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Acute and Sub-acute Oral Toxicity Study of Drepanoalpha® (A Poly-Herbal Formula Used in the Management of Sickle Cell Disease) in Guinea-pigs

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Authors' contributions

This work was carried out in collaboration between all authors. Author PTM designed the study. Author FMK wrote the first draft of the manuscript. Author KNN performed the statistical analysis. Authors FB and JNK wrote the protocol. Authors PBM and DSTT managed the analyses of the study. Author BZG managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To deepen the toxicological investigation of Drepanoalpha®, a poly-herbal formula used for its antisickling and radical scavenging properties in Congolese traditional medicine.
Study Design: To evaluate lethality, biochemical alterations, behavioral disturbances in guinea-pigs orally given increasing doses of Drepanoalpha® aqueous extracts.
Place and Duration of Study: Faculty of Medicine and Pharmacy, Official University of Bukavu, Bukavu, DR Congo, between June to December 2015.

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Methodology: The extracts were prepared as aqueous decoctions of Drepanoalpha powder. Animals were set to groups of 3 animals each. The doses varied from 250 mg to 16000 mg/kg body weight. Acute toxicity had to be estimated by LD50. Sub-acute toxicity was carried out by giving fractionated doses of Drepanoalpha extract during 2 weeks. Changes in blood biomarkers and behavioral signs were evaluated from baseline values of control untreated animals.
Results: Up to 16000 mg/kg, no death was recorded in acute toxicity. Thus, LD50 was not calculated. No significant changes in animals behavioral were observed during sub-acute testing. Instead, body weight gain, blood cell counts increase, BUN and ALT decrease, no change in serum creatinine were noted.
Conclusion: The findings comfort the safety of Drepanoalpha as found previously studies in rats.

Keywords: Drepanocytosis; Drepanoalpha®; acute toxicity; blood biomarkers; guinea-pigs.

1. INTRODUCTION

A large proportion of populations in many developing countries rely heavily on traditional practitioners and herbal plants to meet their primary healthcare needs [1]. The World Health Organization (WHO) estimates that 70 to 80% of the people in developing countries use traditional medicine as a major source of health care. So, herbal drugs have received greater attention as an alternative to clinical therapy and the demand for these herbal remedies has greatly increased recently. Indeed, medicinal plants have been used for years in daily life to treat diseases all over the world and still remain the basis for development of modern drugs [2-15]. Nowadays, researches about traditional knowledge on medicinal plants use have provided a lot of important drugs [2-4,8].

However, many people underestimate the toxicity of natural products and cannot realize that these agents could be as toxic as (or more than) synthetic drugs. Despite the usage of plants in folk medicine over ages, it is only lately that pharmacological and toxicological studies begun gaining attention from scientists [16-24]. Many plants have been reported to be toxic to both humans and animals [16-24]. Thus, the assurance of safety, efficacy and quality of herbal products has become a key issue for the WHO [22-24].

The Democratic Republic of the Congo (DRC) harbors almost 47% of African tropical forests and more than 80% of its population relies on medicinal plants for the treatment of various diseases among which sickle cell disease (SCD) also called drepanocytosis, a genetic blood disorder arising from a mutation in the ß-globin gene that leads to the replacement of glutamic acid residue by valine at the sixth position of the ß-chain of hemoglobin [6,7,13]. Up to 2% of the Congolese population is affected by this chronic disease [6,12]. Our research team has identified around 100 medicinal plants used by traditional healers to manage SCD [6,7,24-30]. A bio-guided based plants selection led the team to the formulation of Drepanoalpha®. This poly-herbal formula is produced from most active composed of a mixture of polyphenols and anthocyanins from edible plants previously described [13,26-29] and is taken three times daily for the treatment of SCD mainly in children. Drepanoalpha® has shown anti-sickling, anti-hemolytic, anti-radical and antioxidant activities *in vitro*. It also showed the capacity to increase red blood cells, hemoglobin build up rate, platelets and white blood cells counts in rats [31]. The present study was undertaken to complete toxicological investigations (acute and sub-chronic toxicities) in other animal species than rats, and guinea-pigs were selected for the purpose.

2. MATERIALS AND METHODS

2.1 Preparation of Drepanoalpha® Decoctions

Each aliquot of 100 g of powder was soaked in 500 mL of distilled water in a flask and the mixture was kept boiling for 15 min. After cooling, the extract was filtered through cotton wool and kept in a clean sealed flask. The filtrate was concentrated by evaporation on a hot plate and then placed in oven at 50℃ for 24 h to make a dry extract. The residue was retaken with physiological saline solution to make different concentrations for oral administration. Unless used the same day, solutions were conserved at 4℃ for further studies.

2.2 Animals

Male and female guinea-pigs, aged 2 to 4 months old and weighing 300 to 410 g were

chosen for this experiment. These animals were kept in the animal boundary of the Faculty of Medicine and Pharmacy of the Official University of Bukavu (UOB), prepared and used according to the standards required for experiment on laboratory animals [32].

2.3 Toxicological Studies

2.3.1 Acute toxicity (LD50)

Healthy animals were randomly assigned into groups of 3 animals each, and were given the extracts by feeding cannula at increasing doses with 2n progression from 250 mg to a maximal limit of 16000 mg/kg BW. The animals were then observed for 96 h. Behavior signs were recorded and the number of deaths in each group was counted to estimate the LD50 graphically by Probit analysis [9].

2.3.2 Sub-acute toxicity

The animals were divided into three groups of 3 animals each according to their body weight. The doses to be administered are generally calculated following arithmetic progression such that the latter experimental group receives 1/10 of the expected LD50 value [20]. In our case, each of test groups received the extract orally at 800 mg, 1200 mg and 1600 mg/Kg twice a day for 21 days. The fourth group was constituted with control animals which received only physiological saline each day. Animals had freely access to water and adequate food.

2.3.3 Biochemical analysis

At the end of observation, the survival animals were sacrificed and blood samples were collected for the determination of biomarkers. White blood cells (WBC) and Red blood cells (RBC) counts were determined by hematocytometer method using Türck's solution and saline solution [33,34]. Blood urea nitrogen (BUN) was measured by Berthelot colorimetric method [33]. The determination of creatinine was made by the method of Jaffe using picric acid and NaOH 0.4 mol/L [35]. Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) were assayed with Emekyn SGOT (AST) and Emekyn SGPT (ALT) Kits Biovision.

2.3.4 Behavioral manifestation

The animals were observed and behavioral manifestations were noted to detect any abnormal signs.

2.4 Statistical Analysis

Differences between test groups and control groups were statistically compared using Windows Excel statistics tool by Independent Sample t-test and one-way ANOVA at the significance of 95% confidence level. The results are presented as mean ± standard deviation.

3. RESULTS AND DISCUSSION

3.1 Evaluation of Acute and Sub-acute Toxicity

Table 1 gives the result of oral acute test in guinea-pigs treated with Drepanoalpha® extracts while Table 2 shows behavioral changes during both the acute and sub-acute testing. As shown, no death or significant behavioral changes occurred up to the dose of 16000 mg/kg. This suggests that Drepanoalpha® formula is relatively harmless at single dose according to Hodge and Sterner classification [24]. In toxicity rating by joint FAO/WHO Expert Committee on Food Additives [22], if at 2 g/kg oral dose no death occurred, it is sufficient to assume the substance to be relatively non-toxic. LD50 could not be determined. This result is consistent with our preceding findings on acute toxicity of Drepanoalpha® in Wistar albino rats [31]. In that study, the acute toxicity assessment revealed that the medium lethal dose (LD50) was higher than 4000 mg/kg. These results indicate that Drepanoalpha® formula has high safety margin through oral route which allows the use of this poly-herbal as nutraceutical in the management of sickle cell disease in humans.

By principle, short-term repeated dosing tests (1-4 weeks) are performed to obtain information on the toxicity of a substance after repeated administration and are generally required for the successful design of sub-chronic studies. The major objective of short-term studies is to determine adverse effects at low doses, dose response, and sometimes to identify target organs [36-39]. During the sub-acute toxicity test in this experiment, it has been observed only a kind of hypoactivity at high doses in some animals, and a mean increase of about 5.60±0.98% of body weight in both control and treated groups. There was however a higher increase in treated animals compared to control group ($p<0.05$). The increase in animals' weight confirmed the nutritional potential of drepanoalpha® as nutraceutical in the management of sickle cell anemia. Children with SCD often have nutritional problems particularly

in Africa; this generally leads to a low weight as compared to normal children. The mechanism by which Drepanoalpha® increases body weight is unknown, but it may be suggested that it might stimulate appetite, increase absorption and utilization of food nutrients. Reduction in body weight gain and internal organ weights would be an indicative simple and sensitive sign of toxicity after exposure to a toxic substance [16-21, 36-39].

3.2 Evaluation of Changes in Blood Biomarkers

Results presented in Table 3 show that Drepanoalpha® significantly increased blood cells counts (RBC and WBC) in animals given doses bigger than 2000 mg/kg ($p<0.05$). This result is consistent with the observation in Wistar albino rats [31]. Moreover, a recent study on nutritional value of Drepanoalpha® showed that this nutraceutical contains many micronutrients (e.g. Fe, Zn, Ca) and good amount of proteins (unpublished results). These elements also can contribute to the increase of hemoglobin and RBC levels. BUN decreased significantly at high doses, AST increased while ALT was decreasing (Fig. 1).

Drepanoalpha® is not toxic towards immune cells. Rather it would strengthen immunity. This could be very important for sickle cell disease patients who are very fragile and susceptible to infections [6,25-30]. Serum creatinine level is a good indicator of renal function since any elevation of serum levels is associated to a marked failure of nephron function [39]. Serum ALT activity is a highly sensitive biomarker of hepatotoxicity, but slight elevations are often observed in rodents in the absence of correlative liver histomorphologic damages suggesting false positive or potentially prodromal signals [40]. When liver cell cytoplasmic membranes are damaged, a variety of enzymes normally located in the cytosol or membrane bound enzymes such as ALP are released into the blood stream. Their measurement provides information on liver function and extrahepatic bile obstruction and intrahepatic cholestasis [31]. No changes or slight decreases reveal the safety profile of Drepanoalpha on the liver and renal function.

Table 1. Acute toxicity test of Drepanoalpha formula in guinea-pigs in 96 hrs of observation

Group	Number of animals	Dose (mg/Kg bw)	Number of deaths	Percentage of deaths
Control	3	0	0	0
Group 1	3	250	0	0
Group 2	3	500	0	0
Group 3	3	1000	0	0
Group 4	3	2000	0	0
Group 5	3	4000	0	0
Group 6	3	8000	0	0
Group 7	3	16000	0	0

Table 2. Behavioral changes in acute and sub-acute toxicity test of Drepanoalpha in guinea pigs

Intoxication signs	Acute test doses (mg/kg BW)								Sub-acute test doses (mg/kg BW)		
	0	250	500	1000	2000	4000	8000	16000	800	1200	1600
Pilosity modification	-	-	-	-	-	-	-	-	-	-	-
Change eyes color	-	-	-	-	-	-	-	-	-	-	-
Breathing difficulty	-	-	-	-	-	-	-	-	-	-	-
Hypoactivity	-	-	-	-	-	+	+	+	-	-	-
Blood in urine	-	-	-	-	-	-	-	-	-	-	-
Diarrhea	-	-	-	-	-	-	-	-	-	-	-
Vomiting	-	-	-	-	-	-	-	-	-	-	-
Convulsion and coma	-	-	-	-	-	-	-	-	-	-	-

Legend: Positive sign (+), Negative sign (-). In acute toxicity a single dose is give; in sub-acute test the dose is given twice a day for two weeks

Table 3. Body weight gain in guinea pigs treated with Drepanoalpha formula during 12 days

Group	Animals	Daily dose (mg/kg)	Mean body weight (g)			
			Before treatment	After treatment	Mean change	% change
Control	3	0	313	326	+13	4.15
Group 1	3	8000 bid	317	337	+20	6.31*
Group 2	3	12000 bid	345	366	+21	6.09*
Group 3	3	16000 bid	410	434	+24	5.85*

**p-value=0.003 significantly different from control group*

Table 4. Changes in blood biomarkers in guinea-pigs treated with Drepanoalpha poly-herbal

Group (dose mg/kg)	WBC (WBC/µL)	RBC (x10^9/L)	Creatinine (mg/dL)	BUN (mg/dL)	AST (U/L)	ALT (U/L)
Control (0)	2200±163	4.0±0.3	2.5±0.3	54.6±1.5	14.9±2.1	40.7±6.9
Group 1 (250)	1866±249†	4.3±0.3	2.2±0.6	66.5±4.0	16.4±1.0	33.6±14.4
Group 2 (500)	2000±163†	3.1±0.7	2.3±0.9	56.0±3.5	16.5±1.8	29.5±11.1
Group 3 (1000)	3000±711*	4.3±0.1	4.5±1.6*	62.6±5.7	26.6±3.2*	38.0±5.5
Group 4 (2000)	4400±565*	3.7±0.5	4.2±1.0*	58.9±3.5	41.7±1.2*	35.5±17.1
Group 5 (4000)	4133±771*	6.5±1.1*	2.7±0.4	27.6±5.2†	67.9±1.2*	38.2±2.9
Group 6 (8000)	3133±188*	8.1±1.1*	2.5±0.3	29.1±5.1†	55.8±11.4*	26.5±3.3†
Group 7 (16000)	5533±340*	7.7±1.5*	2.4±0.1	28.1±4.3†	63.9±11.0*	24.2±3.4†

*Legend: †significant reduction from control; *significant increase from control*

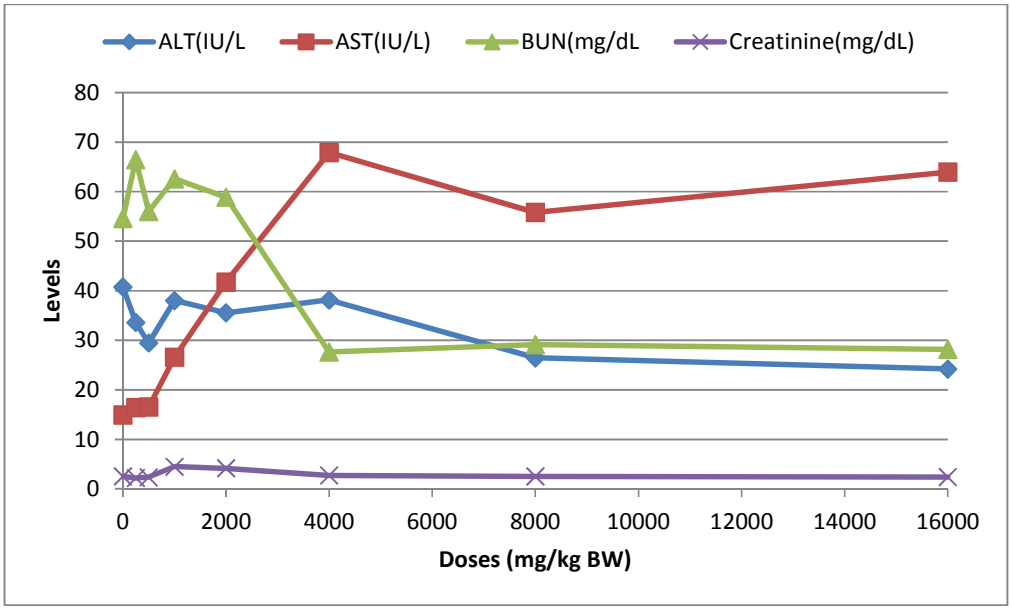

Fig. 1. Tendencies as mean values for blood biomarkers change at different doses

4. CONCLUSION

The acute and sub-acute oral toxicity studies of the aqueous extract of Drepanoalpha® in guinea-pig did not show any mortality up to the dose of 16000 mg/kg during the treatment and observational periods of 12 days. These results suggested that the oral administration of this poly-herbal does not induce any toxic effects and gives assurance for its use. Body weight increase of treated animals suggests nutritional potential of this poly-herbal formulation. Further study is needed to evaluate its chronic toxicity.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that Principles of laboratory animal care (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. WHO Monographs on Selected Medicinal Plants. 1999;1:295. Available:www.who.int/medicinedocs/en/d/Js2200e/ Dec 17, 2015
2. Makunga NP, Philander LE, Smith M. Current perspectives on an emerging formal natural products sector in South Africa. J. Ethnopharmacol. 2008;119: 365-75.
3. Fabricant DS, Farnsworth NR. The value of plants used in traditional medicine for drug discovery. Environ. Health Persp. 2001;109(Suppl 1):69-75.
4. Kaufman DW, Kelly JP, Rosenberg L. Recent patterns of medication use in the ambulatory adult population of the United States. The Slone Survey JAMA. 2002; 287:337-444.
5. Ates DA, Erdogrul OT. Antimicrobial activities of various medicinal and commercial plant extracts, Turk. J. Biol. 2003;27:157-162.
6. Mpiana PT, Tshibangu DST, Shetonde OM, Ngbolua KN. *In vitro* antidrepano-cytary activity (anti-sickle cell anaemia) of some Congolese plants. *Phytomedicine*. 2007;14:192-195.
7. Mpiana PT, Ngbolua KN, Mudogo V, Tshibangu DST, Atibu EK. The potential effectiveness of medicinal plants used for the treatment of Sickle cell Disease in the Democratic Republic of Congo folk medicine: A review. In: Progress *in* Traditional and Folk herbal medicine, Gupta VK, Singh GD, (eds), Daya Publishng House, New Delhi; 2012.
8. Mpiana PT, Masunda TA, Longomba BF, Tshibangu DST, Ngbolua KN. Anti-hyperglycemic activity of *Raphia gentiliana* De Wild. (*Arecaceae*). European Journal of Medicinal Plants. 2013;3(2):233-240.
9. Kasali FM, Kadima JN, Mpiana PT, Ngbolua KN, Tshibangu DS. Assessment of antidiabetic activity and acute toxicity of leaf extracts from *Physalis peruviana* L. in guinea-pig. Asian Pac. J. Trop. Biomed. 2013;3(11):885-890.
10. Kasali FM, Mahano AO, Bwironde FM, Amani AC, Mangambu JD, Nyakabwa DS, et al. Ethnopharmacological survey of plants used against diabetes in Bukavu city (DR CONGO). The Journal of Ethnobiology and Traditional Medicine, Photon. 2013;119:538-546.
11. Kasali FM, Mahano AO, Nyakabwa DS, Kadima JN, Misakabu FM, Tshibangu DST, et al. Ethnopharmacolical survey of medicinal plants used against malaria in Bukavu city (DR Congo). European Journal of Medicinal Plants. 2014;4(1): 29-44.
12. Ngbolua KN, Benamambote BM, Mpiana PT, Muanda DM, Ekutsu E, Tshibangu DST, et al. Ethno-botanical survey and Ecological Study of some Medicinal Plants species traditionally used in the District of Bas-Fleuve (Bas-Congo Province, Democratic Republic of Congo), Research Journal of Chemistry. 2013;1(2):1-10.
13. Ngbolua KN, Modogo V, Mpiana PT, Malekani MJ, Rafatro Herintsoa, Urverg Ratsimamanga S, et al. Evaluation de l'activité anti-drépanocytaire et antipaludique de quelques taxons végétaux de la République démocratique du Congo et de Madagascar, Ethnopharmacologia. 2013;50:7-12.
14. Ngbolua KN, Mpiana PT, Mudogo V, Ngombe NK, Tshibangu DST, Ekutsu E, et al. Library search, ethno-pharmacological survey and Floristical study of some medicinal plants traditionally used to treat Infectious and parasitic pathologies in the Democratic Republic of Congo. The Journal of Medicinal Plants, Photon. 2014;106:454-467.

15. Katemo M, Mpiana PT, Mbala BM, Mihigo SO, Ngbolua KN, Tshibangu DST, et al. Ethnopharmacological survey of plants used against diabetes in Kisangani City (D.R. Congo). J. Ethnopharmacol. 2012; 144:39-43.
16. Atsafack SS, Kuiate JR, Mouokeu RS, Koanga Mogtomo MLK, Tchinda AT, Tamokou JD, et al. Toxicological studies of stem bark extract from *Schefflera barteri* Harms (Araliaceae). BMC Complementary and Alternative Medicine. 2015;15:44. DOI: 10.1186/s12906-015-0581-z
17. Barnes J. Quality, efficacy and safety of complimentary medicines: Fashions, facts and the future. Part 1. Regulation and quality. J. Clin. Pharmacol. 2003;55: 226-33.
18. Kripa KG, Chamundeeswari D, Thanka J. Acute and sub-acute toxicity evaluation of ethanolic extract of *Leucas aspera* (Lamiaceae) in experimental rats. Int. J. Drug Development Res. 2011;3(3):339-347.
19. Haja SS, Vedhaiyan N, Sengottuvelu Singaravel S. Acute and sub-acute toxicity studies of some indigenous medicinal plants. Int. J. Pharm. Phytopharmacol. Res. 2013;3(2):166-169.
20. Ramírez JH, Palacios M, Tamayo O, Jaramillo R, Gutiérrez O. Acute and subacute toxicity of *Salvia scutellarioides* in mice and rats. J. Ethnopharmacol. 2007; 109:348- 353.
21. Goudah A, Abo-El-Sooud K, Yousef MA. Acute and subchronic toxicity assessment model of *Ferula assa-foetida* gum in rodents. Veterinary World. 2015;8:584-589.
22. WHO. Specifications for identity and purity and toxicological evaluation of food colours. WHO/Food Add/66.25 Geneva WHO; 1966.
23. Mbaka GO, Adeyemi OO, Oremosu AA. Acute and sub-chronic toxicity studies of the ethanol extract of the leaves of *Sphenocentrum jollyanum* (Menispermaceae). Agric. Biol. J. N. Am. 2010;1(3):265-272.
24. Hodge HC, Sterner JH. Determination of substance acute toxicity by LD50. American Industrial Hygien Association 1943;10:93.
25. Mpiana PT, Mudogo V, Tshibangu V, Kitwa EK, Kanangila AB, Lumbu JBS, et al. Antisickling activity of anthocyanins from *Bombax pentadrum, Ficus capensis, Zizyphus mucronata*: Photo-degradation effect. Journal of Ethnopharmacology. 2008;120:413-418.
26. Mpiana PT, Mudogo V, Tshibangu DST, Ngbolua KN, Shetonde OM, Mangwala PK, Mavakala BK. *In vitro* antisickling activity of Anthocyanins extracts of a Congolese plant: *Alchornea cordifolia M.* Arg. Journal of Medical Sciences. 2007;7(7):1182-1186.
27. Ngbolua KN, Herintsoa R, Hajatiana R, Mudogo V, Tshilanda DD, Tshibangu D ST and Mpiana PT. *In vitro* anti-erythrocyte sickling effect of lunularic acid of natural origin. International Blood Research & Reviews. 2015;4(3):1-6.
28. Mpiana PT, Misakabu FM, Tshibangu DST, Ngbolua KN, Mwanangombo DT. Antisickling activity and membrane stabilization effect of anthocyanins extracts from *Adansonia digitata* L. barks on sickle blood cells. International Blood Research and Reviews. 2014;2(5):198-212.
29. Mpiana PT, Misakabu FM, Kintadi JM, Ngbolua KN, Tshibangu DST, Tsalu PV, et al. Antisickling activity and physic-chemical stability of anthocyanin extracts from *Hypoxis angustifolia* Lam bulbs (Hypoxidaceae). in: Noboru M, Editor, Anthocyanins: Occurrence, structure, biosynthesis and health benefits based on their evidences of medicinal phytochemicals in vegetables and fruits. Nova Science Publishers, Inc, Yew York; 2014.
30. Tshilanda DD, Mpiana PT, Onyamboko DNV, Mbala BM, Ngbolua KN, Tshibangu, DST et al. Antisickling activity of butyl stearate isolated from *Ocimum basilicum* (Lamiaceae). Asian Pac J Trop Biomed. 2014;4(5):393-398.
31. Ngbolua KN, Mpiana PT, Tshibangu DST, Mazasa PP, Gbolo BZ, Atibu EK, Kadima JN, Kasali FM. *In vitro* antisickling and radical scavenging activities of a poly-herbal formula (Drepanoalpha®) in sickle cell erythrocyte and acute toxicity study in Wistar albino rats. European Journal of Medicinal Plants. 2014;4(10):1251-1267.
32. EEC Directive. Guide for the care and use of laboratory animals; 1986. Accessed on 18 November, 2015. Available:http://ec.europa.eu/food/fs/aw/aw_legislation/scientific/86-609-eec_en.pdf
33. Roussel M, Davis BH, Fest T, Wood BL. International Council for Standardization in Hematology (ICSH). Toward a reference

method for leukocyte differential counts in blood: Comparison of three flow cytometric candidate methods. Cytometry A. 2012; 81(11):973-982.
34. Craig SB, Smythe LD, Graham GC, Burns MA, McMahon JL, Dohnt MF, et al. Haemoglobin and red cell counts in leptospirosis patients infected with different serovars. Rev. Soc. Bras. Med. Trop. 2013;46(2):237-240.
35. Nagaraja P, Avinash K, Shivakumar A, Krishna H. Quantification of creatinine in biological samples based on the pseudoenzyme activity of copper-creatinine complex. Spectrochim. Acta. A. Mol. Biomol. Spectrosc. 2012;92:318-324.
36. Wilson AB. Experimental design. In: Anderson D, Conning DM, editors. Experimental toxicology: The basic principles. 2nd ed. Cambridge: The Royal Society of Chemistry; 1990.
37. Eaton DL, Klaasen CD. Principles of toxicology. In: Klaasen CD, editor. Casarett and Doull's toxicology: the basic science of poisons. New York, NY: McGraw-Hill; 2001.
38. Gadaga LL, Tagwireyi D. Critical review of the guidelines and methods in toxicological research in Africa. In: Kuete V, edotor. Toxicological survey of African Medicinal plants. Elsevier, First Edition; 2014.
39. Lameire N, Van Biesen W, Vanholder, R. Acute renal failure. Lancet. 2005; 365(9457):417-430.
40. Ozer J, Ratner M, Shaw M, Bailey W, Schomaker S. The current state of serum biomarkers of hepatotoxicity. Toxicology. 2008;245(3):194-205.

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