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Effect of Dihydroartemisinin on contractility to Drugs in isolated smooth muscle Preparations in guinea pig *in vitro* models

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Abstract

This study was aimed at evaluating the effect of Dihydroartemisinin (DHA) on contractility to drugs in ileum isolated smooth muscle preparations in guinea pigs using *in Vitro* experimental procedures / standard protocols in an organ bath set up. dihydroartemisinin (1.0×10^{-8} - 1.0×10^{-5} mg/mL) when applied alone and separately excited marked variable effects on guinea pig ileum. In some preparation it showed no response, while in others it produced slight phasic contraction when external calcium (Ca^{2+}) ion was introduced. The slight phasic contractile activity was abolished by verapamil (5×10^{-3} mg/mL). Dihydroartemisinin (1.0×10^{-6} - 1.0×10^{-5} mg/mL) caused marked significant induced contraction – dependent inhibition of acetylcholine, potassium chloride and histamine in depolarizing tyrode solution ($p < 0.05$). The Inhibitory response maxima of dihydroartemisinin on acetylcholine induced contraction is 17.0 ± 0.1 mm (65.56% inhibition); and is moderate when compared to the inhibitory effect of atropine (10^{-5} M). The result shows that dihydroartemisinin seems to act via non-specific receptor mechanism, with appreciable calcium channel blocking activity and it is safe at therapeutic doses.

Keywords: Dihydroartemisinin; Contractility; Smooth muscle; *Plasmodium berghei bergiei*, *in vitro*; toxicity

1. Introduction

Malaria is a tropical disease; and remains a major public health problem in endemic regions ^{1,2}. World malaria report indicate that there were 219 million cases of malaria globally in 2019 and 438,000 malaria deaths; currently there are estimated 619,000 malaria death in 2021 ¹. *Plasmodium falciparum*, is the most clinically significant causative organism and has been reported to demonstrated an unusual propensity to acquire resistance to antimalarial therapy ^{3,4}.

The unprecedented spread of chloroquine-resistant strains of *Plasmodium falciparum* had severely weakened the range of drugs available to treat the disease and has increased interest in newer agents such as the Artemisinin Derivatives. Drugs so far used for the treatment of malaria range from the ancient chloroquine, mefloquine, primaquine, sulfadoxine, pyrimethamine, proguanil, amodiaquine, quinine and agents like doxycycline, among others.

Currently, there seems to be increasing interest in the use of Artemisinin derivatives (Artemether, Arteether, Artesunate and Dihydroartemisinin) which are a new series of antimalarial drugs with a high level of activity against chloroquine resistant strains of malaria parasite [2,5].

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World Health Organization (WHO) recommended treatment schedule containing an Artemisinin-combination therapy (ACTs) is now the recently used standard treatment in the world for *plasmodium falciparum* malaria. As a response to increasing levels of resistance to antimalarial medicines, WHO recommends that all countries experiencing resistance to conventional monotherapies, such as chloroquine, amodiaquine or sulfadoxine and pyrimethamine, should use combination therapies, preferably those containing artemisinin derivatives (ACTs – artemisinin-based combination therapies) for *falciparum malaria*.

Currently artemisinin and its derivatives (artemether, arteether, artesunate and dihydroartemisinin) present a new series of antimalarial drugs with a high level of activity against chloroquine resistant strains of malaria parasite⁵ These artemisinin-based combination therapy (ACT) are recommended as first line treatment for uncomplicated malaria, which has also resulted to increase in the use of artemisinins⁶. Artemisinins generally have good permeability; artemether is lipid –soluble and poorly water-soluble⁷. Furthermore, treatment of malaria with artemisinins has been implicated with various unwanted effect or adverse effects, including that of muscle weakness, malaise, vomiting, diarrhoea etc. some of these effects are on smooth muscles activities.

Research work on the mechanical activity of muscles on ancient antimalarials such as chloroquine, mefloquine, quinine in animal models had been carried out extensively^{8,9,10,11}. studied the effects of chloroquine on the rat urinary bladder strip. On the contrary, few studies so far have been carried out on the effect of Artemisinin derivatives on muscle contractility; Ejirofor *et al.*, studied the effects of artemether on uterine muscle¹², The effect of artesunate on guinea pig ileum was investigated¹³.

This present work was designed to investigate the effect of dihydroartemisin on isolated ileum smooth muscle contractility in guinea pigs model with a view of elucidating the probable mechanism of the drug action and to ascertain the safety profile of dihydroartemisin in ACTs Combination.

2. Materials and methods

2.1. Experimental Animals

The research was carried out using matured experimental animals (guinea pigs) of both sexes namely: healthy and diseases-free male and female guinea pigs (320-370 g) were purchased from the animal house of the department of Pharmacology and Toxicology University of Uyo; the guinea pigs were used for *in vitro* organ bath study, while the mice (19-24g) were utilized for the *in vivo* models; the mice were divided randomly into groups (n=5) according to their body weights in a proper range.

The animals were fed with a standard laboratory feed pellet-growers mash from agro feeds Limited, Lagos and provided distilled water for drinking *ad libitum*. All animals were well acclimatized having been kept in clean wooden cages with a solid bottom to protect the guinea pigs feet and laboratory-grade pine shavings were added as beddings; the cage, contained in well-ventilated house; also since temperature fluctuations are hard on guinea pigs, they were maintained under standard conditions (temperature: “22±3 °C”; and were shielded from direct unlight; photoperiod: 12-h natural light and 12-h dark cycle; humidity: 35-60%) for two weeks prior to drug treatments.

2.2. Drugs/Chemicals and their Sources

The drugs purchased include pure dihydroartemisin powder (Afrab Chem. Ltd., Lagos), Nigeria. Atropine, Calcium channel blockers e.g. Verapamil, α -adrenoceptor blocker such as Phentolamine etc. Acetylcholine Chloride were obtained from Sigma chemical Co.(USA), Propranolol, from Macclesfield (Great Britain). Calcium chloride (Copharm) : Magnesium chloride (Hopkin Williams, U.K). Barium chloride (BDH, UK); histamine phosphate and Potassium chloride (Sigma, USA).

The Physiological solutions used in this study were Tyrode solution and De jallon solution. All chemicals were of high analytical grade and were dissolved in either deionized distilled water at the required concentrations.

2.3. Equipment

Organ bath apparatus (Orchid Scientifics), Aerator (Type r. 301. USA), Slow-moving kymograph (C.F Palmer LTD England), Triple beam balance (New Jersey, USA), Mettler balance P165 (Gallenkamp, Germany, UK). Dissecting set, Dissecting board, Ohaus triple beam balance (USA), Microscope, Microchemical (top-loading) balance (USA).

2.4. Preparation of Drugs

The completely homogenous test drug - dihydroartemisinin were administered to all the animals in the test groups, with the aid of 23G stainless steel oropharyngeal cannula.

The method of calculation of volume (ml) of the drug to be given to each animal as follows:

$$\text{Volume administered (ml)} = \frac{\text{Weight of rat (kg)} \times \text{Required dose (mg/kg)}}{\text{Concentration of the drug}}$$

The doses used for their *in vivo* studies were determined from the data obtained based on standard doses used for animal models relating to body weight in previously established dosage in similar studies - on the effect of Artemisinin-Based Combination therapy in wistar albino rats which suggested treatment dose of between 3 mg /kg - 6 mg /kg of Body weight.¹⁴.

2.5. Parasite inoculum preparation

The method of Peters,¹⁵ as described by Udobang¹⁶, was adopted; each mouse that was used in the *in vivo* experiment (test group) was inoculated intraperitoneally with 0.2ml of infected blood containing about 1×10^7 *Plasmodium berghei berghei* parasitized erythrocytes. In order to achieve this, the parasitized blood donor with high parasitaemia was obtained by first anaesthetizing the mouse with chloroform, and through cardiac puncture blood was collected with the aid of sterile syringe and heparinized bottles. The percentage parasitaemia was determined by counting the number of parasitized red blood cells against the total number of red blood cells.

2.6. Sub-grouping of the test Animals

The test group animals were demarcated into three (3) sub-groups containing four (4) mice per group infected with *Plasmodium berghei berghei*; this order was followed for the three set of drug treatments - i.e. dihydroartemisinin 2.0, 3.0 and 5.0 mg/kg for low, moderate and high doses regimen respectively.

The animals in the control groups were also divided into 3 subgroups; For -1. Non-infected/ Non-treated mice, 2. Non-infected /Treated mice, animals were sacrificed under chloroform anaesthesia and by cervical dislocation. All drugs were given twice daily via oral route for five (5) consecutive days adding up to 120 hours. The animals of all the groups were observed and, 3. Infected mice /Non-treated according to the dosage regimens respectively. At the close of all treatment exposures for 2 days post administration. The animals were fasted overnight and sacrificed on the 8th day. The mice were also assessed for the integrity of muscles using standard histological tissues staining procedures.

2.7. Histopathological Studies and Collection of samples.

Samples were collected in the *in vivo* experiment after treatment as follows: clear incision were made into the abdominal cavity of the mice up till the border near the tail of the rats. Fresh ileum, uterus, and portion of large intestine were removed from the rats and immediately fixed in 10 % formalin in specimen containers for 3 days (72 hours). These organs were cut laterally and longitudinally to examine the internal structure as describe by Yakubu¹⁷. They were processed for histological evaluation by pathologist in the University of Uyo Teaching Hospital, Uyo, Nigeria.

2.8. Experimental procedures *in vitro* animal models

This study was carried out using standard experimental procedures as described by – Unekwe,¹⁸ Nwafor¹⁹; which were applicable in the use of Organ bath with a slow moving kymograph, a basic instrument for measuring muscle tension. The organ bath was properly washed using distilled water and filled with appropriate physiological solutions.

A vertical strand of isolated muscle tissue of 2 cm were be picked gently using forceps, needle and white thread was passed through the tissue and tied through the arm of the frontal lever to the tissue holder. The tissue holder was then placed in the tissue organ bath. The tissue were observed closely for the contractile response in the Tyrodes solution alone; It was allowed to stabilized for about thirty (30) to sixty (60) minutes before investigation commences.

2.9. Recordings of Contractile Responses against the Concentration of the Acetylcholine

The methods as described by¹⁰ were adopted. A vertical strand of isolated ileal smooth muscle tissue of about 2 cm was gently picked using forceps, needle and white thread was passed through the tissue and tied through the arm of the frontal lever to the tissue holder. After equilibrium, the concentration response test to acetylcholine alone at a concentration of 4×10^{-8} to 10^{-5} M was conducted separately before the addition dihydroartemisinin. The least dose (10^{-5}

M) was added first to the fluid bathing the tissues and the effect was observed for 0.5-2 minutes and this was followed by 2-3 washings, after which the next higher dose of the agonist was added and the procedure was repeated for about 5 doses of the agonist

2.10. Recordings of Contractile Responses against the Concentration of the Acetylcholine

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3. Results and discussion

The result of this study shows that dihydroartemisinin at a concentration of 1.0×10^{-8} to 1.0×10^{-5} g/ml produced no significant contractile responses on the isolated ileum smooth muscles within 30-45 minutes of drug contact in the organ bath containing the appropriate physiological solution.

3.1. Effect of Dihydroartemisinin on acetylcholine induced contraction on guinea pig ileum

Dihydroartemisinin at a concentration of 1.0×10^{-5} g/ml significantly antagonised the contraction induced by acetylcholine at concentration range of 4×10^{-8} to 4×10^{-7} mg/mL and 4×10^{-6} to 4×10^{-5} mg/mL respectively. * $P \leq 0.05$, when compared to control. (Table 1 and Table 2)

3.2. The Effect of Dihydroartemisinin on Muscle tissues in Mice

Administration of Dihydroartemisinin (5 mg/kg, 3 mg/kg and 2.0 mg/kg of body weight) to mice for 5 days in non-infected/ non-treated mice, infected/ treated mice and in infected/ non-treated mice did not produce any significant effect on the integrity of the ileum smooth muscles in the range of 2.0 mg/kg and 5.0 mg/kg doses administered in this study (Figure 3 to 6).

Table 1 Effect of Dihydroartemisinin on acetylcholine-induced contractions in isolated ileum strip in guinea pig models

				Responses of dihydroartemisinin on acetylcholine induced contractions.		
FBC Acetylcholine (Ach.) (mg/ml) Control	- log	* Maximum height (mm)	% of max	FBC of DHA (1.0×10^{-5} mg/ml) on acetylcholine induced contractions	- Log(M)	* Response Max. of Ach in the presence of DHA (mm)
4.0×10^{-8}	- 7.4	12.32 ± 0.12	24.80	4.0×10^{-8}	-7.4	2.33 ± 0.7
4.0×10^{-7}	- 6.4	31.67 ± 0.05	64.20	4.0×10^{-7}	-6.4	4.0 ± 0.5
4.0×10^{-6}	- 5.4	35.00 ± 0.10	71.05	4.0×10^{-6}	-5.4	08.0 ± 0.3*
4.0×10^{-5}	- 4.4	49.65 ± 0.12	100.00	4.0×10^{-5}	-4.4	17.0 ± 0.1*

Control response: graded concentration of acetylcholine (mg/mL), Test response: response concentration of dihydroartemisinin in mg/ml; $\bar{X} \pm$ SEM of 4 values, * $p \leq 0.05$; maximum inhibitory height by DHA 17.0 ± 0.1 mm (65.56%) on Ach. induced contractions

Table 2 Effect of Dihydroartemisinin on histamine induced contractions in isolated ileum strip in guinea pig models

				Responses of Dihydroartemisinin/piperazine alone .			
FBC Histamine (mg/ml) Control	- log	* Maximum height (mm) of contractions	% of max	FBC of DHA/Pip. 10 ⁻⁶ on histamine contractions	- Log(M)	* Maximum inhibitory height (mm) of relaxation	% of max
1.0x10 ⁻⁸	-8	18.67 ± 0.1	31.5 ± 0.1	1.0x10 ⁻⁸	-8	9.67 ± 0.16	23.43
1.0 x10 ⁻⁷	-7	37.33 ± 0.1	62.9 ± 0.1	1.0x10 ⁻⁷	-7	21.67 ± 0.22	52.10
1.0 x10 ⁻⁶	-6	42.00 ± 0.1	70.7 ± 0.1	1.0x10 ⁻⁶	-6	26.7 ± 0.06*	64.83
1.0 x10 ⁻⁵	-5	59.34 ± 0.1	100 ± 0.0	1.0x10 ⁻⁵	-5	41.33 ± 0.1*	100.00

Control response: graded concentration of Histamine (mg/ml), Test response: responses concentration of Dihydroartemisinin in mg/ml; $\bar{X} \pm \text{SEM}$ of 4 values, *p ≤ 0.05: maximum relaxation height 41.33 ± 0.1 mm (30.4%) (of DHA on Histamine).

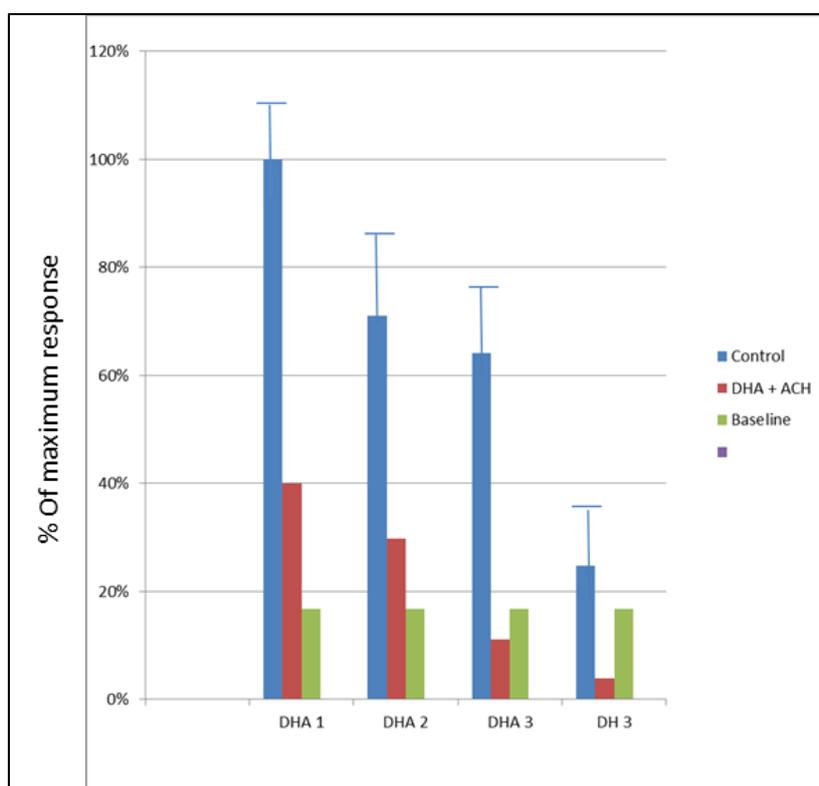


Figure 1 Histogram showing the % of maximum inhibitory response effect of DHA on smooth muscle of the ileum in guinea pig model (DHA1- low- dose, DHA2 – middle dose and DHA3 higher doses).

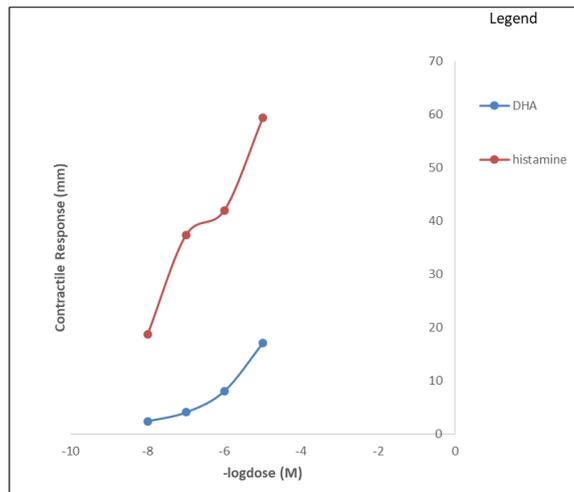


Figure 2 Effect of DHA on histamine induced contraction on guinea *p ≤ 0.05 significant relative to the control

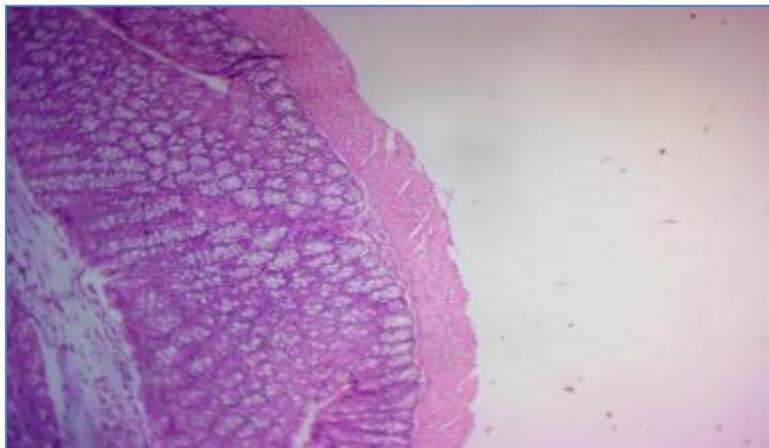


Figure 3 Photomicrographs of smooth muscle of the Ileum of non-Infected and non – treated at mag. Control. A (x400)stained with H& E method. Revealed normal ileum cellular profile



Figure 4 Photomicrographs of smooth muscle of the Ileum of Infected and treated with 3 mg/kg of dihydroartemisinin at mag. A(x100) & B(x400)stained with H& E method.Revealed normal cellular profile

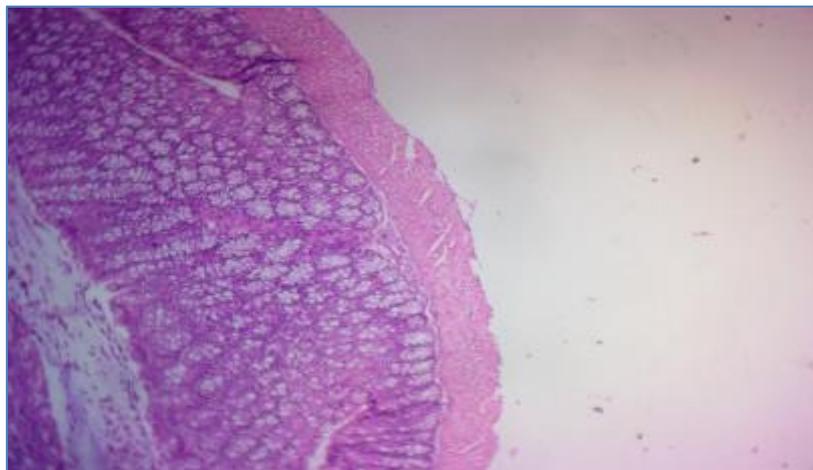


Figure 5 Photomicrographs of smooth muscle of the ileum of Infected and treated with 5 mg/kg of dihydroartemisinin at mag. A(x100) & B(x400) stained with H& E method. Revealed mild to moderate inflammation of cellular profile

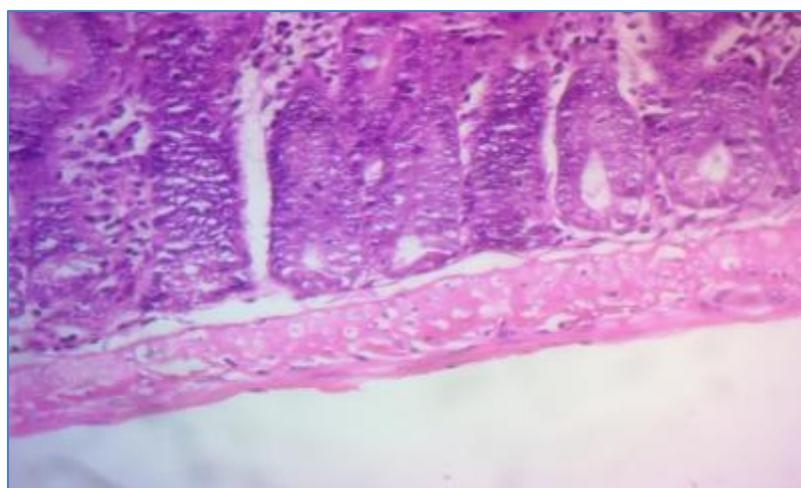


Figure 6 Photomicrographs of smooth muscle of the ileum of non-Infected and treated with 2 mg/kg of dihydroartemisinin at mag. A(x100) & B(x400) stained with H& E method. Revealed normal ileum cellular profile

The histopathological examination revealed no abnormality seen in the ileum smooth muscle profiles at the dose of 3mg/Kg and at 5mg/Kg of DHA. smooth muscle profiles at the dose of 3mg/Kg and at 5mg/Kg of DHA.

Dihydroartemisinin (DHA) alone and DHA/piperazine at a concentration of (1.0×10^{-8} - 1.0×10^{-5} g/mL) produced no contractile responses on the isolated guinea pig ileum muscle strips within 30 – 60 minutes of drug-tissue contact in the organ bath containing Tyrode's solution. In some preparations it showed no response, while in others it produced slight phasic contraction when external calcium (Ca^{2+}) ion was introduced. The slight phasic contractile activity was abolished when verapamil (5×10^{-3} mg/ml) was added to the organ bath fluid. This observed results of no contractile responses when dihydroartemisinin was applied alone, was similar with report on chloroquine which produced no contractile responses when applied on the rat urinary bladder strip under baseline conditions [10]. The antagonism by dihydroartemisinin in each instance, for example (Ach. > Histamine) was non-competitive, this is basically proven by the agonist – concentration response curves which were clearly displaced to the right in asymmetric non – parallel fashion, with depressed maxima (Figure 1 and 2). It had been an established principle that, contractile responses induced by acetylcholine is influenced mainly by the stimulation of muscarinic receptors [11]; On the other hand, the observed slight contractile responses, were reversibly abolished due to the introduction of zero Ca^{2+} in physiological solution. KCl-induced contractions are largely reported to be due to a depolarizing action on the plasma membrane of the rat urinary bladder, as a result of which extracellular Ca^{2+} influx occurs via voltage – dependent Ca^{2+} channels (VOCs); [18, 19, 20; 21]. also, dihydroartemisinin (1.6×10^{-6} – 1.6×10^{-3} mg/mL) when applied alone and separately had little or no

marked variable contactile effects on ileum smoothmuscles strips: the effect of dihydroartemisinin/piperazine (1.6×10^{-4} mg/ml) on acetylcholine (4.0×10^{-10} – 4.0×10^{-6} mg/mL.) induced contractions was markedly inhibitory ; these inhibition was significant ($P < 0.01 - 0.05$), (Table 1 and 2). This findings is similar with report that artemether (48 - 480 ug/ ml) had no agonist effects on the isolated uterine smooth muscles of both non-pregnant and pregnant rats [12]; similarly artesunate (4.0×10^{-6} – 4.0×10^{-5} mg/mL.) had been reported to caused marked significant dose-dependent inhibitory contraction of acetylcholine, potassium chloride and histamine in depolarizing Tyrode's solution [13]. This observed results can further be justified based on earlier reports that, KCl- induced contractions were due to a depolarizing action on the plasma membrane of the guinea pigs and rats isolated ileum, as a result of which extracellular Ca^{2+} influx occurs via voltage – dependent Ca^{2+} - Channels , [19, 20; 21]. The availability of Ca^{2+} is a basic determinant for smooth muscle contraction [22]. The observed result of inhibition by dihydroartemisinin on agonists induced contractile responses were not inhibited by phentolamine and atropine in the different set of study- which might likely suggest non-specific antagonism. The histopathological examination revealed no abnormality seen in the ileum smooth muscle profiles at the dose of 3mg/Kg and at 5mg/Kg of DHA.

4. Conclusion

The results from this study revealed significant inhibitory contractile responses of dihydroartemisinin and DHA/piperazine combination on acetylcholine induced contraction in isolated ileum smooth muscle tissue in guinea pig in a dose dependent manner. Dihydroartemisinin seems to be acting by interference with extracellular Ca^{2+} influx and possibly with mild interference with transmembrane ion fluxes by a non-specific processes; histopathological studies also revealed that DHA may possess safe pharmacological properties.

Compliance with ethical standards

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Disclosure of conflict of interest

There is no conflict of interest existing among the Authors in this research work.

Statement of ethical approval

These studies involve the use of animals, all the animal used received humane care the study protocol was designed to comply with the institutions guidelines for used of laboratory animals (Faculty of Pharmacy, University Uyo, Nigeria, ethical committee's clearance was obtained), in line with the principle of laboratory animal care of National institute of health –NIH publication guidelines.[23].

Contribution of the Authors

Author 1 is the chief investigator, Author 2 is the main supervisor authors 3 and 4 are the co-investigators ; while Authors 5 is our undergraduate student on research work and author 6 assisted in the data analysis.

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