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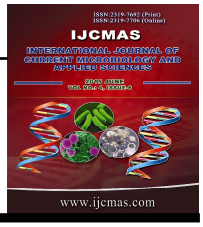

Original Research Article

Toxicological Evaluation of Aqueous Extracts Leaves of *Vepris heterophylla* (Engl.) R. Let. (Rutaceae) in Rat

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Keywords

Vepris heterophylla, Acute, Sub acute, Toxicity, Safety

A B S T R A C T

Experiments were carried out to assess the toxicity of aqueous extract and essential oil from leaves of *V. heterophylla* used in the treatment of arterial hypertension. Rats were divided into 7 groups of 10 (5 males and 5 females) in the acute study and 3 groups for sub acute toxicity. Extract were administered by orally at the dose of 3-18 g/kg in the acute study and 300-1200 mg in sub acute toxicity. The behavioral modifications, food and water intake, mortality and biochemical and hematological parameters were monitored. At the dose of 18 g/kg, the mortality was 100% after 24 hours and the lethal dose 50 (LD_{50}) was determined to be 12.6 g/kg. The AST, ALT, ALP, the total protein, increased while the RBC, haemoglobin, and hematocrit appreciably decreased. There was an increase of creatinin, urea, protein as well as ions Na^+, Cl^-, K^+ and Mg^{2+} while uric acid, Ca^{2+}, and the inorganic phosphate showed a reduction. These results showed that *V. heterophylla* aqueous extract has a safe margin for the therapeutic use.

Introduction

The search for new drugs from natural sources for the treatment of the cardiovascular diseases is increasing worldwide. This can be considered as an alternative to tackle resistance to conventional drugs. With the scrutiny of developing phyto-drugs that will be more effective in the treatment of arterial hypertension, *Vepris heterophylla* (Rutaceae) also known as Kounikoutchoum (Guiziga, Mofou), Hohoum (Zoulgo), Gougouvetche (Mafa), Kotokolhi (fulfulde); in the North of Cameroon was selected based on the results of the ethnobotanic investigations (Hamawa *et al.*, 2010). *Vepris heterophylla* is a medicinal plant used in

traditional pharmacopeia of Cameroon in the treatment of various illnesses including hypertension (Burkill, 1997; Ngamo *et al.,* 2001). Several authors have previously reported the medicinal values of this plant (Letouzey, 1968; Moulis *et al.,* 1994; and Keita and Ouattara, 1995). Ntchapda et al (2013a) demonstrated the vasorelaxant effects of the methanol extract from leaves of *V. heterophylla on* rat aorta. The identification of active ingredients from plant extracts is capital because it allowed the development of essential drugs. A flavonoid (6,8-dihydroxy-4'-methoxyflavone) isolated from *V. heterophylla* was found to induced nitric oxide-dependent vasodilation in rat aorta (Ntchapda et al 2013b). On the same line a triterpen (3β-16β, 23, 29-tetrahydroxyoleane-12-ene) was found to increase intracellular Ca^{2+} in rat aorta endothelial cells (Ntchapda *et al.,* 2013c). Alkaloids such as Kokusaginine isolated from leaves of this plant possess antimicrobial properties against bacteria and fungi (Kuete *et al.,* 2008). Cytotoxic activities of skimmianine another compound isolated from this plant was demonstrated against breast cancer cells and epidemic tumors (Cui *et al.,* 2003). The efficacy of *V. heterophylla* leaf extract in bringing relieve to patients with cardiovascular diseases was previously demonstrated by Ntchapda et al (2013d). The purpose of this study was to evaluate the acute and subacute toxicity of *V. heterophylla* aqueous extract in rats.

Materials and Methods

Material plant

The leaves of *V. heterophylla* were collected in the locality of Kaliyao (Far North Region-Cameroon) in August 2013. The identification was done in the National Herbarium of Cameroon where a voucher specimen was kept under the N° 61615/HNC. Leaves were washed with tap water, dried and crushed into powder.

Animals

Wistar rats of both sexes, of 9 weeks old and weighing 178.35±5.46 g at the start of the experiment were used to evaluate the acute and subacute toxicity. Strains of animals were from Center Pasteur in Yaoundé. They were reared in the Department of Biological Sciences, Faculty of Sciences (University of Ngaoundéré). The animals were housed under controlled temperature (24 ± 2°C) and relative humidity (45 ± 10%). Moreover, they had free access to food (pellets from LANAVET [Laboratory NVS]) and filtered tap water. The animal handling was under the control of the veterinary surgeon of the Science Veterinary Surgeon and Medical School of the University Ngaoundéré. Experimental protocols and procedures were approved by the Institutional Animals Care and Use Committee, and the research was approved by the Animal Ethics Committee of the University of Ngaoundéré.

Preparation of the aqueous extract

One thousand grams of fresh leaves of *V. heterophylla* was steeped in 1 L of distilled water for 12 h at room temperature. The macerate was filtered through Whatman filter paper No. 3, and the filtrate concentrated in a rotary evaporator at 40°C for 24 h. This process repeated several times and yielded 102.6 g of concentrated crude extract. The extract was stored at −20°C until further use.

Acute toxicity of the aqueous extract of the leaves of *Vepris heterophylla*

Rats were divided into 6 groups of 10 each. Males and females rats in each group were

housed separately in Plexiglass cages. Rats were acclimatized in the laboratory environment 7 days before the start of the experiment. The mice were fasted for 12 h prior to the experiment with free access to water. Rats were orally administered; a single dose of *V. heterophylla* aqueous extracts (3-18 g/kg) or distilled water for the control group. Animals from the same batch received the same dose of extract once daily. The animals were observed during the first 2 h after administration of the extract and were supplied with food. Mortality was recorded after 24 h. Food and water intake and body weight of surviving animals were evaluated up to 14 days. Dead animals were autopsied for macroscopic observation of internal organs. The surviving animals were sacrificed by decapitation. Arterio-venous blood was collected in heparinized tubes and was centrifuged with 4900 tr/min. Collected plasma was preserved at -20^{o}C for hematologic and biochemical analysis. The liver, the kidney, and the heart were collected; fat removed; weighed and preserved at -20^{o}C for the histological cuts and the biochemical analyses.

Subacute toxicity of the aqueous extract of the leaves of *V. heterophylla*

Rats were divided into 4 groups of 10 each (5 males and 5 females). The control group was orally treated with distilled water, and the other 3 groups were administered the plant extract at the dose of 300, 600 and 1200 mg/kg. The doses were selected from the literature as suitable doses to evaluate the hepato-protective activity (Donfack *et al.*, 2008, Atsamo *et al.*, 2011). The extract was administered by oral route once daily for 6 weeks. During this period, the behavior of the animals was observed and recorded. The weight, water, and food consumption were monitored at the end of each week. The last day of treatment, the animals were placed individually in metabolic cages for 24 h. Urine were collected; the pH was evaluated and stored at −20°C for biochemical analyzes. The survivors were anesthetized with chloroform and sacrificed. The arterio-venous blood was collected in heparinized tubes and centrifuged at 4900 rpm for 20 min. The collected plasma was stored at -20°C for biochemical analyzes. Liver, kidney, and heart were removed, cleared of fat material, weighed and stored at -20°C for biochemical analyzes and a portion preserved in formalin for histological analysis. The liver and the kidneys separately were crushed then homogenized in KCl (150 mm) in a Teflon potter on top of the ice. After centrifugation at 4900 rpm during 20 min, the supernatant was collected and preserved at -20^{o} C for the biochemical analyses.

Parameters

Urinary and plasma electrolyte concentrations were determined using a flame photometer (JENWAY PFP 7, Japan) according to standard methods described before (Henry *et al.*, 1974). Concentrations of creatinine, urea, glucose, albumin, and electrolytes in the plasma and urine samples were evaluated using a two-way digital spectrophotometer (SECOMAM RS 232C, Germany). Hematological and biochemical analyzes were performed by means of an automatic device type Toshiba 200 FR NEO (TOSHIBA Co., Japan). For hematological analysis, parameters like red blood cell, mean corpuscular volume, etc., were measured as described by Lahlou *et al.* (2008). Alanine transaminase (ALT), aspartate transaminase and alkaline phosphatase (ALP) were evaluated in serum and urine. Kidney functioning index was assessed by determination of the concentration of creatinine, urea, uric acid, Na^{+}, K^{+}, and Cl^{-}. The kidneys, liver and

heart, dissected out and fixed in 10% formalin fluid underwent histological cuts; stained with hematoxylin and eosin following the standard procedures.

Phytochemical study

The preceding studies mentioned the presence of several primary and secondary metabolites. The phenolic compounds, triterpenes, essential oils, sterols, alkaloids, fatty acids, flavonoïdes, athraquinones, coumarins, catecho tannins, are present in the extract. The presence of alkaloids and flavonoïdes is remarkable (Ntchapda *et al.*, 2013b).

Statistical analyses

Results were expressed in form of means ± ESM. Comparison of means was made using the Student's t-test and one-way ANOVA of Origin Graph software (Microcal Origin 6.0, Microcal, MA USA) software version 6.0. The difference was considered significant when $P < 0.05$.

Results and Discussion

Acute toxicity

Movement of the animals decreased during the first two hours following the administration of a single dose of extract. The animals treated with *V. heteropylla* aqueous extract at higher doses (9, 12, 15, and 18g/kg) remained calm. Moreover, sensitivity of the animals to noise, touch and aggression decreased. This effect was pronounced in animals treated with doses ≥ 9 g/kg. After 48 hours, all the surviving rats gained back their faculties. It should be mentioned that all the animals that succumbed to treatment presented convulsions, disordered jumps and ended up suffocating. A modified aspect of the feces (liquid) occurred with animals treated with the doses of 12, 15 and 18g/kg. The oral administration of the aqueous extract of *V. heterophylla* caused a reduction of food and water intake and body weight at the doses of 6, 9, 12 and 18g/kg. The DL_{50} was determined to be 12.6 g/kg**.** The maximum tolerated dose was determined to be 6g/kg in both males and females. Organ weights were not significantly modified by the treatments when compared to the controls treated with distilled water (Table 1).

Subacute toxicity

Effects of the aqueous extract of *V. heterophylla* on the urinary volume of excretion and the pH for 100g of body weight

A single administration of *V. heterophylla* aqueous extract (300, 600, and 1200 mg/kg) was able to provoke 24 h later a significant increase ($P < 0.05$) and dose-dependent volume of urinary excretion. Urine volume increased from 26.38 ± 2.13 ml/100g/24h in controls (distilled water) to 43.22 ± 2.11 ml/100g/24h at the dose of 300 mg/kg that represents an increase of 63.83%. The dose of 600 mg/kg increased urine volume to 56.81 ± 2.24 ml/kg/24h represent an increase of 115.35%. For the highest dose (1200 mg/kg), the volume of urinary excretion went from 26.38 ± 2.13 ml/100g/24h in controls to 69.80 ± 2.65 ml/100g/24h in the treated group (Figure 1), which represents an increase of 165.59%. The pH values of urine of animals treated with the extract of *V. heterophylla* at the dose 300 mg/kg presented significant modification ($P < 0.05$). The dose of 1200 mg/kg also showed a significant ($P < 0.05$) increase in pH values (8.12 ± 1.22). The pH value of the urine of animals treated with the extract at dose of 600 mg/kg was higher (7.35 ± 1.12) than that of the control group (6.45 ± 1.34) (Figure 1).

Effect of *V. heterophylla* on body weight, food and water intake

Rats' body weight progressively increased during the six weeks of treatment. It went from 237.78 ± 2.57 to 249.25 ± 2.7 with the dose of 300 mg/kg representing an increase of 4.82% in males and 2.57% in females. With the dose of 1200 mg/kg, males' increase was at 5.72% while females' increase was 3.97% (Table 2). Food intake in rats treated with the dose of 300 mg/kg went from 92.24±2.43 g/rat in the first week to 134.47±3.16g/rat in the last week, representing an increase of 39.90% (Table 2). An increase of water consumption was observed in all groups. This volume increased by 7.19% at the males and 8.79% in the females at the dose of 300 mg/kg.

Effect *V. heterophylla* on the relative organs weight

The aqueous extract of *V. heterophylla* had no significant effect ($P < 0.05$) on the heart. However, the dose of 1200 mg/kg, increased significantly ($P < 0.05$) the relative weight of liver, kidney, testis, and epididymis in males whereas, in females, the weight of kidney, uterine, and ovarian was significantly increased 6 weeks after administration of a daily dose of the *V. heterophylla* extract (Table 3).

Effects of *V. heterophylla* on the hematological parameters

Table 4 presents the variation of eosinophiles, basophiles, lymphocytes and monocytes. The eosinophiles varied from 1.52±0.04% for the control to 1.54±0.32% in the males and 1.55 ±0.03% for the control to 1.56±0.37% in the females at the dose 300mg/kg, representing an increase of 1.32% and 0.64% for males and females respectively.At the doses of 600 and 1200mg/kg, a dose-dependent decrease of eosinophiles was observed. The control values of 1.52±0.04% in males varied to 1.51±0.43% in males treated with plant extract at the dose 600 mg/kg, and from 1.55 ±0.03% in control females to 1.53±0.41% in the females treated with the dose of 600 mg/kg.

The lymphocytes rate increases dose-dependently during the period on treatment in males as well as in the females. Values went from 82.69±2.31% in control males to 86.61±1.96% in treated males and 84.25±1.9% in control females to 87.46±2.4% in treated females, representing an increase of 4.74% and 3.81% respectively in males and females at the dose of 300 mg/kg. The basophiles and the monocytes decreased dose-dependently in both sexes. The values went from 1.58±0.46% in the control to 1.53±0.55% and 7.12±0.59% in the control to 6.35±0.71% in males respectively for the basophiles and monocytes, with an increase rate of 3.16% and 12.13%. In the females, the values went from 1.61±0.45% in the control to 1.57±0.37% and 6.75±0.62% in the control to 6.03±0.67%, representing a reduction rate of 2.48% and 10.67% respectively for the basophiles and monocytes**.**

Neutrophiles and MCH decreased dose-dependently during the treatment period.. This reduction went from 16.45±0.61(pg/red cell) in the control to 14.97±0.79 (pg/red cell) and from 14.79±1.58% in the control to 12.37±2.15% in males respectively for the MCH and Neutrophiles at the dose 300 mg/kg, showing a reduction of 9% and 16.36%. Whereas in the females, it went from 16.21±0.36 (pg/red cell) in the control to 15.76±0.53 (pg/red cell) and from 15.01±2.68% in the control to 12.76±1.94% for the MCH and Neutrophiles respectively, showing a reduction of 2.78% and 14.99%.

On the other hand, the WBC and the platelets increased dose-dependent during the treatment period. The values passed from 7.58±0.15 K/µl in the control to 7.98±0.16 K/µl and from 789.64±3.25 k/µl in the control to 826.34±2.74 K/µl in the males treated with 300 mg/kg of extract respectively for WBC and platelets, representing 5.28% and 4.68% increase in both males and females.

Table 4 shows that the RBC and the MVC decreased dose-dependently. This decrease, from 9.16±0.45 ($x10^6$/µl) in the control to 8.96±0.37 ($x10^6$ /µl) and of 51.64±2.63 (fl/red cell) in the control to 49.34±3.02 (fl/red cell) in the males treated with the dose 300 mg/kg respectively for the RBC and the MVC, representing a reduction of 2.18% and 4.45%. Whereas in the females, the values went from 8.54±0.51($x10^6$/µl) in the control to 8.33±0.59 ($x10^6$/µl) and from 56.2±2.15 (fl/red cell) in control to 55.31±2.03 (fl/red cell), representing 2.46% and 1.58% respectively for RBC and MVC at the dose 300 mg/kg. As for hematocrits and hemoglobin's, they increased dose-dependently. The values went from 44.13±2.17% for the control to 45.86±3.24% and 16.93±0.84 (g/dl) for the control to 17.24±0.52 (g/dl) in males treated with 300mg/kg of extract respectively for the hematocrit and hemoglobin, with a percentage of 3.92% and 1.83%. Whereas in the females, the values went from 45.28±2.6% in the control to 47.64±2.51% and of 16.87±0.56 (g/dl) in the control to 17.01±0.94 (g/dl) for the hematocrit and hemoglobin respectively, representing an increase of 5.21% and 0.83% at the same dose (Table 4).

Effects *V. heterophylla* on index of liver function

Table 5 shows that the rate of the enzymes, ALT, AST, ALP increased dose-dependently. The ALT values of 39.15±3.9 (U/l) in the controls increased to 41.25±2.5 (U/l) in males and of 36.45±2.71 (U/l) in female controls increased to 37.8±2.46 (U/l) in females treated with the dose of 300 mg/kg, representing an increase of 5.36% and 3.7% respectively. AST values went from 45.23±3.1(U/l) in the controls to 47.25±2.31(U/l) and from 39.27±2.64 (U/l) in the controls to 40.1±2.65 (U/l) respectively in males and females at the dose 300 mg/kg, exhibiting an increase of 4.47% and 2.11%. As for the ALP, the rate passed from 31.54±4.5 (U/l) in the controls to 32.67±2.16 (U/l) in the males and from 26.31±2.15 (U/l) to the controls to 27.34±3.14 (U/l) in the females, representing an increase of 3.58% and 3.91% respectively. The lipids, TC and the LDL were reduced opposing the TG and HDL, which reduced dose-dependently. TC values went from 61.35±3.14 (mg/dl) in the controls to 58.34±2.16 (mg/dl) in males and from 70.21±4.11 (mg/dl) in the control to 66.38±3.16 (mg/dl) in the females at dose of 300 mg/kg, exhibiting a reduction of 4.91% and 5.45% respectively. The LDL went from 17.23±2.31 (mg/dl) in the controls to 15.62±3.17 (mg/dl) in males and from 22.03±3.14 (mg/dl) in the control to 19.36±2.54 (mg/dl) in females at the dose 300 mg/kg, representing a reduction of 9.34% and 12.12% respectively in males and females. The TG values went from 87.35±3.54 (mg/dl) in the control to 90.01±3.14 (mg/dl) in males and from 95.12±2.16 (mg/dl) in female controls to 97.85±2.67 (mg/dl) in the females treated with the dose of 300mg/kg, displaying an increase of 3.04% and 2.87% in males and females respectively. The HDL values passed from 21.35±1.37 (mg/dl) in the controls to 22.01±2.04 (mg/dl) in males and from 19.76±3.2 (mg/dl) to the female controls to 20.34±1.25 (mg/dl) in treated females, showing an increase of 3.09% and

2.85%. The proteins, the TP and Albumin were in rise, however, bilirubine reduced dose-dependently. The TP passed from 6.49±0.26 (mg/dl) in the controls to 6.98±0.22 (mg/dl) in males and of 7.63±0.31 (mg/dl) in the controls to 7.86±0.37 (mg/dl) in females treated with the dose of 300mg/kg, representing an increase of 7.55% and 3.01% respectively in males and females. Albumin passed from 5.94±0.45 (mg/dl) in the controls to 6.02±0.4 (mg/dl) in males and from 6.17±0.32 (mg/dl) in the controls to 6.35±0.26 (mg/dl) in treated females (300mg/kg); representing a rise of 1.35% and 2.92%. As for bilirubine, it passed from 1.3±0.31 (mg/dl) in the controls to 1.1±0.23 (mg/dl) in males and from 0.8±0.2 (mg/dl) in the controls to 0.7±0.17 (mg/dl) in females treated with the dose of 300mg/kg, showing a reduction of 15.38% and 12.5%. Sugars like glucose underwent an increase dose-dependently. It went from 87.53±2.97 (mg/dl) in the controls to 89.23±2.11 (mg/dl) for the males and of 90.12±2.39 (mg/dl) in the controls to 91.58±2.3 (mg/dl) in treated females (300 mg/kg); representing a rise of 1.94% and 1.62% (Table 5).

Effects *V. heterophylla* on index of kidney function

Table 6 shows the increase in urinary parameters. The rate of creatinin passed from 3.09±0.45 (mg/dl) to the control to 3.14±0.42 (mg/dl) in the males and of 3.24±0.62 (mg/dl) to the controls to 3.3±0.53 (mg/dl) in the females treated with the dose of 300 mg/kg, showing an increase of 1.62% and 1.85%. The urea passed from 2.35±0.12 (mg/dl) in the controls to 2.38±0.19 (mg/dl) in males and from 2.39±0.16 (mg/dl) in the controls to 2.4±0.14 (mg/dl) in treated females (300 mg/kg), showing an increase of 1.28% and 0.42%. The proteins, passed from 0.15±0.01 (mmol/l) in the controls to 0.2±0.00 (mmol/l) in males and of 0.2±0.01 (mmol/l) in the controls to 0.3±0.04 (mmol/l) in females treated with the dose of 300mg/kg, showing an increase of 25% and 50%. Na^+ passed from 87.36±0.97 (mmol/l) in the control to 88.95±1.73 (mmol/l) in treated males and from 89.25±1.37 (mmol/l) in the controls to 91.53±1.52 (mmol/l) in the treated females (300 mg/kg), showing an increase of 1.8% and 2.55%. The ion Cl^- passed from 92.46±1.12 (mmol/l) in the controls to 94.19±1.73 (mmol/l) in the treated males and from 94.2±1.51 (mmol/l) in female controls to 97.24±2.18 (mmol/l) in treated females, representing an increase of 1.87% and 3.23%. The ion K^+ passed from 19.2±0.31 (mmol/l) in the controls to 20.16±0.62 (mmol/l) in treated males and from 17.14±0.33 (mmol/l) in the controls to 18.15±0.53 (mmol/l) in treated females (300 mg/kg), showing a rise of 5% and 5.89%.

Effects *V. heterophylla* on the markers of the oxydative stress

The aqueous extract of *V. heterrophylla* decreased the glutathion in the males as well as in the females. This rate passed from 0.0196±0.002 µM in the controls to 0.0032±0.0013 µM in treated males (300 mg/kg), showing a reduction of 83.67%. In the females this rate passed from 0.0222±0.0025 µM in the controls to 0.0054±0.0011 µM in treated animals, representing a reduction of 75.67%. The aqueous extract of *V. heterophylla* decreased dose-dependently the rate of glutathion in the serum. In the same way, the rate of malondialdehyde passed from 0.004±0.001 µM to 0.0498±0.019 µM, with a significant increase ($P<0.05$) of 11.45% in males; whereas in the females this rate passed from 0.0058±0.0013 in the controls to 0.068±0.016 with an increase of 10.72%. The aqueous extract of *V. heterophylla* increased dose-dependently the rate on malondialdehyde in blood. The superoxyde

dismutase passed from 0.498±0.051 µM in the controls to 0.46±0.078 µM with a reduction of 7.63% in males whereas this rate passed from 0.474±0.032 µM in the controls to 0.466±0.071 µM in females with a reduction rate of 1.68% (Table 7). The aqueous extract of *V. heterophylla* decreased dose-dependently the rate on superoxyde dismutase in the rats.

Effect *V. heterophylla* on the histopathology of the liver and the kidneys

The evaluation of histopathology cuts of the liver and the kidneys of the rats treated with 1200 mg/kg in the subacute toxicity showed congestion on the hepatic cells, with a nuclear fragmentation. It was also observed on the liver the Caryopynose and a caryorrhexis. The kidney on the other hand presented a glomerular sclerosis and edemas (Figure 2).

The toxicological evaluation of the aqueous extract of the leaves of *V. heterophylla* was necessary due to its importance in the traditional pharmacopeia of Cameroon. In this work, we evaluated acute toxicity by oral administration of a single dose of the extract leaves of *V. heterophylla*. It was demonstrated that single dose administration (≥ 6 g/kg) of the aqueous of extract of *V. heterophylla* provoked in the following minutes deterioration of rat behavior. The animals presented a reduction in aggressiveness, locomotion, sensitivity to the touch, and the noise. The modification of the aspect of the feces could be explained by an overdose, which could modify the mechanism of transformation of nutrients in the intestines, also coupled with gastro-intestinal metabolic disorders and motricity. Animals that died following the administration of a single dose of *V. heterophylla* presented convulsions, diarrheas and the ultimate stage was anarchic jumps followed by disordered in respiratory movements. The modification of the behavior in the rats was due to the active compounds present in the plant as shown by Bafor *et al.* (2009) when studying the toxicity of *Ficus exasperata* in the rats. These results show that the aqueous extract of *V. heterophylla* could have a depressive action on the central nervous system as reported in the case of many other medications. The convulsions observed in the animals could be due to the increase in the excitability of the neurons (Schmitt, 1976).

Mortality went from 10% at the dose of 9g/kg to 100% at the dose of 18g/kg. The DL_{50} determined by calculation was of 12.6g/kg. Cases of death observed with dose higher or equal to 6g/kg suggest the presence of toxic compounds existing in small quantities in the extract and of which the proportion would increase with the dose (Kerharo and Adam, 1976). This study showed that the male rats were more resistant to the extract than the female rats. The hormonal differences, which exist between the two sexes, could be responsible of this difference in behavior. The body weight, the food and water intakes were reduced starting from the animals treated with the dose of 9g/kg. It should be noted that this reduction could be explained by the action of the extract. Therefore the loss of body weight could be explained by the reduction in the food and water intakes due to an action of the extract on the appetite. Such results were reported by Vimala (1999); and Kanjanapothi *et al.* (2004); when studying the toxicity of the aqueous extract of *Kaempferia galanga* in the rats. The growth slow-down could be the consequence of metabolic or appetite disorders, which, provoked a reduction of interest in food.

Table.1 Effects of the aqueous extract leaves of *V. heterophylla* on the behavior of the animals 14 days after administration of the extract

Dose (g/kg)	% Mortality		Latency	Symptoms	% Weight change (g)		% Food intake		% Water intake	
	Males	Females			Males	Females	Males	Females	Males	Females
0	0/5	0/5	-	none	26.51±2.13	26.36±2.14	42.67±1.13	41.88±1.24	25.09±0.52	23.14±1.15
3	0/5	0/5	-		25.95±3.04*	27.48±1.96*	45.08±3.24*	43.21±2.91*	27.94±2.3*	24.85±3.17*
6	0/5	0/5	-		17.19±2.20	17.90±2.13	30.97±2.57	29.81±3.17	29.26±3.08*	24.06±3.08*
9	0/5	1/5	>6h<8h	During the first two hours, the locomotion of the animals decreased following the administration of dose of extract. The animals having received the extract of *V. heteropylla* to the high dose of 9, 12, 15 and 18g/kg remained calm. The sensitivity of the animals to the touch and the sound also decreases according to the dose. This reduction was pronounced with subjects having received a dose of extract higher or equal to 9g/kg.	13.30±2.13	14.05±2.14	30.02±2.82	29.66±3.45	14.64±3.12	14.46±2.60
12	2/5	3/5	>4h<6h		12.54±2.16	12.49±2.18	31.26±2.31	32.06±2.13	14.20±2.30	14.21±2.15
15	3/5	4/5	>2h<8h		7.82±3.15	5.35±1.77	28.85±3.18	31.68±4.12	14.06±1.97	14.43±3.16
18	5/5	5/5	>30min<1h		0	0	0	0	0	0

Each value represents the average ± ESM, n=5

* P < 0.05, significant difference compared to the control

Table.2 Effect *V. heterophylla* on the body weight, food and water intake

		1-14 days		15-28 days		29-42 days	
	Doses mg/kg	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
Body weight	**0**	252.3±3.34	250,7±2,59	259.61±2.90	256.12±3.60	262.24±4.48	260.51±2.91
	300	237.78±2.57*	240.52±3.90*	241.15±2.67*	242.46±2.14*	246.71±2.41*	249.25±2.70*
	600	229.34±3.43*	230.15±3.41*	236.21±3.27*	235.45±4.18*	239.02±4.16*	238.98±3.49*
	1200	219.37±2.75*	224.35±3.28*	226.11±3.52*	228.29±2.14*	231.94±2.84*	233.25±3.67*
Food intake	**0**	96.55±2.17	100.47±1.96	121.06±3.02	130.63±2.51	144.29±2.12	151.69±2.43
	300	92.24±2.43*	97.74±3.46*	111.87±2.56*	121.29±2.49*	129.05±3.14*	134.47±3.16*
	600	79.67±2.45*	82.66±2.15*	96.68±3.41*	105.64±2.49*	111.06±3.25*	116.36±3.17*
	1200	60.25±4.12*	62.84±4.01*	73.89±2.67*	82.27±2.71*	87.97±3.18*	91.88±2.94*
Water intake	**0**	134.76±4.11	136.43±3.61	137.74±3.26	140.25±3.14	142.51±3.44	146.64±3.17
	300	119.47±3.25*	122.68±2.43*	124.63±3.42*	124.44±3.16*	125.07±2.26*	126.50±4.03*
	600	99.87±2.14*	100.25±3.12*	104.26±302*	104.28±4.38*	112.49±2.45*	113.43±3.61*
	1200	76.25±2.64*	78.46±3.17*	80.04±3.16*	80.04±3.16*	72.52±4.35*	76.25±2.64*

Each value represents the average ± ESM, n=5
* P < 0.05, significant difference compared to the control

Table.3 Effects of aqueous extract of *V. heterophylla* on relative organs weight

Organs	**Males**				**Females**			
(mg/kg)	**Control**	**300**	**600**	**1200**	**Control**	**300**	**600**	**1200**
Liver	3.11±0.11	3.35±0.13	3.56±0.22	3.86±0.12*	3.13±0.02	3.22±0.21	3.34±0.12	3.44±0.21
Kidney	0.64±0.02	0.65±0.04	0.66±0.03	0.68±0.02*	0,59±0,03	0.59±0.04	0.57±0.02	0.62±0.03*
Heart	0.30±0.01	0.31±0.04	0.31±0.03	0.32±0.03	0.33±0.01	0,34±0,01	0.34±0.02	0.35±0.03
Lung	0.70±0.02	0.71±0.02	0.73±0.03	0.71±0.04	0.67±0.02	0,68±0,01	0.72±0.05	0.70±0.02
Spleen	0.29±0.01	0.29±0.03	0.33±0.01	0.29±0.04	0.28±0.02	0,29±0,02	0.28±0.02	0.30±0.03
Testis	0.60±0.01	0.60±0.02	0.62±0.02	0.64±0.02*	-	-	-	-
Epididymis	0.25±0.01	0.26±0.01	0.28±0.01	0.29±0.04*	-	-	-	-
Uterus	-	-	-	-	0.28±0.01	0.32±0.01	0.36±0.01	0.39±0.02*
Ovary	-	-	-	-	0.03±0.00	0.03±0.00	0.04±0.00	0.038±0.00*

Each value represents the average ± ESM, n=5
* P < 0.05, significant difference compared to the control

Table.4 Effects of *V. heterophylla* on hematological parameters

Organs		Males				Females			
	Normal range	**Control**	**300**	**600**	**1200**	**Control**	**300**	**600**	**1200**
RBC (x 10^6/µL)	5-10	9.16±0.45	8.96±0.37*	8.53±0.39*	7.74±0.63*	8.54±0.51	8.33±0.59*	8.03±0.43*	7.42±0.51*
WBC (x 10^3/µL)	1-5	7.58±0.15	7.98±0.16*	8.24±0.13*	8.49±0.14*	7.65±0.18	8.02±0.23*	8.27±0.19*	8.56±0.21*
Platelets (x 10^3/µL)	600-1100	789.64±3.25	826.34±2.74*	859.42±2.56*	872.36±3.25*	778.95±3.54	823.16.39±4.02*	866.45±2.56*	887.38±3.11*
Haemoglobin (g/dL)	11-19	16.93±0.84	17.24±0.52*	15.36±0.59*	14.41±0.91*	16.87±0.56	17.01±0.94*	15.28±0.79*	14.3±0.47*
Haematocrit (%)	35-57	44.13±2.17	45.86±3.24*	43.11±2.34*	42.05±2.26*	45.28±2.60	47.64±2.51*	44.39±2.07*	43.27±3.21*
RDW (%)	12-18	14.71±1.18	12.27±1.12*	10.25±2.12*	8.32±2.96*	15.01±2.68	13.56±1.44*	11.14±3.33*	9.32±2.32*
MCV (fL)	46-65	51.64±2.63	49.34±3.02*	48.12±1.97*	47.26±2.28*	56.2±2.15	55.31±2.03*	54.76±3.11*	52.43±2.36*
MCH (pg)	18-23	16.45±0.61	14.97±0.79*	14.03±0.61*	11.87±0.91*	16.21±0.36	15.76±0.53*	14.17±0.82*	12.84±0.59*
MCHC (g/dL)	31-40	34.13±2.22	35.86±3.15*	36.11±2.43*	38.05±2.27*	35.28±2.4	37.64±2.63*	38.39±2.16*	39.27±3.33*
Neutrophil (%)	2-20	14.79±1.58	12.37±2.15*	9.85±3.12*	7.2±1.96*	15.01±2.68	12.76±1.94*	10.04±3.06*	8.07±2.14*
Basophile (%)	0-7	1.58±0.46	1.53±0.55*	1.47±0.51*	1.38±0.35*	1.61±0.45	1.57±0.37*	1.42±0.39*	1.34±0.49*
Eosinophil (%)	0-1	1.52±0.04	1.54±0.32	1.51±0.43*	1.48±0.62*	1.55 ±0.03	1.56±0.37	1.53±0.41*	1.50±0.36*
Lymphocytes (%)	65-94	82.69±2.31	86.61±1.96*	89.73±2.58*	81.13±2.16	84.25±1.90	87.46±2.4*	92.43±3.21*	84.27±2.15
Monocytes (%)	0-6	7.12±0.59	6.35±0.71*	4.13±0.81*	3.05±0.48*	6.75±0.62	6.03±0.67*	4.76±0.53*	3.58±0.64*

Each value represents the average ± ESM, n=5

* $P < 0.05$, significant difference compared to the control

Table.5 Effects *V. heterophylla* extract on index of liver function

Doses (mg/kg)	0		300		600		1200	
	Males	Females	Males	Females	Males	Females	Males	Females
Glucose (mg/dL)	87.53±2.97	90.12±2.39	89.23±2.11*	91.58±2.30*	90.83±3.01*	93.14±2.17*	92.35±2.34*	93.56±3.21*
ALT (IU/l)	39.15±3.90	36.45±2.71	41.25±2.50*	37.80±2.46*	42.35±3.11*	39.49±2.94*	43.02±3.15*	40.87±2.53*
AST (IU/l)	45.23±3.10	39.27±2.64	47.25±2.31*	40.10±2.65*	48.35±2.49*	42.37±3.15*	50.12±4.2*	43.52±2.17*
ALP (IU/l)	31.54±4.50	26.31±2.15	32.67±2.16*	27.34±3.14*	34.26±4.56*	29.30±3.27*	35.97±2.56*	30.97±4.26*
TP (g/dL)	6.49±0.26	7.63±0.31	6.98±0.22*	7.86±0.37*	7.03±0.24*	8.07±0.36*	7.12±0.28*	8.16±0.34*
Albumine (mg/dL)	5.94±0.45	6.17±0.32	6.02±0.40*	6.35±0.26*	6.14±0.41*	6.43±0.25*	6.19±0.37*	6.45±0.38*
Totalbilirubine (mg/dL)	1.30±0.31	0.80±0.20	1.10±0.23*	0.70±0.17*	0.90±0.13*	0.60±0.11*	0.70±0.15*	0.40±0.09*
TC (mg/dL)	61.35±3.14	70.21±4.11	58.34±2.16*	66.38±3.16*	55.69±4.05*	62.37±3.60*	50.12±3.62*	57.96±2.54*
TG (mg/dL)	87.35±3.54	95.12±2.16	90.01±3.14*	97.85±2.67*	93.24±3.39*	99.60±2.64*	94.21±3.26*	102.3±4.18*
LDL (mg/dL)	17.23±2.31	22.03±3.14	15.62±3.17*	19.36±2.54*	14.11±3.29*	16.64±2.57*	11.29±2.56*	13.89±3.27*
HDL (mg/dL)	21.35±1.37	19.76±3.20	22.01±2.04*	20.34±1.25*	22.95±2.03*	20.96±2.17*	23.15±3.01*	21.09±1.62*

Each value represents the average ± ESM, n=5

* P < 0.05, significant difference compared to the control

Table.6 Effects *V. heterophylla* on index of kidney function

Doses en (mg/kg)	0		300		600		1200	
	Males	Females	Males	Females	Males	Females	Males	Females
Creatinine (mg/dl**)**	3.09±0.45	3.24±0.62	3.14±0.42*	3.30±0.53*	3.21±0.39*	3.34±0.51*	3.26±0.53*	3.40±0.62*
Protein (mg/dl**)**	0.15±0.01	0.2±0.01	0.20±0.00*	0.30±0.04*	0.26±0.05*	0.40±0.06*	0.30±0.04*	0.50±0.06*
Urea (mg/dl**)**	2.35±0.12	2.39±0.16	2.38±0.19*	2.40±0.14*	2.41±0.17*	2.42±0.20*	2.45±0.15*	2.50±0.22*
uric Acid (mg/dl**)**	45.21±3.11	42.20±2.64	37.25±2.33*	40.10±2.65*	28.35±2.42*	37.39±2.15*	25.12±1.20*	26.32±1.16*
Na^+(mmol/l**)**	87.36±0.97	89.25±1.37	88.95±1.73*	91.53±1.52*	90.05±1.30*	93.12±1.65*	91.13±1.94*	95.20±2.10*
Cl^-(mmol/l**)**	92.46±1.12	94.20±1.51	94.19±1.73*	97.24±2.18*	95.00±1.92*	99.24±2.11*	97.25±2.13*	104.30±2.18*
K^+(mmol/l**)**	19.20±0.31	17.14±0.33	20.16±0.62*	18.15±0.53*	22.23±0.44*	19.37±0.46*	22.84±0.50*	19.72±0.65*
Ca^{2+} (mmol/l**)**	81.32±0.31	77.28±0.29	77.11±0.43*	73.70±0.37*	68.29±0.43*	68.62±0.31*	63.71±0.45*	61.96±0.39*
Mg^{2+} (mmol/l**)**	15.14±0.15	15.54±0.22	16.37±0.24*	16.44±0.23*	20.16±0.31*	18.56±0.25*	17.39±0.23*	17.49±0.43*
Pi (mmol/l**)**	31.35±0.24	30.26±0.11	28.34±0.36*	32.34±0.16*	30.69±0.35*	29.37±0.22*	28.32±0.62*	27.96±0.34*

Each value represents the average ± ESM, n=5

* P < 0.05, significant difference compared to the control

Table.7 Effects *V. heterophylla* on the markers of the oxidative stress.

	Malondialdehyde (µM)		Glutathion (µM)		Superoxyde dismutase (µM)	
Treatments	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
Control	0.004±0.001	0.006±0.001	0.019±0.002	0.022±0.002	0.498±0.051	0.474±0.032
300mg/kg	0.049±0.019*	0.068±0.016*	0.003±0.001*	0.005±0.001*	0.460±0.078*	0.466±0.071*
600mg/kg	0.006±0.001*	0.007±0.000*	0.017±0.001*	0.017±0.002*	0.448±0.051*	0.456±0.023*
1200mg/kg	0.004±0.001	0.006±0.001*	0.020±0.002	0.024±0.004	0.284±0.027*	0.288±0.033*

Each value represents the average ± ESM, n=5
* P < 0.05, significant difference compared to the control

Figure.1 Effects of the aqueous extract of *V heterophylla* on the urinary excretion volume of and the pH for 100g of body weight. Each value represents the average ± ESM, n=5, * P < 0,05, significant difference compared to the control

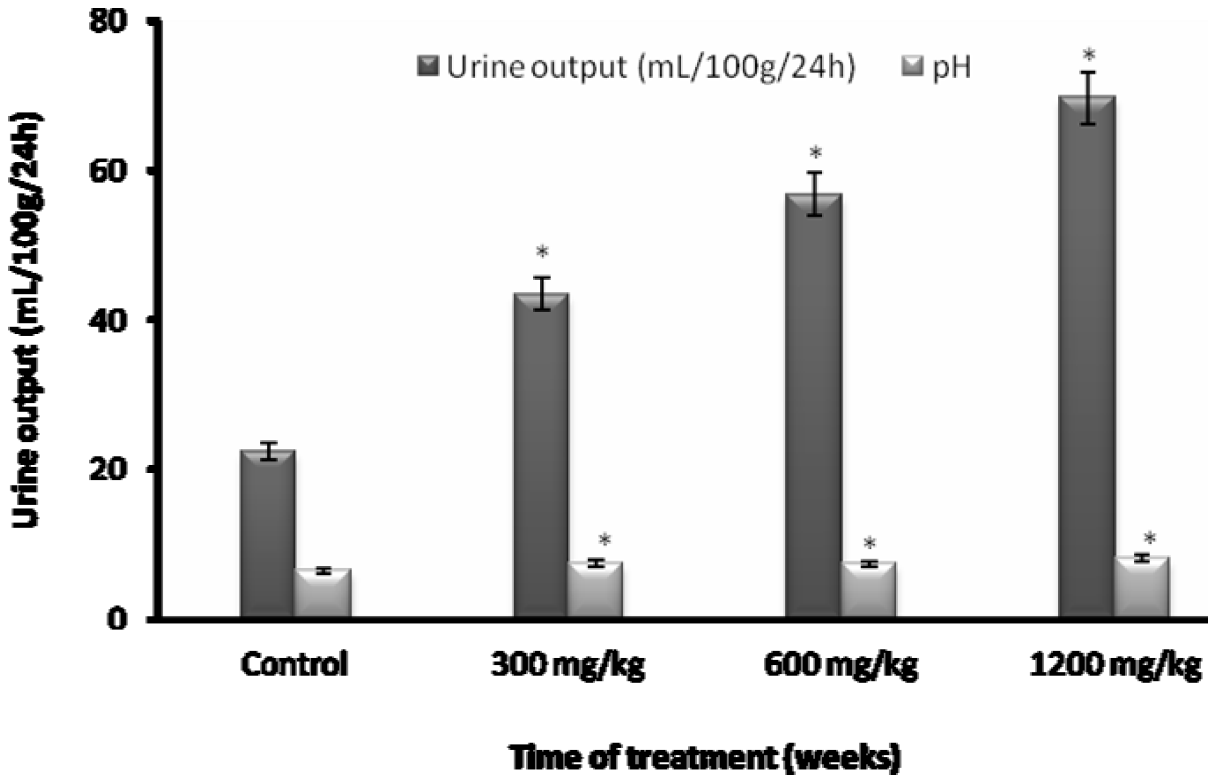
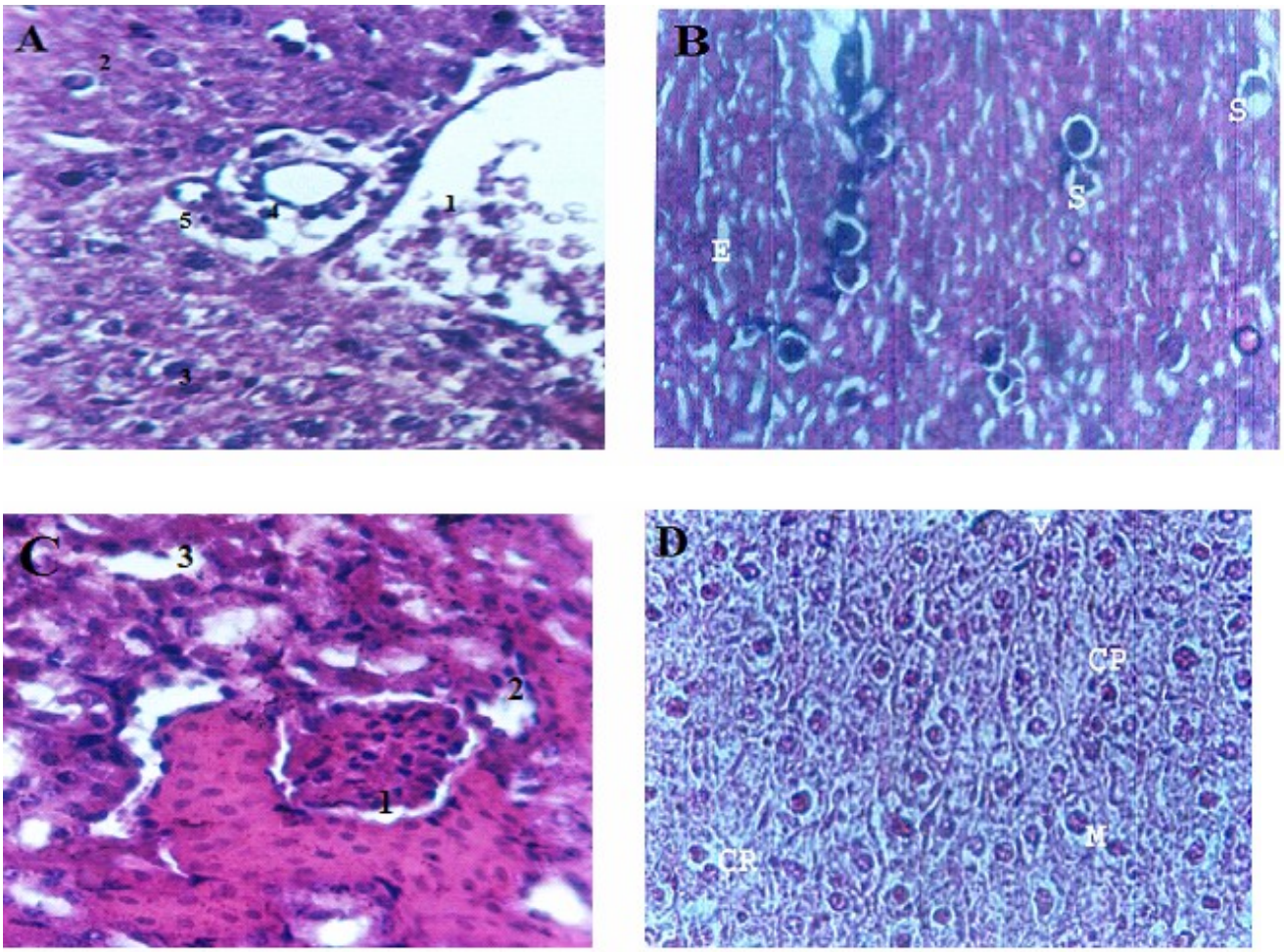


Figure.2 Effect *V heterophylla* on hepatic and renal tissues. The figure 2A shown the normal architecture of the control liver observed under photonic microscope H.E (X 400) presenting a hepatic Parenchyma at normal architecture with portals vein (1), spaces portals (2), hépatocytes and sinusoids (3), hepatic artery (4), and a bile canal (5).The figure 2B shows the liver of rat treated at the dose 1200 mg/kg (X 400) presenting a congestion (V) followed Mitosis (M), caryopynose (CP) and caryorrhexis (CR).The figure 2C shows the normal architecture of kidney observed under photonic microscope H.E (X 400), with a corpuscle of Malpighi made up of a cluster (1) wrapped in the capsule of Bowman. Proximaux (2) and distal (3) circumvented tubes distinct. The figure 2D presents a kidney of rat treated at the dose 1200 mg/kg (X 400) with a glomerular sclerosis (S) and an oedema (E).



The study of the subacute toxicity of the aqueous extract revealed that the administration of a repeated doses do not provoke the deterioration of the behavioral reactions. It was observed an increase in the food and water intakes. This increase could be explained by the progressive increase in the body weight. After 6 weeks of treatment it was noted a reduction in the hematologic parameters like the RBC, hemoglobin, hematocrit, at the higher doses. The reduction in RBC suggests that *V. heterophylla* in repeated administration could cause acute or chronic anemia. The general reduction in values of these hematologic parameters can be due to the direct destruction of the cells or the loss in circulation of mature cells per hemorrhage,

or by capillary escapes or then could also be due to a reduced blood cells production (Nunia *et al.,* 2007). The increase in enzymes ASAT, ALAT, ALP could be due to the long term toxic effects compared to those observed in acute toxicity (Al-Mamary *et al.,* 2002). These results suggest a toxicity and a significant serious prejudicial effect in the long term use of *V. heterophylla* aqueous extract since the AST is present in high concentrations in a many organs, such as the liver and kidneys, while the ALT is mainly limited to the cytosol of the hepatocytes, its high concentration can provide a quantitative evaluation of the degree of damage supported by liver (Al-Mamary *et al.,* 2002).

The increase in proteins could explained by hepatocytes cell membrane dislocations which would have contributed for a reduction of the use of proteins and consequently to an increase of stock of these one in the liver. The increase of lipids, creatinine, urea, uric Acid could slow down the tubular function by inducing a renal failure (Nimenibio-Uabia, 2003; Ijeh and Agbo, 2006). The decrease of ions could be due to the osmotic balance of membrane potential. Regarding urinary parameters, the increasing rate of creatinine could be explained by an enormous transformation of creatine which is a nitrogenous organic acid that occurs naturally in vertebrates and helps to supply energy to all cells in the body, primarily muscle. The increased creatinine could acquaint on the level of activity of the animals. In the same way the high concentration of urea could expressed the high activity of the kidney busy evacuating toxic metabolites from the plant extract. The presence of protein in the urine is the cause of a renal dysfunction; the tubular reabsorption is not quite assured. The increase in the rate of the ions could be due to ensure the osmotic balance but also could be due to the diuretic activity of the plant extract (Ntchapda *et al.* 2014). The assessment of the histopathological cuts of the liver and the kidneys of the rats treated with the dose of 1200 mg/kg showed anomalies on the liver and kidneys. It was observed congestion in the hepatic cells, with a nuclear fragmentation. It was also observed on the liver a caryopynose and caryorrhexis. The kidneys on the other hand presented a glomerular sclerosis and edemas.

In conclusion, these results showed that *V. heterophylla* has a safe margin for the therapeutic use.

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