International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
IJCS 2017; 5(5): 2079-2083

Received: 05-07-2017
Accepted: 06-08-2017

Etim EI
Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmacy, University of Uyo, Nigeria

Attih EE
Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmacy, University of Uyo, Nigeria

Owaba DAC
Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmacy, Niger Delta University, Wilberforce Island, Bayelsa State, Nigeria

Correspondence
Etim EI
Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmacy, University of Uyo, Nigeria

Structural modification of dihydroartemisinin and antimicrobial assessment of the deoxy and disulphide derivatives

Etim EI, Attih EE and Owaba DAC

Abstract
Dihydroartemisinin is the active metabolite of all artemisinin compounds and is active against all human malaria parasites including multi-drug resistant *Plasmodium falciparum* strains. Isosteric structural modification of dihydroartemisinin was done by replacing the peroxide bond with disulphide, assessing the characteristics of the products formed and the *in vivo* antiplasmodial efficacy using mice was confirmed. The present study was aimed at assessing the *in vitro* antimicrobial potency or otherwise of the deoxy and disulfide derivatives of dihydroartemisinin. Pure dihydroartemisinin solution was chemically reduced using hydrogen generated *in situ* from zine pellet and dilute hydrochloric acid. Chloroform was used to extract the organic phase. The reduced product was dissolved in dimethylsulphoxide and oxidized using hydrogen sulphide gas. The presence of sulfur in the synthesized compounds was confirmed using standard tests. GC-MS properties of the original starting material dihydroartemisinin, the deoxy and disulphide derivatives were analyzed. The *in vitro* antimicrobial test of the synthesized drugs using agar diffusion method by pour plate showed activity against *Pseudomonas aeruginosa* and *Aspergllus niger* for deoxyartemisinin while, disulfide derivative showed activity against *Escherichia coli, Pseudomonas aeruginosa* and *Aspergillus niger*. The minimal Inhibitory Concentrations of the drugs against the susceptible organisms were also established. The synthesized drugs inhibited the microbial growth of Gram negative organisms (*Pseudomonas aeruginosa* and *Escherichia coli*) and *Aspergillus niger* (a fungus), indicating their potential as antimicrobial agents against gram negative organisms and some fungi species.

Keywords: Dihydroartemisinin, Deoxy and Disulphide derivatives, Antimicrobial potency.

Introduction
Malaria represents a global threat to health which is difficult to keep under control. It remains an important public health concern in countries where transmission is all year round, as well as in areas where transmission has been largely controlled or eliminated [1, 2]. Artemisinin, also known as qinghaosu and its semi-synthetic derivatives are groups of drugs that possess the most rapid action of all current drugs against *P. falciparum* malaria [3]. It was extracted from the leaves of *Artemisia annua L.,* also known as sweet wormwood that displayed outstanding antimalarial activity [4, 5]. Its discovery has lead to the treatment of chloroquine resistant malaria. Artemisinin is poorly soluble in water and oil, but its active metabolite and synthetic derivative dihydroartemisinin (DHA) is soluble and is a more potent blood schizontocide [6, 7]. Artemisinin and its derivatives have a short systemic half life and a high rate of recrudescence. The disulphide derivative of dihydroartemisinin was synthesized by removal of the peroxide bond and replacing it with a disulphide. The synthesized disulphide derivative of DHA was confirmed of having antiplasmodial activity similar to that of DHA using mice [8]. Sulfur based products are used in fungal treatment and as chemotherapeutic agents. The present study was aimed at assessing the antimicrobial potentials or otherwise of the synthesized deoxy and disulphide derivatives of dihydroartemisinin, so as to justify further research of its use in clinical practice.

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 Chemicals - Pool England through their Nigeria

representatives. A Cecil spectrophotometer CE 7200 and GC-MS-QP2012 plus - Shemadzu Japan were used for analysis.

Chemical Reduction of Dihydroartemisinin

Dihydroartemisinin powder 500mg was dissolved in 200ml of chloroform in a flat-bottomed flask. 10.0g of zinc dust was placed in a 500ml beaker and 100ml of dilute hydrochloric acid was added to produce hydrogen gas. The DHA solution was poured into the Zn/HCl mixture for the hydrogen gas produced to reduce the peroxide bond *in situ* [8]. The Zn-HCl-DHA mixture was stirred continuously under subdued light for two hours. To the mixture, 10ml of dilute sodium hydroxide was added and stirring continued for another 10 minutes. The mixture was transferred into a separating funnel. The chloroform phase containing the deoxydihydroartemisinin (RDHA) was collected in a beaker and kept in a dark cupboard for the chloroform to evaporate, leaving yellowish powder RDHA.

Synthesis of Sesquiterpene Lactol Endodisulphide

Deoxydihydroartemisinin 10mg was dissolved in 10ml of dimethyl-sulphoxide in a large tube. Hydrogen sulphide gas from Kipps apparatus was bubbled into the solution for two hours in a fume cupboard. Precipitate formed was allowed to settle and decanted. The residue was washed with water and allowed to dry in a desiccator [8].

Test for the Presence of Sulphur in the Synthesized Endodisulphide: (Sodium fussion test): Small quantity of the synthesized disulphide was placed in a combustion tube and small quantity of sodium metal was added. The tube was heated to red hot for the component to fuss. The resulting solid was dissolved in water and filtered. To a potion of the filtrate few drops of sodiumnitroprusside was added and to another portion lead acetate solution was added [7, 8].

GC-MS Analysis: Hyphenated technique of gas chromatography coupled with mass spectrophotometer (GC-MS), Shimadzu, Japan was employed to obtain the chromatogram. The analysis was performed at a column oven temperature of 250ºC and a pressure of 100.2 kpa with a total flow rate of 6.2 ml/minute. The infrared spectra were recorded on Fourier Transform infrared spectrophotometer, model 8400s shimodz, Japan [8].

Collection of test organisms: The following test organisms and clinical isolates were obtained from the Pharmaceutical Microbiology Laboratory, Faculty of Pharmacy, University of Uyo, Nigeria: *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Aspergillus niger* while, *Candida albicans and Escherichia coli* were obtained from the Microbiology laboratory department, University of Uyo Teaching Hospital (UUTH) Uyo.

Culture media: The media used in culturing the microorganisms (microbes) were Mueller-Hinton Agar (MHA) and Sabouraud Dextrose Agar (SDA). The media were prepared according to the manufacturer's instruction [9].

Standardization of test organisms: Standard suspensions of the bacterial culture were prepared by sub-culturing exactly 0.01ml of the overnight culture of each test organisms in a sterile Nutrient Broth and incubated at 37ºC for 16 – 24 hours while fungal cultures were sub-cultured in a sterile Sabouraud Dextrose Broth (SDB) at 28ºC for 24 – 48 hours. The turbidity of each of the broth cultures was aseptically adjusted by ten-fold serial dilutions using sterile Nutrient Broth (for bacteria isolated) and Sabouraud Dextrose Broth (for fungi isolates) to that of 0.5 McFarland Nephelometer standards with an estimated cell density of 1 x 10^8CFU/ml [10,11].

Prearation of plates, inoculation of organisms and incubation: The plates (Petri-dishes), pipettes, test tubes, Pasteur pipettes, cork-borer and other apparatus to be used were sterilized and allowed to cool before use. A preliminary antimicrobial activity of the synthesized drugs; deoxy and disulfide derivatives of dihydroartemisinin were evaluated against *Pseudomonas aeruginosa*, (Gram-negative bacteria) *Staphylococcus aureus* and *Bacillus subtilis* (Gram-positive bacteria) *and Aspergillus niger* (fungus). 5.0mg each of the deoxy and disulfide derivatives of dihydroartemisinin were weighed out and dissolved in 5ml of 15 % Tween 80 to obtain a concentration of 1mg/ml, since the synthesized drugs were insoluble in distilled water [8]. The sterile plates were divided into four quadrants and labeled appropriately with the synthesized drugs, and the standard antibiotic and antifungal. A 0.01ml of each test organism was seeded to the surface of the plates using sterile Pasteur pipettes. 20ml of Mueller-Hinton Agar (MHA) and Sabouraud Dextrose Agar (SDA) each were measured into different plates. The plates were gently swirled for proper mixing of the agar and the organisms. The mixtures in the plates were allowed to solidify on the bench. A – 4mm diameter sterile cork borer was used to bore four holes each on the quadrant in the solidified plates [9].

The antimicrobial activities of the synthesized drugs and commercial drugs (Ceftriaxone for injections USP 1g, Ketoconazole tablet USP 200mg and Nystatin oral tablet USP 500,000IU) were tested using agar diffusion techniques by pour plate method as described above. 10mg/ml of Ceftriaxone (antibiotic), 25mg/ml of Ketoconazole and 25,000IU of Nystatin (antifungal) were used as standards. 0.01ml of each standard drug was introduced into their appropriate labeled holes on each plate representing a particular organism, while 0.01ml each of the synthesized drugs were carefully introduced using a sterile Pasteur pipette. The prepared plates with the synthesized drugs and standard drugs were allowed to remain at room temperature for one hour to ensure the proper diffusion of the agents into the medium. The bacteria cultures were incubated for 24hours at 37ºC while that of *Aspergillus niger* was incubated at 28ºC for 24-48hours before examination for zones of inhibition. The zones of inhibition were measured using a vernier caliper. For the second antimicrobial test, the following organisms were used: *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus and Candida albicans.* The quantity of the synthesized drugs was increased to 100mg each.

100mg each of the deoxy and disulfide derivatives of dihydroartemisininin was weighed and dissolved in 2ml of 15% Tween-80 to produce a stock concentration of 50mg/ml. Two-fold serial dilution was made from the stock concentration to give further concentrations of 25mg/ml, 12.5mg/ml, 6.25mg/ml, 3.125mg/ml, 1.5625mg/ml, and 0.78125mg/ml, respectively. The procedure above was repeated while, 10mg/ml of Ceftriaxone antibiotic, 10mg/ml of Ketoconazole and 10,000IU of Nystatin were used as standards. 0.01ml of each standard drug was introduced into their appropriate labeled holes on each plate representing a particular organism, while 0.01ml of different concentration of the synthesized drugs was carefully introduced using a

sterile Pasteur pipette. The prepared plates with the synthesized drugs and standard drugs were allowed to remain at room temperature for one hour to ensure the proper diffusion of the agents into the medium. The bacteria cultures and *Candida albican* were incubated for 24hours at 37ºC before examination for zones of inhibition. The zones of inhibition were measured using a vernier caliper.

Determination of minimum inhibitory concentrations (MIC): The Minimum inhibitory concentration of the synthesized drugs for the test organisms was determined using the tube dilution method [10]. The standardized organisms above that showed inhibition zones were used for the analysis. 125mg each of the synthesized drugs, (deoxy and disulphide derivatives of dihydroartemisinin) was weighed and dissolved in 5ml of 15% Tween-80 as a diluents to get a stock concentration of 25mg/ml. Two fold serial dilution of the stock concentration was made to produce 12.5mg/ml, 6.25mg/ml, 3.125mg/ml, 1.5625mg/ml, 0.78125mg/ml, 0.390625mg/ml, 0.1953125mg/ml and 0.09766mg/ml respectively. The two fold serial dilution was done using Nutrient Broth and Sabouraud Dextrose Broth (SDB) as diluents i.e. 1.0 ml of the broth and 1.0 ml of the synthesized drugs until the desired concentration was reached. The tubes were inoculated with a loop-full of the respective organisms, the tubes were appropriately labeled and incubated at 37ºC for 24hours (for bacteria) and at 28º for 24-48hours for the fungus (*Aspergillus niger*). The tubes were observed for the presence or absence of growth [9].

Results

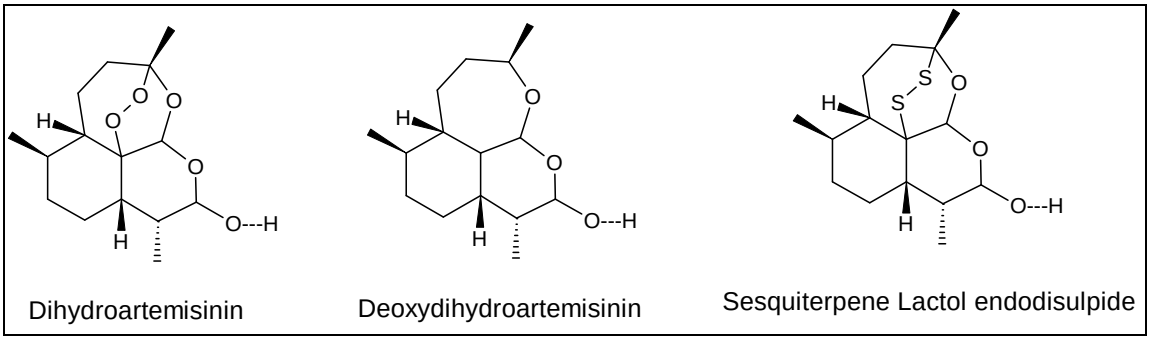
Synthesis: The result for the chemical reduction of DHA and subsequent addition of sulfur was confirmed by the nitroprusside test which gave a blue precipitate in the DDHA but not with DHA and RDHA, and lead acetate which gave a black precipitate with DDHA and not with DHA and RDHA. This confirms that the sulfur derivative was produced [8].

GC-MS analysis: The fragmentation pattern for the test compounds is as shown;

Compound 1: Dihyroartemisinin (DHA): $C_{15}H_{24}O_5$; MS [ES+-MS] m/z (relative intensity): 284 $[M]^+$ (28.45 %), 270 $[M- CH_2]^+$ (45.24 %), 266 $[M-H_2O]^+$, 256 $[M-CO]^+$ (20.07 %), 254 $[M-(CH_3)_2]$ (2.50%)$^+$ 41 $[M-C_{13}H_{24}O_4]^+$ (100.00 %) (Base peak),

Compound 2: Deoxydihydroartemisinin (RDHA)**:** $C_{15}H_{24}O_5$; MS [ES+-MS] m/z (relative intensity): 254 [M]+ (1.05 %), 253 [M-H]+ (2.82 %), 238 [M-O]$^+$ (1.67 %), 226 [M- CO]$^+$ (52.77 %), 222 [M-2O]+ (24.84 %), 220 $[M-H_2O_2]^+$ (21.53 %); 212$[M-C_2H_2O]^+$ (10.53 %), 184$[M-C_2H_2O_2]^+$ (21.53 %), 43$[M-C_{13}H_{23}O_2]^+$ (100.00 %, base peak).

Compound 3: Sesquiterpene lactol endodisulphide (DDHA)**:** $C_{15}H_{24}O_3S_2$; MS [ES+-MS] m/z (relative intensity): 316 [M]+ (1.35 %), 298 $[M-H_2O]$+ (62.82 %), 282 $[M-H_2S]^+$ (31.67 %), 256$[M-CH_2COOH]^+$ (21.53 %), 254$[M-H_2S$ - 32$]^+$ (21.53 %), 248 [M- $(H_2S)_2]^+$ (12.77 %), 91$[M-C_{13}H_{23}O_2]^+$ (100.00 %, base peak).



The dihydroartemisinin, the reduced deoxydihydroxyartemisinin and the new compound; sesquiterpene lactol endosulphide were subjected to mass spectrometry (MS). The obtained MS data were matched with library data of organic compounds. The compounds were identified by comparing their diagnostic peaks with library data of these compounds. The ES+-MS of 1 showed diagnostic peaks such as $[M]^+$ at m/z 284 $[M]^+$ (28.45 %) while 270 (45.24 %), 256 (20.07 %) and the base peak at 41 (100.00 %) represent ES the losses of - CH_2, -H_2O, and - $C_{13}H_{24}O_4$ units respectively from the $[M]^+$. Many fragmented ions also appeared in the MS of **2** but those that could readily be identified include: $[M]^+$ at m/z 254 (1.05 %) while 220 (21.53 %) indicates the loss of -H_2O_2 and $C_{13}H_{23}O_2$, the base peak. The MS fragments of **3** showed $[M]^+$ at m/z at 316 (1.35 %), loss of –H_2S and S at m/z 254.

$^+$-MS data were run on Kratos MS 8 [8].

Antmicrobial results

Table 1: Area of inhibition by the synthesized drugs: deoxy and disulfide derivatives of dihydroartemisinin(1.0mg/ml) and the standard drugs in mm^2

Organisms	Deoxy Derivative 1mg/Ml	Sulfide Derivative 1mg/Ml	Ceftriaxone 10 Mg/Ml	Nystatin 25,000 Iu	Keto-Conazole 25 Mg/Ml
Pseudomonas auroginosa	7	9	38	-	-
Staphylococcus aureus	-	-	38	-	-
Bacillus subtilis	-	-	21	-	-
Aspergillus niger	18	20	-	21	23

Table 2: Area of inhibition by the synthesized drugs: deoxy and disulfide derivatives of dihydroartemisinin(25mg/ml) and the standard drugs mm^2.

Organisms	Deoxy Derivative 25mg/Ml	Sulfide Derivative 25mg/Ml	Ceftriaxone 10 Mg/Ml	Nystatin 10,000 Iu	Keto-Conazole 10 Mg/Ml
Pseudomonas auroginosa	14	28	38	-	-
Escherichia coli	-	22	39	-	-
Staphylococcus aureus	-	-	25	-	-
Candida albicans	-	-	-	22	25

Table 3: Minimal inhibitory concentration (MIC) of the deoxy derivative of dihydroartemisinin.

Organisms	Concentrations of the deoxy derivative in (mg/mL)							
	12.5 mg/ml	6.25 mg/ ml	3.125 mg/ml	1.5625 mg/ml	0.78125 mg/ml	0.390625 mg/ml	0.195313 mg/ml	0.097656 mg/ml
Pseudomonas aeruginosa	-	+	+	+	+	+	+	+
Aspergillus niger	-	+	+	+	+	+	+	+

Table 4: Minimal inhibitory concentration (MIC) of the sulphide derivative of dihydroartemisinin.

Organisms	Concentrations of the sulfide derivative in (mg/mL)							
	12.5 mg/ml	6.25 mg/ml	3.125 mg/ml	1.5625 mg/ml	0.78125 mg/ml	0.390625 mg/ml	0.195313 mg/ml	0.097656 mg/ml
Pseudomonas aeruginosa	-	-	+	+	+	+	+	+
Esherichia coli	-	-	-	-	-	+	+	+
Aspergillus niger	-	+	+	+	+	+	+	+

Fig 1: plate showing zones of inhibition by disulfide derivative of dihydroartemisinin against *Pseudomonas aeruginosa* at conc. 25mg/ml (S1), 12.5 mg/ml (S2)and 6.25mg/ml (S3).

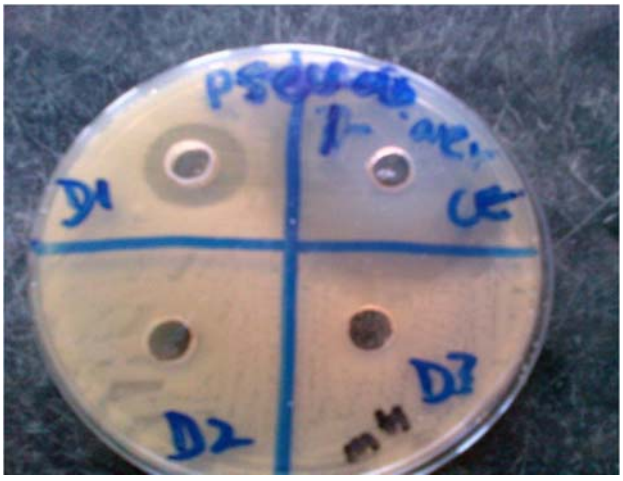


Fig 2: plate showing zone of inhibition by deoxydihydroartemisinin against *Pseudomonas aeruginosa* at a concentration of 25mg/ml (D1), 12.5 mg/ml (D2) and 6.25mg/ml (D3).

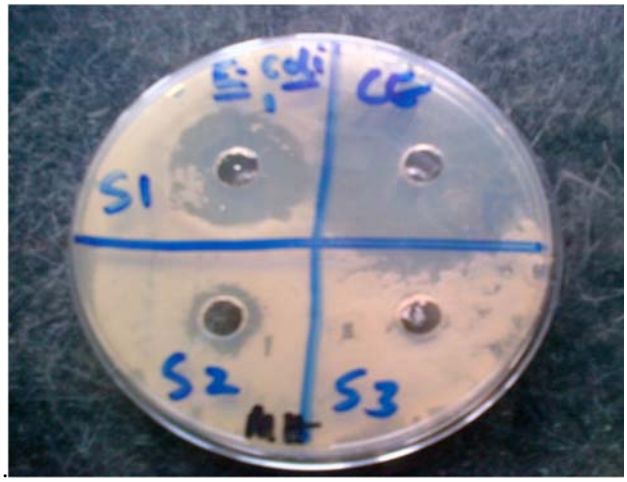


Fig 3: Plate showing zones of inhibition by disulfide derivative of dihydroartemisinin against *Escherichia coli* at concentrarions of 25mg/ml (S1) and 12.5 mg/ml (S2), 6.25mg/ml (S3).

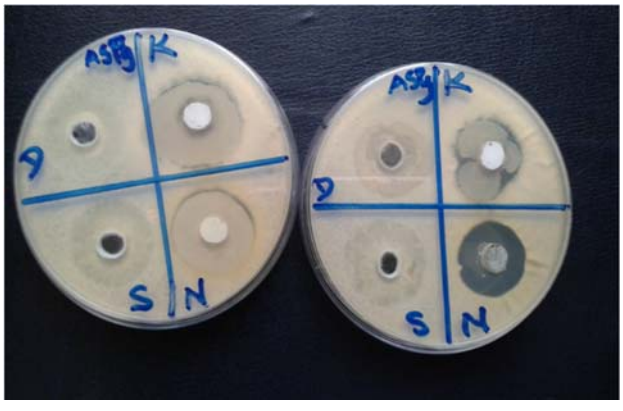


Fig 4: Plate showing zones of inhibition by deoxy and sulfide derivatives of dihydroartemisinin against *Aspergillus niger* at a concentration of 1mg/ml (S3).

S : sulfide derivative of artemisinin
D : deoxyartemisinin
CE : ceftriaxone (an antibiotic)
N : Nystatin (an antifungal)
K : Ketoconazoloe (an antifungal)

Discussion

Isosteric substitution of the peroxide bind produced the disulphide derivative with different physicochemical properties, stability and toxicity from the parent compound [8]. The GC-MS expected molecular ion for the sesquiterpene lactol endodisulphide, sulphur substituted product should be 316 or 317 allowing for isotopic sulphur. The detected ion fragment results from the loss of a molecule of water M^+ (-18). This ion fragment is only present in the spectrum of sulfur substituted compound. The loss of neutral molecule H_2S from molecular ion 316 gives the fragment of mass 282. In the pure DHA with molecular ion M+284. The loss of CH_3C^+=0 fragment from M^+ gave the fragment 241. From above fragmentation, it was observed that all the compound follow a similar pattern in mass fragmentation [6].

In the evaluation of the antimicrobial potency of the synthesized drugs against the test organisms: *Staphylococcus aureus* and *Bacillus subtilis* (Gram positive organisms), *Pseudomonas aeruginosa* (Gram negative organism) and *Aspergillus niger* (Fungus), the result obtained showed that when a concentration of 1mg/ml was used as a preliminary susceptibility test, the deoxy and disulfide derivatives showed activity against *Pseudomonas aeruginosa* and *Aspergillus niger* (Table 1). This was indicated by the appearance of zones of inhibition on the plates. While, no zones of inhibition was seen on the plates inoculated with *Staphylococcus aureus* and *Bacillus subtilis* (Gram positive organisms, Figure 4).

When the concentration of the synthesized drugs was increased to 50mg/ml stock concentration with a two folded serial dilutions to obtain further concentrations, These organisms were tested using the different dilutions; *Escherichia coli*, and *Pseudomonas aeruginosa* (Gram negative organisms), *Staphylococcus aureus* (Gram positive) and *Candida albicans* (fungus). Sulfide derivative at 25mg/ml, 12.5mg/ml and 6.25mg/ml concentrations showed inhibition zones and the deoxyartemisinin showed inhibition zone at 25mg/ml concentration against *Pseudomonas aeruginosa*. (Figure 1). The disulfide derivative gave inhibition zone against *Escherichia coli,* at 25mg/ml and 12.5mg/ml concentrations but, the deoxy derivative did not. No zone of inhibition was seen on the plate inoculated with *Staphylococcus aureus* (Gram positive) and *Candida albicans* (fungus). The standard drugs showed greater and clearer zones of inhibition when compared with the synthesized drugs.

Further antimicrobial evaluation was done to determine the Minimal Inhibitory Concentration (MIC) of the drugs against the organisms susceptible to them. The results obtained revealed that, deoxyartemisinin showed MIC against *Pseudomonas aeruginosa* and *Aspergillus niger* at a concentration of 12.5mg/ml while, the sulfide derivative gave MIC against *Pseudomonas aeruginosa* at 6.25mg/ml concentration. While, the MIC for *Escherichia coli* was seen at 0.78125mg/ml concentration and *Aspergillus niger* at 12.5mg/ml concentration (Tables 3 and 4).

Conclusion

The research work revealed that the synthesized drugs: deoxydihydroartemisinin and disulfide derivative of dihydroartemisinin inhibited the microbial growth of Gram negative organisms (*Pseudomonas aeruginosa* and *Escherichia coli*) tested against them and *Aspergillus niger* (a fungus). This indicates that, they have potential for use as antimicrobial agents against Gram negative bacteria and some fungi species.

Acknowledgement

The authors are grateful to Mr. Nsikan Malachy of the animal House Department of Pharmacology and Toxicology, Mrs. Ekaete Umoh, the Chief Technologist of the department of Pharmaceutical and Medicinal Chemistry and Mr. A Akpan, the Chief Technologist of the Department of Pharmaceutical Microbiology, Universtiy of Uyo for their technical assistance.

References

1. World Health Organization. World malaria report, Geneva, Switzerland, 2014, 32-42.
2. Hien, TT. An overview of the clinical use of artemisinin and its derivative in the treatment of falciparium malaria in Viet Nam. Trans. R. Soc. Trop. Med Hyg. 1999; 88(1):87-88.
3. White NJ. Delaying antimalarial drug resistance with combination chemotherapy. Parassitologia. 1999; 41:301-308.
4. Kuhn T, Wang Y. Artemisinin - an innovative cornerstone for antimalaria therapy. Prog Drug Res. 2008; 66(383):385-422.
5. Ho W. Studies on molecular mechanism of action and synthesis of new derivatives of antimalarial drug artemisinin, Hong Kong: Hong Kong University of Science and Tech. (*Thesis-Dphill*). 2004; 1(38):309-337.
6. Coker HAB, Chukwuani CM, Ifudu ND, Aina BA. The Malaria scourge-concept in disease management. Nigerian Journal of Pharmacy. 2001; 32:19-47.
7. Udobre AS, Etim EI, Udobang JA, Udoh AE, Akpan AE, Ekpo NA. Antiplasmodial effect of the methanol extract of the stem bark of Nauclea latifolia. Indo American Journal of Pharmaceutical Research. 2013; 3(8):6484-6489.
8. Etim EI, Johnson EC, Bassey UB, Essien EE. Sythesis characteristics and antiplasmodial studies of the deoxy and disulphide derivatives of dihydroartemisinin. Chemistry Research Journal. 2017; 2(4):47-54.
9. Hugo WB, Russel AD. Pharmaceutical microbiology, 2nd ed. Blackwell Scientific publishers, Oxford, 134-135.
10. Tilton RC, Howard BJ. Antimicrobial susceptibility testing in: BJ Howard (ed.), Clinical Pathogenic Microbiology. CV. Mosby, Missouri USA, 121-156.
11. Black Jacquelyn. Microbiology: Principles and Exploration (8th edition). John Willey and sons, 2012, 68.