

# Acute, Sub-Chronic Toxicity in Wistar Rats and Cytotoxicity Studies of Hydroethanolic Root Extract of *Cassia Sieberiana* DC

BAH Fatoumata<sup>1</sup>, KANE Yaghoub<sup>2</sup>, FALL Alioune Dior<sup>3</sup>, CABRAL Mathilde<sup>1</sup>, TOURE Aminata<sup>1</sup>, LAM-FAYE Absa<sup>1</sup>, DIOP Cheikh<sup>1</sup>, DIENG Serigne Ibra Mbacke<sup>3</sup>, DIOUF Amadou<sup>1</sup> and FALL Mamadou<sup>1\*</sup>

<sup>1</sup>Laboratoire de Toxicologie et Hydrologie, Université Cheikh Anta Diop, Dakar, Senegal

<sup>2</sup>Service de Pathologie médicale, d'anatomie pathologique et clinique ambulante à l'Ecole Inter-états des Sciences et Médecine Vétérinaires de Dakar (E.I.S.M.V.)

<sup>3</sup>Laboratoire de Pharmacognosie et de Botanique, Université Cheikh Anta Diop, Dakar, Senegal

\*Corresponding author: FALL Mamadou, Email: mamadou3.fall@ucad.edu.sn

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## Abstract

Acute and sub-chronic toxicity studies of hydroethanolic root extract of *Cassia sieberiana* (Caesalpinaceae), a plant widely used in traditional medicine in Senegal, were carried out in Wistar rats. Cytotoxicity of this extract was studied on vervet monkey kidney cells by the MMT [3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide] method concurrently. In the acute toxicity study, five female rats (260 ± 10 g) were each administered a single oral dose (5000 mg/kg) of the hydroethanolic extract of *C. Sieberiana* root bark and observed for signs of acute toxicity for 14 days. In the sub-chronic toxicity study, the hydroethanolic extract at 500, 1000 and 2000 mg/kg was administered to sets of 6 Wistar rats daily for 28 days. Each set consisted of 3 males (278 ± 13g) and 3 females (260 ± 10 g). Urinalysis, haematological and biochemical analyses were carried out on urine, whole blood and serum samples. Histological studies of the liver, heart, and kidney tissues were also performed. The results showed that the acute and subchronic toxicity studies of hydroalcoholic root extract of *C. sieberiana* did not result in death among the animals; there were no signs of toxicity. However, the histological study showed the presence of necrotic and inflammatory cells in the hepatic parenchyma and a sinusoidal stasis at the end of 28 days. The cytotoxicity study on cell viability showed that the plant seems to be cytotoxic only at very high doses.

**Keywords:** *Cassia sieberiana*, *in vivo*, *in vitro*, Toxicity

## Introduction

*Cassia sieberiana* DC (*Caesalpinaceae*) is a very common plant in all Sudanese savannah. It is widely distributed throughout the southern Sahel and Sudan savannah from Senegal to Cameroun, up to Sudan and the Republic of Congo [1]. It is also found in most parts of Nigeria. It is a perennial savannah legume shrub or tree, up to 20 m high. The leaves, which are spirally arranged, contain short hairs and can be elliptical to ovate in shape. The fruit is a cylindrical pod which contains rusty to dark brown ellipsoid seeds. The whole plant has therapeutic virtues but it is almost always the roots which serve for preparations. Phytochemical analysis of the root and fruit pulp of the plant shows the presence of four principal active groups: tannins, saponins, quinones, which are diversely concentrated [2].

The aqueous decoctions of the root, stem bark and the fruit pulp have been used traditionally for the treatment of many diseases: inflammatory conditions, tiredness and joint pains, fever, malaria,

diarrhoea, leprosy, bilharzia and stomach pains [2,3]. Other uses including improvement of lactation after childbirth and treatment of rheumatic conditions are noted (15). Authors have shown that *C. sieberiana* extracts possess antimicrobial, antifungal, antioxidant and anti-ulcerogenic activity [4-7].

Ethanolic and aqueous root extracts of *C. sieberiana* also have analgesic, anti-inflammatory, antiparasitic, myorelaxant and antispasmodic activity [8,9].

In a study, Obidah et al. [10] reported that oral administration of aqueous extract of *C. sieberiana* stem bark to rats resulted in hepatotoxicity at lower doses (20-60 mg/kg) and nephrotoxicity at higher doses (180 mg/kg). Earlier, Tamboura *et al.* [3] found that the lethal dose (LD<sub>50</sub>) of the aqueous leaves extract in mice was 24.4 mg/kg. Many studies of *C. sieberiana* have been performed on different organs [11-14]. Though the root extract of the plant is widely used for medicinal purposes, little data is available regarding its toxicity. Only an acute single-dose toxicity study has been reported [7,15]. However, the extract may have deleterious effects on the liver at high doses with prolonged administration [15]. In this study we investigated the *in-vivo* acute and subacute toxicity in rats as well as the *in-vitro* cytotoxicity.

## Materials & Methods

### Materials

**Plant material:** The root bark of *Cassia sieberiana* was collected from the Garden for Experimentation of useful Plants of the Faculty of Medicine, Pharmacy and Dentistry at the University of Cheikh Anta Diop of Dakar (UCAD) and at Hann National Park of Dakar. It was identified and authenticated in the Botany and Plant Biology Laboratory of the Faculty of Medicine of UCAD.

**Reagents and Chemical Test kits:** aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), creatinine and urea were purchased from BioSystems (Spain). Urine test strips, Labistix, were obtained from Laboratory Diagnostics (Siemens, France). Minimal Essential Medium (MEM Highveld Biological), Berberine chloride (CAS: 633-65-8, purity: >95%) solution of MTT, phosphate buffered saline (PBS) without Ca + Mg, DMSO were purchased from Sigma-Aldrich (France).

**Animals:** Female and male Wistar albino rats weighing 260 ± 10 g were used. The animals were housed three per cage maximum. They were fed a normal commercial pellet diet, they were given water *ad libitum* and maintained under laboratory conditions (temperature 22-24°C, relative humidity 60-70 %). The research was conducted in accordance with the internationally accepted principles for laboratory animal use and care.

**Cells:** VERO cells (from a vervet monkey kidney of Africa) purchased from the "Department of Veterinary and Tropical Diseases" (University of Pretoria, South Africa)

### Methods

#### Preparation of hydroethanolic extract

The plant material was dried and grounded to a fine powder. One hundred grams (100 g) of the powdered root bark was mixed with 500 mL of water-ethanol mixture (20/80; v/v). The mixture was boiled at 70°C for 15 minutes. It was allowed to cool and then filtered using N° 1 Whatman filter paper. The obtained filtrate was concentrated by evaporation using a rotary evaporator. The pasty extract was completely dried at room temperature to obtain a dry extract which was then grounded into powder. The dry extract obtained (hydroethanolic

extract: HEE) was dissolved in distilled water, and the resultant mixture was homogenized by stirring (5-10 min) using a magnetic stirrer. The volume of the solution administered to the animals was determined using their body mass.

### In vivo study

**Acute toxicity studies:** Acute toxicity of the extract was determined by gavage in rats according to the Organisation for Economic Co-operation and Development (OECD) chemical tests guideline no. 420 adopted on December 17, 2001 (16). Five Wistar female rats weighing  $260 \pm 10$  g were obtained from the animal unit (Toxicology Laboratory of Dakar University). A single oral dose of the extract was administered at 5000 mg/kg to each rat with an oral gavage needle. Mortality and general behaviour of the animals were observed over a 48-hour period. They were monitored for clinical signs of toxicity including piloerection, lachrymatory, locomotor and respiratory activities for a period of 14 days.

**Sub-chronic toxicity studies:** Four groups of six rats (3 females:  $260 \pm 10$  g and 3 males:  $278 \pm 13$  g) were force-fed by mouth daily for 28 days. Group 1 was kept as control and received normal tap water. Group 2, 3 and 4 were treated respectively with 500, 1000 and 2000 mg/kg of hydroethanolic extract of *C. sieberiana*. Animals in each group were weighed on day zero (baseline) and weekly thereafter. Rat blood samples in each group were collected into tubes with or without anticoagulant at the end of treatment; they were centrifuged at 4000 rpm for 5 minutes and the resulting serum taken for biological tests.

**Urinalysis:** Rat urine samples were collected and analysed every week for glucose, ketones, and proteins, using urine reagent strips (Labistix, Siemens) from Diagnostic Laboratory Inc., France.

**Haematological analysis:** Red blood cells (RBC), mean cell volume (MCV), haematocrit (HCT), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), white blood cells (WBC), haemoglobin (HGB) and platelet counts (PLT) were determined with an automated haematology analyser (MIN-BC-3000Plus auto hematology analyzer, Mindray).

**Serum biochemical analyses:** Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood sugar and creatinine of samples were determined according to the protocols of Biosystems diagnostic kits (Spain) with a semi-automated blood chemistry analyser (A15 Biosystems, Barcelone).

**Histology:** At the end of treatment, rats were sacrificed by cervical dislocation and the heart, liver and kidney were excised. All organs were stored in 10 % neutral formalin and dehydrated in ethanol. The tissues were then cleared with chloroform and impregnated into paraffin

wax. Five  $\mu$ m thick sections were mounted on slides and stained with haematoxylin and eosin for light microscopic examinations.

### In vitro study: Cytotoxicity assay

Cytotoxicity of hydroethanolic root extract of *Cassia sieberiana* was determined using a tetrazolium-based colorimetric assay (MTT assay) described by Mosmann (1983) (18). Vero monkey kidney cells were harvested and centrifuged at  $200 \times g$  for 5 minutes. The pellet was re-suspended in growth medium and seeded at a density of  $2.4 \times 10^3$  cells/mL using Minimal essential medium (MEM) (Highveld Biological, South Africa) supplemented with 0.1% gentamicin (Sigma) and 10% foetal calf serum (Highveld Biological, South Africa). After 24 hours of incubation at 37°C, the medium on cells was removed and 200  $\mu$ L of test plant extract or berberine chloride (Sigma) (positive control) was added at various concentrations (quadruplicate dilutions prepared in growth medium). Cells were further incubated at 37°C in a 5% CO<sub>2</sub> incubator for 5 days. Thereafter, 30  $\mu$ L MTT (stock solution of 5 mg/mL in phosphate-buffered saline) was added to each well and the plates were incubated for 4 hours at 37°C. The medium was carefully removed by aspiration and the MTT crystals were dissolved into formazan by adding 50  $\mu$ L of DMSO to each well. The absorbance, proportional to the amount of MTT reduced, was measured at 570 nm using a microplate reader (Versamax). The LC<sub>50</sub> values were calculated as the concentration of plant extract resulting in a 50% reduction of absorbance compared to untreated cells from a linear regression equation. LC<sub>50</sub> values are expressed as the mean of three independent triplicate assays and values were expressed as mean of quadruplicate assays.

### Statistical analysis

Results were analysed using Student's t test. A p-value less than 0.05 was considered significant compared to the control.

## Results

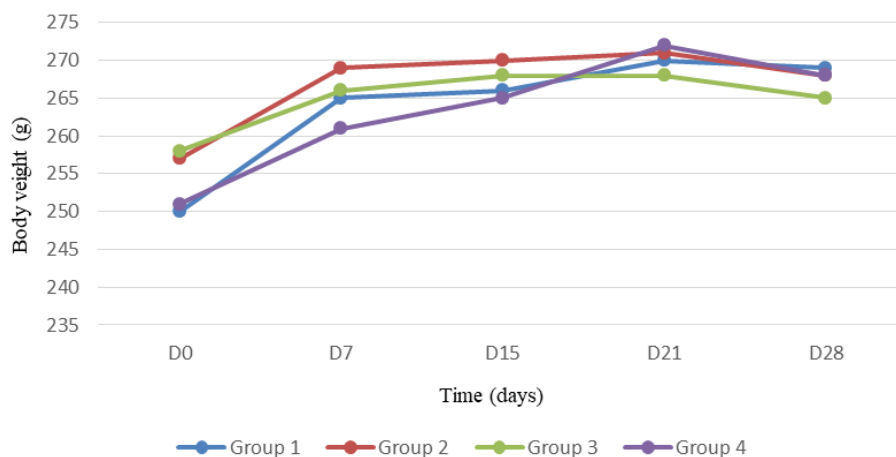
### In vivo study

**Acute toxicity studies:** No animal mortality was induced by the single dose of the extract given at 5000 mg/kg. Furthermore, the animals did not show toxicity signs or visible changes in locomotion and respiration.

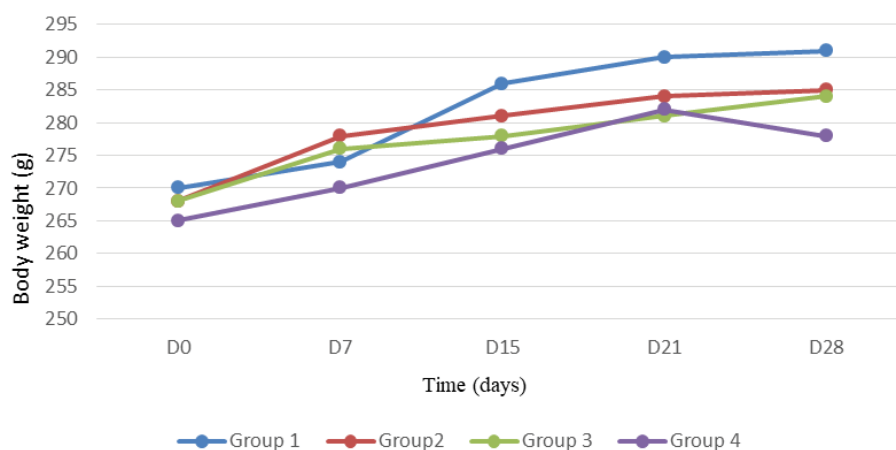
**Sub-chronic toxicity studies:** No deaths were observed; no significant clinical relevant changes were observed in general behaviour and other physiological activities in this study.

### Body weight

The evolution of body weight during the treatment is shown in Figure 1 and Figure 2. The results showed no significant difference



**Figure 1:** Effect of oral administration of hydroethanolic root extract of *Cassia sieberiana* on female rats' body weights ( $n=3$ ). Treated groups were compared to controls using student's t test. Data are mean  $\pm$  SEM and  $P$  values less than 0.05 were considered significant.



**Figure 2:** Effect of oral administration of hydroethanolic root extract of *Cassia sieberiana* on male rats' body weights ( $n=3$ ). Treated groups were compared to controls using student's t test. Data are mean  $\pm$  SEM and  $P$  values less than 0.05 were considered significant.

**Table 1:** Effect of hydroethanolic root extract of *Cassia sieberiana* on biochemical serum parameters.

MALES				
	Control (n=3)	500 mg/kg (n=3)	1000 mg/kg(n=3)	2000 mg/kg(n=3)
Glucose(g/L)	0.73 $\pm$ 0.04	0.72 $\pm$ 0.11	0.72 $\pm$ 0.05	0.72 $\pm$ 0.05
Creatinine (mg/L)	7.35 $\pm$ 0.23	6.9 $\pm$ 1.27	7.5 $\pm$ 0.96	6.1 $\pm$ 0.28
ASAT (UI/L)	336 $\pm$ 164	206 $\pm$ 27.5	287 $\pm$ 120	211 $\pm$ 33.8
ALAT(UI/L)	86 $\pm$ 8.48	119 $\pm$ 67.8	118 $\pm$ 43.3	96.5 $\pm$ 14.9
FEMALES				
	Control (n=3)	500 mg/kg (n=3)	1000 mg/kg (n=3)	2000 mg/kg(n=3)
Glucose (g/L)	0.83 $\pm$ 0.03	0.77 $\pm$ 0.13	0.77 $\pm$ 0.06	0.69 $\pm$ 0.07
Creatinine (mg/L)	8.26 $\pm$ 0.30	8.83 $\pm$ 0.85	7.5 $\pm$ 0.23	7.35 $\pm$ 0.07
ASAT(UI/L)	131 $\pm$ 57.1	244 $\pm$ 96.1	265 $\pm$ 114	228 $\pm$ 53.5
ALAT (UI/L)	108 $\pm$ 54.7	188 $\pm$ 79.1	88.9 $\pm$ 9.75	108 $\pm$ 7.63

**Table 2:** Effect of hydroethanolic root extract of *Cassia sieberiana* on haematological parameters.

MALES				
	Control group (n=3)	500 mg/kg (n=3)	1000 mg/kg (n=3)	2000 mg/kg (n=3)
WBC ( $10^3/uL$ )	7.8 $\pm$ 0.66	7.23 $\pm$ 0.64	8 $\pm$ 2.20	8 $\pm$ 1.35
RBC ( $10^3/uL$ )	7.85 $\pm$ 0.31	6.85 $\pm$ 0.18	7.14 $\pm$ 1.22	7.09 $\pm$ 0.39
HGB (g/dL)	15.5 $\pm$ 1.4001	13.4 $\pm$ 0.64	13.4 $\pm$ 2.20	13.9 $\pm$ 0.85
HCT(%)	40.9 $\pm$ 2.96	36.2 $\pm$ 1.30	37.4 $\pm$ 5.4	37.5 $\pm$ 2.32
MCV (fL)	52.1 $\pm$ 1.77	52.9 $\pm$ 1.06	52.53 $\pm$ 1.15	53 $\pm$ 1.9
MCH (pg)	19.7 $\pm$ 0.5	19.6 $\pm$ 0.56	18.8 $\pm$ 0.12	19.5 $\pm$ 0.75
MCHC (g/dl)	37.9 $\pm$ 0.32	37.1 $\pm$ 0.42	35.8 $\pm$ 0.56	37 $\pm$ 0.12
PLT(G/l)	651 $\pm$ 56	692 $\pm$ 91	536 $\pm$ 97	525 $\pm$ 45
FEMALES				
	Control group (n=3)	500 mg/kg (n=3)	1000 mg/kg (n=3)	2000 mg/kg (n=3)
WBC ( $10^3/uL$ )	8.1 $\pm$ 3.55	7.06 $\pm$ 1.25	7.13 $\pm$ 0.43	9.23 $\pm$ 3.59
RBC ( $10^3/uL$ )	6.77 $\pm$ 0.57	6.4 $\pm$ 0.12	7.08 $\pm$ 0.30	7.45 $\pm$ 0.44
HGB (g/dL)	13.5 $\pm$ 1.2	12.5 $\pm$ 0.75	13.7 $\pm$ 0.32	13.7 $\pm$ 1.60
HCT (%)	37.4 $\pm$ 2.85	34.4 $\pm$ 1.9	37.1 $\pm$ 0.80	38.8 $\pm$ 1.4
MCV (fL)	55.5 $\pm$ 0.47	53.7 $\pm$ 2.01	52.5 $\pm$ 1.19	51.4 $\pm$ 0.8
MCH (pg)	19.9 $\pm$ 0.15	19.5 $\pm$ 0.9	19.3 $\pm$ 0.49	19.1 $\pm$ 0.17
MCHC (g/dL)	36.1 $\pm$ 0.61	36.4 $\pm$ 0.62	36.7 $\pm$ 0.23	36.4 $\pm$ 1.3
PLT (G/L)	545 $\pm$ 326	615 $\pm$ 47	648 $\pm$ 119	390 $\pm$ 78

( $p>0.05$ ) in body weight changes between *C. sieberiana*-treated and control animals.

### Biochemistry analysis

The results of biochemical serum parameters at the end of treatment are shown in Table 1. These results show that parameters of the treated animals did not significantly differ from those of control rats. ( $p <$

0.05). Similarly, there were no significant changes in the urinalysis data between *C. sieberiana* root-treated animals and controls.

### Haematological results

The effect of sub-chronic administration of hydroethanolic extract of *C sieberiana* on haematological indices is shown in Table 2. The results show that there were no significant differences ( $p>0.05$ ) in

all parameters measured between control and *C. sieberiana*-treated animals.

### Histological results

Macroscopic examination of tissues shows that *C. sieberina* extract did not affect liver, kidney and heart morphology.

Minor histopathological effect was seen only in liver as scant centrolobular necrosis, slight inflammatory response and stasis (Figure 3a, 3b and 3c).

### Cytotoxicity study

Results of cytotoxicity are shown in Table 3.

It is noted that cell viability decreased as and as the dose of extract increases. The hydroethanolic root extract of *C. sieberiana* had a  $LC_{50}$  value higher than that of berberine used as positive control ( $12.76 \pm 0.91 \mu\text{g/ml}$  versus  $9.99 \pm 0.54 \mu\text{g/ml}$  respectively) as shown in Figure 4.

### Discussion

In the present work, we evaluated the acute & sub-chronic toxicity of root extract of *C. sieberiana* in Wistar rats and cytotoxicity on Vero cells. The limit dose of 5000 mg/kg did not result in mortality or any clinical sign of acute toxicity in animals in the short-term (48 hours) and long-term (14 days) observatory periods, suggesting that the oral  $LD_{50}$  of the extract is more than 5000 mg/kg in rats. The limit test is used in situations where the investigator has information indicating that the test material is likely to be non-toxic or of low toxicity [16]. The same result was found by Donkor et al. [15] who had evaluated the

acute and sub-chronic toxicity of aqueous extract of *Cassia sieberiana* D.C root bark in rodents (rats and mice), [15]. In a previous study, the oral  $LD_{50}$  of root of *C. sieberiana* in rats was estimated to be more than 2000 mg/kg [7]. Therefore, the result of the acute oral toxicity study suggests that *C. sieberiana* root tested at the limit dose is essentially non-toxic (scale of Hodge and Sterner) [17].

In the subchronic toxicity study, the extract did not affect the normal growth of the animals as evidenced by comparing the body weight gain in both control and treated animals over the 28-day treatment period. There were no significant changes in the macroscopic aspects of heart, kidney and liver. The liver and kidney are important organs of the body which play a vital role in metabolic processes. The liver detoxifies substances that are harmful to the body. The kidney helps in maintaining homeostasis of the body by reabsorbing vital substances and excreting waste products [18]. In the present work, the biochemical analysis showed no significant elevation in serum ALT, AST, glucose and creatinine in treated animals compared to controls. However, the histological study showed necrosis of hepatic cells, which could be caused by the substance (specify name of substance you're referring). This is confirmed by Donkor et al in their study [15], which showed elevated levels of ALP and total bilirubin in blood [15]. The serum ALT and AST are sensitive indicators of hepatocellular damage [19]. High levels are present within hepatocytes (Within healthy or damaged hepatocytes? Consider omitting this whole part since this is being repeated in detail in the next phrase) and plasma levels rise as hepatocyte membrane integrity is disturbed during hepatocellular injury [20,21]. Creatinine is the most reliable renal marker and increases only when the majority of renal function is lost [22].

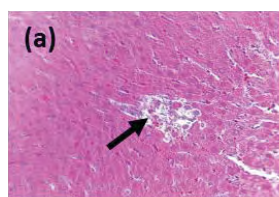


Figure 3a: Histological of rat liver showing isolated necrotic focus in the liver parenchyma (X 40)

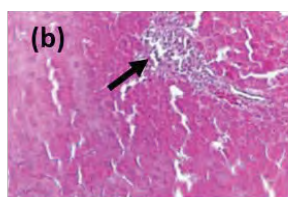


Figure 3b: Inflammatory cells in rat liver portal space (X 40)

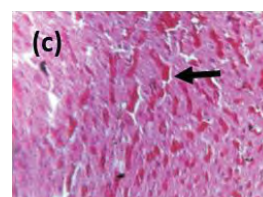


Figure 3c: sinusoidal stasis in rat liver

**Figure 3:** (a), (b) and (c): Histological features of Rat liver. Minor lesion changes observed on livers of the rats orally received hydroethanolic root extract of *Cassia sieberiana*.

**Table 3:** Percentage of viability of kidney cells between products and control.

	1000	100	10	1
HEE	$4.13 \pm 0.94\%^*$	$26.38 \pm 1.66\%^*$	$56.15 \pm 1.74\%^*$	$75.14 \pm 2.05\%^*$
Berberine	nd	$3.52 \pm 0.03\%^*$	$76.09 \pm 12.29\%^*$	$85.82 \pm 6.44\%^*$

HEE = hydroethanolic extract; nd= not determined; \*Statistically significant different between treated cells and control



HEE: Hydroethanolic extract ; BERB: Berbérine

∗: Différence significative par rapport au témoin.

\*Statistically significant different between product and control

**Figure 4:** LC50 (in  $\mu\text{g/ml}$ ) extract and berberine.

In contrast, Obidah et al. [10] have demonstrated that oral administration of the stem bark extract to rats resulted in significant increase in ALT and AST levels. This difference could be explained by the part of the plant used.

There were no significant differences between urinalysis parameters of treated and control animals. The extract may not have deleterious effects on the haematological system since the parameter values (Table 2) did not vary when compared to control rats.

Histological examination of hepatocytes of the treated rats, stained with haematoxylin and eosin, revealed slight centrilobular necrosis, inflammatory cells and sinusoidal stasis in the liver parenchyma. Thus, the extract may have a deleterious effect on the liver after 28 days of exposure to *C. sieberiana* root extract. A result obtained by Donkor et al. [15] indicated a slight necrosis centrilobular of liver cells after 3 months of treatment at the highest dose. This suggests that root extract of *Cassia sieberiana* may be toxic to the liver in the short or long term. The kidney and heart did not show noticeable morphological or microscopic changes in all the treated rats.

Several studies have shown that the MTT assay is sensitive, accurate, convenient, fast and economical for the study of cytotoxicity. In this work, the MTT assay showed an  $IC_{50}$  at 12.76  $\mu\text{g/ml}$ . This means that the extract was less toxic than the positive control with 9.99  $\mu\text{g/ml}$ . Berberine, which has been used as a reference, is a natural toxin of several plants of the family *Berberidaceae*. It is found in *Coptis sinensis*, used as a medicinal plant in China, and in *Hydrastis canadensis* as a dietary supplement [23]. This suggests that its content in these plants commonly used by humans does not present any danger.

The inhibition of cell viability effect increases proportionally to concentration. This suggests that the extract may be toxic at high doses.

## Conclusion

We concluded that the oral toxicity of the hydroethanolic root extract of the *C. sieberiana* in rats was found to be low (oral  $LD_{50} > 5000 \text{ mg/kg}$ ). However, the extract may have deleterious effects on the liver at high doses with prolonged administration. These results suggest that its usage in traditional medicine should be limited to moderate doses on a short-term basis.

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