

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20

Original Research Article
In-vivo Anti-Tumor Evaluation of
Dihydroartemisinin-Derived Endodisulphide on
MNU-Induced Liver Cancer in Sprague-Dawley Rats

Imoh Emmanuel Udoh¹, Jonah Sydney Aprioku^{2*}, IyeopuMinakiri Siminialayi³

¹ Department of Clinical Pharmacy and Biopharmacy, Faculty of Pharmacy, University of Uyo, Uyo, Nigeria

² Department of Experimental Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, University of Port Harcourt, Port Harcourt, Nigeria

³ Department of Pharmacology, Faculty of Basic Medical Sciences, University of Port Harcourt, Port Harcourt, Nigeria

21 * Corresponding author

22 Email: svdaprio@yahoo.com

23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
ABSTRACT

54 **Aims:** The study evaluated the antitumor potentials of a disulphide-substituted derivative of
55 dihydroartemisinin (sDHA) on chemically induced cancer of the liver in Sprague-Dawley rats in
56 comparison with its parent compound, dihydroartemisinin (DHA) and a standard anticancer drug.

57 **Study Design:** Animals are divided into ten groups (n=10 per group), and cancer is induced in all
58 followed by administration of experimental agents, vehicle or distilled water.

59 **Place and Duration of Study:** Study was done in Faculty of Pharmacy, University of Uyo, Nigeria in
60 2015-2016.

61 **Methodology:** *N*-methy-*N*-nitrosourea, MNU (50 mg/kg) was administered intravenously as single
62 dose to induce cancer in animal groups, followed by oral treatment with sDHA (37.42, 74.83 or 112.25
63 mg/kg/day), DHA (57.45, 114.89 or 172.34 mg/kg/day), cyclophosphamide (0.71 mg/kg/day) or Tween
64 30 (0.3 ml/day) for 28 days. Control group received distilled water (0.3 ml/day), while a group received
65 only MNU (positive control). Drug treatments commenced 10 days after MNU injection and animals
66 were observed for 52 days after drug treatments and sacrificed. Serum levels of CA-27-29, 8-OHdG
67 and SOD were measured using ELISA method; and using immunohistochemical tissue staining
68 techniques, Bcl-2 and Ki67 protein expressions were analyzed in hepatic cells.

69 **Results:** MNU caused elevation ($P < .0001$) in CA-27-29 and 8-OHdG; and reduction ($P < .0001$) in
70 SOD. Hepatic cells of MNU alone treated rats demonstrated strong immunoreactivity for Bcl-2 and
71 Ki67 ($\geq 75\%$). Oral treatments of sDHA or DHA resulted in dose-dependent reductions of MNU-
72 induced CA-27-29 and 8-OHdG elevations ($P < .001$) but had no effect on SOD. Additionally, MNU
73 induced Bcl-2 and Ki67 positive immunoreactive expressions were reduced to 25-50% by sDHA and
74 DHA; DHA showing greater effect. Cyclophosphamide reversed all the MNU induced toxic effects.

75 **Conclusions:** sDHA possesses antitumor activity against liver cancer; has lesser efficacy than DHA,
76 but both drugs are less effective than cyclophosphamide.

77 **Keywords:** *Artemisinin, Bcl-2, CA-27-29, cyclophosphamide, immunohistochemistry*

78

79

80 1. INTRODUCTION

81 Cancers are among the leading causes of morbidity and mortality globally[1]. Different treatment
82 approaches exist for the disease, but chemotherapy (the use cytotoxic drugs) remains the first line of
83 treatment for many cancers[2,3]. Although, standard anticancer drugs can produce good responses
84 and survival, many unfortunately, are expensive, require frequent administration and cause serious

85 adverse effects (especially in combination). Thus, the search for anticancer drugs that are affordable
86 with high safety profiles while maintaining their efficacies continues.

87

88 Artemisinin derivatives are clinically used as antimalarial agents and have relative low host
89 toxicity[4,5]. They have equally been shown to possess potent anticancer activity against a variety of
90 human cancer models[6-8].The presence of endoperoxide group in their molecules, have been shown
91 to be responsible for this anti-cancer activity, but their short half-lives[5], may be a limitation in their
92 utilization in cancer chemotherapy. It is envisaged that superoxide dismutase (SOD) enzyme, which is
93 present in human blood, readily breaks down the pharmacophore (endoperoxide group) of
94 artemisinin via its anti-peroxide action and this is thought to contribute to artemisinin's short half-
95 lives[9]. In view of the above, it is hypothesized that structural modification of the endoperoxide ring of
96 artemisinin via substitution of the peroxide oxygen atoms with suitable atoms may resist SOD effect
97 and extend their half-lives, which has led to the synthesis of a disulphide derivative of DHA, the active
98 metabolite of artemisinin[10]. The new drug was synthesized through structural modification of DHA
99 by replacing its peroxide oxygen atoms with disulphide, or persulphide[10]. With the synthesis of this
100 new compound, it would be reasonable to evaluate its pharmacokinetic, toxicity and anticancer
101 profiles. Acute toxicity evaluation of the disulphide derivative of DHA has been done in our earlier
102 study[11]. It has also been demonstrated to exhibit remarkable dose related anti-malaria effect in rats
103 which compared favorably with DHA[10], but it is not yet known whether it would maintain or possess
104 higher anticancer activity with respect to the parent drug.

105

106 This study was therefore intended to conduct an *in-vivo* evaluation of the antitumor potential of
107 disulphide substituted derivative of DHA on *N*-Methyl-*N*-Nitrosourea (MNU) induced liver cancer in
108 Sprague-Dawley rats in comparison with the parent drug.

109

110

111 **2. MATERIALS AND METHODS**

112 **2.1 Materials**

113 **2.1.1 Equipment**

114 Microplate reader (Biotek, USA), pH meter (Denver Instrument, Model: 215, New York, USA),
115 centrifuge (Zeny Inc., Model: 800-1, Salt Lake, USA), UV/Visible spectrophotometer (Biotek, Model:

7305, Winooski, USA), rotary microtome (Leica Biosystem, Model: Leica RM 2125, Germany), wax dispenser (MH 85523B, United Kingdom), Microfield digital laboratory oven (Model: SM90234, England), automatic tissue processor (Leica TP1020, Germany), digital Olympus microscope (Model: XXT/300, Germany) and autoclave (LifesterwarePvt Ltd., Delhi, India) were among the equipment used for the study.

121

122 **2.1.2 Drugs**

123 Pure powder of dihydroartemisinin, DHA (UV spectral ultraviolet-visible absorption spectra absorption-
124 0.55, max wavelength- 270nm; GC-MS fragmentation showed a significant molecular ion of
125 284; Melting point- 146-148°C); and disulphide substituted derivative of DHA, sDHA (UV spectral
126 ultraviolet-visible absorption spectra absorption- 0.268, max wavelength, 320nm; GC-MS
127 fragmentation showed a significant molecular ion of 316; Melting point- 140-142°C) were obtained
128 from the Department of Medicinal and Pharmaceutical Chemistry, Faculty of Pharmacy, University of
129 Uyo, Nigeria.

130

131 Cyclophosphamide (Endoxan[®]) tablets (Zyus Biogen- Baxter, Mumbai, India), was purchased from
132 the Pharmacy Unit of the University of Uyo Teaching Hospital, Uyo, Nigeria.

133

134 **2.1.3 Reagents**

135 CA 27-29 assay kit (Cell Biolabs Inc., San Diego, CA 92126, USA), 8-OHdG assay kit (Cell Biolabs
136 Inc., San Diego, CA 92126, USA), SOD assay kit (Cell Biolabs Inc., San Diego, CA 92126, USA), Bcl-
137 2 assay kit (BioVision Inc. 155 South Milpitas Blvd., Milpitas, California 95035, USA), Ki67 assay kit
138 (BioVision Inc. California 95035, USA) and haematoxylin and Eosin stain kit (Sigma- Aldrich Inc. St.
139 Louis, MO 63178. USA) were among reagents used for the study.

140

141 **2.1.4 Chemicals**

142 *N*-Methyl-*N*-Nitroso urea, MNU (Sunglong Biotech Co. Ltd, Zhejiang, China), ketamine HCl (Merck,
143 Darmstadt, Germany), xylazine (Taj Pharmaceutical Limited, Mumbai, India), polysorbate (Tween®)
144 80 (Sigma-Aldrich and Merck, KGaA, Darmstadt, Germany), paraffin wax (Megha Group of
145 Companies, Meghalaya, India), TRIS-acetate-EDTA buffer (Sigma-Aldrich Inc. St. Louis, MO

63178. USA), and phosphate buffer saline (Sigma- Aldrich Inc. St. Louis, MO 63178. USA), xylene (Taj Pharmaceutical Limited, Mumbai, India) were among chemicals used for the study.

2.1.5 Animals

Healthy, pathogen free male and non-pregnant female Sprague-Dawley rats, aged 6-8 weeks old, weighing 180-200 g were used for the study. They were housed in plastic cages (3 per cage) and maintained under standard conditions (12 h light-dark cycle, and room temperature). The rats were given free access to clean water and commercial rodent chow and allowed to acclimate for two weeks before commencement of experiment. Experimental procedures were approved by the Ethics Committee on Research, University of Port Harcourt, Nigeria. Animals were handled according to standard guidelines for care and use of laboratory animals[12]

2.2 Methods

2.2.1 Experimental Design

The rats (50 females and 50 males) were randomly distributed into ten groups (labelled as groups 1-10) containing 10 animals of same sex per group. Seven groups were administered *N*-Methyl-*N*-Nitrosourea (MNU) as a single dose (50 mg/kg) through the jugular vein to induce cancer [13], and ten days afterwards treated orally with DHA (57.45, 114.89 or 172.34 mg/kg body weight), sDHA (37.42, 74.83 or 112.25 mg/kg body weight) or cyclophosphamide (0.71 mg/kg body weight) continuously for 28 days. The eighth group (positive control group) received only MNU treatment and experienced the maximal number of tumors. The ninth group (negative control) received only distilled water (0.3 ml) and generated no spontaneous tumors during the entire period of the study. The tenth group was given the vehicle of the drugs (Tween 80, 0.3 ml) and used as the experimental control group. Dosing was performed once daily between 4 and 6 pm. MNU was dissolved in physiological saline (1 % w/v) buffered to pH 5.0 with 3 % acetic acid, whereas the drugs, except cyclophosphamide were dissolved in Tween 80 (30 %). Cyclophosphamide was dissolved in distilled water. The doses of sDHA and DHA corresponded to 10, 20 and 30 % of their LD₅₀[11], while the dose of cyclophosphamide was equivalent to its therapeutic dose for cancer chemotherapy[14].

175 After the drug administrations, the rats were observed without treatment for additional 52 days. On the
176 91st day of the experiment, the animals were anesthetized with ketamine/xylazine (150/10 mg/kg, **ip**)
177 and sacrificed by cervical dislocation. Blood was collected by cardiac puncture and blood serum was
178 used to analyze specific biochemical cancer biomarkers: cancer antigen (CA-27-29), 8-
179 hydroxydeoxyguanosine (8-OHdG) and superoxide dismutase (SOD). Livers were carefully removed
180 and fixed in neutral buffered formalin for less than 24 h and immunohistochemically analyzed
181 afterwards.

182

183 **2.2.2 Measurement of Cancer Biomarkers**

184 The cancer biomarkers analyzed were- CA 27-29, 8-OHdG and SOD. Briefly, blood serum (10 µl) and
185 the sample dilution buffer (40 µl) was mixed in an antibody pre-coated micro ELISA strip plate specific
186 for CA 27-29, 8-OHdG or SOD and incubated at 37°C for 30 min. The wells of the plates were washed
187 and horseradish peroxidase (HRP)-conjugated antibody (50 µl) specific for CA 27-29, 8-OHdG or
188 SOD was added and incubated at room temperature. TMB substrate solution- chromogenic solution A
189 and B (50 µl each) was then added and incubated at 37°C for 15 min after proper mixing with sample
190 solution in wells. Finally, the reaction was terminated with 0.16M sulfuric acid (stop solution) and CA
191 27-29, 8-OHdG or SOD concentration was measured spectrophotometrically at 450 nm.

192

193 **2.2.3 Immunohistochemical Analysis (Bcl-2 and Ki67)**

194 Immunohistochemistry was performed with the following primary and secondary antibodies: (1) Bcl-2
195 primary antibodies- mouse anti-Bcl-2 monoclonal antibody, C-2, **10µg/ml** (Santa Cruz Biotechnology,
196 Santa Cruz, CA 95060). (2) Ki67 primary antibodies- mouse anti-rat Ki-67 antibody, MIB-5, **0.01**
197 **µL/ml** (Dakocytomation corporation Carpinteria, CA 94010). (3) Bcl-2 and Ki67 secondary antibodies-
198 biotinylated horse anti-mouse IgG, H+L (Vector Laboratories Inc., Burlingame, CA 94010). Tissue
199 sections of liver (5-µm) were deparaffinised in 3 changes of xylene, hydrated through graded ethyl
200 alcohol and rinsed in 3-4 changes of buffered phosphate, ending with a distilled water rinse. After
201 removing excess water, 3 % hydrogen peroxide was applied to the tissues and incubated for 5 min.
202 The slides used were previously preheated (95°C) in a target retrieval solution for 20 min, allowed to
203 cool for 20 min at room temperature, rinsed gently, and placed in Tris buffer bath for 5 min. Primary
204 antibodies for Bcl-2 or Ki67 were applied unto the tissue specimens and incubated for 10 min. Slides

205 were washed in buffer and incubated with the secondary antibodies for 10 min. Sufficient freshly
206 prepared 3,3'-diaminobenzidine (DAB) solution (the chromogen) was applied and incubated for 5 min
207 for color detection. Tissues were lightly counterstained with Mayer's haematoxylin, blued in warm tap
208 water, dehydrated through ascending grade of alcohol, cleared in xylene, cover-slipped and mounted
209 with a compatible mounting medium. Positively stained cells for Bcl-2 or Ki-67 expression were
210 identified by brown nuclear or cytoplasmic staining against light blue background, respectively.
211 Specimens were considered immunopositive when more than 10% of cells showed clear evidence of
212 immunostaining.

213

214 **2.2.4 Statistical Analysis**

215 Analysis of data was carried out using GraphPad Prism Version 6 and all data were expressed as
216 mean $\pm$ standard error of mean (mean $\pm$ SEM). Differences among treatment groups were analyzed
217 using one way analysis of variance (ANOVA) followed by Newman-Keuls Multiple Comparison Test
218 for intra group comparisons. Values were considered significant at $P < .05$.

219

220 **3. RESULTS AND DISCUSSION**

221 **3.1 Results**

222 **3.1.2 Serum Levels of Cancer Biomarkers**

223 The results showed that there were no significant differences ($P > .05$) between the serum levels of CA
224 27-29 (cancer antigen), 8-OHdG (DNA oxidative product) and superoxide dismutase (SOD) in Tween
225 80 (vehicle) treated group and distilled water treated (control) group (Figures 2-4). In addition, serum
226 levels of CA 27-29 and 8-OHdG were significantly increased ($P < .001$) in MNU-treated group of rats,
227 while SOD was decreased ($P < .001$) compared to control group (Figures 2-4).

228

229 Dihydroartemisinin (DHA) or disulphide-substituted derivative of DHA (sDHA) treated groups, the
230 serum levels of CA 27-29 and 8-OHdG were decreased ($P < .001$) compared to MNU-treated group.
231 When compared with control, the CA 27-29 value that was obtained in high dose (172.34 mg/kg) DHA
232 treated group was not significantly different, but all other values were higher (Figures 2 and 3). CA
233 27-29 and 8-OHdG concentrations were equally reduced ($P < .001$) in cyclophosphamide treated rats
234 compared to MNU alone treated rats, and the values were although, higher but not significantly

different ($P = .05$) when compared with control (Figures 2 and 3). The serum levels of SOD in all DHA or sDHA administered rats were lower ($P < .001$) than control (Figure 4). The values obtained were also not significantly different from the MNU-induced level, except in the group that received high dose DHA, which had a higher ($P < .001$) level (Figure 4). SOD level was increased in cyclophosphamide ($P = .01$) treated rats compared with MNU treated rats, but the value was lower ($P < .001$) when compared with control (Figure 4).

Further, when DHA and sDHA effects were compared, sDHA inhibition of MNU-induced serum elevations in CA 27-29 and DNA 8-OHdG was lower, mostly at the low and mid doses, $P = 0.01$, $P < .001$ (Figures 2 and 3). Also, only DHA inhibited MNU's effect on SOD, which was observed only in the high dose treated group (Figure 4). However, the effects of DHA and sDHA were significantly lower ($P < .001$) when compared with cyclophosphamide (Figures 2-4).

3.1.2 Immunohistochemistry

Liver cells of rats treated with distilled water (control) showed negative expression of Bcl-2 and Ki67 (<5 % positivity), whereas MNU treatment caused strong positive immunoreactive expressions (>75 % positivity) of Bcl-2 and Ki67 (Figures 5 and 6). Liver cells of rats that received DHA or sDHA showed positive Bcl-2 expression; reactivity obtained was <75, <50 and <25 % positivity, respectively (Figure 5). Ki67 was expressed differently in DHA and sDHA treated rats: positive expression was observed in all sDHA treated rats (reactivity <60, <50 and <50 % positivity, respectively), whereas it was positive only in rats that received low and mid doses of DHA, <25, <25 and <10 % positivity, respectively (Figure 6). In addition, the liver cells of cyclophosphamide treated rats showed negative Bcl-2 and Ki67 expressions (<5 or 10 % positivity) as shown in Figures 5 and 6.

3.2 Discussion

This study reports the antitumor potential of a newly synthesized derivative of dihydroartemisinin (DHA) in comparison with the parent drug. The new compound being investigated is a sesquiterpene disulphide substituted derivative of DHA (sDHA) believed to have an extended half-life than the parent drug. Preliminary studies on the new drug have shown that the drug exhibits an antiplasmodia effect

264 in rats which compared favorably with the parent drug[9], but its anticancer activity was yet to be
265 investigated prior to this study.

266

267 In this study, three doses (low, medium and high) of sDHA and DHA, equivalent to 10, 20 and 30 % of
268 their LD₅₀, respectively were used, while cyclophosphamide was used as a standard anticancer drug.
269 Further, *N*-Methyl-*N*-Nitrosourea (MNU) was used to induce cancer in the liver of Sprague-Dawley
270 rats in this study. This model has been used extensively to test the preventive potential of a variety of
271 chemical compounds and interventions[12,15]. Specific biochemical and immunohistochemical cancer
272 biomarkers were evaluated which included- CA 27-29, 8-hydroxydeoxyguanosine (8-OHdG),
273 superoxide dismutase (SOD) enzyme, B-cell lymphoma 2 (Bcl-2) and Ki67.

274

275 From our results, MNU induced marked elevations in serum levels of CA 27-29 and 8-OHdG and
276 reduction in SOD enzyme, indicative of cancer development in target tissues[16-19]. The
277 immunohistochemistry results showed that the liver cells of rats that received only distilled water
278 demonstrated negative Bcl-2 and Ki67 expressions (<5 % positive); whereas there were very strong
279 positive immunoreactive nuclear/cytoplasmic expressions of Bcl-2 and Ki67 (>75 % positive) in the
280 hepatic cells of MNU-injected rats without drug treatment. Bcl-2 is a protein that regulates cell death
281 (apoptosis) in the body, basically by either inducing (proapoptotic) or inhibiting (anti-apoptotic)
282 apoptosis. Bcl-2 is specifically considered an important anti-apoptotic protein and is thus classified as
283 an oncogene[20]. Ki-67 is a specific nuclear marker for cell proliferation, and overexpression is
284 frequently seen in a variety of malignant tissues[21-23]. The strong positive expressions of Bcl-2 and
285 Ki-67 in MNU treated rats therefore confirms the presence of cancer in livers of the treated rats.

286

287 sDHA or DHA treatment decreased the MNU-induced elevations of CA 27-29 and 8-OHdG dose-
288 dependently. However, both drugs failed to normalize levels of these cancer biomarkers, except CA
289 27-29 that was reduced to comparable level as control by DHA at the high dose. On the other hand,
290 the drug treatments generally produced no effect on the MNU-induced reduction of SOD, except the
291 high dose of DHA which was not sufficient to normalize SOD level. These findings indicate that DHA
292 and sDHA can ameliorate cancer development, but may have little effect on cancers predominantly
293 mediated by superoxide anion oncogenic activity, as SOD enzyme was minimally affected by both

294 drugs. The effects of the two drugs were generally comparable, although, DHA exhibited higher
295 effects at few doses. Cyclophosphamide, which was used as a reference drug in this study, produced
296 greater levels of inhibition of the MNU-induced biochemical toxicities than sDHA or DHA, and equally
297 reversed the MNU effects. Furthermore, sDHA and DHA treatments reduced Bcl-2 and Ki67
298 expressions in liver in dose-dependent manners. High dose treatment of both drugs completely
299 reversed MNU induced Ki67 expression but not Bcl-2 in liver. The results also showed that DHA
300 produced greater inhibitory activity of Bcl-2 and Ki67 expressions in the liver and may therefore be
301 more effective than sDHA. Cyclophosphamide treatment ameliorated Bcl-2 and Ki67 expressions
302 better than DHA or sDHA, as it reversed MNU induced expressions of the two cancer biomarkers.

303

304 The overall results indicate that DHA and sDHA possess ability to reduce MNU-induced tumor
305 activities in the liver, but lack recovery and may not compete favorably with cyclophosphamide in
306 efficacy in rats. However, the results are indicative of a possibility of obtaining greater effectiveness
307 using higher dose levels of the drugs. This is a potential area of future study. MNU has multiple organ
308 targets, and acts as carcinogen across species [24, 25]. Although, the target of MNU differs among
309 species, its organ targets are fairly similar in rodents [25] and the carcinogenic action of MNU on
310 liver cells in rats can be applicable for development of human disease. The present findings can
311 therefore be used to identify therapeutic interventions.

312

313 4. CONCLUSION

314 The study reveals that anticancer activity is not lost in disulphide-substituted dihydroartemisinin, a
315 new drug obtained through structural modification of dihydroartemisinin. The new drug possesses
316 potential antitumor activity against MNU-induced liver cancer. Over the dose range used, the drug has
317 lesser efficacy than the parent drug (dihydroartemisinin), and neither the new drug nor
318 dihydroartemisinin has comparable efficacy with cyclophosphamide. Future studies using higher
319 doses of the new drug is recommended.

320

321

322 ACKNOWLEDGEMENTS

323 The authors thank Mr. Sampson A. Oyebadejo for support in the immunohistochemical analysis.

324

325 **COMPETING OF INTERESTS**

326 None

327

328

329 **AUTHORS' CONTRIBUTIONS**

330 All authors contributed in the design of the study and participated in conducting the experiments.

331 Udoh managed the literature searches. Aprioku performed the statistical analysis, wrote the protocol

332 and the first draft of the manuscript. All authors read and approved the final manuscript.

333

334

335 **CONSENT**

336 Not applicable.

337

338

339 **ETHICAL APPROVAL**

340 The experimental procedures were approved by the Research Ethics Committee of University of Port

341 Harcourt, Nigeria.

342

343 **REFERENCES**

344 1. WHO- World Health Organization. Cancer; Fact Sheet No. 297. Geneva, Switzerland; 2015.

345 2. Leigh NB. Treatment paradigms for patients with metastatic non-small-cell lung cancer: first-,
346 second-, and third-line. *CurrOncol*. 2012;19(Suppl 1):S52-8.

347 3. Li F, Zhao C, Wang L. Molecular-targeted agents combination therapy for cancer: Developments
348 and potentials. *Int J Cancer* 2014;134:1257–69.

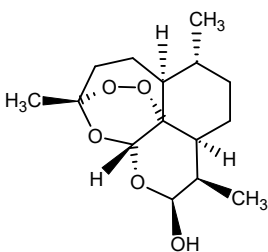
349 4. deVries, PJ, Dien TK. Clinical pharmacology and therapeutic potential of artemisinin and its
350 derivatives in the treatment of malaria. *Drugs* 1996;52(6):818-36.

351 5. Na-Bangchang KS, Krudsood U, Silachamroon P, Molunto O, Tasanor K, Chalermrut N, *et al*. The
352 pharmacokinetics of oral dihydroartemisinin and artesunate in healthy Thai volunteers. *Southeast*
353 *Asian J Trop Med Public Health* 2004;35(3):575-82.

354 6. Jiao Y, Ge CM, Meng HG, Cao JP, Tong J, Fan SJ. Dihydroartemisinin is an inhibitor of ovarian
355 cancer cell growth. *ActaPharmacol Sin*. 2007;28(7):1045–56.

- 356 7. Lu YY, Chen TS, Qu JL, Pan WL, Sun L, Wei XB. Dihydroartemisinin (DHA) induces caspase-3-
357 dependent apoptosis in human lung adenocarcinoma ASTC-a-1 cells. J Biomed Sci. 2009;16:16.
- 358 8. Crespo-Ortiz MP, Wei MQ. Anti-tumor activity of artemisinin and its derivatives: From a well-
359 known anti-malarial agent to a potential cancer drug. J Biomed Biotech. 2012;15:1705-21.
- 360 9. Marnett LJ, Riggins JN, West JD. Endogenous generation of reactive oxidants and electrophiles
361 and their reactions with DNA and protein. J Clin Invest. 2003;111:583-93.
- 362 10. Bassey UB. Synthesis, characteristics and antimalarial study of the deoxy and disulphide
363 derivatives of dihydroartemisinin. M.Sc Thesis, Department of Pharmaceutical and Medicinal
364 Chemistry, Faculty of Pharmacy, University of Uyo, Nigeria. Supervisor: Prof. E.E. Essien; 2015.
- 365 11. Udoh IM, Aprioku JS, Siminialayi IM. Acute and sub-acute toxicity evaluation of disulphide
366 derivative of dihydroartemisinin. World JPharm Res. 2016;6(1):77-95.
- 367 12. CCAC- Canadian Council On Animal Care. The Care and Use of Farm Animals in Research,
368 Teaching and Testing. 12-15. Ottawa; 2009.
- 369 13. Parikh RR, Gildener-Leapman N, Narendran A, Lin H, Lemanski N, Bennett JA, *et al.* Prevention
370 of N-Methyl-N-Nitrosourea-Induced Breast Cancer by a-Fetoprotein (AFP)-Derived Peptide, a
371 Peptide Derived from the Active Site of AFP. Clin Cancer Res. 2005;11:8512-20.
- 372 14. BNF- British National Formulary. London, UK: BMJ Group and Royal Pharmaceutical Society;.
373 2013.
- 374 15. Verma S, Bahorun T, Singh RK, Aruoma OI, Kumar A. Effect of Aegle marmelos leaf extract
375 on N-methyl N-nitrosourea-induced hepatocarcinogenesis in Balb/c mice. Pharm Biol. 2013;51(10):
376 16. Clinton S, Beason K, Bryant S, Johnson J, Jackson M, Wilson C, *et al.* A comparative study of
377 four serological tumor markers for the detection of breast cancer. Biomed SciInstrum.
378 2003;39:408-14.
- 379 17. Valavanidis A, Vlachogianni T, Fiotakis C. 8-hydroxy-2'-deoxyguanosine (8-OHdG): a critical bio-
380 marker of oxidative stress and carcinogenesis. J Environ Sci Health C Environ
381 CarcinogEcotoxicol Rev. 2009;27:120–39.
- 382 18. Alscher RG, Erturk N, Heath LS. Role of superoxide dismutases (SODs) in controlling oxidative
383 stress in plants. J Exp Bot. 2002;53(372):1331–41.
- 384 19. Irani K, Xia Y, Zweier JL, Sollott SJ, Der CJ, Fearon ER, *et al.* Mitogenic signaling mediated by
385 oxidants in Ras-transformed fibroblasts. Science 1997;275(5306):1649–52.

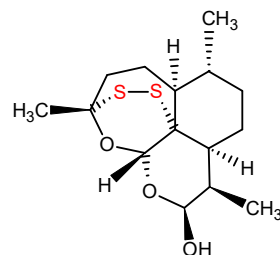
- 386 20. Cleary ML, Smith SD, Sklar J. Cloning and structural analysis of cDNAs for bcl-2 and a hybrid bcl-
 387 2/immunoglobulin transcript resulting from the (14;18) translocation. Cell 1986;47(1):19-28.
- 388 21. Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. J Cell Physiol.
 389 2000;182(3):311-22.
- 390 22. Johannessen AL, Torp SH. The clinical value of Ki-67/MIB-1 labeling index in human
 391 astrocytomas. PatholOncol Res.2006;12:143-7.
- 392 23. Stuart-Harris R, Caldas C, Pinder SE, Pharoah P. Proliferation markers and survival in early
 393 breast cancer: a systematic review and met-analysis of 85 studies in 32,825 patients. Breast
 394 2008;17:323-34.
- 395 24. Swann PF, Magee PN. Nitrosamine-induced carcinogenesis: The alkylation of nucleic acids of the
 396 rat by *N*-methyl-*N*-nitrosourea, dimethylnitrosamine, dimethyl sulphate and methyl-
 397 methanesulphonate. Biochem J 1968;110:39-47.
- 398 25. Tsubura A, Lai Y, Miki H, Sasaki T, Uehara N, Yuri T, Yoshizawa K. Animal models of *N*-Methyl-
 399 *N*-nitrosourea-induced mammary cancer and retinal degeneration with special emphasis on
 400 therapeutic trials. In Vivo 2011;25:11-22.



$C_{15}H_{24}O_5$

Sesquiterpene lactol endoperoxide

Dihydroartemisinin



$C_{15}H_{24}S_2O_3$

Sesquiterpene lactol endodisulphide

Disulphide substituted derivative of dihydroartemisinin (SDHA)

Fig. 1. Chemical structures of dihydroartemisinin and its disulphide substituted derivative (Structures drawn by chem.draw format)

418
419
420
421
422
423
424
425
426
427
428
429
430
431
432
433
434
435
436
437
438
439
440
441
442
443
444
445
446
447
448
449
450
451
452
453
454
455
456
457
458
459
460
461
462
463
464
465
466
467
468
469
470
471
472
473
474

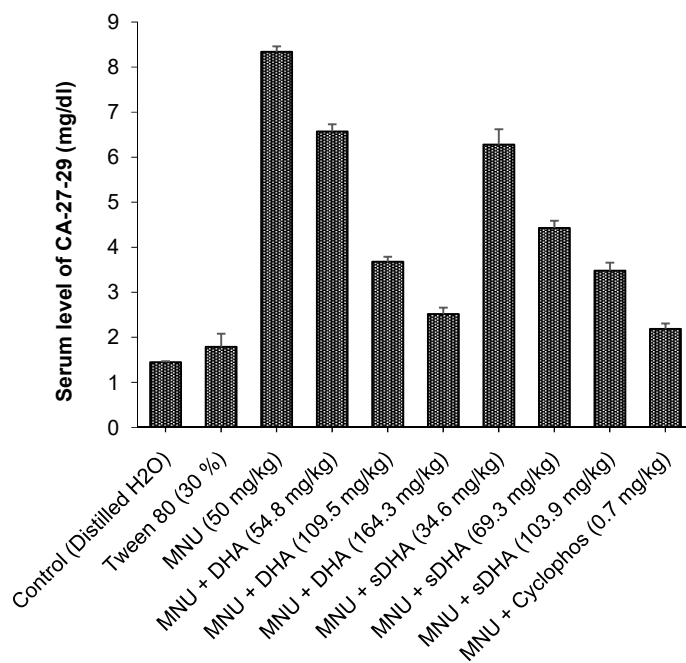


Fig 2. Effects of 28 days administration of dihydroartemisinin (DHA), disulphide-substituted DHA (sDHA) and cyclophosphamide (cyclophos) on MNU-induced elevation of serum CA-27-29 antigen level in Sprague-Dawley rats
*Test drugs, significant from control, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$*
Test drugs, significant from MNU, ^a $P = 0.01$; ^b $P < 0.001$
sDHA treatment, significant from DHA, ^c $P = 0.01$
Test drugs, significant from cyclophosphamide, ^d $P < 0.05$; ^e $P < 0.001$
Mean $\pm$ S.E.M = Mean values $\pm$ Standard error of means of ten experiments

475
476
477
478
479
480
481
482
483
484
485
486
487
488
489
490
491
492
493
494
495
496
497
498
499
500
501
502
503
504
505
506
507
508
509
510
511
512
513
514
515
516
517
518
519

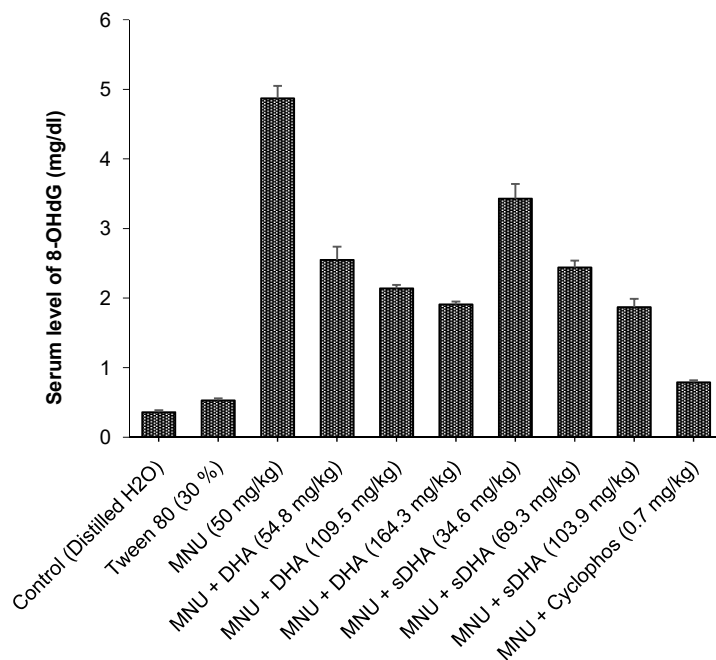


Fig 3. Effects of 28 days administration of dihydroartemisinin (DHA), disulphide-substituted DHA (sDHA) and cyclophosphamide (cyclophos) on MNU-induced elevation of serum 8-hydroxydeoxyguanosine, 8-OHdG (cell DNA damage marker) level in Sprague-Dawley rats
*Test drugs, significant from control, * $P < 0.001$*
Test drugs, significant from MNU, [§] $P < 0.001$
sDHA treatment, significant from DHA, [†] $P = 0.001$
Test drugs, significant from cyclophosphamide, [#] $P < 0.001$
Mean $\pm$ S.E.M = Mean values $\pm$ Standard error of means of ten experiments

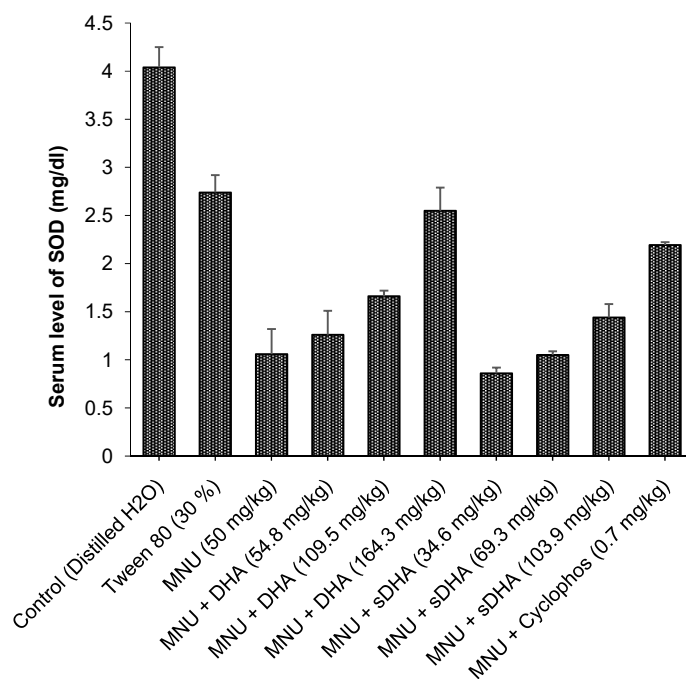


Fig. 4. Effects of 28 days administration of dihydroartemisinin (DHA), disulphide-substituted DHA (sDHA) and cyclophosphamide (cyclophos) on MNU-induced reduction of serum superoxide dismutase (SOD) enzyme level in Sprague-Dawley rats

Test drugs, significant from control, * $P < 0.001$

Test drugs, significant from MNU, ^a $P < 0.01$; ^b $P < 0.001$

sDHA treatment, significant from DHA, [†] $P < 0.01$

Test drugs, significant from cyclophosphamide, [‡] $P < 0.05$; [#] $P < 0.01$

Mean $\pm$ S.E.M = Mean values $\pm$ Standard error of means of ten experiments

580

581

58

58

58

58

58

58

58

58

59

59

59

59

59

59

59

59

59

60

601

60

60

60

60

60

60

61

61

61

61

61

61

61

61

61

61

61

62

621

62

62

62

62

62

62

62

62

63

63

63

63

63

63

63

63

63

63

63

63

63

63

63

63

63

63

63

63

63

63

63

63

63

63

63

63

63

63

63

63

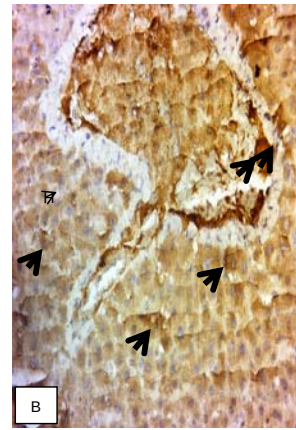
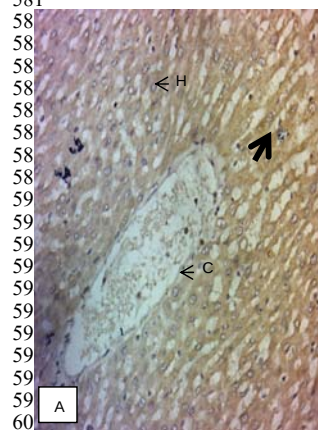
63

63

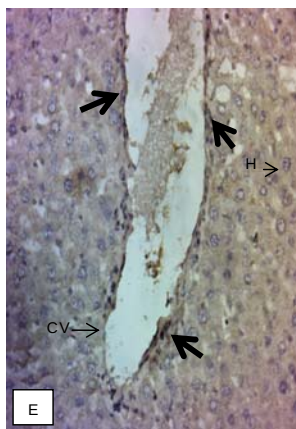
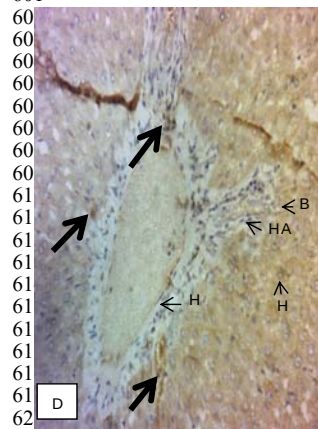
63

63

63



C



H

H

CV

CV

BD

HV

H

I

H

I

H

I

H

I

H

I

H

I

H

I

H

I

H

639 **Fig 5. Photomicrographs showing hepatic cell expression of Bcl-2 antigen in MNU treated and**
640 **MNU plus DHA, sDHA or cyclophosphamide treated Sprague-Dawley rats (Bcl-2**
641 **immunohistochemical method, x400)**
642

643
644 *A: Liver of animals treated with distilled water (Control), showing negative immunoreactive nuclear*
645 *expression for Bcl-2 (less than 5 % positivity).*

646
647 *B: Liver of animals treated with MNU (50 mg/kg) alone, showing strong positive immunoreactive*
648 *nuclear expression for Bcl-2 (more than 75 % positivity).*

649
650 *C: Liver of animals treated with MNU plus cyclophosphamide (0.7 mg/kg), showing negative*
651 *immunoreactive nuclear expression for Bcl-2 (less than 5 % positivity).*

652
653 *D: Liver of animals treated with MNU plus DHA (54.8 mg/kg), showing strong positive immunoreactive*
654 *nuclear expression for Bcl-2 (less than 75 % positivity).*

655
656 *E: Liver of animals treated with MNU plus DHA (109.5 mg/kg), showing strong positive*
657 *immunoreactive nuclear expression for Bcl-2 (less than 50 % positivity).*

658
659 *F: Liver of animals treated with MNU plus DHA (164.3 mg/kg), showing positive immunoreactive*
660 *nuclear expression for Bcl-2 positive (less than 25 % positivity).*

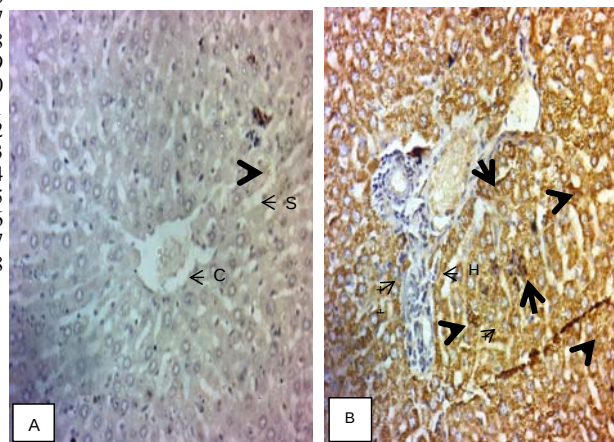
661
662 *G: Liver of animals treated with MNU plus sDHA (34.6 mg/kg), showing strong positive*
663 *immunoreactive nuclear expression for Bcl-2 (less than 75 % positivity).*

664
665 *H: Liver of animals treated with MNU plus sDHA (69.3 mg/kg), showing strong positive*
666 *immunoreactive nuclear expression for Bcl-2 (less than 50 % positivity).*

667
668 *I: Liver of animals treated with MNU plus sDHA (103.9 mg/kg), showing positive immunoreactive*
669 *nuclear expression for Bcl-2 (less than 25 % positivity).*

670
671 *MNU: N-methyl-N-nitrosourea, DHA: Dihydroartemisinin, sDHA: Disulphide-substituted*
672 *dihydroartemisinin*
673

674
675 **Key:** Central vein (CV), Hepatocyte (H), Bcl-2 (→), Bile duct (BD), Hepatic Artery (HA), and Hepatic
676 *vein (HV).*
677



H
H
B

H

699
700
701
702
703
704
705
706
707
708
709
710
711
712
713
714
715
716
717
718
719
720
721
722
723
724
725
726
727
728
729
730
731
732
733
734
735
736
737
738
739
740
741
742
743
744
745
746
747
748
749
750
751
752
753
754
755
756
757

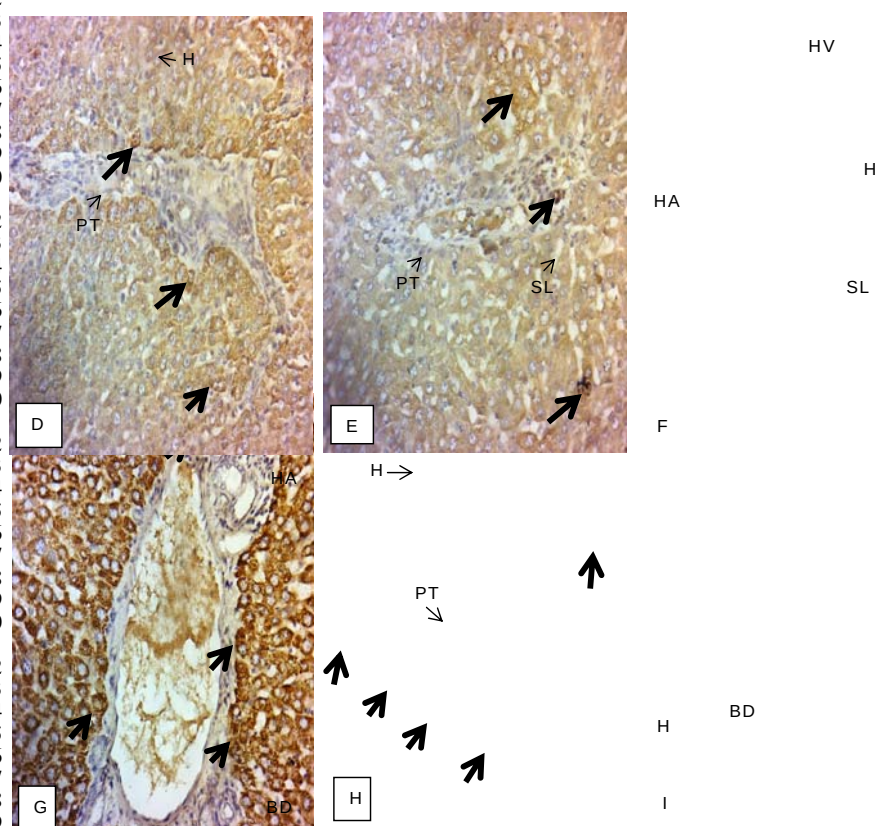


Fig. 6. Photomicrographs showing hepatic cell expression of Ki67 in MNU treated and MNU plus DHA, sDHA or cyclophosphamide treated Sprague-Dawley rats (Ki67 immunohistochemical method, x400)

A: Liver of animals treated with distilled water (Control), showing negative immunostained cytoplasmic expression for Ki67 (less than 5 % positivity).

B: Liver of animals treated with MNU (50 mg/kg) alone, showing strong positive immunostained cytoplasmic expression for Ki67 (more than 75 % positivity).

758 C. Liver of animals treated with MNU plus cyclophosphamide (0.7 mg/kg), showing negative
 759 immunostained cytoplasmic expression for Ki67 (less than 5 % positivity).
 760
 761 D: Liver of animals treated with MNU plus DHA (54.8 mg/kg), showing positive immunostained
 762 cytoplasmic expression for Ki67 (less than 25 % positivity).
 763
 764 E: Liver of animals treated with MNU plus DHA (109.5 mg/kg), showing positive immunostained
 765 cytoplasmic expression for Ki67 (<25 % positive cells).
 766
 767 F. Liver of animals treated with MNU plus DHA (164.3 mg/kg), showing negative immunostained
 768 cytoplasmic expression for Ki67 (<10 % positive cells).
 769
 770 G: Liver of animals treated with MNU plus sDHA (34.6 mg/kg), showing very strong positive
 771 immunostained cytoplasmic expression for Ki67 (<60 % positive cells).
 772
 773 H: Liver of animals treated with MNU plus sDHA (69.3 mg/kg), showing positive immunostained
 774 cytoplasmic expression for Ki67 (<50 % positivity).
 775
 776 I. Liver of animals treated with MNU plus sDHA (103.9 mg/kg), showing positive immunostained
 777 cytoplasmic expression for Ki67 (< 50 % positivity).
 778
 779 MNU: N-methyl-N-nitrosourea, DHA: Dihydroartemisinin, sDHA: Disulphide-substituted
 780 dihydroartemisinin
 781
 782 **Key:** Hepatocyte (H), Hepatic vein (HV), Hepatic Artery (HA), Bile duct (BD), Ki67 (→), Central vein
 783 (CV) and Sinusoidal lining (SL)
 784
 785
 786
 787
 788