01	0.22 ± 0.02	0.21 ± 0.02
(g/100 g)	0.07 ± 0.01	0.02 ± 0.01	0.13 ± 0.02	0.09 ± 0.01
Seminal vesicles (g)	0.23 ± 0.03	0.11 ± 0.01	0.29 ± 0.03	0.24 ± 0.06
(g/100 g)	0.08 ± 0.01	0.06 ± 0.01	0.12 ± 0.01	0.10 ± 0.03

Values represent the mean ± S.E.M. (n = 12/group).

TABLE 6. EFFECTS OF THE SAAE (0.25, 0.5AND1.0G/KG) ON ABSOLUTE AND RELATIVE MASSES OF FEMALE SWISS MICE ORGANS TREATED FOR 30 CONSECUTIVE DAYS.

Organs	Control	SAAE 0.25 g/kg	SAAE 0.5 g/kg	SAAE 1.0 g/kg
Heart (g)	0.170± 0.005	0.160± 0.008	0.170± 0.004	0.160± 0.006
(g/100 g)	0.080± 0.005	0.060± 0.005	0.060± 0.005	0.060± 0.005
Lungs (g)	0.230± 0.009	0.220± 0.020	0.200± 0.010	0.170± 0.007
(g/100 g)	0.100± 0.006	0.080± 0.010	0.080± 0.008	0.070± 0.004
Liver (g)	1.76 ± 0.05	1.44 ± 0.11	1.58 ± 0.07	1.59 ± 0.08
(g/100g)	0.81 ± 0.04	0.60 ± 0.06	0.62 ± 0.05	0.62 ± 0.05
Stomach (g)	0.320± 0.008	0.300± 0.010	0.320± 0.007	0.310± 0.010
(g/100 g)	0.150± 0.010	0.160± 0.010	0.150± 0.007	0.170± 0.006
Kidneys (g)	0.390± 0.010	0.380± 0.010	0.400± 0.009	0.390± 0.010
(g/100 g)	0.180± 0.010	0.170± 0.010	0.190± 0.020	0.180± 0.008
Adrenal glands (g)	0.030± 0.003	0.020± 0.001	0.020± 0.002	0.020± 0.003
(g/100 g)	0.010± 0.002	0.010± 0.002	0.020± 0.001	0.020± 0.002
Spleen (g)	0.19 ± 0.02	0.18 ± 0.02	0.17 ± 0.02	0.19 ± 0.01
(g/100 g)	0.09 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.09 ± 0.01
Uterus (g)	0.17 ± 0.02	0.10 ± 0.01	0.13 ± 0.03	0.14 ± 0.02
(g / 100 g)	0.070± 0.007	0.060± 0.005	0.070± 0.006	0.070± 0.008
Ovaries (g)	0.060± 0.007	0.040± 0.005	0.060± 0.009	0.060± 0.004
(g/ 100 g)	0.170 ± 0.005	0.160± 0.008	0.170± 0.004	0.160± 0.006

Values represent the mean ± S.E.M. (n = 12/group).

The histological analysis showed discrete lymphocytic infiltrate in the heart of males treated with the dose of 1.0 g/kg **(Figure 8)**, discrete hepatic steatosis in the females treated with doses of 0.5 and 1.0g/kg **(Figure 9)** and increased renal capsular space **(Figure 10)** and hypertrophy of gland adrenal espongiocytes **(Figure 11)** in both sexes treated with 1.0 g/kg of SAAE.

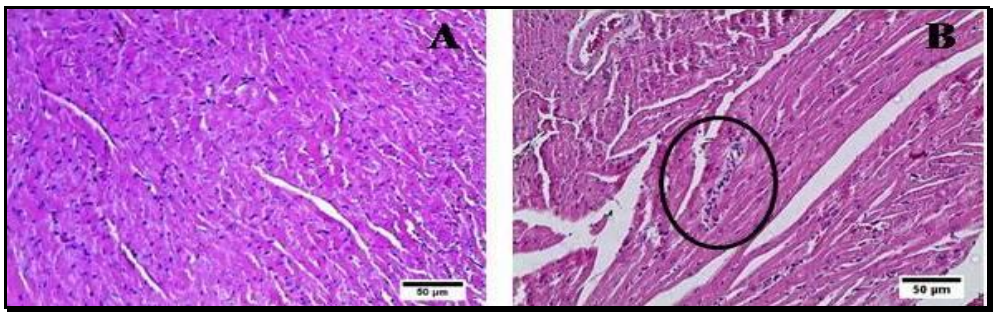


FIGURE 8: PARAFFIN SECTIONS (HE, 10X INCREASED) OF MALE MICE HEART TREATED WITH SAAE DURING 30 CONSECUTIVE DAYS. A – CONTROL; B – ANIMALS TREATED WITH SAAE AT DOSE 1.0 G/KG. THE CIRCLE INDICATES DISCRETE LYMPHOCYTIC INFILTRATE.

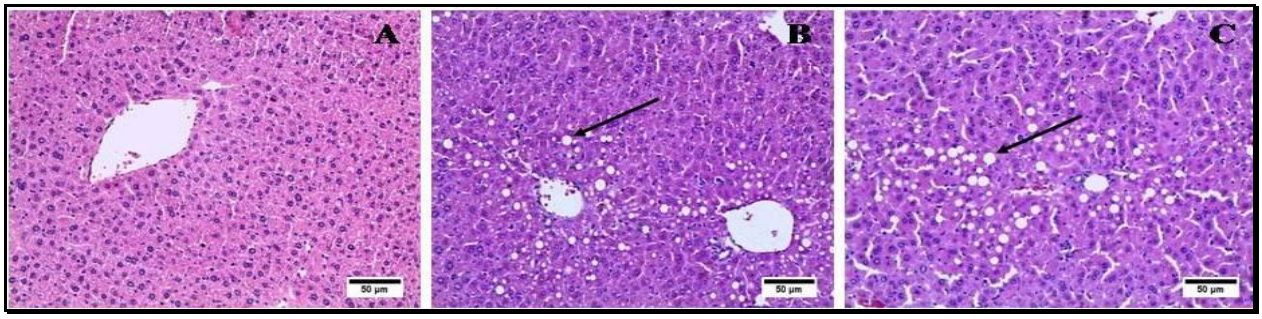


FIGURE 9: PARAFFIN SECTIONS (HE, 10X INCREASED) OF FEMALE MICE LIVER TREATED WITH SAAE DURING 30 CONSECUTIVE DAYS. A – CONTROL; B – ANIMALS TREATED WITH SAAE AT DOSE 0.5 G/KG; C – ANIMALS TREATED WITH SAAE AT DOSE 1.0 G/KG. THE ARROWS INDICATE DISCRETE HEPATIC STEATOSIS.

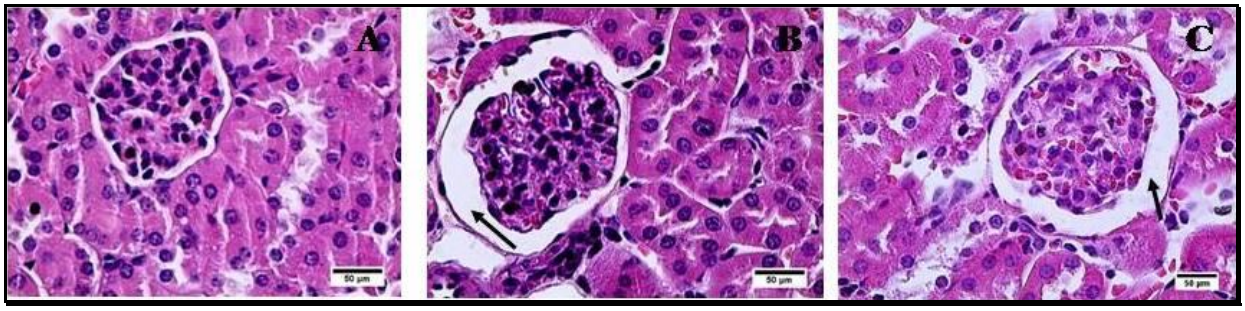


FIGURE 10: PARAFFIN SECTIONS (HE, 10X INCREASED) OF RENAL CAPSULAR SPACE OF THE ANIMALS TREATED WITH SAAE DURING 30 CONSECUTIVE DAYS. A – CONTROL; B – MALES TREATED WITH SAAE AT DOSE 1.0 G/KG; C – FEMALE TREATED WITH SAAE AT DOSE 1.0 G/KG. THE ARROWS INDICATE INCREASED CAPSULAR SPACE.

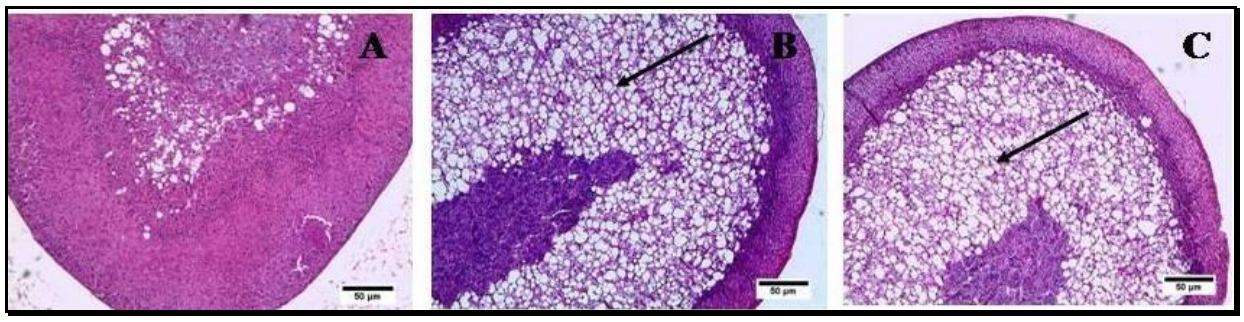


FIGURE 11: PARAFFIN SECTIONS (HE, 10X INCREASED) OF ADRENAL ESPONGIOCYTES OF THE ANIMALS TREATED WITH SAAE DURING 30 CONSECUTIVE DAYS. A – CONTROL; B – MALES TREATED WITH SAAE AT DOSE 1.0 G/KG; C – FEMALE TREATED WITH SAAE AT DOSE 1.0 G/KG. THE ARROWS INDICATE HYPERTROPHY OF ESPONGIOCYTES.

DISCUSSIONS: The present study evaluated the acute and subacute toxicities of the aqueous extract of *Simaroubaamara* stem bark, since there is a lack of pharmacological and toxicological studies for the species. The HPLC analysis quantified the main metabolites observed in the preliminary phytochemical screening and the catechins were the major components quantified in the extractive solution. Complementary phytochemical data were provided in this study as the presences of hydrolysable and condensed tannins, chlorogenic and cafeic acids. In previous phytochemical screening studies were isolated alkaloids, triterpenes, quassinoids and tannins[11]from the stem bark of *S. amara*.

The results of the acute toxicity study indicated that the oral administration of SAAE in a single oral dose of 2.0g/kg did not produce any signs of toxicity or mortalities in the rats, suggesting a LD_{50} above this dose. According to the OECD[7], acute oral toxicity refers to those adverse effects occurring following oral administration of a single dose of a substance or multiple doses given within 24 hours. Substances with LD_{50} values greater than 2.0 g/kg by the oral route may be considered practically non-toxic. Similarly, sub acute toxicity tests with the SAAE during 30 consecutive days did not caused either visible clinical signs of toxicity.

Although the food intake by females did not decrease with the treatment, the animals of both sexes treated with the highest dose showed a significant reduction in the body mass gain in the last week. McRae et al.[12] have shown that tannins interact with salivary and mucosa-associated proteins producing astringency which is reflect in reduced feed intakes. Moreover, studies indicate that the major effect of tannins is not relation to their inhibition on food intake but rather the decreased efficiency in converting the absorbed nutrients[13].

It is worth noting that males treated with all doses of SAAE presented proteinemia and albuminemia. Although the percentage of absorption from the gut is reduced in animals treated with the highest dose Waghorn[14] demonstrated that lower concentrations of condensed tannins in herbage ingested by sheep was able to improve the nutritive value of herbage by binding to plant proteins and protecting them from excessive degradation in the rumen, increasing the amount of essential amino acids absorbed by 20-50% compared to plants without condensed tannins. In contrast to this, consumption of tannins higher concentration to long-term may induce histological changes in the intestinal mucosa including abnormal villous structure and disruption of cellular network of communication.

The exact mechanism whereby the *S. amara* constituents could damage vital body organs cannot be derived from the present investigation, but the possible tissues injuries could be reflected through of the analysis of serum enzymes. The increased levels of alkaline phosphatase (AF) and total bilirubinin in the males treated with doses of 0.5 and 1.0 g/kg of SAAE reflect possibly an impairment of bile flow to the intestine since those studies in rats have indicated biliary excretion of some green tea catechins as epigallocatechin-gallate, epigallocatechin and epicatechin[15]. Although the AF is an on specific enzyme, it is also localized in the bile canaliculi membranes and may be regurgitated in to the circulation when there is impairment of bilirubin excretion, with consequent hyperbilirubinemia[16].

Despite the RDW increased levels in the females treated with the highest dose, these values were within the reference limits for the species according Restel[17]. In relation to biochemical parameters, the females treated with doses of 0.5 and 1.0 g/kg showed hyperuricemia and those treated only with the highest dose had hypertriglyceridemia. This fact could be related to hormonal factors since in the males there is no change in these parameters. However, the estrogen is uricosuric which reduces uric acid serum levels and, initially, estrogen decreased levels would not be considered, which could explain the hyperuricemia, due to increased synthesis of triglycerides which is a physiological action of this hormone[18].

Our findings also contradict the results of Ho et al.[19] whose xanthine oxidase activity, enzyme responsible for the acid uric formation, was inhibited in male mice treated with the ethyl acetate fraction and hydrolysable tannins isolated from *Balanophora laxiflora*. Thus, an explanation for

this fact requires more detail in the future investigations.

Besides the hormonal influence, the hypertriglyceridemia could also be result of increased intestinal incorporation of lipids by chylomicrons due to treatment with SAAE. Arhelgeret al.[20] verified an increased hepatic uptake of chylomicron in rabbits treated with a single dose of a 40% tannic acid solution. The hypertriglyceridemia, however, could be the cause of the discrete hepatic steatos is viewed in the female treated with the SAAE at doses of 0.5 and 1.0 g/kg. Thus, the steatos is would not be result of a hypoproteinemia that would reduce the lipoprotein formation and the triglycerides excretion, but it would occur due to an inadequate caloric intake with subsequent fatty acids mobilization from the adipose tissue to the liver[21].

The discrete lymphocytic infiltrate viewed in the heart of the males treated with the highest dose could result from the tannin effects. Meloet al.[22] verified that treatment of Swiss female mice with tannin-enriched fractions from the aqueous extract of *Mascagnia rigid* Griseb. caused changes in the enzymatic profile of heart muscle with significant increases in myocardial enzyme CK-MB (creatine kinase fraction MB), which is released from damaged myocardial tissue. This injury and inflammation process could be related with the leukocytosis observed in these animals.

However, the renal effects of the treatment were the most pronounced since was similar for both sexes. The increase of the capsular space viewed in males and females treated with the highest dose could be consequence of diuretic effects of tannins. Experimental studies have demonstrated that tannins are endowed with both diuretic and vasodilator actions, factors that may contribute to an increased urinary excretion. The increased urinary excretion could result from an increase in the total filtration pressure in the glomeruli, which would force a large volume of fluid into the capsular space[23].

The hypertrophy of espongiocytes, cells located in the fasciculate zone of adrenal gland, was verified in males and females treated with the highest dose and could be related to tannins. There have been several suggestions that deficiency of Na^+ is exacerbated by tannins present in the diets of herbivores. Hastings et al.[24]showed that inclusion of tannic acid in the diet of male domestic mice (*Musdos mesticus*) increased symptoms of Na^+ stress such as altered morphology of the adrenal gland. Brient-Johnson et al.[25] showed that the size of the fasciculate-reticular is zone was significantly larger in voles provided diets with 1.5% tannic acid.

CONCLUSIONS: Our data showed that acute administration of the aqueous extract of *Simaroubaamara* stem bark in a single oral dose of 2.0 g/kg was non-toxic and that subacute treatment produced some significant histological changes in the highest dose. However, the highest dose employed in the present study was equivalent to five times the dose used in the traditional medicine, when calculated according to its yield. Thus, further studies should be conducted in order to elucidate as a long term treatment would act on these organs committing them.

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