In vivo Evaluation and *in silico* Prediction of the Toxicity of Drepanoalpha Hard capsules

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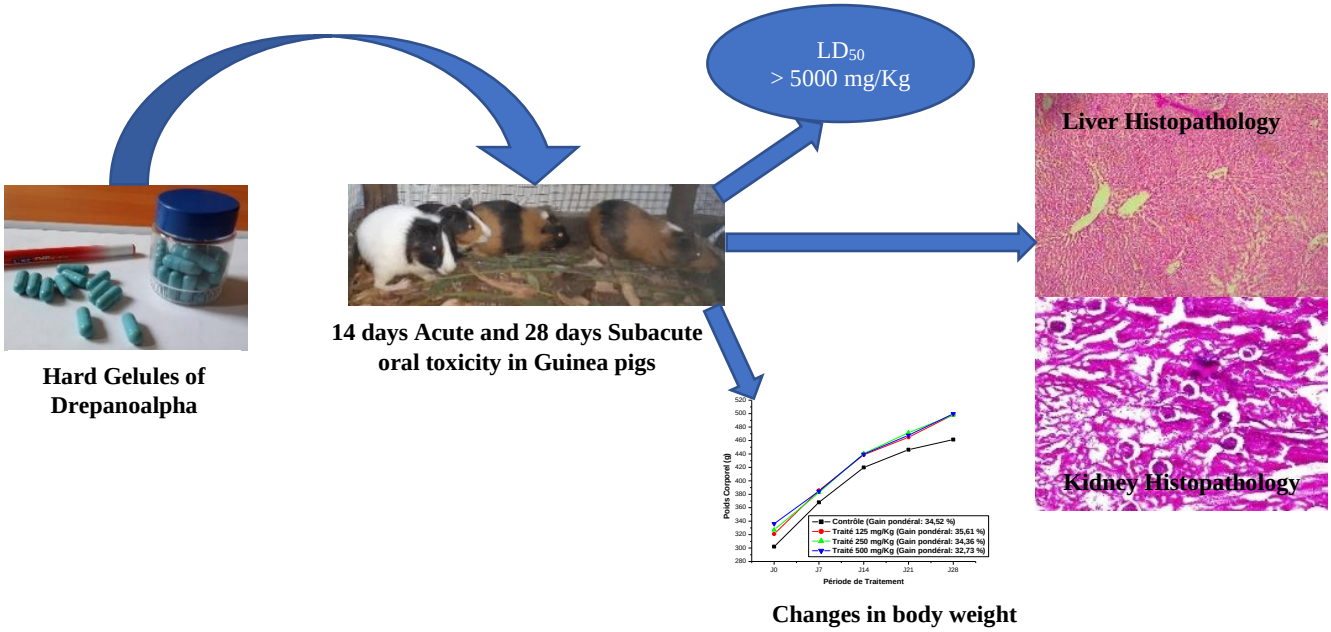
GRAPHICAL ABSTRACT



ABSTRACT

This study was carried out in order to investigate the safety of Drepanoalpha hard capsules, a phytomedicine used for the management of sickle cell disease in the Democratic Republic of Congo, by acute and sub-acute administration in Guinea pigs. The hard capsules were dissolved in normal saline solution (NaCl 9‰). The animals were randomly selected, marked and divided into 2 groups of 5 animals each (3 males and 2 females) for acute toxicity and 4 groups of 3 animals each for sub-acute toxicity. They received by gavage a single dose of 5000 mg/ kg of body weight (B.W.) of Drepanoalpha hard capsules for acute toxicity and 125 mg/ kg, 250 mg/ kg and 500 mg/ kg of B.W. twice daily for 28 days for sub-acute toxicity. Normal saline solution was used as control. Hematological, biochemical and histopathological analyses were performed and the behavior of the animals was observed after treatment. The results showed that the median lethal dose (LD_{50}) is greater than 5000 mg/ kg of B.W., and the relative weights of vital organs (kidney, liver, lungs, and heart) collected from Guinea pigs at the end of treatment on D_{14} (acute toxicity) and D_{28} (sub-acute toxicity) did not undergone significant changes ($p>0.05$). The results of haematological (Red Blood Cells, White Blood Cells, Haemoglobin, Haematocrit) and biochemical (ALT, AST, Albumin, Total Protein) tests did not

show significant differences between control and test groups (0.05 for acute toxicity), while the histopathological study revealed no damage to the various organs excised.

In conclusion, the results confirm the safety of Drepanoalpha, as shown in previous studies in rats and Guinea pigs using lyophilizate and decoction.

Keywords: Acute Toxicity, Sub-acute Toxicity, Drepanoalpha

1. INTRODUCTION

Herbal medicine continues to gain popularity and interest in green medicine is increasing because it represents alternative to modern medicine. Traditionally, plants and plant extracts are used to treat many diseases and disorders but their use can represent a serious health issue because of their potential toxicity. Indeed, plant extracts can be very effective therapeutically, but some can reveal themselves to be toxic with a risk of poisoning as well (Ngbolua et *al.*, 2011a, b). To prevent such a risk, evaluation of plant extracts toxicity of plants with proven therapeutic use is recommended since toxicity data are mandatory to ensure safety in the use of medicinal products (Chanda et *al.*, 2015).

According to the World Health Organization (WHO), more than 80% of the population in poor regions of Africa depend mainly on traditional medicine (Mpiana et *al.*, 2016; Chanda et *al.*, 2015). Thus, in its 2014-2023 strategy, WHO encourages African countries to develop and modernize Traditional African Medicine (TAM) as an integral part of their health care system (WHO, 2013). It has therefore endorsed the use of herbal products in national policies and drug regulatory measures to strengthen research and evaluation of the safety and efficacy of herbal products. The use of plants for curative purposes is becoming increasingly popular as they are considered beneficial and without side effects for some of them (Chanda et *al.*, 2015). However, this use of medicinal plants as medicines is simply based on traditional folklore use that has been perpetuated over several generations. With the increasing use of medicinal plants, a thorough scientific study of these plants is becoming imperative, based on the need to validate their folkloric use (Mpiana et *al.*, 2016; Sofowora, 1989). Plants are supposed to be safe are often mistakenly considered less toxic because they are "natural", but several dangerous and fatal side effects have been reported (Whitto et *al.*, 2003; Izzo, 2004)(Chanda et *al.*, 2015). Indeed, these products contain bioactive principles that may cause adverse effects (Bent and Ko, 2004). Thus, ensuring the safety, efficacy and quality of herbal products has become a key issue for the WHO (Mpiana et *al.*, 2016).

The Democratic Republic of Congo (DRC) is home to nearly 47% of Africa's tropical forests and more than 80% of its population depends on medicinal plants for the treatment of various diseases including sickle cell disease, a genetic blood disease resulting from a mutation of the ß-globin gene that causes the replacement of a glutamic acid residue with valine in the sixth position of the ß-chain of haemoglobin (Gbolo et *al.*, 2017a; Ngbolua et *al.*, 2013a).

Up to 2% of the Congolese population is affected by this haemoglobinopathy (Gbolo et *al.*, 2017a; Ngbolua et *al.*, 2013b). Our research team has identified nearly 100 medicinal plants used by traditional healers to treat sickle cell disease (Gbolo et *al.*, 2017a; Ngbalua et *al.*, 2014a-c). A bioguided selection of these plants led us to the formulation of Drepanoalpha®, which is a multi-herb formula produced from a mixture of bioactive plants. (Gbolo et *al.*, 2017a; Mpiana et *al.*, 2014a,b). Pre-clinical studies have reported that Drepanoalpha is endowed with antifalcemic, antihemolytic, antiradical and antioxidant properties *in vitro* (Gbolo et *al.*, 2017b-c; Ngbolua et *al.*, 2014d). It has also shown the ability to increase red blood cell count, hemoglobin accumulation rate and platelets in rats, Guinea pigs and sickle cell patients (Gbolo et *al.*, 2017b-c; Ngbolua et *al.*, 2014d) and has no toxic effect on either rats or Guinea pigs (Mpiana et *al.*, 2016; Ngbolua et *al.*, 2014).

The overall objective of this study is to evaluate the toxicity of hard capsules containing ethanolic extracts of Drepanoalpha in Guinea pigs.

The specific objectives assigned to this research are:

- Evaluation of the LD_{50},
- Determination of haematological and biochemical parameters,
- Histopathological study of a few vital organs (heart, stomach, liver and kidneys).
- Prediction of the ADMET (Absorption, Distribution, Metabolism, Excretion and Toxicity) profile of some compounds contained in Drepanoalpha.

2. MATERIALS AND METHODS

2.1. Acute Toxicity Study (LD_{50})

2.1.1. Selection and preparation of the animal species

Healthy, nulliparous and non-pregnant Guinea pigs were randomly selected, individually marked for identification purposes and were acclimated to laboratory conditions for 3 days.

2.1.2. Test limit of 5000 mg/kg of B.W.

Ten Guinea pigs were divided before fasting into two groups of 5 animals each (3 males and 2 females) according to their weight: a control group (mean weight: 307.6±32.83 g) and a treatment group (weight: 339.69±4.05 g). The test drug (prepared some time prior to administration) was administered by gavage in a single dose using a gastric tube. All animals had free access to tap water and food throughout the experiment but were shortly fasted (withholding food, but not water) for 24 h before the oral administration of the single dose of Drepanoalpha hard capsules drug. The control group received a normal saline solution (NaCl 9‰) and the treated group received a single dose of 5000 mg/kg of body weight of "Drepanoalpha capsule" dissolved in the normal saline solution according to the dose adjustment method of the Organization for Economic Cooperation and Development (OECD) guidelines 425 (OECD, 2008). Four hours after dosing, the animals had free access to water and food. Every day, the weight of all animals was measured.

2.2. Sub-acute toxicity study

Twelve female Guinea pigs (OECD, 2008) were divided into 4 groups of 3 each. The administered doses were selected and distributed using arithmetic regression so that the highest administrated dose was equal to 1/10 of the LD_{50} value. The 1/10 of the LD_{50} for the case in point is the $1/10^{th}$ dose of the highest dose administered for acute toxicity. Thus, the experimental doses administered to the Guinea pigs of three groups were 125 mg/kg, 250 mg/kg and 500 mg/kg administered twice daily for 28 days, respectively, and the fourth group was the control group which received only a NaCl 9‰ solution.

2.2.1. Signs of mortality and clinical signs (general behavior)

During the experimental period, all animals were observed daily for behavioral disturbances (difficult movement, drowsiness, slowed respiration and convulsions), latency and/or mortality were noted after dosing at 30 min, 1 hour, 2 hours, 5 hours, 24 hours and daily for 14 days for acute toxicity and 28 days for sub-acute toxicity.

The LD_{50} is less than the test dose (5000 mg/kg) if at least three animals died and is greater than the test dose (5000 mg/kg) if at least three animals were able to survive.

2.2.2. Body and organ weight measurement

The body weight of each animal was measured with a sensitive scale (METTLER PM600) during the acclimatization period, once before the start of dosing and every day until the day of sacrifice.

At the end of the experiment, all Guinea pigs were shortly fasted (but free access to tap water) overnight from 6:00 p.m. to 6:00 a.m. and then were weighted. Three Guinea pigs per group were sacrificed by decapitation preceded by immobilization with ethanol.

The liver, kidneys, stomach and heart of each animal in the different groups were excised immediately after blood sampling. After excision, the organs were immediately weighed (paired organs were weighed together) and photographed and preserved with buffered formalin for histological examination. The organ weight ratio was calculated by the relationship :

$$OWR = \frac{OW(g)}{BW(g)} \times 100$$

With OWR (organ weight ratio), OW (organ weight) and BW (body weight)

2.2.3. Evaluation of histological damage

The liver, kidneys, stomach and heart of dissected animals were excised and preserved with buffered formalin for histopathological analysis according to the basic techniques described by Lee and Luna (1968). The individual incised organs were cut into several fragments and placed in cassettes and preserved in 10% formalin during fragmentation. The tissue blocks were sectioned in ribbons at a thickness of 4 μm with Leitz microtome (Leitz 1512) after inclusion in paraffin. Finally, the organs were stained with haematoxylin-eosin, fixed between slide and lamina before being observed using a microscope equipped with a Leica ICC50 W camera.

2.2.4. Biochemical and Haematological analysis

Blood samples from each sacrificed animal were collected after decapitation in EDTA (Ethylene-Diamine-Tetra-Acetic acid) tubes and non-EDTA dry tubes. EDTA blood was used to determine the hematological parameters where the red blood cells (RBC), hemoglobin (HGB), white blood cells (WBC) and hematocrit (HTC) were evaluated with an automated hematology analyzer (Coulter ABX Electronic). Serum was separated from non-EDTA blood after centrifugation at 3000 rpm for 5 min and the following parameters were evaluated: alanine amino-transferase (ALT), aspartate amino-transferase (AST), urea, creatinine, total protein by spectrophotometric methods. The assays were carried out using the human brand biochemical automaton with Biobase biochemical kits.

2.3. ADMET Profile

Twelve molecules (table 1 below) isolated from Drepanoalpha's plants were selected according to the literature on their biological activities. These molecules include polyphenols (including

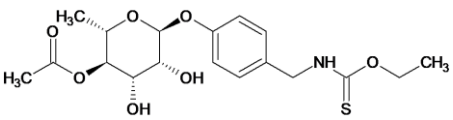
flavonoids), fatty acid esters, triterpenoids, known for their documented antisickling activity (Lukyamuzi et *al.*, 2019 ; Tshilanda et *al.*, 2016; Tshibangu et al, 2011), alkaloids known for their analgesic and anti-inflammatory activity (Hayfaa, 2013), thiocarbamates selected for their anti-inflammatory and antitumor activity (Cheenpracha et *al.*, 2010; Murakami, 1998). Calculations for *in silico* prediction of the pharmacokinetic properties of these twelve bioactive compounds were performed using the pkCSM web server (http://biosig.unimelb.edu.au/pkcsm). These properties are absorption, distribution, metabolism, excretion and toxicity (ADMET). The selected compounds were drawn using ChemDraw software, and their SMILES were generated for calculations using the pkCSM web server.

Table 1 : List of selected compounds

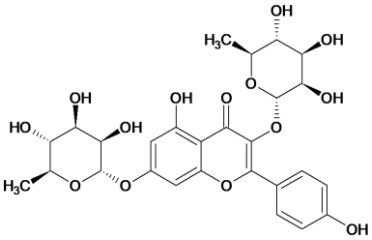
C. Nu	**C. Na**	**R. F.**	**M. W.**	**N°** CAS
1.	Niaziminin	$C_{18}H_{25}NO_7S$	399.46 g/mol	147821-56-5
2.	Kaempferitrin	$C_{27}H_{30}O_{14}$	578.52 g/mol	482-38-2
3.	Quinindoline	$C_{15}H_{10}N_2$	218.25 g/mol	243-38-9
4.	Vasicine	$C_{11}H_{12}N_2O$	188.23 g/mol	6159-55-3
5.	Ellagic acid	$C_{14}H_6O_8$	302.19 g/mol	476-66-4
6.	1,2,3-triolein	$C_{57}H_{104}O_6$	885.43 g/mol	122-32-7
7.	3,5,3′-trihydroxy-7,4′-dimethoxyflavone	$C_{17}H_{14}O_7$	330.29 g/mol	529-40-8
8.	3,5,7-trihydroxy-4′-methoxyflavone	$C_{16}H_{12}O_6$	300.26 g/mol	57396-72-2
9.	5,7-dihydroxy-3,4′-dimethoxyflavone	$C_{17}H_{14}O_6$	314.29 g/mol	4712-12-3
10.	5,4′-dihydroxy-7-methoxyflavanone	$C_{16}H_{14}O_5$	286.28 g/mol	2957-21-3
11.	β-sitostérol	$C_{29}H_{50}O$	414,71 g/mol	83-46-5
12.	Stigmastérol	$C_{29}H_{48}O$	412,69 g/mol	83-48-7

***C. Nu : Compound Numbers ; C. Na : Compound Name ; R. F. : Raw Formula ; M. W. : Molecular weight**

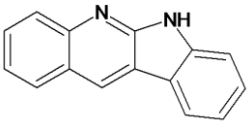
The following figures show the structures of the selected molecules



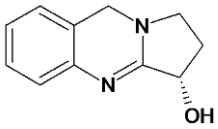
01. Niaziminin



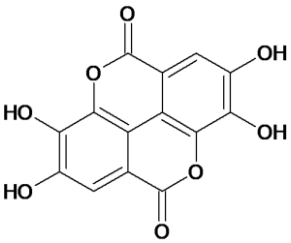
02. Kaempferitrin



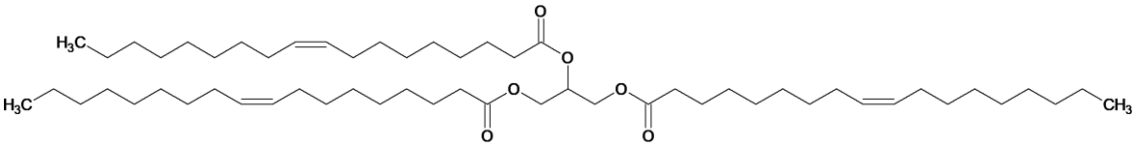
03. Quinindoline



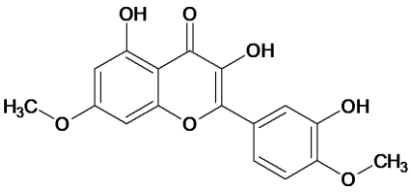
04. Vasicine



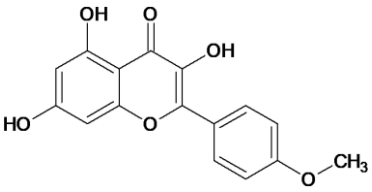
05. Ellagic acid



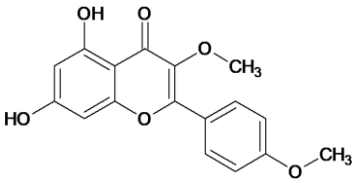
06. 1,2,3-triolein



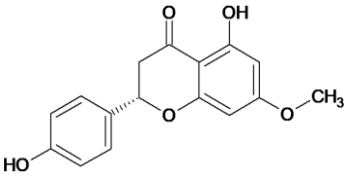
07. 3,5,3′-trihydroxy-7,4′-dimethoxyflavone



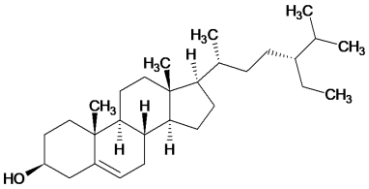
08. 3,5,7-trihydroxy-4′-methoxyflavone



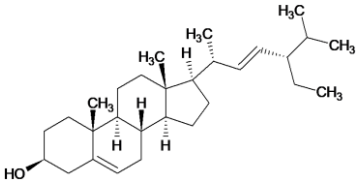
09. 5,7-dihydroxy-3,4′-dimethoxyflavone



10. Sakuranetin



11. β-sitostérol



12. Stigmastérol

Figure 1. Structures of molecules **1-12** derived from Drepanoalpha's constituent plants

2.4. Statistical analysis

The statistical analyses of the results were performed using Origin version 6.1 software. The values are presented as an average ± SD. Significance levels between treatment and control groups were measured by the Student t-test. If $p<0.05$, the difference between the values is considered significant.

2.5. Ethical considerations.

The study was conducted after receiving the approval of the ethical and scientific committee of the School of Public Health of the Faculty of Medicine of the University of Kinshasa, with the highest level of requirement for humanity and compassion in the use of animals in biomedical research (Approval No.: ESP/CE/237/2019). The animals used in this study were not subjected to unnecessarily painful and frightening situations. The procedure was carried out by a well-trained person. The animals were protected from pathogens and placed in an appropriate environment. The number of animals was kept to the minimum possible, without compromising the scientific objectives of the study (Richmond, 2000, Veissier, 1999).

3. RESULTS

3.1.Acute toxicity study of Drepanoalpha hard capsules in Guinea pigs

The various signs of toxicity observed in **Guinea** pigs are described in **Table 1**.

Table 1. Acute toxicity of "Drepanoalpha" hard capsules administered by oral treatment

Toxic symptoms	**Control (2mL NaCl (9‰)**			**Drepanoalpha (5000 mg/kg)**		
	D_0	D_7	D_{14}	D_0	D_7	$_{14}$
Changing the Pilosity	Yes	None	None	Yes	None	None
Ability to feed	Yes	Yes	Yes	Yes	Yes	Yes
Weight gain	None	Yes	Yes	None	Yes	Yes
Changing eye colors	None	None	None	None	None	None
Breathing Difficulty	None	None	None	None	None	None
Hypoactivity	Yes	None	None	Yes	None	None
Blood in urine	None	None	None	None	None	None
Diarrhea	None	None	None	None	None	None
Vomiting	None	None	None	None	None	None
Convulsion/coma	None	None	None	None	None	None
Death	None	None	None	None	None	None

This table shows that during the acute toxicity observation period, none of the treated animals succumbed. No signs of toxicity such as tremor, change of step, blood in urine, convulsion, salivation, diarrhea, coma ... were noted. However, a partial hypoactivity and a slight increase in the respiratory frequency in treated Guinea pigs was observed but these observations ended after about ten minutes. In addition to this hypoactivity and the transient change in respiratory rate, a modification of pilosity after a few minutes was also observed, which would reflect the stress felt during feeding. It took a few minutes for the animals to realize that they were not in any danger.

Table 2 gives changes in the body and organs weight of Guinea pigs after acute oral treatment with "Drepanoalpha" hard capsules

Table 2. Changes in the body and organs weight of Guinea pigs after acute oral treatment with "Drepanoalpha" hard capsules

	Control (NaCl 9‰)	Drepanoalpha (5000 mg/kg)
Body weight (g)		
Initial	305.03 ± 3.99	326.14 ± 3.29
Final	408.28 ± 22.67	411.38 ± 33.48
Weight gain (%)	25.29	20.72
Organ weights (g) and (OWR)		
Heart	1.77 ± 0.28 (0.43 ± 0.02)	2.10 ± 0.08 (0.51 ± 0.04)
Liver	12.02 ± 0.87 (2.95 ± 0.16)	11.66 ± 0.35 (2.84 ± 0.24)
Stomach	10.50 ± 1.37 (2.57 ± 0.14)	13.97 ± 0.19 (3.41 ± 0.24)
Kidneys	2.91 ± 0.47 (0.48 ± 0.04)	3.5 ± 0.19 (0.85 ± 0.29)

The comparison of the initial mean weight at D_0 of the treated group to final mean weight at D_{14} (Table 2) shows a significant difference in the weight ($p = 0.042$, $t = 4.717$). As for the weight gain between animals in the control group compared to those treated with Drepanoalpha hard capsules, there was no significant difference ($p>0.05$).

Figure 2 displays the vital organs observed after dissection.

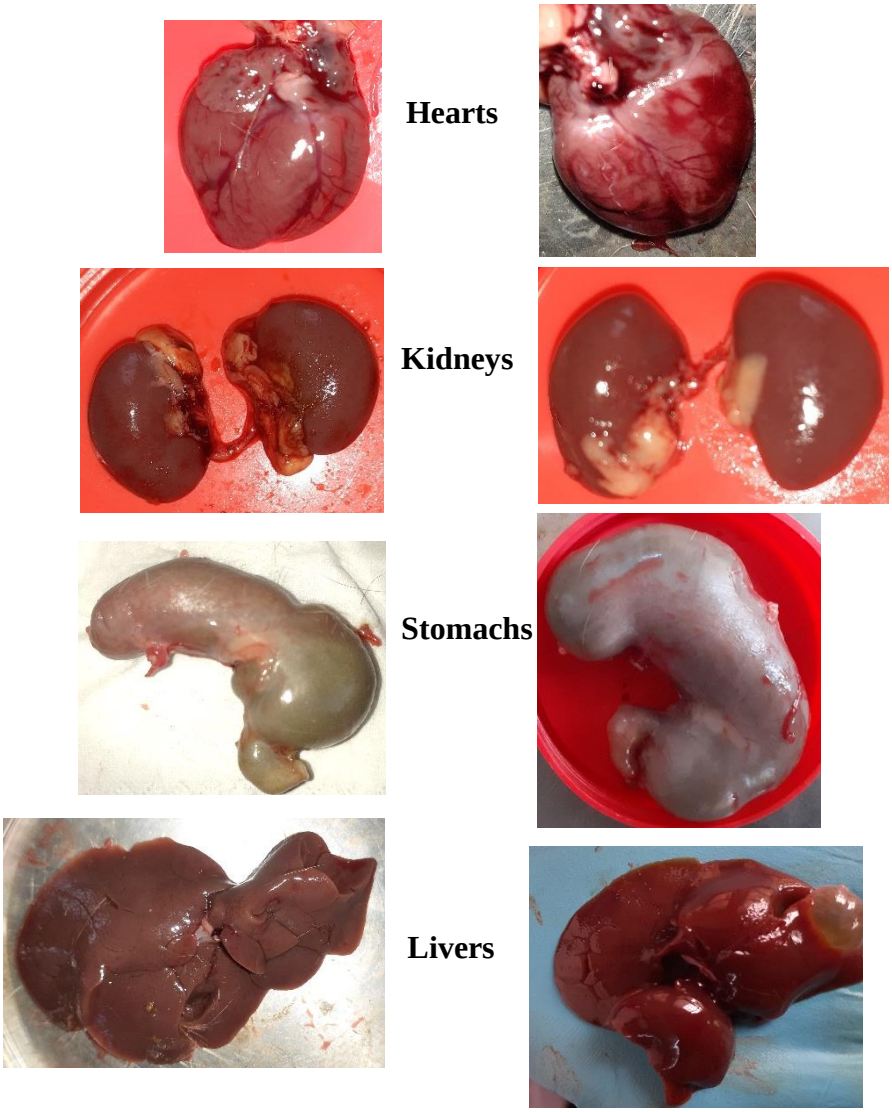


Figure 2. Photograph of vital organs of no treated (left) and treated (right) Guinea pigs

Table 3. Effects of Drepanoalpha on hematological parameters in acute toxicity (14 days).

Haematological	**Control (NaCl** 9‰)	**Drepanoalpha (5000 mg/kg)**
HB (g/dl)	16.3±0.3	16.7±0.0
WBC ($x10^3/mm^3$)	12.6±4.7	11.6±1.1
RBC ($10^6/mm^3$)	4.1±0.9	4.6±0.0
HCT (%)	49.0±1.0	50.0±0.0

Statistical analysis applied to data presented in **Table 3** above shows that on day D_{14} post-treatment, there was slight decrease (non-significant; $p > 0.05$) in WBCs relative to control. This decrease is good evidence that Drepanoalpha would be an effective product for the management of sickle cell disease. However, at the same dose, Drepanoalpha hard capsules

slightly increase other parameters such as red blood cell count, hemoglobin, and hematocrit, although these differences were not statistically significant at the 5% probability level.

This result shows that Drepanoalpha acts on hematopoiesis and compensates for the chronic erythropenia (anemia) and hyperhemolysis that characterize sickle cell disease subjects.

Table 4. Effects of Drepanoalpha on biochemical parameters in acute toxicity (after 14 days).

Biochemical	**Control (NaCl 9‰)**	**Drepanoalpha (5000 mg/kg)**
GPT (UI/l)	72.1±10.3	71.0±11.8
GOT (UI/L)	74.3±33,5	78.2±26.1
Urea (g/L)	0.2±0.1	0.36±0.1
Creatinin (mg/L)	7.3±2.4	8.6±2.5
Direct bil. (mg/L)	3.4±0.4	0.7±0.05
Total bil. (mg/L)	5.6± 0.4	3.7±0.1

Table 5. Effects of Drepanoalpha on blood proteins in acute toxicity (after 14 days).

	Control (NaCl 9‰)	**Drepanoalpha (5000 mg/kg)**
Total protein (g/L)	53.0±6.9	54.3±2.0
Albumin (g/L)	46.7±5.8	47.3±2.5

Inspection of **Table 4** shows a small increase in serum GOT and a decrease in GPT in animals treated with Drepanolapha hard capsules. However, it is well known that an increase in serum transaminase values is pathological when it is 1.5 times the upper limit of normal and persists for 6 months. In this case, no significant difference was found ($p>0.05$) between the control and treated group. The transaminase level varies depending on parameters such as sex, age, body temperature, body mass index (BMI) and to the technique used in the medical analysis laboratory as well. Since the increase observed in the control group does not exceed pathological norms, we conclude that Drepanoalpha hard capsules are not toxic to liver cells as indicated by the mean values of the marker enzymes.

Statistical analysis applied to results presented in **Table 4**, with the probability threshold equal to 0.05, showed that on day D_{14} post-treatment, no significant difference was observed between the mean values of creatinine and urea for the two groups. These results show that Drepanoalpha hard capsules are not toxic to the kidneys.

Scrutiny of **Table 5** shows that Drepanoalpha hard capsules do not disturb the mean values of total protein and albumin on day D_{14} after treatment. This result was confirmed by statistical analysis which did not reveal a significant difference at the 0.05 probability threshold between the two groups. This is experimental evidence that the biosynthetic capacity of the liver is maintained intact and therefore the product is not hepatotoxic.

3.2.Histological Analysis

Figures below show the optical micrographs of organs in the control and test groups.

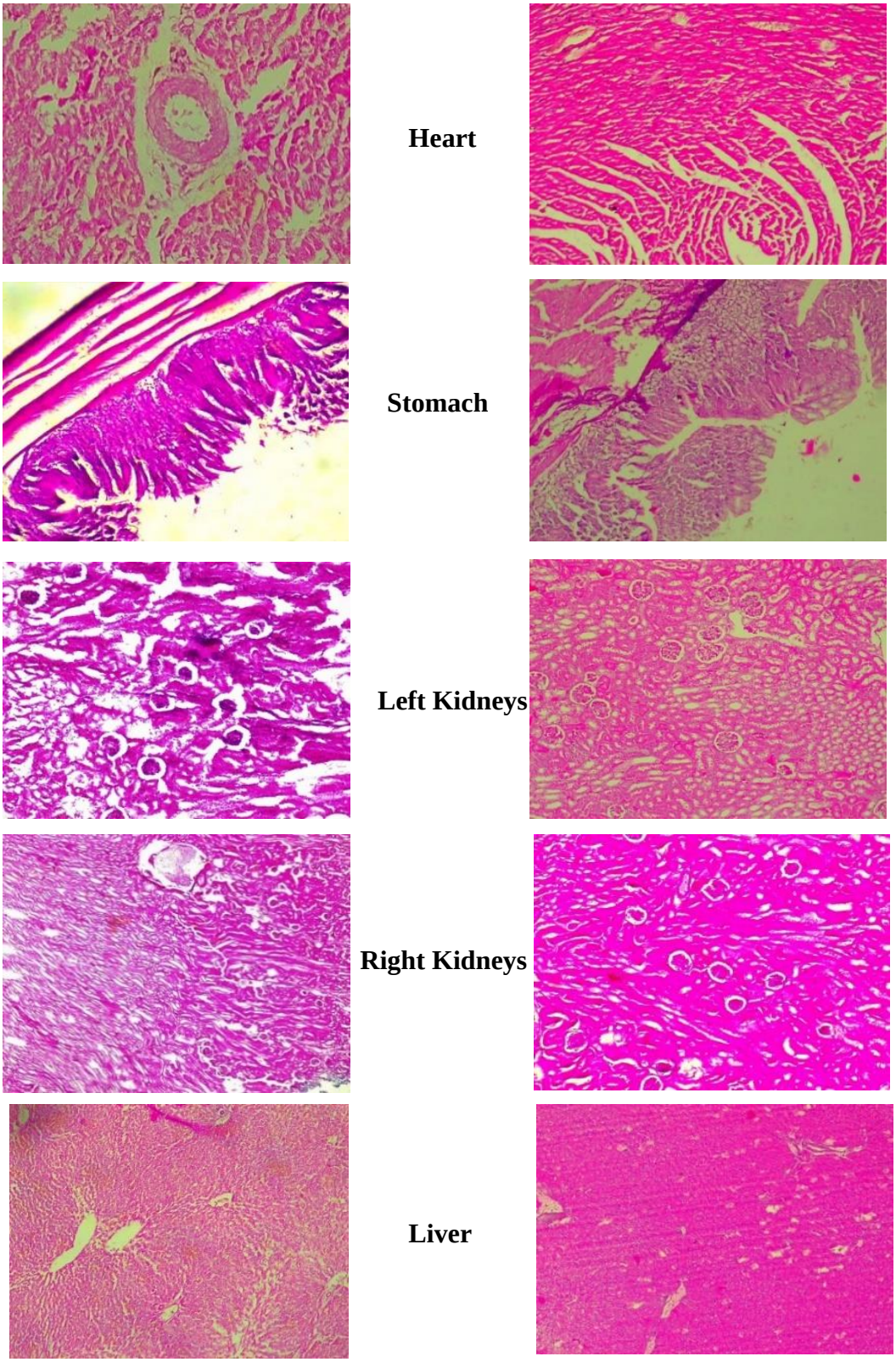


Figure 3. Comparison of histological sections between Control Group (left) and Treated Group (Right)

Histological analysis of different organs of Guinea pigs treated with Drepanoalpha hard capsules shows a normal morphology in all rodents.

3.3. Sub-acute toxicity study of Drepanoalpha hard capsules in Guinea pigs

Figure 3 shows the effects of "Drepanoalpha" hard capsules on body weight (g) and weight gains of Guinea pigs in sub-acute toxicity.

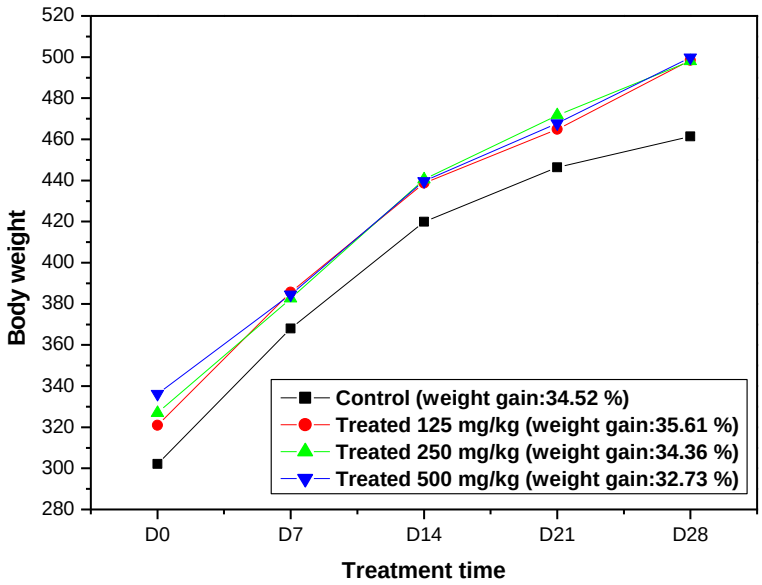


Figure 4. Effect of "Drepanoalpha" hard capsules on guinea pig body weight (g) and weight gain (%) in subacute toxicity study (28 days)

Comparison of the initial mean weight of Guinea pigs on day D_0 of the treated groups with the final mean weight of the control group on D_{28} (**Figure 3**) shows no significant difference in their weight in both groups. The weight gain between animals in the control group and those in the Drepanoalpha capsule-treated groups at different doses for 28 days shows no statically significant difference ($p>0.05$).

Table 6. Effect of "Drepanoalpha" hard capsules on organ weights (g) of Guinea pigs in sub-acute toxicity (28 days).

Parameters	**Control (NaCl** 9‰)	**125 mg/kg**	**250 mg/kg**	**500 mg/kg**
Heart	2.49 ± 0.13	2.66 ± 0.13	2.70 ± 0.07	2.67 ± 0.01
Liver	16.95 ± 1.09	18.93 ± 1.20	18.73 ± 0.88	18.29 ± 0.03
Stomach	10.63 ± 0.61	11.83 ± 0.29	11.62 ±0.51	11.34 ± 0.12
Kidneys	3.87 ± 0.20	4.25 ± 0.35	4.24 ± 0.23	4.14 ± 0.01

The 28-days sub-acute toxicity oral administration of Drepanoalpha hard capsules in Guinea pigs induced no adverse effects on target vital organ weights. The comparison of organ weights of guinea pigs treated with Drepanoalpha hard capsules at different concentrations with those of the control group showed no statically significant difference ($p>0.05$).

Table 7. Effect of different doses of Drepanoalpha hard capsules on hematological parameters in sub-acute toxicity (28 days).

Parameters	**Control (NaCl** 9‰**))**	**125 mg/ kg**	**250 mg/kg**	**500 mg/kg**
HB (g/dL)	13.23 ± 0.51	14.23 ± 1.07	14.43 ± 0.84	15.33 ± 0.67
WBC ($x10^3/mm^3$)	16.62 ± 0.16	12.10 ± 0.66	10.83 ± 1.53	11.50 ± 1.50
RBC ($10^6/mm^3$)	4.75 ± 0.22	5.42 ± 0.21	5.74 ± 0.30	6.37 ± 0.25
HCT (%)	39.67 ± 1.53	42.67 ± 3.21	43.33 ± 2.52	46.00 ± 2.00

The 28-days oral sub-acute toxicity administration of Drepanoalpha hard capsules in Guinea pigs shows a statistically significant ($p > 0.05$) decrease in WBC counts in all treated groups compared to the control one. At the same doses, Drepanoalpha hard capsules significantly increased the values of other parameters such as red blood cell count, hemoglobin, and hematocrit ($p<0.05$: HCT: $t = 4.3589$; $p = 0.01207$; HB: $t = 4.42173$; $p = 0.0115$; WBC: $t = -5.87459$; $p = 0.00419$ and RBC: $t = 8.34465$; $p = 0.0011$).

Table 8. Effect of different doses of Drepanoalpha hard capsules on biochemical parameters in sub-acute toxicity (28 days).

Parameters	**Control (NaCl** 9‰)	**125 mg/kg**	**250 mg/kg**	**500 mg/kg**
GPT (UI/l)	60.63 ± 10.35	53.35 ± 7.36	47.28 ± 12.05	41.63 ± 3.15
GOT (UI/L)	32.52 ± 7.30	24.1 ± 3.24	25.95 ± 5.64	22.81 ± 6.01
Urea (g/L)	27.37 ± 9.48	23.58 ± 3.25	24.07 ± 5.32	23.48 ± 9.22
Creatinin (mg/L)	8.1 ± 1.2	6.7 ± 0.4	6.17 ± 0.2	6.17 ± 0.2
Direct Bil. (mg/L)	3.07 ± 2.2	2.47 ± 0.6	2.53 ± 0.4	2.67 ± 0.8
Total Bil. (mg/L)	5.77 ± 2.1	3.67 ± 1.6	4.2 ± 1.1	4.23 ± 0.3

Biochemical analysis of the 28-day oral sub-acute toxicity test in guinea pigs showed a decrease in all parameters at different doses compared to the control. Application of Student's t-test

comparing the means between the parameter values of the control group to those of the Drepanoalpha capsule groups at different doses shows a non-significant difference at the 0.05 probability threshold. Only the GPT values at 500 mg/kg of body weight showed a significant difference $p<0.05$ ($t = -3.04161$; $p = 0.03834$).

Table 9. Effect of different doses of Drepanoalpha hard capsules on blood proteins in sub-acute toxicity (28 days).

Parameters	**Control (NaCl** 9‰)	**125 mg/kg**	**250 mg/kg**	**500 mg/kg**
Total protein (g/L)	53.00 ± 6.90	54.33 ± 2.10	48.33 ± 2.50	46.00 ± 4.40
Albumin (g/L)	38.00 ± 1.00	29.67 ± 3.80	31.33 ± 2.10	29.33 ± 5.90

Drepanoalpha hard capsules do not interfere with the mean values of total protein and albumin on day D_{28} post-treatment in oral sub-acute treatment. This result was confirmed by statistical analysis which did not reveal a significant difference at the 0.05 probability threshold between the different doses of the treated groups and the control group.

3.4.ADMET Profile

Table 10. ADMET profiles of molecules **1-12**

Parameters	**Compound number**											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Absorption & Distribution												
Caco2 (log Papp in 10^{-6} cm/s)	0.522	0.225	1.57	1.595	0.335	-0.471	0.548	1.084	1.11	1.209	1.275	1.287
HIA (%)	61.853	35.385	93.815	86.22	86.684	82.434	69.767	93.383	95.376	91.347	95.474	95.981
Skin permeability (log Kp)	-3.12	-2.735	-2.735	-2.827	-2.735	-2.735	-2.735	-2.984	-2.75	-3.102	-2.728	-2.726
P-glycoprotein substrate	Yes	Yes	Yes	No	Yes	No	Yes	Yes	Yes	Yes	No	No
P-glycoprotein I inhibitor	No	No	No	No	No	No	No	No	No	No	No	Yes
VDss (log L/kg)	-0.464	1.487	0.144	0.08	0.375	-1.08	0.529	0.113	-0.057	-0.229	0.243	0.223
BBB (log BB)	-1.036	-1.823	0.956	-0.127	-1.272	-1.661	-1.45	-0.392	-0.57	-0.239	0.815	0.805
Metabolism												
CYP2D6 substrate	No	No	Yes	No	No	No	No	No	No	No	No	No
CYP3A4 substrate	No	No	Yes	Yes	No	Yes	No	No	No	No	Yes	Yes
CYP1A2 inhibitior	No	No	Yes	No	Yes	No	Yes	Yes	Yes	Yes	No	No
CYP2C19 inhibitior	No	No	Yes	No	No	No	No	Yes	Yes	Yes	No	No
CYP2C9 inhibitior	No	No	Yes	No	No	No	No	No	Yes	No	No	No
CYP2D6 inhibitior	No	No	No	No	No	No	No	No	No	No	No	No
CYP3A4 inhibitior	No	No	No	No	No	No	No	No	Yes	No	No	No

Parameters	**Compound number**											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Excretion												
Total clearance (log ml/min/kg)	0.037	-0.102	0.784	0.58	0.537	2.634	0.611	0.132	0.668	0.163	0.628	0.618
Renal OCT2 substrate	No	No	No	No	No	No	No	No	No	No	No	No
Toxicity												
Ames test	No	No	No	Yes	No	No	No	Yes	No	No	No	No
Hepatotoxicity	Yes	No	No	No	No	No	No	No	No	No	No	No
Max. tolerated dose (log mg/kg/day)	0.176	0.519	0.205	0.204	0.476	0.439	0.757	0.238	0.561	0.696	-0.272	-0.318
hERG I inhibitor	No	No	No	No	No	No	No	No	No	No	No	No
hERG II inhibitor	No	Yes	No	No	No	No	No	No	No	No	Yes	Yes
Oral rat acute toxicity (LD50, mol/kg)	2.718	2.587	2.363	2.697	2.399	2.592	2.352	2.334	2.323	2.108	2.474	2.454
Oral Rat Chronic Toxicity (LOAEL, log mg/kg_bw/day)	2.248	3.228	1.028	1.427	2.698	0.81	2.678	1.736	2.016	2.041	1.108	1.125

4. DISCUSSION

Phytotherapy is used by up to 80% of the population in developing countries. Despite its widespread use, few scientific studies have been undertaken to verify the safety and efficacy of traditional remedies (Hilalya et *al.*, 2004). To assess the safety of Drepanoalpha, our improved traditional medicine for human use, toxicological evaluation in experimental models was used in order to assess its toxicity and to provide indications for the choice of the minimum tolerated dose for humans. The choice of rodents for this purpose is related to the fact that these animals express the same side effects as humans in terms of hematological, gastrointestinal and cardiovascular effects (Chukwuma and Obioma, 2014; Ogbonnia et *al.*, 2010).

Acute toxicity study

The acute toxicity test is therefore the first step in the toxicological evaluation of drugs. It consists of treating animals with a single dose within a short period of time and observing the adverse effects and mortality of the animal (Joshua et *al.*, 2008). In this study, Drepanoalpha hard capsules showed no mortality at the limit dose of 5000 mg/kg of B.W. during the observation period of more than 24 hours. However, slight changes in behavior, respiration and hypoactivity were observed in treated Guinea pigs for a few minutes after dosing. This transient hypoactivity is thought to be related to the viscosity of the extract. The animals would need a resting time necessary for the absorption of the extract (Ngbolua et *al.*, 2016). It should also be noted that these slight behavioral deviations in these animals would be due to an adaptation following ingestion of a product that is not part of their diet. Moreover, it is well known that when the active ingredients contained in plants are in excess in a body, certain chemical compounds can, beyond their expected therapeutic activity, cause certain dysfunctions (Lohoues et *al.*, 2006). The slowing down of the respiratory frequency, as well as convulsions can be attributed to the intoxication with phenolic compounds; whereas hypoactivity could be attributed among others to the sedative, anaesthetic and especially psychoactive properties of alkaloids (Luis Blanc et *al.*, 1988 in Lohoues et *al.*, 2006). All these secondary metabolites are present in Drepanoalpha.

The test limit for acute oral toxicity is generally considered to be 5 g/kg of body weight. If no mortality is observed at this dose level, a higher dose is generally not required (Hayes, 2011; OECD, 2008). The absence of mortality at this dose level indicates that there are no phytomedication-related adverse effects and that Drepanoalpha hard capsules are not toxic. These results are in agreement with those obtained by Ngbolua et al (2016) and Mpiana et al

(2014) who worked on the lyophilisate and decoction of Drepanoalpha powder, respectively. Consequently, these results suggest that the median lethal dose (LD_{50}) is greater than 5000 mg/kg. These results show that Drepanoalpha hard capsules are practically non-toxic or non-lethal after acute exposure. And its excipients (lactose and aérosil) have no influence on toxicity. They have a GRAS status (Generally Recognized As Safe) (Roth, 2020; Fine Chemical, 2018). According to Kennedy et al (1986) and Diezi (1989), substances with an oral LD_{50} greater than 5 g/kg can be considered practically non-toxic; furthermore, according to the Hodge and Sterner (1980) scale, Drepanoalpha has a toxicity index of 5, thus almost non-toxic. This suggests safety for this latter product (Kotué et *al.*, 2013; Diezi, 1989).

Changes in body weight are generally used as an indicator of the side effects of drugs and chemicals compounds (Tofovic and Jackson, 1999; Raza et *al.*, 2002; Teo et *al.*, 2002; Hilalya et *al.*, 2004; Nandy et *al.*, 2012; Kingsley et *al.*, 2012), any weight gain indicates that the drug does not alter carbohydrate, lipid and protein metabolism. The increase in body weight of the animals suggests that they have increasingly accumulated calories from their normal diet (Chukwuma and Obioma, 2014). Although the used animals have a diet rich in nutrients, Drepanoalpha hard capsules could have allowed a good absorption and use of these nutrients because they have properties to add some trace elements to the nutrition of the consumer (Gbolo et *al.*, 2017). The weight gain observed in treated groups is evidence that Drepanoalpha hard capsules are therefore safe. Indeed, the extract is toxic when it leads to a weight loss of about 10% or more (Schilter et *al.*, 2003, Chanda, 2015). These results corroborate with those obtained previously on lyophilisate and Drepanoalpha powder (Mpiana et *al.*, 2016; Ngbolua et *al.*, 2014).

In general, 14 days after the single dose administered to Guinea pigs, weight growth was observed in all rodents. This weight gain would therefore be related to their ability to feed. In addition, we found that Guinea pigs in the control group showed a high weight gain compared to the treated groups. These results corroborate our previous work and those obtained by Kotué et al, who worked with Hemodya on wistar rats (Kotué et al, 2013; Ngbolua et al, 2014; Mpiana et al, 2016).

Organ weight is also an important indicator of the physiological and pathological status of the animal. Indeed, it makes it possible to demonstrate whether or not the organ is attacked after drug exposure (Mukinda and Syce, 2007; Ramesh et *al.*, 2009).

Heart, liver, kidneys are organs that can be damaged during intoxication. In addition, it should be noted that the liver is the key organ in the metabolism and detoxification of xenobiotic compounds (Clarke and Clarke, 1977; Viau and Tardif, 2003; Ramesh et *al.*, 2009). The observed difference in the shape and increase in liver weight of rats in the treated group compared to the control group is believed to be primarily due to the difference in body weights between the two groups. It is well established that organ weights vary with age and/or body weight. This difference can also be attributed to a high rate of metabolism following exposure of this organ to the extract containing several secondary metabolites (Jothy et *al.*, 2001). The heart is organ that can be affected by chemical compounds resulting from metabolism (Oduola et *al.*, 2010). In this study, no macroscopic signs of toxicity (no mortality and organ damage) were observed during the study period on the tested organs (heart, stomach, kidneys, liver).

Analysis of hematological parameters is also a good indication in the toxicological risk assessment of drugs. They can provide information on the toxic effects of drugs on blood constituents because the hematopoietic system is one of the most sensitive targets for toxic compounds and an important indicator of physiological and pathological status in both humans and animals (Jothy et *al.*, 2001, Adeneye, 2006; Mukinda and Syce, 2007, Chanda, 2015).

The slight increase in parameters such as WBC, HCT and HB, observed in Guinea pigs on day D_{14} post-treatment can be explained by an increased production of haematopoietic regulatory factors (Kafaie et *al.*, 2012). These observations show that Drepanoalpha would prevent painful attacks in sickle cell disease patients and are in perfect agreement with our previous work on Drepanoalpha (Mpiana et *al.*, 2016; Ngbolua et *al.*, 2014; Gbolo et *al.*, 2017).

Further, biochemical parameters are involved in the determination of overall toxicity because values of markers such as urea, creatinin and transaminases are abnormally elevated in the blood when drugs are toxic to vital organs such as the liver and kidneys (Chukwuma and Obioma, 2014; Ngbolua et *al.*, 2014). Urea and creatinine are considered better indicators of kidney dysfunction and failure. This is because the kidneys normally eliminate most of the urea, which is then excreted in the urine. When the kidneys are damaged, less urea is eliminated and therefore remains in the bloodstream, where it accumulates as it passes through the body. Creatinin, on the other hand, is a good indicator of the function of the glomerular filtration of the kidneys. High levels of creatinin in the blood indicate poor kidney function (Gnanamani et *al.*, 2008). In this study, no alterations in the values of these two parameters were observed.

With regards to the hepatocyte lysis, cytosolic enzymes are released into the blood. Evaluation of the activity of serum enzymes such as ALT (GPT) and AST (GOT) is a conventional means of ensuring the functional integrity of the liver (Aliyu et *al.*, 2006). ALT is a cytosolic enzyme secreted into liver cells from which it is released into the bloodstream in the event of hepatic cell necrosis (Gome et *al.*, 2011; Dufour et *al.*, 2000). It is a liver-specific enzyme, making it an important and highly sensitive indicator of hepatotoxicity (Pratt and Kaplan, 2000; AlHabori et *al.*, 2002). AST is also an indicator of hepatocyte destruction, although in addition to the liver it is found in the heart, skeletal muscle, lungs and kidneys (Dufour et *al.*, 2000). Indeed, ALT and AST are intracellular enzymes involved in amino acid metabolism. These enzymes are highly concentrated in muscle, liver and brain. Increasing in the concentration of these enzymes in the blood are an indication of necrosis in these tissues (Hussein and Abdel-Gawas, 2010; Forchielli et *al.*, 2012). ALT and AST levels rise rapidly when the liver is damaged for a variety of reasons including hepatic cell necrosis, hepatitis, cirrhosis and hepatotoxicity of certain drugs (Pratt and Kaplan, 2000; Dufour et *al.*, 2000).

In the case of myocardial infarction, AST usually increases by 10 to 20 times the normal mean at most, ALT is normal or only slightly increased (Iwuanyanwu et *al.*, 2012). Our results indicate that Drepanoalpha hard capsules did not significantly alter these parameters.

Serum proteins are part of a general health check-up, to determine nutritional status, or to look for certain liver or kidney disorders. Albumin (made by hepatocytes) represents 60% of the proteins present in the blood. It is used to transport many endogenous (originating inside the body) and exogenous (originating outside the body) substances in the blood and it helps to maintain oncotic pressure (the force that attracts water towards the proteins). High albumin levels in the blood can be the result of hemoconcentration due to dehydration, fluid loss or diabetes insipidus. But it will also indicate poor transport of substances (Viau and Tardif, 2003). While a low albumin level in the blood reflects undernutrition related to anorexia nervosa or tumours; hepato-cellular insufficiency; various severe inflammations. In this study, our results show that Drepanoalpha hard capsules do not alter serum protein values.

Histopathological study of the kidney, liver, stomach and heart recovered from treated and control animals showed normal architecture (figure 3), suggesting that no detrimental changes and morphological disturbances were caused by the single oral administration of Drepanoalpha. These results corroborate those of the biochemical analyses on kidney and liver function.

Subacute toxicity study

After 28 days of subacute toxicity testing and observation, no signs of intoxication were noted at the administered Drepanoalpha hard capsules doses. Three experimental groups were given successively per dose (twice daily), 125 mg/kg, 250 mg/kg and 500 mg/kg. Weight gain was also observed in all guinea pigs. This is justified by the fact that the guinea pigs acquired an ability to feed which would be stimulated by the vitamins present in this product. But it should also be noted that this weight gain would be due to the proteins contained in Drepanoalpha. These results on sub-acute toxicity confirm those of our previous work (Mpiana et *al.*, 2016).

The metabolism of xenobiotics is made possible thanks to certain enzymes including cytochromes P450 (CYP) which play a central role in the biotransformation, metabolism and/or detoxification of these foreign compounds in the body. The latter are divided into families (CYP 1-2-3) and subfamilies (CYP 1A - 2C - 2D - 3A), the main ones involved in drug metabolism being CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP3A4. Since drug metabolism is essentially hepatic, it most often involves several CYPs and more rarely a single or preferential CYP: it is under these circumstances that the risk of interaction is highest (Guéguen et al., 2006).

In vivo toxicological studies (acute and sub-acute toxicity) in rodents and humans have shown that Drepanoalpha is not toxic. However, in order to prevent drug interactions, twelve molecules isolated from the constituent plants of this phytomedicine were analyzed *in silico* (virtual simulation) for their pharmacokinetic and toxicological properties (ADMET).

Eight of these twelve compounds (1, 2, 3, 5, 7, 8, 9 and 10) are substrates for the P-glycoprotein. The metabolism of xenobiotics breaks down into 3 phases (functionalization: oxidation-reduction and hydrolysis, conjugation and transportation) that ultimately lead to the elimination of foreign substances in bile and urine. Glycoprotein P is involved in the third phase, that of transport through the membranes of xenobiotics, and especially of conjugated derivatives (Guéguen et *al.*, 2006).

Compound 3 is an inhibitor of cytochromes CYP1A2, CYP2C19 and CYP2C29. Compound 9 is an inhibitor of CYP1A2, CYP2C19, CYP2C29 and CYP3A4, while compounds 5, 7, 8 and 10 are inhibitors of CYP1A2 and compounds 8 and 10 are also inhibitors of CYP2C19. These results show that the presence of two or more compounds at high concentrations in the phyotomedine can lead to interactions with conventional drugs metabolized by CYP1A2, CYP2C19 and CYP2C9. Indeed, drug interactions may reduce the activity of the concerned enzymes, in particular those of the intestinal epithelium by inhibition or may increase metabolic

activity by induction. Such interactions can extend the range of variability to about 400-fold. However, these differences in metabolism, as well as differences associated with plasma drug concentrations, do not translate into linear differences in drug concentrations, effects depending on the relationship between drug concentration, the response and adverse effects (Wilkinson, 2005).

In addition, at high concentrations, compound 1 can cause hepatoxicity, compounds 2, 11 and 12 can be cardiotoxic while compounds 4 and 8 can be genotoxic.

At the therapeutic doses, Drepanoalpha is non-toxic, hence the need for strict adherence to the dosage.

The expression of these compounds in the plant being a function of abiotic factors such as climate, geological environment of the plant harvesting site, etc. and biotic factors such as the presence of predators and/or parasites as well as interspecific competitions within ecosystems and the specificity of each plant taxon related to its genetic constitution (Ngbolua, 2012).

5. CONCLUSION

Acute oral toxicity studies of Drepanoalpha hard capsules in Guinea pigs showed no mortality at 5000 mg/kg during treatment and 14-days observation periods. These results suggest that oral administration of this formulation does not induce any toxic effects. This shows that regardless of the change in galenic formulation, Drepanoalpha is safe and provides assurance for its use. The increase in body weight of treated animals suggests the nutritional potential of this polyherbal formulation.

Conflict of Interest

The authors declare that there is no conflict of interest.

Contributions of the authors

Gbolo Benjamin designed and carried out the study, conducted the experiments, analysed the data and wrote the manuscript. Mpiana PT, Pierre Duez, Ngbolua KN, Tshibangu DST, Memvanga Patrick and Tshilanda DDT helped design the study and supervised it. Lebwaze Bienvenu assisted with the histopathological study. Aristote Matondo and Jason T. Kilembe

helped to carry out calculations on ADME-T properties. All authors read and approved the final manuscript.

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