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Toxicological evaluation of *Hydrocotyle bonariensis* Comm. ex Lamm (Araliaceae) leaves extract

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Abstract

Background: The present study was designed to determine the cytotoxic, acute and sub-chronic oral toxicity profile of the hydroethanol extract of *Hydrocotyle bonariensis* leaves. The toxicity studies are preceded by a phytochemical screening to characterize the phytochemical compounds contained in this extract.

Results: The phytochemical screening allowed to identify the presence of alkaloids, tannins, flavonoids and saponosids. The cytotoxicity study allowed retaining that this extract does not present cellular toxicity. The acute oral toxicity study was performed at a limit dose of 5000 mg/kg body weight by the oral route. The extract showed no signs of toxicity or mortality during the 14-day daily observation period. Repeated-dose oral toxicity studies were conducted by administering 500 mg/kg and 1000 mg/kg body weight of the extract orally daily to the treated batches for 28 days. The extract did not reveal any toxicity either on the weights of the animals or on the relative weights of the organs taken. The analysis of biochemical and hematological parameters revealed no significant difference between the indicators of the batches treated with 500 mg/kg and 1000 mg/kg, compared to the control batch during the 28 days of treatment.

Conclusion: These results show that the hydroethanol extract of *Hydrocotyle bonariensis* leaves is relatively safe toxicologically when administered orally.

Keywords: *Hydrocotyle bonariensis*, Cytotoxicity, Acute and subacute toxicity

1 Background

Plants are a valuable source of life support within the animal kingdom as they serve as sustenance, therapeutic substance and shelter material. Nearly 80% of people in developing countries use plants to treat themselves [40]. These medicinal plants are thus widely used and can also be important resources for new substances with therapeutic potential and high costs. The obvious interest in the frequent use of these medicinal plants calls for the notion of toxicity, which is defined as the set of harmful

effects caused by a substance introduced at a single relatively high dose or at small doses repeated for a long time on a living organism [7, 16]. The study of the toxicity of a substance is the set of pharmacological tests, which determine the degree or harmfulness of the latter in order to regulate its use. *Hydrocotyle bonariensis* Comm. ex Lam. is one of the medicinal plants of the Araliaceae family, which is highly valued in the traditional treatment of several pathologies in Africa, Asia and South America. Practitioners of traditional Togolese medicine to treat mainly high blood pressure and diabetes use it. It is also used as a sedative, for kidney and liver problems, tuberculosis, rheumatoid arthritis and many other ailments [5]. Some pharmacological studies of this plant have

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revealed its anti-oxidant and anti-inflammatory properties [24]. Despite the wide use of *H. bonariensis* in Africa, Asia and South America, this plant has not yet been the subject of scientific studies of its toxicity, allowing allowing the regulation of its use. There is no sufficient scientific data, collected based on experimental studies on this plant, to analyze its toxicological impact. The multiplicity of its virtues as well as its large geographical distribution would increase the frequency of its use and consequently would make a significant part of the population susceptible to the potential risk of toxicity linked to its use. The objectives were to (1) determine the qualitative composition of secondary metabolites of *H. bonariensis*, (2) evaluate the cytotoxicity on cepid larvae (*Artemia salina* LEACH), (3) evaluate the acute toxicity and (4) the subacute toxicity of this extract in Sprague Dawley rats.

2 Methods

2.1 Plant material

The leaves of *Hydrocotyle bonariensis* were collected at the test station of the higher school of agronomy of Lome University. They were identified at the Laboratory of Botany and Plant Ecology of the Faculty of Sciences where the sample was deposited under the number "TG 15,183". These leaves were washed with tap water and then dried under air conditioning at 20 °C.

2.2 Preparation of the hydro-ethanolic extract

The leaves of *Hydrocotyle bonariensis* after 3 weeks of drying were pulverized and then 320 g were macerated for 72 h with intermittent stirring in a hydro-ethanolic solvent in a ratio of equal volumes (1:1). After maceration, the solution was filtered through filter paper and cotton. The filtrate obtained was evaporated under vacuum using a rotavapor at 45 °C. 68.28 g of extract was obtained, giving a yield of 21.34%. The extract obtained was kept in a cool place for the preparation of the different concentrations to be used in the tests.

2.3 Animal material

The animals used for the experiments are Sprague Dawley (SD) rats of average body weight of $250 \text{ g} \pm 20$ coming from the animal house of the Physiology-Pharmacology laboratory of the Faculty of Science of the University of Lome. They were fed a standard diet and maintained at 24 ± 2 °C, and subjected to a 12 h light/dark cycle with free access to food and water. Twelve hours before experimentation, food was removed but water remained available.

2.4 Phytochemical screening

Qualitative phytochemical screening was performed on hydroethanolic extract of *Hydrocotyle bonariensis* leaves (HEHBL), using the standard method based on staining and precipitation reactions as described by Houghton and Raman [17]. Table 1 shows the different chemical groups sought and the tests for their detection.

2.5 Evaluation of cellular toxicity

The cytotoxicity test, based on the survival of shrimp larvae (*Artemia salina* LEACH) in seawater in the presence of the test solution, which was performed following the protocol adopted by Djengue et al. [8]. *A. salina* eggs were cultured in an Erlenmeyer flask containing seawater collected from the sea of Togolese coastline and filtered before use [27]. The whole set was left in continuous agitation for 48 h. During this time, the eggs hatched into young larvae. A stock solution of the HEHBL was prepared at a concentration of 50 mg/ml of distilled water. A series of 10 successive 1:2 dilutions were made with seawater from the stock solution of the extract. The concentrations expressed in mg/ml of the dilutions contained in test tubes numbered 1–10 were 25 mg/ml; 12.5 mg/ml; 6.25 mg/ml; 3.125 mg/ml; 1.582 mg/ml; 0.781 mg/ml; 0.391 mg/ml; 0.195 mg/ml; 0.098 mg/ml; 0.049 mg/ml respectively. Using a cone micropipette, a colony of 16 live larvae was brought into contact with the series of solutions of progressive concentrations of the HEHBL. These media and controls were left under agitation and

Table 1 Chemical compounds sought in the hydroalcoholic extract of *H. bonariensis*

Secondary metabolites	Reagents	Observation (positive result)
Flavonoids	Ferric chloride 1	Orange pink
	1/10 NaOH	Red
Tannins	Ferric chloride 1	Blue-black coloration
Alkaloids	Copper sulfate	
	Mayer	Yellowish white precipitate
	Dragendorf	Red–orange precipitate
Saponosides	Foam index test	Important presence of foam of at least 1 cm

live larvae are counted 24 h after incubation. To ensure that the death observed in the assays is attributed solely to the extract concentrations and not to starvation, these tubes are compared to control tubes containing larval solutions only knowing that *A. salina* shrimp larvae can live up to 48 h without food due to feeding on their yolk sac [28]. The experiment was repeated three times.

The percent mortality of shrimp larvae of each concentration of HEHBL was determined. For each sample, the lethal concentration that causes 50% larval death (LC50) was calculated with a 95% confidence interval by regression and logarithmic analysis. This value is obtained from the best fit curve giving the concentration equivalent to the death of half of the larvae. The degree of toxicity of the extract was evaluated based on the correspondence table established by Mousseux [26] (Table 2) and used by Houmènou et al. [18].

2.6 Acute toxicity

Acute test: The acute test was conducted using the OECD 425 (2008) "dose adjustment" method at 5000 mg/kg of HEHBL. The test was performed on 6 female SD rats and their behavior was observed as well as the number of deaths over a period of 14 days. After 15 h of fasting, they were divided as follows: control lot consisting of three females receiving distilled water at 10 ml/kg; experimental lot consisting of three females receiving the extract at 5000 mg/kg. Behavioral observation was performed 3 h after administration of the substances. Afterwards, hydration and feeding were performed daily for 14 days. During this period, signs of toxicity were noted, including changes in coat, motility, tremors, mass, grooming, respiration, stool appearance, mobility and death.

2.7 Subacute toxicity

The study was conducted according to OECD guideline 407 (2008). It was conducted on 18 female SD rats divided into three equal batches of 6 rats as follows: batch I, receiving distilled water at a rate of 1 ml/100 mg body weight (control batch); batches II and III receiving a solution of the HEHBL at a rate of 500 and 1000 mg/kg body weight respectively. The treatment lasted 28 days. Rats

were fed and hydrated ad libitum and weighed every 2 days. At the end of the treatment, the rats were fasted for 24 h, and then blood was drawn, followed by dissection after administration of 80% ethyl urethane at 1.5 mg/kg. The organs collected were liver, kidney, spleen and heart. These were rinsed with 0.9% saline and weighed. The relative weight of each organ was calculated according to the formula: ((Pr: relative organ weight (g/100 g); Po: organ weight (g); Pa: rat body weight (g). $Pr = Po/Pa \times 100$). Several biochemical and hematological parameters were determined.

2.8 Statistical analysis

The results are expressed as mean ± standard error on the mean. They are processed with GraphPad Prism 7.0 software. Analysis of variance (ANOVA) is used to compare the means. The results are significant if the *p* value < 0.05.

3 Results

3.1 Phytochemical screening of HEHBL

Qualitative tests for the determination of chemical compounds show the presence of flavonoids, alkaloids, saponosides and tannins in HEHBL (Table 3).

3.2 Evaluation of cytotoxicity of HEHBL

The analysis of LC50 values allows us to say that HEHBL is non-cytotoxic because the average lethal concentration (LC50) obtained is 0.12 mg/ml, higher than the limit set (0.1 mg/mL).

3.3 Assessment of acute toxicity

Oral administration of HEHBL did not result in the death of rats in all treated batches. Observations revealed no signs of asthenia, somnolence, anorexia, diarrhea or reduced mobility during the experimental period. The lethal dose 50 (LD50) is therefore assumed to be greater than 5000 mg/kg (Table 4).

Table 2 Correspondence between LC50 and toxicity [26]

CL ₅₀	Toxicity
CL ₅₀ ≥ 100 µg/mL or 0.1 mg/mL	-
100 µg/mL > CL ₅₀ ≥ 50 µg/mL ou 0.05 mg/mL	+
50 µg/mL > CL ₅₀ ≥ 10 µg/mL ou 0.01 mg/mL	++
CL ₅₀ < 10 µg/mL	+++

-: non toxic; +: low toxicity; ++: moderate toxicity; +++: high toxicity

Table 3 Chemical compounds contained in the hydroethanolic extract of *H. bonariensis*

Secondary metabolites	Reagents	Relative presence
Flavonoids	Ferric chloride 1 1/10 NaOH	+ +
Tannins	Ferric chloride 1 Copper sulfate	+ -
Alkaloids	Mayer Dragendorf	+ -
Saponosides	Foam index test	+

+ : present; - : absent

Table 4 Observed parameters during the assessment of acute toxicity

Parameters	Treatments	
	DW (10 mL/kg)	HEHBL (5000 mg/kg)
Number of animals	3	3
Mobility	N	N
Aggressiveness	N	N
Stool condition	N	N
Trembling	A	A
Sleepiness	A	A
Sensitivity to pain	N	N
Vomiting	A	A
Vocalization	A	A
Pilo-erection	A	A
Ptosis	A	A
Vigilance	N	N
Number of deaths	0	0

N No, A absent, DW distilled water

3.4 Evaluation of sub-chronic toxicity

3.4.1 Body weight changes in rats

As shown in Table 5 the body weights of rats that received HEHBL at 500 mg/kg and 1000 mg/kg orally for 28 days continued to increase normally each week until day 28 of treatment in proportion to the control rats. But no significant difference was observed in comparison to the body weight of treated rats to control rats from D0 to D28.

3.4.2 Relative organ weights

The results obtained are presented in Fig. 1. It appears from this figure that HHEHB at doses of 500 mg/kg and 1000 mg/kg does not significantly influence the relative weights of noble organs (heart, liver, spleen, kidneys).

3.4.3 Effects of the extract on biochemical and hematological parameters

Analysis of biochemical and hematological parameters revealed no significant difference between the indicators

Table 5 Average weight of rats per week

	Control	HB 500MG/KG	HB 1000MG/KG
D0	119,5 ± 2,8	120,5 ± 4,9	119,3 ± 3,6
D7	129,8 ± 2,6	131,6 ± 5,1	130,1 ± 3,1
D14	133,6 ± 3,0	135,3 ± 5,3	134,3 ± 3,0
D21	146,6 ± 4,9	146,8 ± 6,5	147,6 ± 3,2
D28	148,5 ± 6,8	149 ± 5,6	149,1 ± 4,2

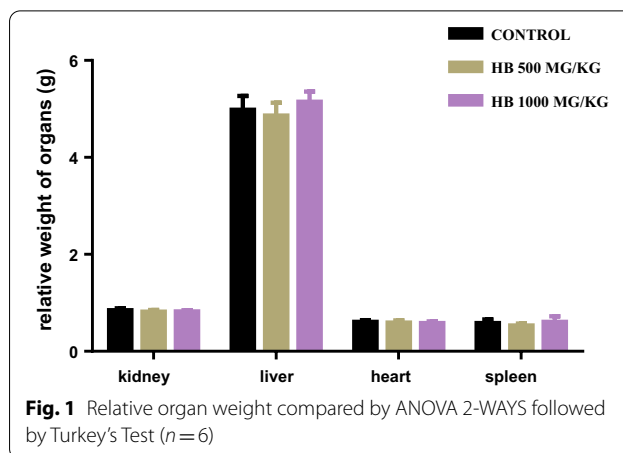


Fig. 1 Relative organ weight compared by ANOVA 2-WAYS followed by Turkey's Test (n = 6)

of the batches treated with 500 mg/kg and 1000 mg/kg, compared to the control batch during the 28 days of treatment (Table 6). However, the liver function tests (SGOT, SGPT, ALP) showed a slight increase in plasma SGOT and SGPT levels at the 1000 mg/kg dose. The analysis of nephrological parameters showed no significant change between the treated and control batches. EHEHB did not induce any significant change in creatinine and urea concentration. Oral administration of the extract days did not induce any significant change in the hematological status in the groups of rats treated at the different doses compared to the control except for a slight increase in the serum white blood cell count at the 500 mg/kg and 1000 mg/kg dose.

4 Discussion

The phytochemical screening of HEHBL revealed the presence of several phytochemical compounds. These include flavonoids, tannins, alkaloids, and saponosides. Previous studies have also revealed similar observations by noting the presence of alkaloids, flavonoids, tannins, phenolic compounds, and saponins in an extract of leaves collected in Nigeria, followed by the isolation of five terpenoid saponins from the underground part of this plant [2], [24], [38]. The secondary metabolites identified in EEHBL are endowed with a multiplicity of biologically active properties: Alkaloids have several biological properties including antibiotic, antiparasitic, anesthetic, antitumor, anticancer, analgesic, and spasmolytic properties [33] [4]. Tannins have a therapeutic action related to astringency, antibacterial, antiviral, antifungal, antiseptic, antioxidant and hemostatic actions [19, 41]. As for flavonoids, they have anti-inflammatory properties, hepatoprotective of a good blood circulation. They have a strong antioxidant or anti-radical,

Table 6 Results of biochemical and hematological parameters

	Control	HB 500MG/KG	HB 1000MG/KG
Creatinine (mg/dL)	0.6 ± 0.03	0.446 ± 0.0	0.5 ± 0.0454
UREA (mg/dL)	25.5 ± 2.0	23.9505 ± 0.539	21.2715 ± 1.66
SGOT (UI/L)	150 ± 13.099	155.5 ± 9.503	181.6667 ± 53.984
SGPT (UI/L)	50.6 ± 7.6	51.33 ± 4.3	61.83334 ± 13.4
ALP (UI/L)	201 ± 31.1	208.5 ± 13.152	200.1 ± 19.708
HAEMOGL (mm ³)	13.7 ± 0.3	11.17667 ± 1.578	7.676 ± 0.237
WBC (mm ³)	8550 ± 600.9	10,350 ± 1665.2	11,880 ± 2413.7
MVC (mm ³)	51.3 ± 0.922	54.3 ± 0.826	52.72 ± 1.122
PLT (mm ³)	424,500 ± 43,678.95	440,000 ± 30,565.78	455,800 ± 32,209.31

antiproliferative and anti-carcinogenic potential [12] and diuretic [35, 36]. Saponosides have surfactant, antifungal, antibacterial and antiviral properties, they exhibit vein and capillary protective activities and then edematous activity with hormonal activity [23]. The richness of secondary metabolites of *H. bonariensis* could explain the wide spectrum of its medicinal use and scientifically proven pharmacological properties [1, 10, 11, 13, 25, 29, 32]. The results of the cell toxicity test applied on *Artemia salina* shrimp larvae revealed variability in lethality rate, observed according to the different concentrations used. The LC₅₀ lethal concentration determined using the polynomial and logarithmic regression curve equations highlights the fact that HEHBL does not exhibit cellular toxicity. Our results are similar to other cytotoxicity studies performed on medicinal plants [6, 18]. The results obtained from the acute toxicology study imply that the LD₅₀ of HEHBL is greater than 5000 mg/kg body weight. According to the 2008 OECD Globally Harmonized Classification System, this extract can be classified as Category 5 and considered a non-toxic substance by the oral route. This high LD₅₀ limit would indicate a wide margin of safety for consumption of this plant. As showed by our results, similar studies revealed the acute non-toxicity of medicinal plant extracts taken orally [21, 31]. Since no toxic effects were observed in the acute study, an additional study was conducted to evaluate the sub-chronic toxicity of HEHBL during a 28-day experiment. One of the key indicators in toxicity studies of bioactive substances is the change in body weights of treated subjects. Weight loss or gain is thought to be a consequence of the disruption of carbohydrate, protein or fat metabolism by these treatment products [20]; [39]. No significant changes in body weight were observed in rats treated with 500 mg/kg and 1000 mg/kg compared to the control after daily treatment for

28 days. This allows us to suggest that oral and sub-chronic administration of HEHBL has no effect on the normal growth of rats. Similarly, administration of the extract had no effect on the normal growth of the liver, kidney, spleen and heart as no significant changes were observed in the weight of these organs. Relative organ weights are considered to be a relatively sensitive indicator in toxicity studies [22]. The liver and kidney are essential in the evaluation of the toxicity of drugs and plant extracts because the liver and kidney are respectively responsible for the metabolism and elimination of any bioactive substance administered to a living organism. The liver has the role of detoxification of the organism, whether the toxins are of internal or external origin, while the kidney has the role of purification of the blood and elimination of waste products [3, 9, 37]. Thus, hematological and biochemical analyses were carried out to evaluate the possible alterations of the hepatic and renal functions caused by the ingestion of the extracts. Subchronic administration of HEHBL at 500 mg/Kg and 1000 mg/kg did not result in any significant changes in biochemical and hematological parameters. However, the slight increase in serum SGOT and SGPT levels at 1000 mg/kg may reflect the onset of damage to at least one of the tissues such as cardiac tissue, hepatopathy, muscular dystrophy, and internal organ damage at this dose. Renal dysfunction can be assessed by simultaneous measurements of urea, creatinine, considered the main markers of nephrotoxicity [9], [20], [14, 34]. In the present study, there were no changes in plasma urea and creatinine levels in rats treated with 500 mg/kg and 1000 mg/kg. Analysis of hematological parameters is relevant because it provides information on hematopoietic function (assessment of myeloid lineage cells), on the occurrence of allergy (white blood cell studies) and on intravascular effects such as hemolysis [15], [30]. The hematological workup showed no significant

difference between treated and control rats. However, the slight increase in white blood cell count at 500 mg/kg and 1000 mg/kg would indicate a slight occurrence of allergy.

5 Conclusions

The phytochemical study of the hydro-ethanolic extract of *Hydrocotyle bonariensis* leaves revealed the presence of the main groups of bioactive chemical compounds such as alkaloids, tannins, flavonoids and saponosides. Cellular toxicity studies applied on shrimp larvae showed that this extract does not show cytotoxicity. The oral administration of the same extract showed neither acute nor subchronic toxicity in wistar rats at the studied doses. However, a chronic toxicity study of this hydroethanol extract should be considered in perspective as well as the toxicity study of the aqueous extract of the leaves and rhizomes of the same plant by considering many more toxicity indicator parameters. Histological studies would also be welcome to complete the toxicological evaluation of this plant.

Abbreviations

OECD: Organisation for Economic Co-operation and Development; SGOT: Serum glutamic-oxaloacetic transaminase; SGPT: Serum glutamic pyruvic transaminase; ALP: Alkaline phosphatase level; WBC: White blood cells.

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Authors' contributions

KK, AM, PB and TP conceived the project and defined research methodology; KK, KMD-Y, MA and B. K performed the experiments; KK, TP, PB and AM wrote the paper.

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Availability of data and materials

The datasets used and/or analyzed during this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

The research was conducted on laboratory rats in accordance with institutional guidelines of Laboratory of Physiology/Pharmacology of University of Lomé (ref:001/2012/CB-FDS-UL).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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