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EFFECTS OF ARTESUNATE ON CONTRACTILITY TO DRUGS AND OTHER ACTIVITIES OF THE SMOOTH MUSCLE PREPARATIONS.

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ABSTRACT
This study was aimed at evaluating the effect and safety profile of artesunate on drugs induced contractility in different isolated smooth muscle preparations in guinea pigs and mice using *in vitro* and*in vivo* experimental procedures/ standard protocols in an organ bath set up. Artesunate (4.0×10^{-6} - 4.0×10^{-3}mg/ml) when applied alone and separately excited marked variable effects on guinea pig ileum and mouse rectum. 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 ($5x10^{-3}$mg/ml). artesunate (4.0×10^{-6} - 4.0×10^{-5}) 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 artesunate on acetylcholine induced contraction is relatively moderate when compared to the inhibitory effect of atropine (10^{-5} M). Subacute toxicity study of oral administration of artesunate (2.0, 2.5, and 5mg/kg b.w), twice daily for 3 days in *Plasmodium bergheiberghei* infected mice revealed a normal cellular profile of smooth muscle structure. The result shows that artesunate seems to act via non-specific receptor mechanism, with appreciable calcium channel blocking activity and it is safe at therapeutic doses.

KEYWORDS: Artesunate, Contractility, Smooth muscle, *Plasmodium bergheiberghei*, and toxic.

1. INTRODUCTION
Despite efforts of the medical practitioners and Pharmacological researches, malaria still remain an enigma; hence, malaria posed an undiminished threat to people living in or traveling to endemic areas, such as Africa.[1] Malaria causes about 250 million cases of fever and approximately one million deaths annually.[2] (In Africa alone between 1 and 2 million children die from the disease each year.[3] The causative parasite of malaria is the *plasmodium* transmitted by the female anopheles mosquitoes, which is further subdivided into five species of *plasmodia parasites*, namely: *Plasmodiumvivax, plasmodium falciparum* (which are widespread); *Plasmodiumovale* is mainly confined to Africa and is less prevalent, *Plasmodiummalariae*, which causes the least severe but most persistent infections, also occurs widely[4] ; and lastly *Plasmodium knowlesi*, this is the zoonotic specie that causes malaria in Macaques.[5]

Plasmodium falciparum, is the most clinically significant causative organism and has been reported to demonstrated an unusual propensity to acquire resistance to antimalarial therapy.[6] The unprecedented spread of chloroquine-resistant strains of *Plasmodium falciparum* had severally weakened the range of drugs available to treat the disease and has increased interest in newer agents such as the Artemisinin Derivatives.

Currently artemisinin and its derivatives (artemether, arteether, artesunate and dihydroarthemisinin) present a new series of antimalarial drugs with a high level of activity against chloroquine resistant strains of malaria parasite.[7] Artemisinin was first isolated in 1972 in China as an active principle in the dried leaves of Artemisia annua L. (Compositae); it has been used since ancient times for the treatment of malaria. Artesunate is the water soluble sodium salt of artemisinin which are administered either orally or by injection. The endoperoxide group in the artemisinins drug molecule is highly essential for antimalarial activity to occur.[8] Other research has also shown that it posseses some anti-

schistosomial activity, though this has not been totally proven.[9]

An acute attack of malaria typically begins with a prodrome of headache, and fatigue (mainly on body musculature), followed by fever. Also, a classic malarial paroxysm includes chills (shivering), high fever, arthralgia, vomiting[10], and then sweats; other common symptoms include abdominal pain, anorexia, nausea, diarrhea (these effects are mainlyrelated to visceral smooth muscles).

This study was designed to investigate the effect of artesunate on contractility to drugs and the sub-acute effect on ileum preparations in experimental animal models, with a view to elucidate the mechanisms of its actions on the smooth muscle tissues and also to ascertain the safety profile.

2. MATERIALS AND METHOD

Drugs/Equipment used

The drugs that were purchased are artesunate (Novartis Pharm., New York), the drug was purchased from Amela Pharmacy, in Uyo, Nigeria. Atropine, verapamil;α-adrenoceptor blocker such as Phentolamine. Acetylcholine Chloride, Potassium chloride and Histamine diphosphate were obtained from Sigma chemical Co. (USA).

The equipment utilized include: Organ bath and slow moving recording Drum/kymograph and, Digital temperature controller (Orchid Scientific USA)

Drugs preparations

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

The calculated volume (ml) of the drug to be given to each animal were determined by its weight and required dose as follows.

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

The doses used for the *in vivo* studies were determined from the data obtained based on previously established standard dosages 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]

Animals

The research was carried out using Adult experimental animals of bothsexes: Mice and guinea pigswere used. Twenty four(24) mice (18-23g), and Six (6) guinea pigs(430 -450g) were purchased from the animal house of the Department of Pharmacology and Toxicology, University of Uyo; theguinea pigs were used for *in vitro* organ bath study.

They were all evaluated to be diseases-free and devoid of deformities. Animals were fed with a standard laboratory pellet diet and provided distilled water for drinking *ad libitum*. The animals were kept in clean metabolic polypropylene / wooden cages with laboratory-grade pine shavings as beddings, contained in well-ventilated house and maintained under standard conditions (temperature: 25 ±3 ^{o}C; photoperiod: 12-h natural light and 12-h dark cycle; humidity: 35-60 %).

Parasite Inoculum Preparation

Each mouse that was used in the in vivo experiment was inoculated intraperitoneally with 0.2ml of infected blood containing about *1x10^{7}plasmodium bergheiberghei* parasitized erythrocytes as described by Peters.[11] In other 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.

Grouping of Animals for *In Vivo* Study

The mice were divided randomly into two groups according to their body weights in a proper range one day before the administration of the test drug. The first set was used as the Control group; this mice received only distilled water (no drug treatment), and the other group comprised the test group animals.

The test group animals were demarcated into three (3) groups comprising five (5) mice per group for the three doses of drug treatments (Artesunate 2.0, 2.5 and 5.0mg/kg for low, moderate and high doses regimen respectively).

Animals in the control group were also divided into 3 subgroups; 1. Non-infected / Non-treated mice, 2.infected mice /Treated, 3. infected mice /Non-treated according to the dosage regimens respectively. The animals of all groups were inspected daily for detection of clinical signs of toxicity as prescribed by.[12]

At the close of all treatment exposures, animals were sacrificed under urethane anaesthesia and by cervical dislocation. All drugs were given via Oral route for three (3) consecutive days adding up to 72 hours. The animals of all the groups were then observed post administration. Four (4) animals each were sacrificed from all the groups, 24 hours after 3 days observation/post-treatment. The mice were assessed for the integrity of smooth

muscles using standard histological tissues staining procedures.

Experimental Protocol for *in vitro* animal models
This study was carried out using standard experimental procedures as 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 was 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 was observed closely for contractile response in the tyrodes solution alone; It was allowed to stabilized for some about thirty (30) minutes before investigation commences.

Isolated smooth muscle preparations from experimental animal models were used for this study e.g. isolated guinea pig ileum and isolated mice rectal muscles.

Recordings of Contractile Responses Versus the Concentration response of Artesunate
Concentration response tests to acetycholine, histamine, potassium chloride and barium chloride was conducted separately before the addition of artesunate. The tissue was pretreated for about 3 minutes with (10^{-3}g/ml) of artesunate and the whole procedure was repeated.

3. RESULTS

Effect of artesunate on guinea pig ileum, cardiac muscle strips
Artesunate alone at a concentration of 4 x 10^{-5} M produced no contractile responses on the isolated guinea pig ileum, and mouse rectal strips within 30 minute of drug contact in the organ bath containing appropriate physiological solutions. 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 ($5x10^{-3}$mg/ml). artesunate (4.0 x 10^{-6} - 4.0 x 10^{-5}) 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 artesunate on acetylcholine induced contraction is relatively moderate when compared to the inhibitory effect of atropine (10^{-5} M). Table 1 and 2: Figure 1 and 3.

Table 1: Effect of artesunate (10^{-5}M) on acetylcholine (Ach.) induced contraction in isolated guinea pig ileum.

FBC of Ach.(M) Control	-Log M Conc.	Response in mm	% of Max. Response	FBC of artesunate (10^{-5}M) on Ach.	-Log M Conc.	Response in mm	% Response
$4.0x10^{-6}$	-5.4	26.1 ± 0.5	51.78	$4.0x10^{-6}$	-5.4	2.0 ±0.4	3.89
$4.0x10^{-5}$	-4.4	41.2 ± 0.6	80.16	$4.0x10^{-5}$	-4.4	16.0±0.7	31.13
$4.0x10^{-4}$	-3.4	51.4 ±0.4	100	$4.0x10^{-4}$	-3.4	41.0±0.3*	79.80*
$4.0x10^{-3}$	-2.4	45.3± 0.2	88.13	$4.0x10^{-3}$	-2.4	46.5±0.2*	90.5*

X=Mean +SEM of 4 values, *maximum of* Acetylcholine=*.51.4mm ± 0.4* *P ≤ 0.05 significant relative to the control; FBC= Final Bath Concentration.

Table 2: Effect of Atropine(10^{-5}) on Acetylcholine induced contractions compared to responses of 10^{-5} M concentration of Artesunate on ACH induced contraction in an isolated Guinea pig ileum.

				Responses of artesunate on ACH, induced contractions.			
FBC of Atropine (10^{-5l} M) on ACH.	-log	* Maximum height (mm)	% of max	FBC OF ArtE	-log	* Maximum height (mm)	% of max
$4.0x10^{-6}$	5.4	00.0 ±0.0	00 ± 0.0	$4x10^{-8}$	5.4	02 ± 0.4	4.35%
$4.0x10^{-5}$	4.4	00.0 ± 0.5	00 ±1.5	$4x10^{-5}$	4.4	16. ±0.7*	34.81%
$4.0 x10^{-4}$	3.4	00.0 ±0.6	00 ±2.5	$4x10^{-4}$	3.4	41 ±0.3*	87.8 %
$4.0 x10^{-3}$	2.4	03.0 ± 0.0	7.7 ±0.0	$4x10^{-3}$	2.4	46.5±0.2	100%

- *Control response: graded concentration of Acetylcholine (mg/ml),*
- *Test response: responses concentration of artesunate in g/kg,* $P \leq 0.05$*

* $\overline{X}$ *±SEM of 4 values; maximum height.46.5 ± 0.2 by ARTESUNATE*

Table 3: Effect of Artesunate(10^{-5}M) on 1M KCL induced contractions in isolated Guinea Pig ileum.

1M` KCL + Artesunate (10^{-5}M) (Dose in ml)	LOG Dose	Response in (mm)	% of Max. Response
0.2	-0.70	20.0±0.5	57.14%
0.4	-0.40	19.0±0.2	54.29%
0.6	-0.22	31.0±0.3	88.57%

N0TE; X=mean +SEM of 4 values ; P ≤ 0.05 ;Emax=% of Max. Response for 1 M KCL alone = 35 mm.

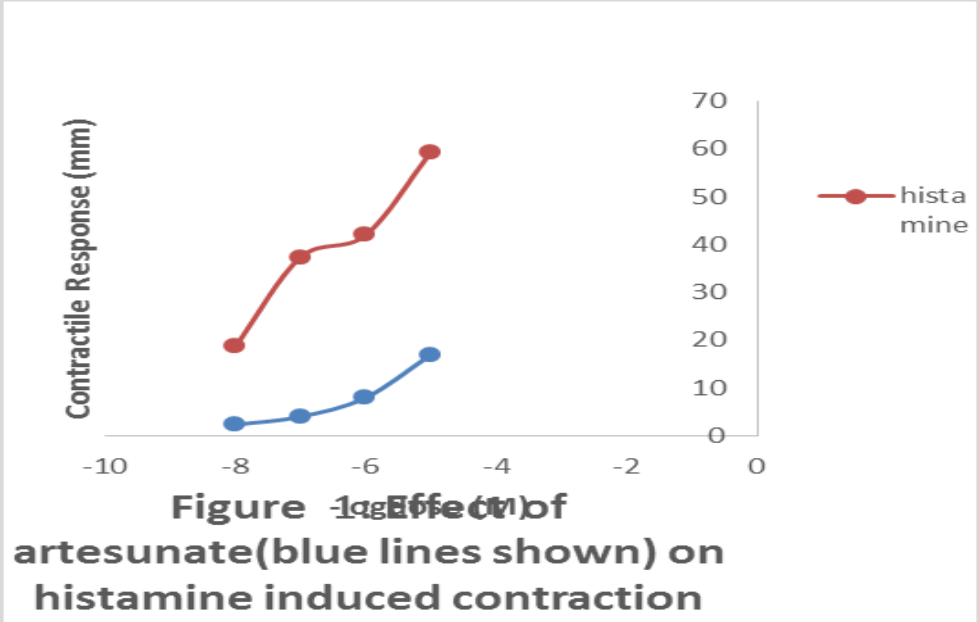

Figure 1: Effect of artesunate(blue lines shown) on histamine induced contraction

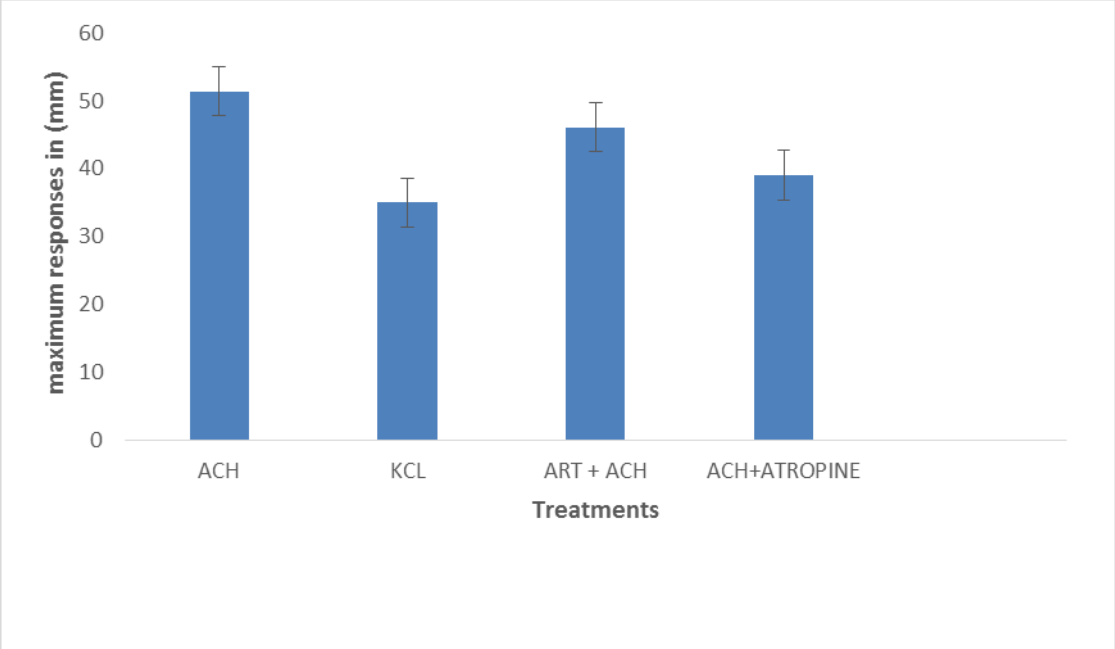

Figure 2: Bar chart showing selected maximum responses (mm) of some agonists and artesunate.

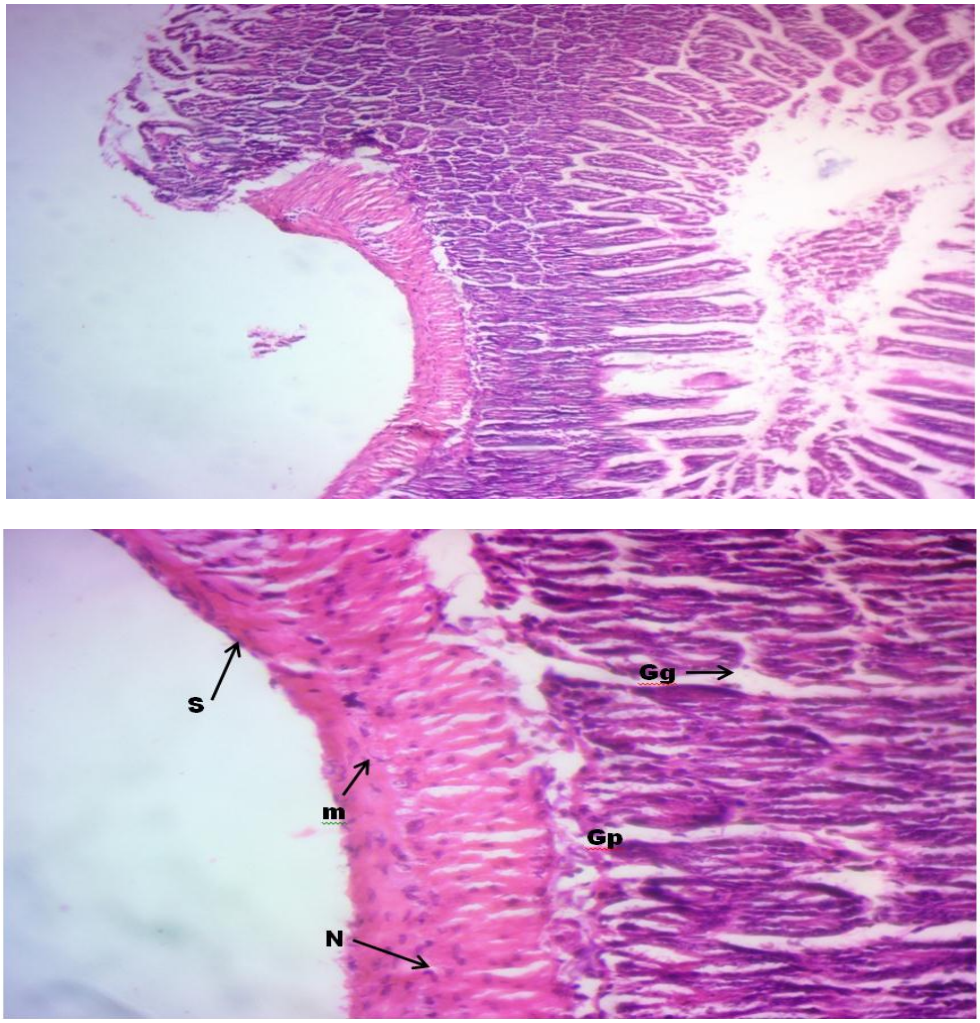

Figure 3: Photomicrographs of smooth muscle of the Ileum infected and non – treated (Negative control) at Magnification of (x100) stained with H& E method.Revealed normal cellular profile. Keys: Lumen (L), Nucleus (N), Gastric gland (Gg), Gastric pit (Gp), Muscularis mucosa (MM) and Serosa(S).

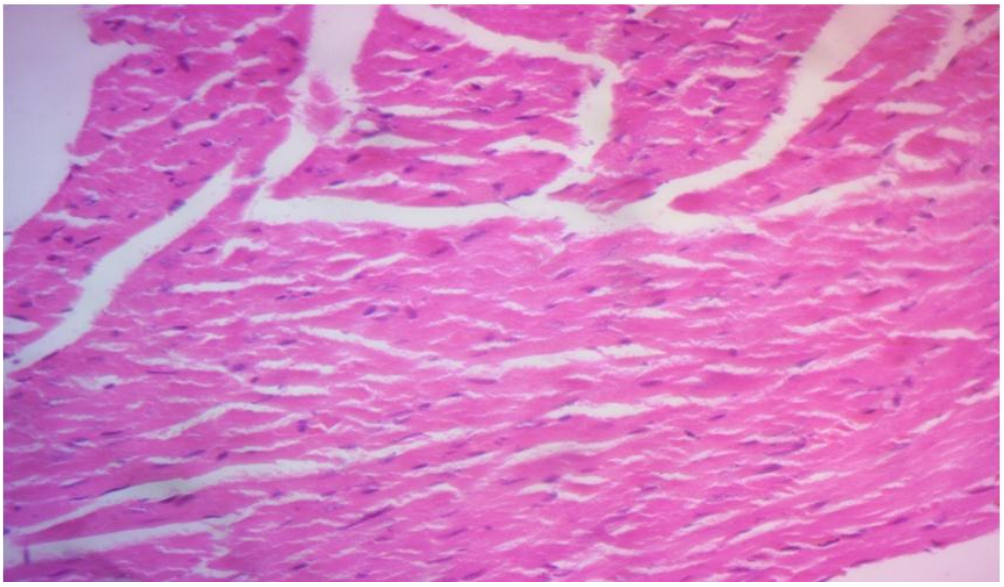

Figure 4: Photomicrographs of smooth muscle of the Ileum Infected + H.D of 6 mg/kg of ARTM at mag. A(x100) & B(x400) stained with H& E method.
Keys: Lumen (L), Nucleus (N), Gastric gland (Gg), Gastric pit (Gp), Muscularis mucosa (MM) and Serosa(S).

Figure 4: Photomicrographs of smooth muscle of the Ileum infected and treated with5 mg/kg of artesunate at magnification(x100) stained with H& E method. Revealed normal cellular and muscle tissue profile.
Keys: Lumen (L), Nucleus (N), Gastric gland (Gg), Gastric pit (Gp), Muscularis mucosa (MM) and Serosa(S).

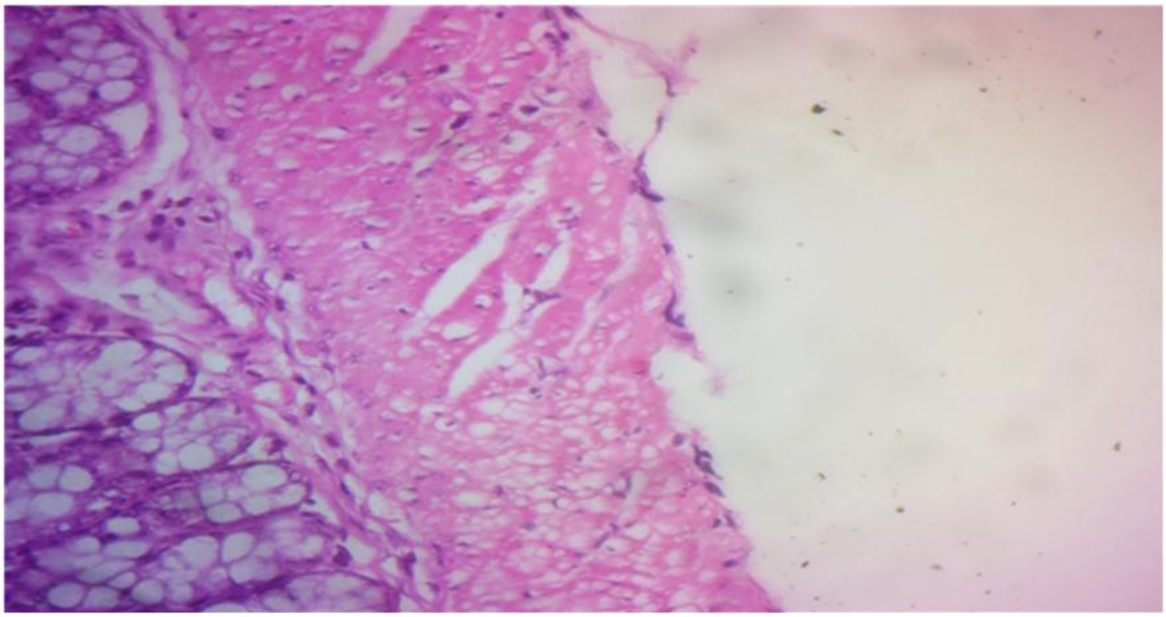

Figure 5: Photomicrographs of smooth muscle of the Ileum Infected and treated with 2.5mg/kg of ART. At mag. (x100) stained with H& E method. Histological sections of smooth muscle of the Ileum Infected with P.berghei and treated with of 2.5 mg/kg of Artesunate- revealed normal cellular profile no abnormality seen.

4. DISCUSSION

This study was designed to investigate the effect of artesunate on drug induced contraction inileum of experimental guinea pig models. The results revealed that artesunate alone at a concentration of $4x10^{-6}$ to $4x10^{-3}$ mg/ml produced no contractile responses, but the same concentration of artesunate significantly inhibited contractile responses induced by acetylcholine, potassium chloride and histamine on isolated smooth muscle tissues in guinea pig ileum models in a dose dependent manner with Emax of 46.1* mm($p<0.05$); (Table 1and 2; Figure 1 and 2). This observed results of no contractile responses when artesunate 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[13], [14],

All the inhibitory effects of the artesunate on induced contractile responses of acetylcholine, potassium chloride and histamine were not antagonized by phentolamine and atropine; in other experiment artesunate produced slight phasic contraction when external calcium (Ca^{2+}) ion was introduced. The slight phasic contractile activity was abolished by verapamil ($5x10^{-3}$mg/ml).This observed results can further be justify 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.[15], [16] [17],[18], The availability of Ca^{2+} is essential for smooth muscle contraction.

Although alternative mechanisms of action have been suggested by way of parasites interacting with haem to produce a carbon-cenered free radicals that alkylate proteins.[19] Another more recent work report thatartemisinin act viaa sarcoendoplasmic reticulum calcium adenosine triphosphatase (SERCA), which is an ortholoque of the mammalian enzyme.[20]

The smooth muscle of infected and non-infected mice treated and non treated with artesunate showed normal cellular profile, hence no abnormality seen,(Figures 3,4,and 5); thus, not affected; this result corroborate with the report of similar studies by Tologbonse on the effect of artemether($2.1x10^{-6}$ to $2.1x10^{-2}$ mg/ml) on the mechanical activities of isolated smooth muscles of non-pregnant uterus and ileum in mice and wister rats, which shows that artemether significant inhibited contractile responses on standard agonists such as acetylcholine potassium chloride and oxytocin.[21]

5. CONCLUSION

We therefore conclude that, artesunate seems to be acting via a non-specific receptor mechanism and mild interference with transmembrane calcium ion fluxes possibly via sarcoendoplasmic reticulum calcium adenosine triphosphatase by a non-specific processes, independent of animal species. The sub-acute toxicity study has also revealed that it is relatively safe in therapeutic doses; thus, artesunate possessed great and safe pharmacological properties that justified their current usage in the treatment of malaria as recommended by the World Health Organization.

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Ethical consideratrion.

All the animals received humane care and the study protocols was designed to comply with the institution's guidelines for use of laboratory animals (Faculty of Pharmacy's ethical committee's clearance was obtained), in line with the 'Principle of Laboratory animal care' (of National Institute of Health-NIH Publication guidelines).[22]

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