International Journal of Medical Science and Clinical Invention 5(01): 3392-3399, 2018
DOI:10.18535/ijmsci/v5i1.03 **ICV 2016: 77.2**
e-ISSN:2348-991X, p-ISSN: 2454-9576

Research Article

Effect of dihydroartemisinin and its disulphide derivative on biochemical, histological and hematological parameters in rat.

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Abstract:

Introduction: The disulphide derivative of dihydroartemisinin was synthesized and confirmed of being effective against Plasmodium berghei berghei, some gramme positive bacteria and fungi.

Objective: The study was designed to compare the effect of the synthesized disulphide derivative of dihydroartemisinin with pure dihydroartemisinin on biochemical, histological and hematological parameters.

Method: Doses of 10%, 20% and 30% of the LD_{50} of pure dihydroartemisinin and its disulphide derivatives were orally administered to each group of rat for 14 days. On the 14th day, the rats were sacrificed, and blood collected by cardiac puncture was used for hematological and biochemical studies. The liver and kidney were removed, weighed and preserved in 10% formalin for histopathology studies.

Results: Using Lork's method, the LD_{50} for pure dihydroartemisinin was 547.72 mg/kg while the disulfide derivative was 346.41mg/kg. There was a significant difference ($p<0.05$) in the concentrations of hematological parameters in treated rats from those of the control group. There was a dose dependent increase in the levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in the treated groups. The cells of the liver and kidney were slightly affected by all the doses of dihydroartemisinin and moderately by the disulphide derivative.

Conclusion: The toxicity of dihydroartemisinin (DHA) and its disulphide derivative (SDHA) are almost the same, making the SDHA not too toxic for animals. The effect of SDHA on the liver, kidney and hematological parameters are more than those of DHA, however they are within tolerated range. Clinical and therapeutic effectiveness should determine the comparative advantages of the newly synthesized SDHA over DHA and vise versa.

Key words: Dihydroartemisinin, Disulphide derivative, Biochemical, Hematological, Organ integrity.

INTRODUCTION

Some enzymes act as indicators of disease states. Enzyme levels in the serum or plasma form an integral part of diagnosis. Increase in the level of enzyme in plasma is indicative of cell damage. Liver disease is the most important

cause of increased alanine aminotransferase (ALT) and aspartate aminotrasferase (AST) activity. In hepatocellular injury or necrosis, there is leakage of cytoplasmic enzymes into the systemic circulation and as such the level of the enzymes in the plasma in higher [1, 2]. Creatinine derived from creatine and phosphocreatine is a major constituent of muscles. When creatinine is released from the muscle into plasma, it is excreted almost exclusively by the kidney through glomerular filtration process. It is neither re-absorbed, secreted, synthesized nor metabolized by the kidney hence the clearance of creatinine is equal to the glomerular filtration rate (GFR). A decrease in the GFR would result in an increase in the plasma creatinine concentration, thus the determination of the plasma creatinine concentration is used in the clinical evaluation of patients with suspected renal disease [3, 4].

Toxicological screening is very important for the development of new drugs and for the extension of the therapeutic potentiasl of existing molecules. Toxicity tests are mostly used to examine specific adverse effects or specific end points [5].

Acute toxicity studies in animals are usually necessary for any pharmaceutical intended for human use. It is the toxicity produced by a pharmaceutical when it is administered in one or more doses during a period not exceeding 24 hours. In acute toxicity testing, single escalating doses are given to small groups of animals and the animals are observed for overt effects and mortality and the Median Lethal Dose (LD_{50}) is calculated [6]. The information obtained from these studies is useful in choosing doses for repeated-dose studies and providing preliminary identification of target organs of toxicity [7].

Repeated-dose toxicity studies are conducted to screen for potential adverse effects of compounds such as pharmaceuticals, pesticides, food additives, or other chemicals using laboratory animals as surrogates for the intended exposed population or target species, most often the human. Repeated-dose studies may be of varying duration, generally 2–4 weeks for sub-acute studies, 3 months for sub-chronic studies, and 6–12 months for chronic studies. Many parameters indicative of the health of the test species are monitored in short-term, sub-chronic, and chronic toxicity studies, resulting in the ability to detect a variety of adverse effects. The doses are selected on the basis of ED_{50} and LD_{50} [8].

Sub-acute toxicity testing is carried out for a minimum of 14 days. The test substance is administered regularly at a specific time, using rodent of any gender and age 5–6 weeks. Baseline parameters such as the behavioural and biochemical parameters of the animals are recorded for use in calculating percentage changes. At the end of the study, tissues from most of the organs are harvested, and histological changes are recorded [9].

Artemisinin and its semi-synthetic derivatives artesunate and dihydroartemisinin (DHA) are frontline drugs recommended by the world health organization (WHO) for use in the treatment of all forms of malaria affecting humans [10, 11] The drugs have the fastest rate of *plasmodium* elimination, and the relieve of malaria symptoms[12] , however the main disadvantage is the short systemic half life and recrudescence hence the recommendation of artemisinin combination therapy (ACT) by the World Health Organisation [11] .

Structural modification of artemisinin and its derivatives produces compounds with diverse therapeutic effect [13,14,15]. The disulphide derivative of dihydroartemisinin (SDHA) was synthesized and reported of having antiplasmodial activity similar to that of pure DHA *in vivo* using rats infected with *Plasmodium berghei berghei* [16]. In another report, the disulphide derivative of dihydroartemisinin (SDHA) was effective against some gramme positive bacteria and fungi [17].

Since the disulphide derivative of dihydroartemisinin (SDHA) has antiplasmodial, antimicrobial and antifungal activities, this studies was designed to compare the effect or otherwise of the newly synthesized SDHA with that of pure DHA on some liver enzymes, creatinine levels, liver and kidney cells integrity as well as hematological parameters during administration of the drugs at low, medium and therapeutic doses (10%, 20% and 30% of LD_{50}).

Materials and Methods

Chemicals: All chemicals used in the study were of analytical grade. Pure dihydroartemisinin was a gift from May and Baker Plc. Lagos, Nigeria. All the reagents were purchased from Sigma Aldrich – Germany or BDH chemical pool England, through their Nigerian representatives.

Animals: Adult virgin Swiss albino mice (20-25g) were used for acute toxicity study and 35 adult wistar albino rats of both sexes (113-120g) were used in the sub-acute toxicity studies. They were maintained under standard environmental condition and had free access to food and water at the animal house of the University. Permission for animal studies was obtained from the animal ethics committee of the University.

Synthesis of Sesquiterpene Lactol Endodisulphide (SDHA): DHA powder was dissolved in chloroform and the solution reduced with hydrogen gas generated *in situ* by the reaction of zinc dust and hydrochloric acid. The chloroform phase was separated, allowed to dry and dissolved in dimethyl sulphoxide. Hydrogen gas was bubbled into the solution to form the disulphide derivative solution which was freeze dried to obtain yellowish white powder (SDHA) [16, 17].

Determination of LD_{50}: Acute toxicity study was carried out as using Lork's method [6]

Experimental design: The experimental animals were divided into seven groups (A-F), with five rats in each group. Group A was control and were given 5.0ml of the vehicle only. Group B, C and D were given 10%, 20% and 30% of LD_{50} of DHA while groups E, F and G were given 10%, 20% and 30% of the LD_{50} of SDHA in mg/kg body weight. The treatment for control and the experimental animals lasted for 14 days. On the 14^{th} day of the experiment, the animals were sacrificed under chloroform anesthesia, two hours after administration of the last doses. Blood was collected by cardiac puncture. Half of the blood was placed into dry herparinized tubes for estimation of hematological indices while the other half was placed in plain centrifuge tubes, and allowed to stand for 30 minutes at room temperature. The blood was centrifuged for 10 minutes at 3000rpm and the supernatant serum was aspirated into plain sample bottles for estimation of biochemical parameters.

The liver and kidney were immediately harvested after blood collection. They were dried, weighed and preserved in 10% formalin for histopathological studies using Haematoxylin and Eosin staining technique [18].

Analysis of serum: The prepared serum was analyzed using BS – 120 Chemistry Analyzer after mixing the serum with different diagnostic kits and incubating at 37^{o}C for time depending on the compound analyzed, following the methods as outlined in Clinical Chemistry Solutions Product List Mindray Brochure. The compounds were measured as follows;

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using ALT and AST diagnostic kit according to International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) without pyridoxal phosphate activation[19,20]. The absorbance was read at 340nm against sample blank which automatically recorded the concentration of ALT and AST in international unit (U/L). Creatinine was measured with creatinine diagnostic kit using modified Jaffe method[19] . The absorbance was measured at 510nm and creatinine concentration was given in µmol/L [3, 4].

Hematological assay: Packed cell volume (PCV), red blood cell (RBC) white blood cell (WBC), hemoglobin, lymphocytes and neutrophils were estimated using standard methods [22]. PCV was measured using microchematocrit method of Coles 1974 [23]. Hemoglobin and WBC count were determined as described by Jain [24].

Statistical analysis: The results of the biochemical and hematological estimations were reported as mean ± SD of mean. Total variations present in a set of date were estimated by one way analysis of variance (ANOVA) followed by the analysis of level of significance between different groups based on ANOVA and Students t-test. Difference among means were determined at 95% confidence level ($p<0.05$).

Results

In the toxicity study, using Lork's method, LD_{50} for DHA was 547.72mg/kg, while SDHA was 345.26mg/kg.

In the sub-acute toxicity studies, intraperitoneal administration of DHA and disulphide derivative SDHA did not produce any physical nor behavioural changes. There were no toxic signs or mortality during the experimental period. During the dosing period and in the last day, the quantity of food and water intake by different dose groups were comparable with the control group. No abnormal deviations were observed. All rats showed significant increase in body weight compared to their initial values. There was no significant difference between the different treatment groups and the control, indicating that DHA and SDHA did not have serious adverse effects on the body weight, which is used to assess the response to the therapy of drug (Table 1).

The weights of organs recorded did not show any significant differences in the treatment and the control group indicating that both DHA and SDHA were not toxic to both liver and kidney (Table 1).

The hematological profile of treated and control group are summarized in Table 2. The results showed that all haematological parameters: red blood cell (RBC) count, white blood cell (WBC) count, differential leukocyte count, platelet count, hemoglobin, packed cell volume (PCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) of the treated rats were significantly different from those of the control group.

Table 1: Effect of Dihydroartemisinin (DHA) and the disulphide derivative (SDHA) on body and organ weights in experimental rats and control.

DOSE	BODY WEIGH (g)			ORGAN WEIGHT (g)	
	DAY 0	DAY 7	DAY 14	LIVER	KIDNEY
CONTROL	114.17±18.34	116.17±18.34	124.17±18.34	6.4±0.45	0.9±0.05
DHA-LD	113.83±13.85	115.83±13.85	123.83±13.85	6.35±0.05	0.9±0.10
DHA-MD	112.83±16.53	114.83±16.53	122.83±16.53	7.65±0.45	0.8±0.23
DHA-HD	109.67±12.57	111.67±12.57	119.67±12.57	5.95±0.05	1.01±0.15
SDHA-LD	101.83±8.35	103.83±8.35	111.83±8.35	5.55±0.05	0.89±0.11
SDHA-MD	93.67±7.91	95.67±7.91	103.67±7.91	5.20±0.11	0.75±0.15
SDHA-HD	102.17±11.28	104.17±11.28	112.17±11.28	6.5±0.30	0.87±0.10

Values are expressed as mean ± SD, n=5, $p<0.05$.
LD low dose 10%, MD medium dose 20%, HD high dose 30% of LD_{50}

Table 2: Effects of Dihydroartemisinin (DHA) and disulphide derivative (SDHA) on hematological parameters of the experimental rats and control.

HAEMATOLOGICAL PARAMETERS	CONTROL	DHA-LD	DHA-MD	DHA-HD	SDHA-LD	SDHA-MD	SDHA-HD
WBC (x 10^3/µL)	5.58±0.77	7.95±1.07*	7.63±0.90*	6.25±0.93*	8.68±0.35*	8.51±0.27*	7.75±0.31*
NEUTROPHILS (x 10^3/µL)	1.33±0.22	1.35±0.17*	1.29±0.44*	1.25±0.01*	2.48±0.30*	2.38±0.37*	2.17±0.33*
LYMPHOCYTES (x 10^3/µL)	4.97±0.93	7.49±0.21*	6.30±0.23*	6.25±0.08*	8.39±0.47*	7.86±0.48*	7.26±0.24*
MONOCYTES (x 10^3/µL)	0.07±0.02	0.16±0.12*	0.10±0.23*	0.08±0.15*	0.14±0.01*	0.14±0.03*	0.12±0.01*
EOSINOPHILS (x 10^3/µL)	0.03±0.01	0.10±0.12*	0.18±0.11*	0.18±0.32*	0.23±0.03*	0.22±0.03*	0.21±0.02*
BASOPHILS (x 10^3/µL)	0.03±0.04	0.22±0.01	0.10±0.03	0.10±0.04	0.23±0.04*	0.12±0.04*	0.1±0.01
RBC (x 10^6/µL)	7.75±0.23	5.63±0.79*	5.15±0.86*	4.65±0.97*	4.42±1.39*	3.93±0.25*	4.13±0.14*
HAEMOGLOBIN (g/dL)	14.48±0.91	15.90±1.07*	11.52±1.33*	10.46±1.28*	10.18±0.65*	10.29±0.60*	12.56±0.54*
MCHC (g/dL)	34.75±0.9	13.23±1.66*	16.13±2.17*	19.12±2.05	14.99±1.35*	17.8±3.16*	27.46±5.62
MCH (pg)	18.88±1.08	10.15±1.09*	13.43±0.78*	15.16±0.09*	11.13±0.26*	11.39±0.33*	13.37±0.60*
PCV (%)	40.25±4.82	36.15±2.63*	40.85±2.17*	44.92±2.15*	23.75±4.97*	28.40±3.01*	30.67±2.36*
PLATELETS (x 10^3/µL)	761.5±77.5	542±70.45*	535±65.87*	633±26.47*	447.5±69.78*	469.8±67.77*	535±25.39*

Values are expressed as mean ± SD, n=5, * significant $p<0.05$.

The biochemical parameters of treated and control group showed that there was a significant increase in the levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in the treated group when compared to control groups, the levels of creatinine was however not significantly affected Table 3

Histopathological examination of the liver and kidney in the control and the treated groups showed differences which

were classified as; not affected, slightly affected, moderately

affected and severely affected, figure 1 and Tables 4 and 5.

Table 3: Comparative Effect of different doses of Dihydroartemisinin (DHA) and disulphide derivative (SDHA) on biochemical parameters of treated and control rats

TREATMENT GROUPS	PARAMETERS		
	AST	ALT	CREATININE
CONTROL	27.70±2.20	14.91±1.31	0.68±0.02
DHA-LD	28.66±2.49	26.62±2.76	0.85±0.01
DHA-MD	41.39±2.78	37.58±2.86	1.08±0.21
DHA-HD	62.64±3.75	51.62±2.77	1.14±0.19
SDHA-LD	76.97±0.73	33.43±0.92	0.71±0.05
SDHA-MD	76.74±0.54	33.98±0.92	0.65±0.07
SDHA-HD	76.81±0.83	33.20±0.68	0.68±0.06

Values are expressed as mean ± SD, n=5, $p<0.05$.

Table 4: Histopathological examination of liver treated with different doses of Dihydroartemisinin (DHA), its disulphide derivative (SDHA) and control.

TREATMENT GROUPS	OBSERVATIONS [at magnification C (x100) and D(x400)]	CONCLUSION
CONTROL	Normal cellular profile of Portal triad, Bile duct, Hepatic Artery, Hepatic vein & Hepatocytes. Nucleus within normal cellular architecture. (Figure 1-D)	Not affected
DHA-LD	Area of inflammation and pyknotic nucleus as compared to control group.	Slightly affected
DHA-MD	Area of inflammation, Vacuolation, Hepatocytic hyperplasia and Pyknotic nucleus as compared to control group.	Slightly affected
DHA-HD	Area of inflammation,Vacuolation,Hepatocytic hyperplasia and Pyknotic nucleus as compared to control group.(Figure 1-E)	Slightly affected
SDHA-LD	Area of inflammation, Vacuolation, Hepatocytic hyperplasia and Pyknotic nucleus as compared to control group.	Slightly affected
SDHA-MD	Area of inflammation, Vacuolation, Hepatocytic hyperplasia and Pyknotic nucleus as compared to control group.	Moderately affected
SDHA-HD	Area of inflammation, Vacuolation, Hepatocytic hyperplasia, Cellular degeneration and Pyknotic nucleus as compared to control group. (Figure 1-F)	Severely affected

Table 5: Histopathological examination of kidney treated with different doses of Dihydroartemisinin (DHA), its disulphide derivative (SDHA) and control.

TREATMENT GROUPS	OBSERVATIONS [at magnification C (x100) and D(x400)]	CONCLUSION
CONTROL	Normal cellular profile of proximal and distal convoluted tubules, adipocytes, collecting ducts, renal corpuscle containing glomerulus and lined with squamous epithelial within normal cellular architecture (Figure 1-A).	Not affected
DHA-LD	Normal cellular profile, no cellular abnormality seen as compared with control group	Not affected
DHA-MD	Normal cellular profile, no abnormality seen as compared with control group.	Not affected
DHA-HD	Slight area inflammation, Glomerular inflammation, vascular degeneration and numerous pyknotic nucleus as compared with control group (Figure 1-B).	Slightly affected
SDHA-LD	Slight area inflammation and numerous pyknotic nucleus, no cellular abnormality seen as compared with control group.	Slightly affected
SDHA-MD	Slight area of inflammation, Glomerular inflammation, vascular degeneration and numerous pyknotic nucleus as compared with control group.	Moderately affected
SDHA-HD	Mild area inflammation, Glomerular inflammation, vascular degeneration, congestion and numerous pyknotic nucleus as compared with control group (Figure 1-C).	Moderately affected

Discussion

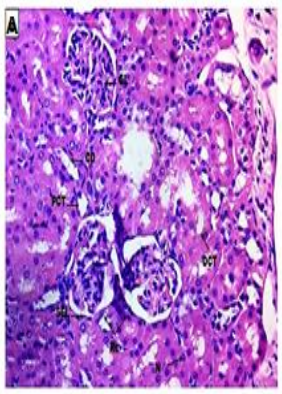
The purpose of this study was to compare the acute and sub-acute toxicity profile of dihydroartemisinin, a known antimalarial, with its synthesized disulphide derivative. The organization for economic cooperation and development (OECD) panel of experts defined acute toxicity as "the adverse effect occurring within a short time of administration of a single dose of a substance or multiple doses given within a span of 24 hours. The purpose of acute toxicity studies is to determine the LD_{50} values which help in determining the safe

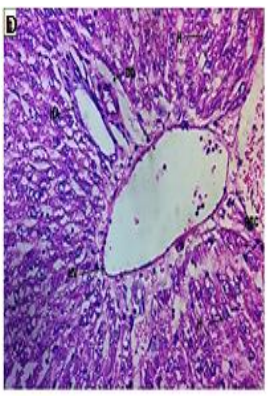
dose range at which the drug can be used such that there is no harmful or lethal effect on the animal, as well as the therapeutic index [25, 26]. In these studies, LD_{50} of DHA was 547.72mg/kg while SDHA was 345.26mg/kg, indicating that DAH has a better safety margin than SDHA.

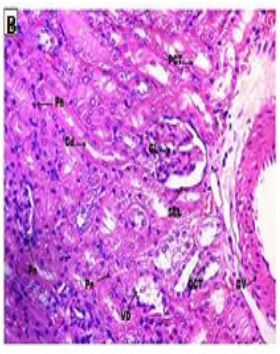
Sub-acute study, gives valuable information on the cumulative toxicity of a substance on target organs or physiological and metabolic effects of the compound at low and therapeutic dose on prolonged exposure. In the sub-acute toxicity test, the dose was selected based on the LD_{50} value of DHA and SDHA respectively (i.e. 10% LD_{50} for low dose, 20% LD_{50} for medium dose and 30% LD_{50} for high dose). In the sub-acute toxicity study, there were no deaths and treatment-related toxicity signs in all the treatment groups of animal.

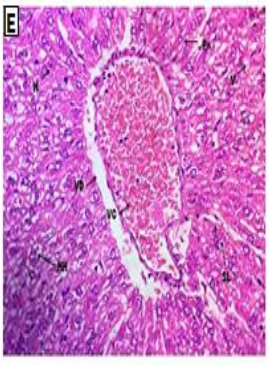
Body weight changes are markers of adverse effects of drugs and chemicals and if the body weight loss occurrence is more than 10% of the initial body weight it is considered statistically significant [27, 28]. In this study all rats showed significant increase in body weight compared to their initial values. There was no significant difference between the different treatment groups and the control, indicating that the DHA and SDHA did not have adverse effects on the body weight.

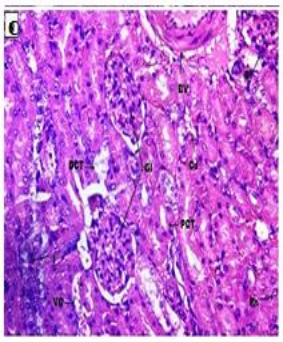
Organ weight also is an important indicator of physiological and pathological status of animals. The relative organ weight is fundamental to confirm whether an organ was exposed to injury or not. The heart, liver, kidney, spleen and lungs are the primary organs affected by metabolic reaction caused by toxicant [26]. The results of this study showed that dihydroartemisinin and its disulphide derivative in different doses administered for 14 days does not have a significant effect on the liver and kidney weight compared to control, this is an indicator of safety of dihydroartemisinin and its disulphide derivatives.

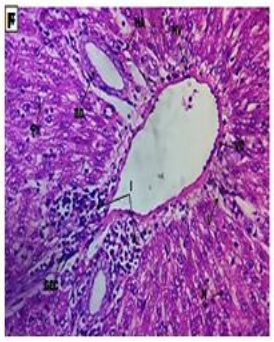












In this study there was significant difference in the haematological parameters of all the treated groups as compared with their respective control groups. Dihydroartemisinin and its disulphide derivative caused significant ($p<0.05$) dose-dependent increase in WBC, neutrophil, lymphocyte, monocyte, eosinophil and basophile counts compared with control, and a dose-dependent decrease in RBC, Haemoglobim, MCHC, MCH, PVC, and Platelet counts. These results suggest that DHA and SDHA may have caused dose-dependent lyses of blood cells and/or inhibition in blood cells synthesis in the treated rats, these was more in the SDHA than DHA [31].

The liver enzymes AST and ALT, are considered markers of liver function. Hepatocellular damage is characterized by a mutual rise in serum levels of AST and ALT. ALT is localized primarily in the cytosol of hepatocytes, this enzyme is considered a more sensitive marker of hepatocellular damage than AST and within limits can provide a quantitative assessment of the degree of damage sustained by the liver [32]. With DHA, there was a dose dependent increase in the levels of AST, while with ALT the increase was not dose dependent, but were all within safety limits suggesting that both drugs are not too toxic for the liver Table 3.

In preclinical toxicity studies, renal changes are particularly liable to occur because of the high doses given and the fact that the kidneys eliminate many drugs and their metabolites [32]. In the present study, creatinine determination was the marker of kidney function. There was no significant differences in serum levels of creatinine in the dihydroartemisin and its disulphide derivative-treated groups compared to control.

Histopathological studies provide supportive evidence for biochemical and hematological observations. The liver is the second largest organ in the body involved in a host of functions including synthesis of clotting factors, detoxification and metabolism of drugs, lipid and carbohydrates. Substantial disruption in its anatomy or function may result in severe alteration in its metabolic roles, and this may adversely affect physiological functions. Release of active oxygen species from dihydroartemisinin bonded with hemoglobin kills the malaria parasite accumulated in the erythrocytic cells. Dihydroartemisinin may indirectly through generation of ROS or directly as toxin to the cells of the liver, affect their cellular integrity and cause defect in membrane permeability and cell

volume homeostasis. In humans hepato-toxicity can be particularly severe if dihydroartemisinin is used in combination with HIV antiretroviral drugs [25]. Histopathological examination of the liver showed that the organ was slightly affected by all dose levels of Dihydroartemisinin when compared to the control group, whereas the effect of Sulphide-substituted Dihydroartemisinin was observed to be dose-dependent (i.e. inflammation and cellular degeneration increased as the dose of the drug increased).

Histopathological examination of the kidney showed little or no effect of Dihydroartemisinin at all dose levels on the kidney, but the diulphide-substituted Dihydroartemisinin had a moderate effect on the kidney. Nevertheless the lack of marked changes in serum level of creatinine implies that the histopathological changes seen were not significant enough to affect kidney function and may be considered as clinically insignificant.

Conclusion: The toxicity of DHA and SDHA are almost the same, making the SDHA not too toxic for animals. The effect of SDHA on the liver, kidney and hematological parameters are more than those of DHA, however they are within tolerated range. Clinical and therapeutic effectiveness as well as stability of the products should determine the comparative advantages of the newly synthesized SDHA over DHA and *vise versa*.

Acknowledgement:

The authors are grateful to Mr. Nsikak Malachy, laboratory technologist, of the animal house of the department of Pharmacology and Toxicology, University of Uyo and Mrs. Ekaete Umoh, the Chief Technologist of the Department of Pharmaceutical and Medicinal Chemistry, University of Uyo for their technical assistance.

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