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ACUTE AND SUB-ACUTE TOXICITY OF *CRASSOCEPHALUM BAUCHIENSE* AQUEOUS EXTRACT IN FEMALE WISTAR RATS

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ABSTRACT

Background: *Crassocephalum bauchiense* is a commonly used medicinal plant in Menoua Division (west-Cameroun) traditional medicine for the treatment of pregnancy and childbirth discomfort, but there is no report on its safety or toxicity. Therefore, we evaluated the toxicity profile of the aqueous Stem and leaves extract of *Crassocephalum bauchiense* in female Wistar rats. **Method:** Acute toxicity test was performed with single oral administration of 5000 mg/kg body weight of *Crassocephalum bauchiense* aqueous extract (CBAE) to rats and the animals were observed for 14 days for signs of toxicity. The subacute toxicity experiment was conducted by oral administration of graded doses (60, 240, and 960mg/kg) of CBAE daily for 28 days. Behavioural changes as well as haematological, biochemical, and histological parameters were then evaluated. **Results:** There was no observable sign of toxicity in the acute toxicity test. There were significant. decreases ($P < 0.05$) in the feed intake on days 4 and 8 of treatment at the doses 960 mg/kg. Also, a significant decrease of lymphocytes percentage associate to an increase of those of granulocytes was observed in all treated groups comparatively to control. Only moderate vascular congestion was registered on liver histology of rats treated with highest doses. CBAE significantly decreased in levels of LDL ($p<0.001$), serum sodium ($P<0.01$; $p<0.05$) urinary potassium ($P<0.001$; $p<0.01$) and increased in levels of triglycerides ($p<0.01$) and liver proteins ($p<0.05$) in all treated animals. For others parameters, there were no treatment related differences. **Conclusions:** Administration of CBAE may be safe at the therapeutic dose but its continuous consumption at the dose of 240mg/kg may be lead to an decrease of the risk of developing cardiovascular diseases.

KEYWORDS: Pregnancy complaints, *Crassocephalum bauchiense*, aqueous extract, acute and sub-acute toxicity, histological section.

1- INTRODUCTION

Pregnancy is the physiological process that is associated with many anatomical, physiological, biochemical and metabolic changes.[1] In the majority of cases, these changes lead to benign discomforts; however, in some cases, these discomforts can induce serious complications that could have serious consequences on the health of the mother and/or the fetus she carries.

For the treatment of some of these pregnancy ailments, modern medicine has implemented various treatments; however, the relatively high costs, side and teratogen effects generated by synthetic drugs have drastically limited their use, promoting a return to herbal medicine,[2] and thus, for all aspects of female reproductive health, a large number of plant species have been and continue to be used by women and traditional practitioners around the world.[3]

Indeed, in Cameroon (Menoua-division of west Region), various recipes are used for the treatment of pregnancy discomfort. *Crassocephalum bauchiense* is among the most used plants in these recipes.[4] It is also used for the treatment of gastrointestinal infections, pain, inflammatory disorders, epilepsy.[5,6] Several studies have shown that different extracts of C. *bauchiense* possess analgesic, antibacterial, antifungal, diuretic and antioxidant properties and that its external use is without toxic effect.[5-8] However, no study has evaluated its oral toxicity, despite the fact that this plant is often consumed in unlimited quantities and over long periods of time, especially by pregnant women. Yet, the administration of natural products to a biological system may induce different types of responses which may be useful or not.[9] Indeed, many studies have reported the toxic effects of herbal medicines.[10,11] And It is known that the

lack of toxicological information on medicinal plants substantially restricts their use in ethnomedecine.[12]

Therefore, we proposed in the present work to evaluate the acute and subchronic toxicity of *C. bauchiense* aqueous extract in order to evaluate the safety of their consumers.

2. MATERIAL AND METHODOLOGY

2.1. Plant material

C. bauchiense (Asteraceae) was collected in its vegetative state in June 2014 in Bamendou village, (Menoua Division, West Region of Cameroon). The taxonomic identification of the plant was done by the Cameroon's National Herbarium (CNH) under the voucher specimen number. 37 884/HNC.

2.2. Animals

Female Wistar albino rats, weighing between 150 and 180 g and of 10 to 12 weeks old were used. They were acclimatized during 10 days, in the Animal House of the Department of Biochemistry (University of Dschang, Cameroon), under standard animal house conditions and allowed free access to food and water for the same period.

2.3. Chemicals

INMESCO (Germany) Kits for aspartate aminotransferase, alanine aminotransferase, alkalin phosphatase, total and direct bilirubin, total cholesterol, HDL-cholesterol, triglyceride, creatinine, and urea were obtained from commercial firm.

2.4. Acute toxicity study

The doses adjustement method of OCDE,[13] for acute toxicity studies was used for the estimation of the lethal dose (LD_{50}) of the plant using female rats. The highest dose recommended by this method (5000 mg/kg) was used because of previous results obtained from cytotoxic study on the *C. bauchiense* aqueous extract (CBAE) which shown that CBAE is not toxic.[14]

Six acclimatized female Wistar albino rats were used in this study. Animal 1 and 2 were used control and received only distilled water (1 ml per 100 g of body weight), while animal 3 to 6 were test group and were treated orally with a single dose of CBAE (5000 mg/kg).The different groups of rats were housed in a separate cages. All animals were fasted for 18 hrs prior to the administration of the plant extract or distilled water. They were continuously and hourly observed during the first day after treatment to detect any signs of toxicity such as: changes in autonomic or behavioral responses (locomotion, aggressiveness), spontaneous activity (reaction to tail pinch and to noise), social interactions, aspect of mucosa and feces, eye coloration and corneal reflex, appearance of hair, trembling, salivation and mortality. When animals are gathered together, it is an indicator of communication (i.e. gathering); they are said to be in activity when they are roaming in the cage; they are say to be reactive when any attempt to touch them, they react by biting; normal reaction to noise is when the rats are unsettled on hearing a noise; the cries of rats when pinched on their tail is an indicator of normal reaction to pinch; the tail is normal when it is flexible (i.e. no rigid); rigid tail is a sign of anger.[15] After the first day following the treatment, animals were supplied with food and water ad libitum, and were further closely observed once daily for 13 days in order to identify signs of toxicity or death. The body weight of each rat was measured every 2 days throughout the observation period. At the end of this period, all survivors were killed to examine macroscopic alterations in their vital organs.

2.5. Sub-acute toxicity study

Twenty female rats were randomly distributed into four (04) groups of five animals each. Animals of groups 2 to 4 (test groups) were treated for 28 days with different doses of CBAE [60 mg/kg (traditional healer dose), 240 mg/kg and 960 mg/kg respectively)] while, animals of group 1 received 1 ml/100 g body weight per day of distilled water (control) for the same period. During treatment, animals were daily weighed, treated with the corresponding dose of extract (test groups) or distilled water (control) before being allowed to food and water (tap water) *ad libitum*.

At the end of the treatment period, prior to sacrifice, animals were subjected to a 12 hours food fasting at the end of which their urine was collected and stored at -20°C for the dosage of proteins, creatinine and urea. Then animals were anesthetized by inhalation of chloroform vapors, dissected and their blood collected by cardiac puncture into sterilized dry test tubes and test tubes containing EDTA. Blood containing EDTA was used for complete blood count while the other was left for 2 hours in refrigerator at 4°c before being centrifuged at 2 500 rpm for 15 minutes. The sera obtained were used for the determination of the effect on toxicity biochemical makers. The liver, lungs, spleen, heart and kidneys of each animal were removed, weighed and their proteins.

2.5.1. Complete blood count

The complete blood count was performed using an automated hematology analyzer. Hematological evaluations included: hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular volume (MCV) and count of red blood cell (RBC), blood platelet (PLT), white blood cell (WBC), lymphocytes and granulocytes.

2.5.2. Preparation of Homogenates and Biochemical Analysis

The homogenates of various organs were obtained by grinding a fixed weight of the organ in 3 ml of phosphate Buffer (pH 7.4; 0.1 M). After centrifugation at 3000 rpm for 15 min, the supernatant was taken and preserved at -20°C.

A lot of biochemical parameters were performed spectrophotometrically using INMESCO (Germany) kits. So, Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline Phosphatase (ALP) activity assays as well as the lipidic profil [total cholesterol (TC), High Density Lipoprotein (HDL) and triglycerides (TG)], creatinine and bilirubin levels were evaluated in accordance with the procedures described by Schumann and Klauke.[16] Urinary proteins were measured by the Bradford method.[17] while total serum and tissues (liver and kidney) protein levels were measured by the Biuret.[18] Ions (Na^+ and K^+) concentrations were measure by Flame photometry. Low Density Lipoprotein (LDL) level and Arterioclerosis Index (AI) were calculated as described respectively by Roeschlau[19] and Ibrahim *et al.*,[20] using the following formulas.

$$LDL = TC - \frac{TG}{5} - HDL$$

$$\text{IA} = (\text{CT} - \text{HDL})/\text{HDL}$$

2.5.3. Histological Cut

Tissue cross sections were done on liver and kidneys fixed in 10% formol as described by Vanhulle *et al.*[21] After sacrificing the animals, small pieces of liver were fixed in 10% formalin, dehydrated in ascending grades of alcohol and cleared in xylene. The fixed tissue were embedded in paraffin wax and sectioned into five micrometres thick with the rotary microtome, then stained with hematoxylin and eosin. Then the sections were examined with light microscope and photographed using a microscopic camera.

2.6. Statistical analysis

All measured variables were expressed as the Mean ± standard error on the mean (SEM). Statistical analyses were performed with SPSS software. The statistical differences between the values were shown by ANOVA (Analysis of Variance) test. Ccomparisons of means were done using the Fisher LSD test and the significance of the differences was established at the 5% level ($p<0.05$).[22]

3. RESULTS

3.1. Acute toxicity

Overall, the study of acute toxicity revealed no adverse change in the behavior of female rat at 5000 mg/kg as compared to the control and no mortality was registered. On the other hand there was no significant change in the body weight, as a toxicity indicator and the macrocospic anatomopathological studies did not show any alteration in the analyzed organs (Table 1). Therefore, the LD_{50} of the extract is over 5000 mg/kg.

Table 1: Acute toxicity study of aqueous extract of CBAE in female rates.

Parameters	Dose of AECB received (mg/kg of BW)	
	0	**5000**
Appearance of the fur	N	N
Trembling	N	N
Grouping and locomotion	N	N
Changes in eyes and mucous membranes	No	No
Shape of the tail	N	N
Appearance of the stool	N	N
Reaction to pinching	N	N
Reaction to noise	N	N
Mortality after 48 hours	0	0
Mortality after 14 days	0	0
Aspect of organs after sacrifice	N	N
LD_{50}		**>5000 mg/kg**

N: Normal;; BW: Body weight

3.2. Sub-chronic toxicity

3.2.1. Effect of CBAE administration on body weight growth and food intake.

Figure 1 shows the evolution of the weight growth of adult rats during 28 days of oral treatment with different doses of CBAE. In general, the percentages increased linearly with the duration of the treatment and independently of the doses of the different aqueous extracts administered. Despite the decrease observed from day 12 of treatment in all treated animals compared to the control, no significant difference in this parameter was observed at the different treatment periods between treated and control animals.

The food consumption of the animals is presented in figure 2. This shows a general decrease in food consumption of all treated animals compared to the control group. This food consumption was significantly reduced only on day 28 of treatment in animals treated with 60 mg/kg and on days 4 and 8 in those treated with 960 mg/kg.

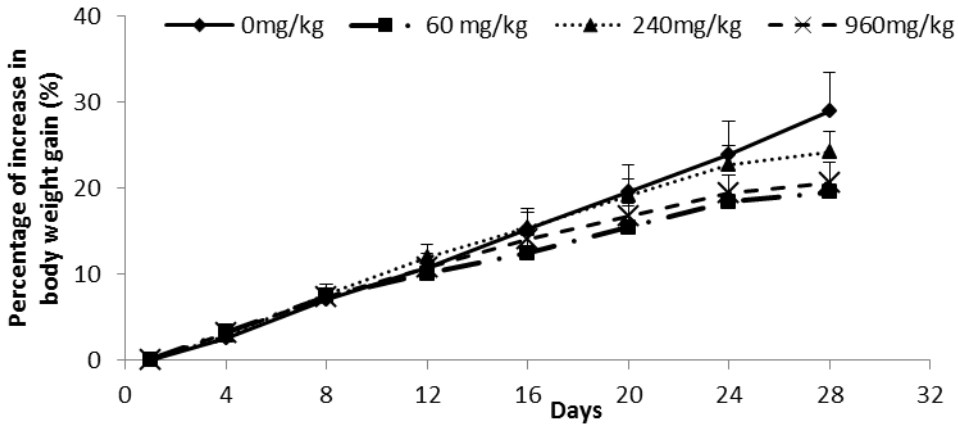

Figure 1: Evolvement of the body weight gain of the animals during the administration period. Each curve represents the mean ± s.e.m. of the values for 5 animals. The values presented are the means of the percentages values of the body weight of each animal relatively to the starting weight.

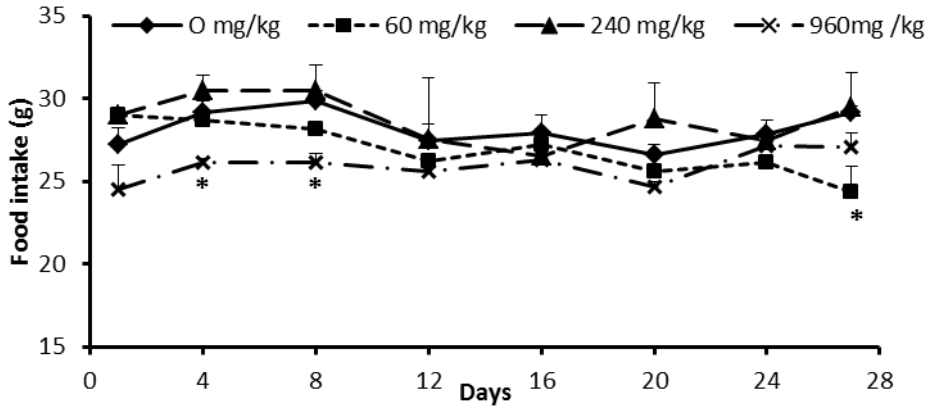

Figure 2: Evolvement of the food consumption of the animals during treatment period. *The value is significantly different at P 0.05 from the control at the corresponding dose. Each curve represents the mean ± s.e.m of the values for 5 animals.

3.2.2. Effects of treatment on haematological parameters

No significant effects of 28-days oral administration of different doses of CBAE were observed on red blood cell and leukocyte number haemoglobin level, haematocrit and mean corpuscular volume. However, a highly significant (P<0.001) increase in granulocyte number and a highly significant (P<0.001) decrease in lymphocyte number were observed in all treated rats compared to the control. Also, a significant increase in the number of blood platelets was recorded in animals treated at 60 mg/kg (Table 2).

Table 2: Effects of CBAE on hematologic parameters of rats.

Parameters	Doses (mg/kg)			
	0	60	240	960
WBC (x 10^3/µl)	7.26±0.68	7,96±1,37	6,22±0,85	5,34±0,93
RBC (x 10^6/µl)	6.19±1.26	7,07±0,62	6,45±0,45	6,58±0,41
Haemoglobin (g/dl)	14.14±0.93	14,90±0,79	14,45±0,58	14,74±1,81
Hematocrit (%)	32.70±5.67	39,04±1,93	33,07±1,98	34,28±2,16
Platelets (x 10^3/µl)	219.76±52.86	430,40±77,20*	232,25±42,42	351,00±73,27
Lymphocytes (%)	84.54±2.14	68,26±1,62***	66,17±3,68***	74,82±2,95*
Granulocytes (%)	9.62±1.52	24,58±1,59***	25,70±3,18***	18,58±2,57*
MCV (fl)	57.84±6.13	56,54±4,33	51,47±0,52	52,24±0,75

*or ** or ***Values significantly different at (p<0.05) or at (p<0.01) or at (p<0.001) from those of the control group (ANOVA and Fisher LSD). Each value represents the mean ± s.e.m. of the values for 5 animals; RBC, Red blood cells. WBC, white blood cells; MCV, mean corpuscular volume.

3.2.3. Effects of treatment on organs relative weights and on liver and kidney histopathology

The effects of treatment on the relative organ weights of treated animals are shown in the following Table 3. It was found that repeated administration of the CBAE resulted in a highly significant (P<0.001) increase in relative ovary weight at 60 mg/kg and a dose-dependent decrease in relative spleen weight which was significant (P<0.05) at the highest dose compared to the control.

The effects of different doses of CBAE on histology of kidneys and liver are shown in Table 4. It appears that compared to the control group, the kidney of treated animals showed mild vascular congestions at the higher dose while the liver of treated animals showed moderate vascular congestions at 240 mg/kg and 960 mg/kg.

Table 3: Effects of sub-chronic oral administration of the CBAE on relative weights of various organs.

	Organs	Doses (mg/kg)			
		0	60	240	960
Relative weight (g/100g of BW)	Liver	3.89±0.23	3,96±0,06	4,16±0,18	4,00±0,13
	Kidneys	0.78±0.02	0,84±0,05	0,88±0,02	0,82±0,03
	Spleen	0.33±0.04	0,29±0,04	0,28±0,01	0,24±0,00*
	Heart	0.39±0.01	0,40±0,01	0,39±0,02	0,37±0,01
	Lung	0.76±0.04	0,63±0,05	0,69±0,08	0,73±0,04
	Ovaries $x10^{-1}$	0.46±0.03	1,16±0,05***	0,53±0,04	0,51±0,02
	Womb	0.27±0.06	0,35±0,05	0,27±0,06	0,24±0,04

***Values significantly different at (p<0.001) from those of the control group (ANOVA and Fisher LSD). Each value represents the mean ± s.e.m of the values for 5 animals;

Tableau 4: Effects of treatment on liver and kidney histology of treated animals compared to controls.

Doses (mg/kg)	Observed abnomalities	
	Kidneys	Liver
Contrôle	No side effect	Slight vasculaires congestions
60	No side effect	Slight vascular congestions
240	No side effect	Moderate vascular congestions
960	Slight vascular congestions	Moderate vascular congestion

3.2.3. Effect of treatment on biochemical parameters

Table 5 shows that compared to control animals, C. bauchiense did not significantly influence the levels of urine and kidney proteins and those of the following seric parameters: ALT, AST, ALP, proteins, creatinine, sodium, potassium, urea and bilirubin.

C. bauchiense extract significantly (p<0.01) decreased HDL levels at 240 mg/kg and LDL levels (p<0.001) in all treated animals. It also increased triglyceride levels (p<0.01) and liver proteins level (p<0.05) in all treated animals.

Also, a significant (P<0.05) increase in urinary creatinine at 240 mg/kg, a significant decrease in urinary urea (P<0.05) at 60 and 960 mg/kg and sodium (P<0.001) at all doses and at 240 mg/kg, respectively were observed. It should be noted that the decrease in urinary sodium was accompanied by a decrease (P<0.01; p<0.05) in serum sodium levels in all treated animals.

Table 5: Effects of CBAE on biochemical parameters of rats.

Parameters	Doses (mg/kg)			
	0	60	240	960
Serum				
Proteins level (mg/ml)	45.28±2.92	52,20±1,38	47,93±1,61	50,79±2,99
ALT (UI/L)	14.19±2.13	13,38±2,52	12,33±1,75	13,84±1,81
AST (UI/L)	66.89±9.04	76,31±6,96	51,88±3,59	76,66±14,97
ALP (UI/L)	22.42±3.37	21,14±3,98	19,48±2,76	21,87±2,87
Total Bilirubin (µmol/l)	2.53±0.37	2,77±0,62	1,79±0,25	3,50±0,50
Direct Bilirubin(µmol/l)	1.08±0.21	0,88±0,34	0,90±0,12	0,74±0,11
Indirect Bilirubin(µmol/l)	1.44±0.55	1,89±0,80	1,06±0,08	2,77±0,48
Creatinine (mg/dl)	0.31±0.07	0,61±0,11	0,63±0,10	0,46±0,05
Urea (mg/dl)	54.29±3.64	51,43±3,50	45,71±4,84	45,71±5,34
Sodium (mg/dl)	49.42±0.24	28,83±1,84**	28,83±1,84**	33,19±3,59*
Potassium (mg/dl)	3.45±0.55	2,30±0,31	2,30±0,31	2,30±0,31
Total cholesterol (mg/dl)	69.85±3.45	53,30±3,97*	47,38±6,13**	57,38±2,51

HDL (mg/dl)	17.01±1.03	14,25±0,74	11,07±0,86**	15,69±1,79
LDL (mg/dl)	52.84±2.90	16,19±0,78***	23,19±4,27***	21,06±1,16***
Triglycerides (mg/dl)	76.55±4.81	101,03±5,71**	100,09±2,63**	102,44±2,33**
AI	3.14±0.20	2,94±0,21	3,45±0,84	2,85±0,50
Urine				
Proteins level (mg/ml)	0.32±0.03	0,31±0,05	0,32±0,04	0,31±0,03
Creatinine (mg/dl)	12.26±0.75	10,64±1,19	16,37±1,32*	10,76±0,82
Urea (g/l)	69.74±3.48	51,94±6,61*	72,14±3,23	41,84±8,03*
Sodium (mg/dl)	13.17±0.65	10,98±1,09	7,80±0,53***	12,23±0,71
Potassium (mg/dl)	1.30±0.23	1,40±0,97	1,62±0,33	2,01±0,39
Liver				
Proteins level (mg/g)	80.96±3.48	113,37±6,06*	122,76±11,05*	97,03±11,23
Kidneys				
Proteins level (mg/g)	8.90±0.60	7,64±0,82	8,77±0,70	9,22±1,19

*or ** or ***Values significantly different at ($p<0.05$) or at ($p<0.01$) from those of the control group (ANOVA and Fisher LSD). Each value represents the mean ± s.e.m. of the values for 5 animals; AI, Arteriosclerosis index

4. DISCUSSION

Tests on animals are still necessary and are applied as a biological marker of risk induced by a synthetic or natural substance.[23]

Considering the previous work on the plant.[6,5,14] in which the plant would be denuous of toxicity, the dose chosen for the acute toxicity test was 5000 mg/kg. Indeed, according to OCDE (2001) the maximum dose of 5000 mg/kg can be used for the acute toxicity test of a xenobiotic when preliminary toxicological data are available on it. Thus, the administration of CBAE at the dose of 5000 mg/kg did not result in any negative change in the behavior or weight of the animals. In addition, no mortality was recorded and gross pathological examination of the toxicity target organs showed no abnormalities. Therefore, the lethal dose 50 (LD_{50}) of CBAE was considered to be greater than 5000 mg/kg. This plant extract could be classified as a non-toxic oral substance, according to the Hodge and Sterner scale or several laws regulating the use and sale of xenobiotics.[24] This result corroborates that of Mouokeu *et al.*,[5] who obtained an LD_{50} greater than 32 g/kg with the ethyl acetate extract of *C. bauchiense* administered dermally.

Administration of CBAE during 28 days resulted in a decrease in food consumption in particular in animals treated with the highest doses on days 4 and 8. However, the reduction in food intake did not produce concomitant decrease in the body weight of the animals. Change in the body weights is one of the first critical signs of toxicity.[25] The weight gained by the animals during the experimental period may be an indication that the extract did not hamper the growth of the animals.[26]

Haematopoietic system is one of the important parameters used to determine the physiological and pathological status of mammals, as it provides information on the reaction of the body to injury.[27] The changes observed in CBAE treated groups, including the significant elevation of the percentage of granulocytes and the significant decrease of the percentage of lymphocytes, were assumed to be toxicologically irrelevant because they were within normal physiological ranges.[28,29]

Hepatic damage is reflected in increased of serum levels of transaminases, total bilirubin and ALP.[30,31] Only an increase in liver protein levels was noted at 60 and 240 mg/kg of *C. bauchiense* extract. Also, the histology and weights of the livers of all treated animals did not show significant differences compared to the control. These observations would show that the CBAE would have no toxic effect on liver function.

Administration of different doses of *C. bauchiense* extract resulted in a significant increase in urinary creatinine levels in animals treated at 240 mg/kg. Also a decrease in serum sodium level at all doses and in urinary urea level at 60 and 960 mg/kg was noted. Creatinine is known to be one of the major indicators of renal function. Kidney dysfunction is generally associated with an increase in serum creatinine, urea and electrolytes levels and decrease of their urinary levels.[32,33] Hence, the results obtained would suggest that CBAE is not harmful to the kidneys. This is especially true since the histological study of the kidneys of treated animals did not reveal any changes. This would confirm that the CBAE is without toxic effect on the kidneys.

Alterations in the concentration of lipids like Total cholesterol, HDL, LDL and triglycerides can provide information on the status of lipid metabolism as well as predisposition of the animals to atherosclerosis.[34] In the present study, although the arteriosclerosis indexes were not affected, we noted a decrease in: serum total cholesterol level at 60 and 240 mg/kg, HDL level at 240 mg/kg and LDL level in all treated animals. Triglyceride levels were increased in all treated animals compared to the control. According to Schaffer and Menche,[32] an excess of bad cholesterol (LDL) and a lack of good cholesterol (HDL) is the major risk factor for cardiovascular disease. Indeed, the oxidation of LDL cholesterol is a gradual process that leads to the

formation of oxidized LDL and this latter plays an important role in the initiation of atherosclerotic plaque formation.[35,36] Hence, the significant decrease in total cholesterol and LDL cholesterol levels observed would reflect a reduced risk of developing cardiovascular disease. However, the increase in triglyceride levels noted at all doses would be a factor in increasing this risk. Since the increase in triglyceride levels in an individual reflects a lack of clearance or overproduction of triglycerides, it could increase the risk of developing cardiovascular disease.[37] However, it is known that during pregnancy, hypertriglyceridemia is necessary not only for fetal growth and development, but also as a source of maternal energy.[38] Therefore, the consumption of CBAE is related to a decreased risk of developing cardiovascular diseases.

5. CONCLUSION

Based on the results obtained in this work it becomes clear that administration of CBAE may be safe at the therapeutic dose but its continuous consumption at the dose of 240mg/kg may be lead to an increase of the risk of developing cardiovascular diseases.

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