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In-vivo and safety studies of artemether and doxycycline entrapped in solid lipid nanoparticles

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Abstract

Nanoparticle has emerged as a promising strategy for the efficient delivery of drugs used for the treatment of some diseases by specific targeting to achieve improved delivery of poorly water-soluble drugs.This is achieved by delivering drug in small particle size to increase the total surface area of the drugs allowing faster dissolution in blood stream. At nanosize range, the properties of materials differ substantially from bulk materials of the same composition, mostly due to the increased specific surface area and reactivity, which may lead to increased bioavailability and toxicity.This research focuses on evaluation of safety and *in vivo study* of artemether-doxycline entrapped in solid lipid nanoparticles.The The safety profile of the formulation was determined in white albino mice. The pharmacodynamics of the drugs were evaluated to determine the suppressive potential of the formulated nanoparticles against established *Plasmodium* infection using a modified 4-day Peter suppressive test. Raw data were analyzed using one-way ANOVA with SPSS software,Significance was accepted at the 5%, 1%, and 0.1% levels.The LD_{50} of the formulated nanoparticle was above 5000 mg/kg. The hematological showed that the percentage parasitemia, PCV, RBC, and HB of all groups treated with artemether-doxycycline-loaded SLNs were in the range of 1.05% to 14.50%, 38.75% to 56.75%, 0.7.05 x 10^{12}/L to 7.30 x 10^{12}/L, and 12.88 g/dL to 18.03 g/dL, respectively.When mean percentage parasitemia of all groups were compared with 22.0 % mean percentage parasitemia of negative control (SLN), it indicates a highly active anti-plasmodial therapeutic effect. While the biochemical and histological results showed that the formulated nanoparticle did not increase the adverse effects of artemether and doxycycline. The artemether-doxycycline loaded solid lipid nanoparticles developed resulted in a safe and effective alternative drug and reduced side effects.

Keywords: *Plasmodium falciparium;* Hematological; Toxicity; Nanoparticle; Formulation.

1. Introduction

The World Health Organization has set an ambitious goal of reducing morbidity and mortality from malaria by 90%. This is especially important for *Plasmodium falciparum* (*P. falciparium*), the species most likely to cause malaria and death. Artemisinin-based combination therapy (ACT) has been effective as a first-lin e treatment for malaria in all age groups. However, when used alone, artemether has not been able to fully clear all asexual and sexual stage parasitemia (Burrrows *et al*., 2017). AL treatment due to re-infection (Kabanywanyi *et al*., 2007), which is a serious concern particularly in areas with very high malaria transmission (Byakika-kibroika *et al*., 2011). Given these limitations, there

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is great interest in the development of new therapeutic approaches, such as the nanoformulation of artemether and doxycycline. This research focuses on an alternative drug delivery system for the combination of artemether and doxycycline.

Artemether is only available in a combination with lumefantrine for the treatment of uncomplicated *P. falciparum* malaria, which is the predominant species in Africa. Artemether produces a reactive metabolite as a result of the interaction between its peroxide bridge and haem iron, which leads to the production of an endoperoxide moiety in the food vacuole of the malaria parasite. This has a secondary effect of inhibiting nucleic acid and protein synthesis within the malaria parasite, resulting in rapidez clearance of parasitemia in the erythrocyte stage of *P. falciparum* and does not exhibit cross-resistance with other antimalarial drugs. However, the poor water solubility of the artemether-lumefantrine combination makes it necessary to consider alternative formulations such as artemether and doxycycline. A combination of poorly water-soluble drugs with lipids can be highly effective in enhancing oral drug absorption and bio-availability (Agbo *et al*., 2016). This solid lipid nanoparticle formulation increases the bio-availability and safety of the drugs.Doxycycline is effective against the asexual erythrocytic forms of *Plasmodium falciparium,* but not against the gametocytes of *P. falciparium*. It is more active against old trophozoites and young schizonts, with a dose and time-dependent relationship for effectiveness of the drug (Dahel, 2006). Doxycycline has a high degree of lipid solubility and a low affinity for calcium binding, which makes it a best fit . However promoting the possiblity to achieve one of the aims of solid lipid nanoparticle; incoporation of lipophilic and hydrophobic drugs.

Solid lipid nanoparticles (SLN) are nano-carriers that are generally spherical in shape with an average diameter of 10-1000 nm, containing a biodegradable solid lipid core matrix. They are not toxic because they are made from physiological lipids and have a number of versatile properties, including high drug payload, controlled release, drug targeting, and the ability to incorporate both hydrophilic and lipophilic drugs. These characteristics make SLN an efficacious nano-carrier system for a wide range of therapeutics that face challenges in this area (Shah *et al*., 2015).

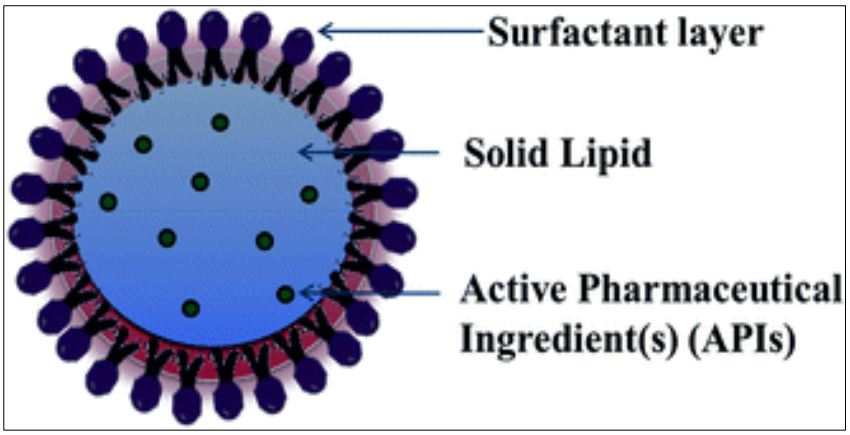

Figure 1 Schematic presentation of the complete structure of solid lipid nanoparticles (https:www//pubs.rsc. org)

2. Materials and methods

2.1. Animals

The experimental animals were white albino mice and rats of either sex, weighing between 20.5 and 49.9 grams for the mice and 150 and 200 grams for the rats. They were between 3 and 4 months old and were obtained from the animal unit at the Faculty of Veterinary Medicine at the University of Nigeria in Nsukka.

2.2. Reagents and chemicals

Methanol, hydrochloric acid, distilled water, and p-nitroaniline were obtained from BDH (UK). Artemether and doxycycline hydrochloride drug samples were voluntarily provided by POKO Pharmaceutical in Awka, Nigeria. Phospholipon® 90G was obtained from Pospholipon GmbH in Cologne, Germany,goat fat from abbatoir and activated charcoal was obtained from BDH Chemical Ltd in England. Sorbitol and Sorbic acid were obtained from Sigma-Aldrich, SYBR® green I was also obtained from Sigma-Aldrich, and Tween 80 was obtained from Guangdong, China.

2.3. Study Site

The formulation and freeze-drying of the samples took place from March to August, 2019 at the Research Center for Biotech and Bio-resources affiliated with UNIPORT. The *in vivo* part of the study was carried out in the Faculty of Veterinary Medicine at the University of Nigeria Nsukka from September to December, 2019.

2.4. Extraction

2.4.1. Extraction and purification of fat from Capra hircus (Goat fat)

To extract the fat from the goat, the adipose tissue was grated and then boiled with half its weight in water on a water bath for 45 minutes. The molten fat was separated from the aqueous phase using a muslin cloth. The fat was further purified by heating with a 2% w/w suspension of activated charcoal (for de-colorization and deodorization) and bentonite (2:1) at 100°C for 1 hour. The suspension was then vacuum filtered using a Buchner funnel (Attama and Nkemnele, 2005).

2.4.2. Characterization of the purified goat fat

The melting point of the goat fat and phospholipion® 90G was determined using a standard procedure. A small quantity of semi-solid creamy goat fat and crystalline phospholipion were each packed separately into thin-walled capillary tubes that had been sealed at or one on end. The capillary tubes and a thermometer were placed in appropriate apertures in a heating block. The temperature was raised uniformly in 10^0C intervals and the melting point was recorded.

2.5. Solid lipid Nano-formulation of artemether/doxycycline

The high shear-pressure homogenization technique (Jenning *et al.*, 2002) was adopted. In each case, the different ratios of the lipid matrix (1:1 and 2:1) were melted separately at 70°C above the heterolipid temperature, and an aqueous phase containing 1 and 2 % w/w Tween 80® (surfactant) was added to the molten lipid matrix with gentle stirring (using a magnetic stirrer). The drugs (artemether and doxycycline) were added to the different ratios of the lipid matrix (1:1 and 2:1) in ratios of 1:2, 1:6 and 1:10 % w/w, 0.1% sorbic acid (preservative) and 4 % sorbitol (cryoprotectant) were also added. Controls containing no drugs (unloaded SLNs) were also formulated to contain the different ratios of lipid matrix, % w/w Tween 80, sorbic acid, sorbitol and sufficient distilled water to up make to 100 % w/w. The mixtures were further subjected to high shear homogenization with a homogenizer (Ultr-Turrax,T18 indu,IKA Germany) at 20, 200 rpm for 15 minutes for 2 cycles to produce a hot oil-in-water pre-emulsion. The pre-emulsion was allowed to cool, leading to crystallization of the lipid and the formation of SLNs. The resulting SLNs were lyophilized using a freeze-dryer to obtain water-free SLNs.

2.6. *In vivo* study of the SLNs

2.6.1. Anti -Parasitemia Test

Plasmodium Berghei suppressive test

A 4-day Peter's suppressive test was modified and used to determine the effect of SLNs on mice. The mice were housed in standard cages, provided with standard feed and water ad libitum, and acclimatized for 2 weeks prior to the experiments. The malaria parasites were maintained by serial blood passage in mice. An infected donor mouse's blood was diluted with isotonic saline to create an inoculum containing infected erythrocytes/mL. Then, 56 mice were each inoculated intra-peritoneally with 0.2 mL of the inoculum before being randomly divided into 14 treatment groups of 4 mice each. A 0.1 g of the drugs were dissolved in 5 mL of the vehicle, which consisted of 0.5 mL of DMSO (Demethyl Sulphoxide) , 1 mL of Tween 80, and 3.5 mL of normal saline. The different batches of the formulation and the pure samples were administered orally to the mice in the respective groups at 4 h (0), 24 h (day 1), 48 h (day 2), 72 h (day 3), 96 h (day 4), 120 h (day 5), 144 h (day 6), and 168 h (day 7). The drugs were administered according to the weights of the mice in the various groups, with the exception of the last group, which was untreated. The stock concentration used was 100 mg/kg/5 mL. On day 7, 24 h after the last treatment, blood was collected from the mice through the tail vein and applied to a clean micro slide to make a thin smear, which was allowed to dry for a few seconds before being fixed with methanol for 2 minutes. The slide was then placed in a staining rack containing giemsa and allowed to sit for 30 minutes before being removed and rinsed with water. The slide was then allowed to dry on a slide rack. For viewing, a drop of oil immersion was placed on the slide and viewed under a ×100 objective (magnification of 1000). During the viewing, both parasitized and non-parasitized blood cells were seen. The viewing was done in a way that allowed for the proper counting of cells, so that the percentage parasitemia could be calculated from the total number of cells counted. Blood samples collected from the animals were subjected to haematological and biochemical tests, after which

the mice were sacrificed and their organs (livers) collected.Thereafter, the histopathological examination was then carried out on the harvested organs.

Figure 2 tween 80 (surfactant)

$$\%\ Parasitemia = \frac{parasites\ RBCs}{total\ RBCS} \times 100 \text{ ------(8)}$$

2.7. Haematological Studies of SLNs in Mice

2.7.1. Total Red Blood Cell Count (TRBC)

The total red blood cell count was determined using the method described by Dacie and Lewis (2000)

2.7.2. Principle

The blood sample was diluted 1:200 with red blood cell diluting fluid and the cells were counted under high power (40X objective) using a counting chamber. The number of cells in the blood was calculated and reported as the number of red cells per micro-liter of whole blood.

2.7.3. Method

The whole blood from the tube was carefully mixed by swirling the bulb. The blood was quickly drawn up to the 0.5 mark using a red blood cell pipette, then excess blood outside the pipette was carefully wiped off with cotton wool. The same pipette was used to draw diluting fluid up to the 101 mark. The cells were allowed to settle for 2 to 3 minutes, then the counting chamber was placed on the stage of the microscope. The microscope was switched to low power (10x objectives) and the light was adjusted to locate the large square in the center with 2 small squares. The microscope was then switched to high power (40x objective). Finally, the red blood cells in the four corner squares and in the center square were counted.

$$\text{Total red blood cells per liter of blood} = \frac{Number\ of\ cell\ counted}{Volume\ counted \times Dilution \times 10} \text{- ------(9)}$$

2.8. Determination of Packed Cell Volume (PCV)

Packed cell volume (PCV) was determined by the methods of Dacie and Lewis (2000)

2.8.1. Principle

Anti-coagulated blood in a glass capillary of specified length, bore size, and wall thickness was centrifuged in a micro-haematocrit centrifuge at RCF 10,000-11,000 rpm for 5 minutes to obtain a constant packing of the red cells. The PCV value was read from the scale of a haematocrit reader or calculated by dividing the height of the red cell column by the height of the total column of blood.

2.8.2. Method

A heparinized capillary tube was filled with blood from an EDTA (Ethylenediaminetetraacetic acid) tube up to three quarters of the tube's capacity. The end of the tube was sealed with a plasticine sealant. The capillary tube was centrifuged for 5 minutes (RCF 10,000-11,000 rpm). Immediately after centrifuging, the PCV was read using a micro-haematocrit reader by aligning the base of the red cell column above the sealant on the 0 line and the top of the plasma column on the 100 line.

$$PCV = \frac{length\ of\ Red\ blood\ Cell\ Count\ (mm)}{Length\ of\ total\ Red\ blood\ cell} \text{-----(10)}$$

2.9. Determination of Haemoglobin (Hb) Concentration

Hemoglobin (Hb) concentration was determined using the hemoglobin cyanide (HiCN) technique as described in the method of Dacie and Lewis (2000).

2.9.1. Principle

Whole blood was diluted 1:20 mL in a modified Drabkin's solution that contained potassium ferricynaide and potassium cyanide. The red cells were hemolyzed, and the hemoglobin was oxidized by the ferricynaide to methaemoglobin. This was converted by cyanide to stable HiCN. The absorbance of the HiCN solution was read at a wavelength of 540 nm. The absorbance obtained was compared with that of a reference HiCN standard solution. Hemoglobin values were obtained from tables prepared from a calibration graph.

2.9.2. Method

A volume of 20 µL of capillary blood was dispensed into 4 mL of Drabkin's neutral diluting fluid in a tube. The tube was stoppered, mixed, and left at room temperature (29-35°C) for 5 minutes. The calorimeter was adjusted to 540 nm wavelength, zeroed with Drabkin's fluid, and the absorbance of the sample was read. Using a table prepared from the calibration graph, the mice hemoglobin values were determined.

$$\text{Concentration of HiCN} = (\frac{mg}{l}) \times \frac{200}{1000} \text{------ (11)}$$

Table 1 Preparation of Calibration Curve for Haemoglobin Test

Tests Tubes	**B**	**1**	**2**	**3**	**4**	**5**
1ml in 20 ml diluted standard	-	4	3	2	1	5
1ml of ammonia water	5	1	2	3	4	-

Six tubes were obtained and labeled as blank B, 1, 2, 3, 4, and 5, respectively. The following reagents were pipetted into the tubes as shown in table below 1.

The calorimeter was adjusted to 540 nm followed by zeroing with Drabkin's neutral fluid in B and reading of the absorbance.

The hemoglobin (Hb) equivalent in g/l of the solution in tubes 1-5 was calculated as follow:

Tube 1 Hb value of HiCN Standard $x\frac{4}{5} =$ Hb g/l
Tube 2 Hb value of HiCN Standard $x\frac{3}{5} =$ Hb g/l
Tube 3 Hb value of HiCN Standard $x\frac{2}{5} =$ Hb g/l
Tube 4 Hb value of HiCN Standard $x\frac{1}{5} =$ Hb g/l
Tube 5 Hb value of HiCN Standard =Hb g/l

A graph of absorbance against concentration was plotted for Hb with values from (20-200) g/l 0r (2-20) g/dl.

2.10. Toxicity Studies

2.10.1. Acute Toxicity study of artemether-doxycycline SLN

Acute toxicity was tested using the method described by Lorke (1983). All treatments were administered orally. The study was divided into two phases. In the first phase, nine mice of either sex were divided into three groups of three mice each. Group 1 received a dosage of 10 mg/kg of artemether-doxycycline SLN, while group II and group III received dosages of 100 mg/kg and 1000 mg/kg, respectively. The animals were observed for signs and symptoms of toxicity and mortality such as severe dazziness, unconciousness, abnormal bleeding, difficulty breathing swelling for 24 hours after treatment. In the second phase, four mice of either sex were divided into four groups of one mouse each. The first

mouse received a dose of 1900 mg/kg, while the second, third, and fourth mice received doses of 2600 mg/kg, 5000 mg/kg ofartemether-doxycycline SLN, respectively. The mice were also observed for 24 hours, and the final results were recorded.

2.11. Biochemical Studies

2.11.1. Liver Function Test

Assay of Alanine Aminotransferase (ALT) Activity

The activity of alanine amino-transferase was assayed using the method of Reitman and Frankel (1957) as described in the Rand-ox kit.

- Principle

The alanine amino-transferase assay, according to this method, is based on the principle that pyruvate is formed by the following reaction: α-oxoglutarate + L-alanine → L-glutamate + pyruvate. Alanine amino-transferase activity was assayed by monitoring the concentration of pyruvate hydra-zone formed with 2,4-dinitrophenylhydrazine.

Assay of Aspartate Aminotransferase (AST) Activity

The activity of Aspartate amino-transferase was assayed using the method of Reitman and Frankel (1957) as described in Rand-ox Kits (UK).

- Principle

The principle of the assay is based on the following reaction: α-oxoglutarate + L-alanine → L-glutamate + pyruvate.

- **Method**

Aspartate amino-transferase activity was assayed by monitoring the formation of oxaloacetate hydra-zone with 2,3-dinitrophenylhydrazine. A volume of 0.5 mL of AST substrate phosphate

buffer was pipetted into both the reagent blank (B) and sample test (T) tubes. A volume of 0.1 mL of distilled water was added to the reagent blank (B). The entire reaction medium was mixed well and incubated for 30 minutes in a water bath at 37°C. After incubation, a volume of 0.5 mL of 2,4-dinitrophenylhydrazine was added to the reagent blank (B) and the sample test tubes. A volume of 0.1 mL of the sample was added to blank (B) only. The medium was mixed thoroughly and allowed to stand for exactly 20 minutes at 25°C. Finally, 5.0 mL of sodium hydroxide solution was added to both blanks.

Assay of Alkaline Phosphate (ALP) Activity

The activity of alkaline phosphatase (ALP) was assayed using the method of Klein *et al*. (1960) as described in the Rand-ox kit (UK). This method is based on the principle that serum alkaline phosphatase hydrolyzes a colorless substrate of phenolphthalein, which at an alkaline pH turns pink in color and whose optical density can be measured spectrophotometrically.

- **Procedure**

A volume of 0.1 mL of distilled water was pipetted into two sets of test tubes labeled SA (sample) and ST (standard), respectively. One drop of a chromogenic substrate was added to the distilled water in each set of test tubes, and their contents were mixed. The tubes were incubated at 37°C for 20 minutes in a water bath. After incubation, a standard solution of 0.1 mL was added to the standard test tube (ST) only, followed by the addition of a serum sample of 0.1 mL to the sample test tube (SA). The contents were mixed and incubated at 37°C for 20 minutes in a water bath. A volume of 5.0 mL of a color developer was added to both sets of test tubes. The absorbance of the sample against the blank (water) was read at a wavelength of 550 nm. The activity of alkaline phosphatase in the serum was calculated using the provided formula.

$$\frac{\text{SAO.D}}{\text{STO.D}} \times 30 = \mu\text{/l of alkaline phosphate-------(12)}$$

Where: SA O.D = Sample Optical Density

ST O.D = Standard Optical Density

Assay of Total Bilirubin Concentration

Bilirubin is formed by the breakdown of hemoglobin in the spleen, liver, and bone marrow. An increase in bilirubin concentration in the serum or tissue causes a condition known as jaundice. Total bilirubin concentration was determined using the method of Jendrassik and Grof (1938) as described in the Rand-ox kit (UK).

- **Principle**

Direct (conjugated) bilirubin reacts with diazotized sulphanilic acid in an alkaline medium to form a blue colored complex. Total bilirubin is determined in the presence of caffeine, which releases albumin-bound bilirubin by reacting with diazotized sulphanilic acid.

- **Method**

A volume of 0.2 mL of sulphanilic acid was pipetted into the sample blank tube and the sample tube. Caffeine (10 mL) and 0.2 mL of the sample were also pipetted into each of the sample blank tube and sample tube. These mixtures were mixed and incubated for 10 minutes at 20-25°C. Finally, 1.0 mL of tit-rant was pipetted into the sample blank tube and sample tube. These mixtures were mixed again and incubated for 30 minutes at 20-25°C. The absorbance was read at 579 nm against the sample blank (578 nm).

$$\text{Total bilirubin (u/mol)} = 185 \times \text{sample blank(578 nm)}$$

2.12. Histopathology studies of the SLN

Afterwards, histopathological examination was performed on the harvested organ. Tissue sections from the livers of experimental animals were fixed in 10% formalin saline and dehydrated in ascending grades of ethanol. The tissue was then cleared in chloroform overnight, infiltrated and embedded in molten paraffin wax. The blocks were trimmed and sectioned at 5-6 um. The sections were deparaffinized in xylene, brought to water, and then stained with hematoxylin and Eosin (H&E) for light microscopy.

2.13. Method of Data Analysis

2.13.1. Statistical Analysis

Results were presented as the mean ± standard deviation (SD) and standard error of mean (SEM) with sample replicates (n=4). For group comparison, raw data were subjected to statistical analysis using one-way ANOVA in the Statistical Package for Social Science (SPSS) 2.10 and Microsoft Excel. Significance was accepted at $P<0.05$, $P<0.01$, and $P<0.001$.

3. Results and discussion

3.1. Toxicity studies

3.1.1. Acute toxicity studies

The LD_{50} of the SLNs in mice was found to be more than 5000 mg/kg body weight. None of the animals died in either phase, and none showed signs of toxicity within 24 hours of constant observation, as illustrated in Table 2. These results indicate that the SLNs are non-toxic at the highest dose tested and can be considered a safe formulation. Acute toxicity studies provide information on the adverse effects of a substance after single or multiple doses given over a period of no more than 24 hours (Al-Affi *et al.*, 2018; Organization for Economic Cooperation and Development, 2002)

Table 2 Acute Toxicity Study (LD 50) of SLNs on Mice

PHASE 2				
Group	**Dosage**	**Mice 1**	**Mice 2**	**Mice 3**
Group 1	10mg/kg	ND and NST	ND and NST	ND and NST
Group 2	100 mg/kg	ND and NST	ND and NST	ND and NST
Group 3	1000 mg/kg	ND and NST	ND and NST	ND and NST
PHASE 2				
Group	**Dosage**	**Mice 1**	**Mice 2**	**Group**
Group 1	1900 mg/kg	ND and NST	ND and NST	Group 1
Group 2	2600 mg/kg	ND and NST	ND and NST	Group 2
Group 3	5000 mg/kg	ND and NST	ND and NST	Group 3

Key: Using 1:2 and 2:1 lipid ratio of SLNs,ND=No Death;NST=No Signs of Toxicity

3.2. *In vivo* Studies

3.2.1. Plasmodium berghei suppressive test and haematological studies of SLNs

The *in vivo* anti-malarial Pharmacodynamic studies indicated a significant, very significant, and very very significant (X = $p < 0.05$, P = $p < 0.01$, Q = $p < 0.001$) release and high anti-plasmodial effect of the drug-loaded SLNs in Plasmodium-infected mice, compared to SLN and artemether-lumefantrine (commercial sample) as shown in Table 3, the results indicated that the mean parasitemia count, PCV, RBC, and HB of all groups treated with artemether-doxycycline-loaded SLNs were in the range of 1.05% to 14.50%, 38.75% to 56.75%, 0.7.05 x 10^{12}/L to 7.30 x 10^{12}/L, and 12.88 g/dL to 18.03 g/dL, respectively. The mean parasitemia count of the negative control (SLN) was 22.0.% The commercial sample (artemether-lumefantrine) reduced parasitemia to 0.75. According to Table 2, there was a significant difference ($p < 0.001$) in the mean percentage parasitemia among the groups. The mean percentage parasitemia in all groups significantly decreased ($p < 0.001$) compared to the mean percentage parasitemia of the mice in groups 10C (artemether), 11C (doxycycline), 13C (artemether-doxycycline), 14C (SLN), and 15C (normal saline). Additionally, there was a significant decrease ($p < 0.001$) in the mean percentage parasitemia of artemether-lumefantrine compared to the other groups. However, there was a significant increase($p <0.001$) in the unformulated artemether-doxycycline (9.75) compared to the artemether-doxycycline-loaded SLNs in groups 1a (1.05) and 3a (1.10). This is not in line with the results obtained by Agbo *et al*. (2018), whose mean parasitemia count of all groups treated with artemether and lumefantrine-loaded SRMS-based SLMs was within the 3.3% to 6.5% range, while the mean parasitemia count of the normal saline in the artemether-doxycycline SLN (negative control) was 37.75. Table 5 also showed that there was a very very significant difference ($p < 0.001$), very significant difference ($p < 0.01$), and significant difference ($p < 0.05$) in the group average packed cell volume (PCV) . This showed that the average packed cell volume of the animals in group 3a was significantly increased ($p < 0.05$) compared to that of the mice in group 14c (SLN). Additionally, the average packed cell volume in group 1a was significantly increased ($p< 0.01$) compared to group 14c (SLN), and it was also observed that the average packed cell volume in group 6a significantly increased compared to group 14c (SLN). However, there was no significant difference ($p > 0.05$) in groups 1b, 3a, 6b, 9a, and 9b when compared to group 14C (SLN). PCV refers to the percentage of whole blood that is composed of red blood cells. It is a measure of the number and size of red blood cells. The haematocrit (PCV) is used to assess anaemia, erythroctosis, hemodilution and hemoconcentration of the blood (Dacie and Lewis, 2000). A low value indicates anemia, while a high haematocrit may indicate dehydration. The significant increase observed in groups 1a, 3a, and 6a could be due to dehydration. On the other hand, the non-significant decrease observed in the parent (pure) drug and the other SLNs suggests that the drugs are unlikely to cause anemia.

The results of the artemether-doxycycline-loaded SLNs and pure drugs on the red blood cell count showed a non-significant increase ($p < 0.05$) in groups 1b, 3b, 6b, 9a, 9b, 11c, 12c, 13c, 15 c, and a significant difference ($p < 0.05$) in group 6a and significant difference in groups 1a and 3a. A high red blood cell count can indicate dehydration, bone marrow disorders, or other health issues. A reduction in red blood cells can be caused by various factors such as anemia, bone marrow failure, nutritional deficiencies, malnutrition, haemolysis due to blood vessel injury, and certain chemotherapy. The significant decrease in the average red blood cell count in group 6a could be due to physiological changes, while the significant increase in groups 1a and 3a could be attributed to dehydration. However, the non-

significant difference ($p > 0.05$) in the majority of the groups (1b, 3b, 6b, 9a, 9b, 11c, 12c, 13c, and 15c) compared to group 14c indicates that the SLNs and the parent drugs are unlikely to cause anemia. Table 2, also showed that there was no significant difference ($p< 0.05$) in the average haemoglobin among the group means of 1b, 3b, 9a, 9b, 10c, 11c, and 15c; a significant difference ($p < 0.05$) in groups 1a, 3a, and 6b; and a very, very significant difference ($p < 0.001$) in groups 13c and 6a. Haemoglobin transports oxygen and gives the blood its red pigment. Therefore, haemoglobin concentration is directly proportional to its ability to transport oxygen throughout the body. The significant increase in the average haemoglobin in groups 1a, 3a, 6a, 6b, and 13c indicates its ability to transport oxygen throughout the body. Thus, the non-significant difference ($p > 0.05$) in the average haemoglobin in the majority of the groups indicates sufficient oxygen, which implies that the SLNs and parent drug do not interfere with the oxygen supply throughout the body.

Table 3 Percentage of parasitemia, red blood cell count, and haemoglobin count in albino mice that were given solid lipid nanoparticles containing artemether and doxycycline, pure artemether, or pure doxycycline.

Group	Parasitaemia (%)	PCV (%)	RBC (x10^{12}/L)	HB (g/dl)
1a	1.05 ± 0.69$^{Q\ a}$	54.0 ± 2.68 $^{p\ c,d}$	7.30 ± 0.55 $^{Q\ d}$	18.03 ± 0.88 ^{xf}
1b	8.50± 4.91$^{Q\ a,b}$	48.25 ± 1.31$^{NS\ a,b,c}$	5..68 ± 0.41 $^{NS\ a,b,c}$	16.23 ± 0.54 $^{NS\ c,d,e,f}$
3a	1.10±0.97$^{Q\ a}$	56.75 ± 1.03 $^{Q\ d}$	8.33 ± 0.67$^{Q\ d}$	18.90 ± 0.83 ^{xg}
3b	4.50 ±2.87 $^{Q\ a,b}$	48.50 ± 4.29 $^{NS\ a,c,d}$	6.60 ± 0.64 $^{NS\ a,b,c}$	16.03 ± 1.39 $^{NS\ c,d,e,f}$
6a	10.25± 2.90 $^{Q\ a,b}$	53.75 ± 0.85$^{x\ b,c,d}$	7.05 ± 0.33$^{x\ b,c,d}$	17.80 ± 0.35$^{Q\ f,g}$
6b	14.00 ± 1.22$^{Q\ a,b}$	38.75 ± 1.03$^{NS\ a}$	4.88 ± 0.63$^{NS\ a}$	12.88 ±0.35 $^{x\ a}$
9a	14.50 ± 4.73 ^{Qb,c}	42.00 ± 1.87$^{NS\ a,b}$	5.18 ±0.18 $^{NS\ a,b}$	14.00 ± 0.68 $^{NS\ a,b}$
9b	13.25±3.42 $^{Q\ b,c}$	46.00 ± 0.82 $^{Ns\ a,b,c}$	5.43 ± 0.13 $^{NS\ a,b,c}$	15.20 ± 0.27 $^{NS\ b,c,d}$
Artemether(10 c)	4.75±0.63 $^{Q\ a,b}$	48.00 ± 1.22$^{x\ a,b,c}$	6.20 ± 0.33 $^{NS\ a,b,c}$	16.1 0 ± 0.35 ^{NSc,d,e,f}
Doxycycline(11c)	12.00 ± 0.41 $^{Q\ a,b,c}$	51.00 ±1.63$^{NSs\ a,b}$	7.00 ±0.45$^{x\ b,c,d}$	17.08 ± 0.51 ^{NSc,d,e,f}
Art + Lumefantrine (12)	0.75 ±0.25 $^{Q\ a}$	42.75 ± 0.25$^{NS\ a,b}$	5.28 ±0.25 $^{NS\ a,b}$	14.00 ± 0.00$^{NS\ c,d,e,f}$
Art + Doxycyline(13)	9.75 ± 3.64$^{Q\ a,b}$	43.75 ± 2.63 $^{Ns\ a,b,c}$	6.25 ± 0.77 $^{NS\ a,b,\ c}$	14.58 ±0.87$^{Q\ a,b,c}$
SLN(14)	22.00 ± 0.48 $^{Q\ c}$	49.75 ± 0.25$^{NS\ a,b,c}$	6.63 ±0.05$^{NS\ a,b,c}$	16.53 ± 0.19 $^{NS\ d,e,f,g}$
Normal Saline (15)	37.75 ± 0.25^{d}	44.75 ± 0.85$^{NS\ a,b}$	5.30 ± 0.08 $^{NS\ a,b}$	15.85 ± 0.27 ^{a,b,c}

Key: Mean and SEM at significance x=($p<0.05$), P = ($P<0.01$), Q=($P<0.001$), Ns-non significant

3.3. Biochemical Studies

Table 4 shows that there was no significant difference ($p>0.05$) in the average ALT level in the group that received artemether-doxycycline loaded solid lipid nanoparticles (SLN) and the group that received the parent drug. However, a significant difference ($p<0.05$) was observed in group 15c, which received normal saline. ALT activity in serum is a reliable and sensitive biomarker of liver disease. It is present in high concentrations in the Cytosol of the liver and to a lesser extent in skeletal muscle, the heart, and the kidney. An increase in ALT can indicate viral or drug-induced hepatitis. It can also be a good indicator of overall health, such as obesity, the metabolic syndrome, and the presence of cardiovascular disease. The non-significant difference ($p>0.05$) in the average ALT level in all groups suggests the absence of hepatocellular damage and other health conditions.

The results of AST levels from Table 4, show a non-significant difference ($p>0.05$) in the average AST in all groups involving artemether-doxycycline loaded SLN and the parent drug. AST is an enzyme present in the liver, nervous tissue, skeletal muscle, and heart. Damage to membranes can release this enzyme into circulation. High levels can indicate liver damage due to toxicity and viral hepatitis, as well as cardiac infection and muscle injury (Varley *et al*., 1991).

According to Table 4, there was no significant difference ($p>0.05$) in the average ALP level in groups 13c (artemether-doxycycline), 14c (solid lipid nanoparticles [SLN]), 1a, 6a, and 9b. However, there was a very significant difference ($p<0.01$) in the average ALP level in groups 3a, 3b, and 9a, and a very, very significant difference ($p<0.001$) in groups

1b, 6a, 10c, 11c, and 12c. ALP is a membrane-bound enzyme that is often used to assess the integrity of the plasma membrane and endoplasmic reticulum. It is present in all tissues throughout the body, but is particularly concentrated in the liver, bile duct, kidney, bone, and placenta. Therefore, it is not a specific marker for the liver. An increase in ALP activity in the serum indicates damage to the tissue membranes (Akanji *et al*., 1993). The non-significance ($p>0.05$) in artemether-doxycycline loaded SLN (1a, 6a, and 9b) and the significant decrease ($p<0.001$, $p<0.01$) in groups 1b, 6a, 3b and 9a suggest that the integrity of the plasma membrane in the cells of various tissues (bone, liver, and kidney) may not have been adversely affected.

Table 4, shows that there was no significant difference ($p>0.05$) in the direct bilirubin level among all groups compared to group 14c, suggesting that there was no hepatocellular damage. Direct bilirubin is a type of bilirubin that is not bound to a specific protein (albumin) in the blood. High levels of bilirubin can indicate different types of liver or bile problems. A significant decrease ($p<0.001$) was observed in group 11c, which could be attributed to dehydration.

The total bilirubin level, as shown in Table 4, can help to identify the cause of health conditions such as jaundice, anemia, and liver disease. There was a very, very significant decrease ($p<0.001$) in groups 1a, 3a, 3b, 6a, and 6b, and a very significant difference ($p<0.01$) in groups 1b, 9b, and 10c compared to group 14c, which could also be attributed to dehydration. There was no significant difference ($p>0.05$) observed in groups 11c, 13c, and 14c.

Table 4 Effect of solid lipid nanoparticles containing artemether and doxycycline, pure artemether, and pure doxycycline on alanine amino transferase (ALT), Aspartate amino-transferase (AST), alkaline phosphatase (ALP), total bilirubin, and direct bilirubin in albino mice

Group	ALT	AST	ALP	DBILIRUBIN	TBILIRUBIN
1a	8.00 ± 2.65 ^{NS}	9.17 ± 2.80$^{NS\ a}$	104.53± 6.13$^{NS\ a,b,c}$	2.43 ±0.30 ^{NS}	10.57 ± 0.35 $^{Q\ a}$
1b	7.50 ± 0.87 ^{NS}	10.33 ±2.80$^{NS\ a}$	52.73 ± 4.28 ^{Qa,b}	3.07 ± 0.15 ^{NS}	13.07 ± 1.15 $^{P\ a,b}$
2a	6.17 ± 0.73 ^{NS}	11.83 ±4.15^{NSa}	79.57 ±20.42 ^{Pa,b,c}	2.37 ± 0.28 ^{NS}	12.03 ± 1.15P ^{a,b}
2b	4.73 ± 0.82 ^{NS}	10.83 ± 2.77^{NSa}	81.67 ± 23.43$^{P\ a,b,c}$	2.20 ± 0.15 ^{NS}	12.23 ± 0.23 Q^{a,b}
3a	13.00 ±4.35 ^{NS}	10.00 ± 3.21$^{NS\ a}$	105.23 ±13.54 ^{NSa,b,c}	2.47± 0.32 ^{NS}	13.93± 0.59 $^{Q\ b}$
3b	5.83 ± 1.01 ^{NS}	11.00 ± 0.50$^{NS\ a}$	48.78± 3.44 ^{Qa}	2.57 ± 0.50 ^{NS}	10.50 ± 1.02 ^{Qa}
9a	14.13 ± 5.44^{NS}	14.50 ± 5.13 ^{NSa}	65.57 ± 23.22 $^{P\ a,b}$	2.97± 0.90 ^{NS}	16.90 ± 2.06 $^{NS\ C}$
9b	11.50± 4.00^{NS}	10.83 ±3.18 $^{NS\ a}$	114.10 ± 10.99 $^{NS\ b,c}$	2.33 ± 0.24^{NS}	23.0 ± 0.58 $^{P\ d}$
Artemether (10)	11.83 ± 2.45^{NS}	12.00 ±3.33 $^{NS\ a}$	48.77 ± 14.49 $^{Q\ a}$	3.00 ± 0.25 ^{NS}	26.0 ± 0.58 $^{P\ e}$
Doxycycline (11)	21.00± 1.73^{Q}	7.77 ± 0.72 $^{NS\ a}$	53.67 ± 7.74$^{Q\ a,b}$	1.87 ±0.88 ^{P}	29.0 ± 0.5 8$^{NS\ f}$
Art + Lumefantrine(12)	12.50 ±4.91^{NS}	12.50 ± 3.33 $^{NS\ a}$	67.87 ± 14.15 $^{P\ a,b}$	2.45 ± 0.14 ^{NS}	31.67 ±0.33^{Qh}
Art + Doxycyline (13)	8.00 ±1.00^{NS}	11.25 ± 2.74 $^{NS\ a}$	91.93 ± 3.44 $^{NS\ a,b,c}$	2.73 ± 0.07 ^{NS}	34.50 ± 0.58 ^{NSg}
SLN (14)	9.75 ± 1.30^{NS}	7.10 ± 1.21 $^{NS\ a}$	135.47 ± 1.82 $^{NS\ c}$	2.70 ± 0.12 ^{NS}	36.50 ± 0.29 ^{NSg}
Normal Saline (15)	10.27 ± 0.15^{X}	10.85 ± 0.74 ^{a}	136.60 ± 0.35 c	2.70 ±000	37.50 ± 0.29 ^{h}

KEY: Mean and SEM at significance X= ($p<0.05$), P = ($P<0.01$), Q=($P<0.001$), Ns-non significant

3.4. Histopathological studies

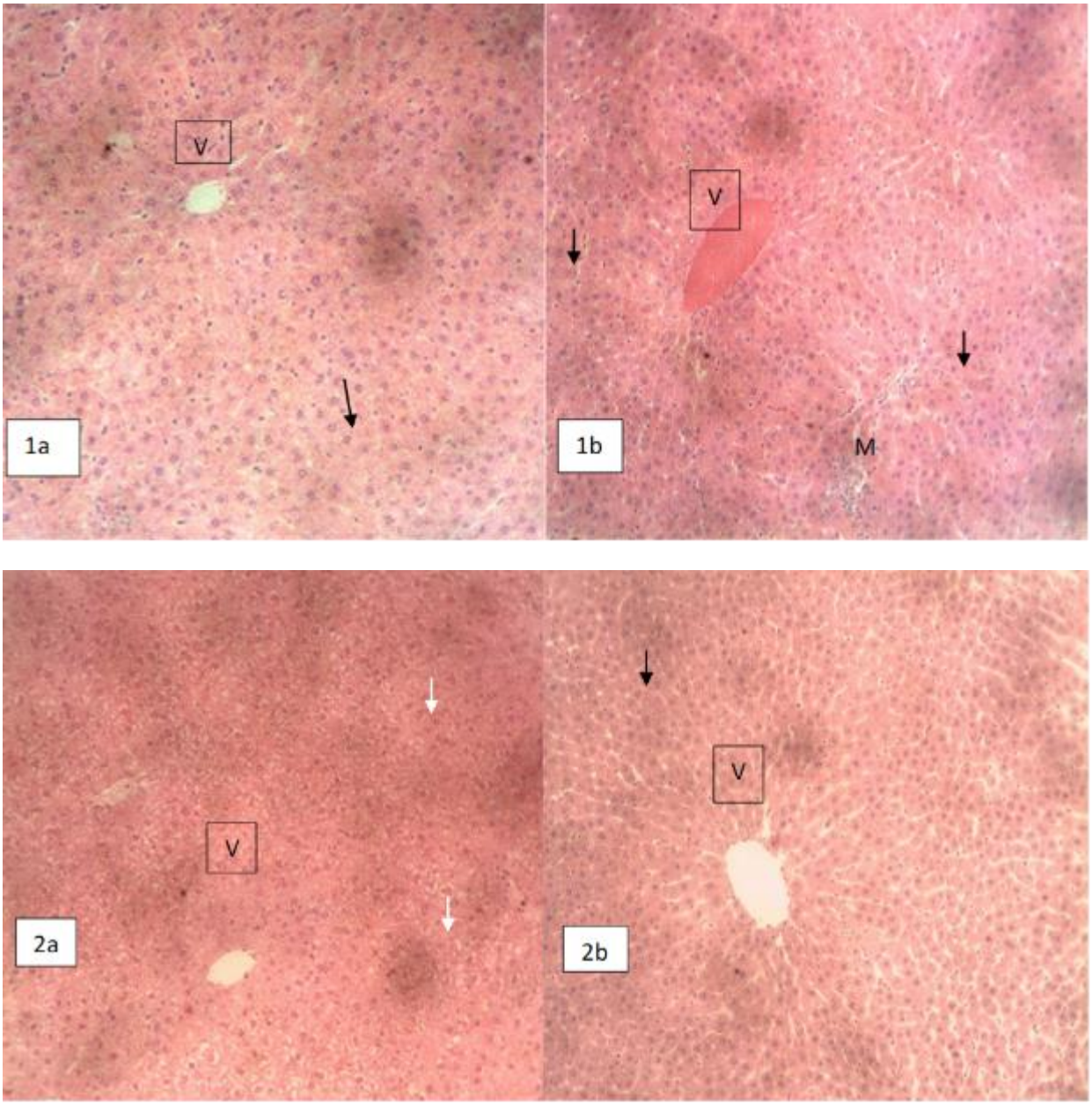

Figure 2 Histo-morphologic sections of the liver in the different groups. The study showed normal hepatic histo-architechture for albino mice in groups 1a and 2b, consisting of normal hepatic lobules with normal hepatocytes (black arrows) arranged in interconnecting cords around the central veins (V). Scanty areas of infiltration of mono-nuclear leukocytes (M, areas of cell swelling) were observed in group 1b, while areas of hepatocellular necrosis (white arrows) were seen in group 2a. H&E x100.

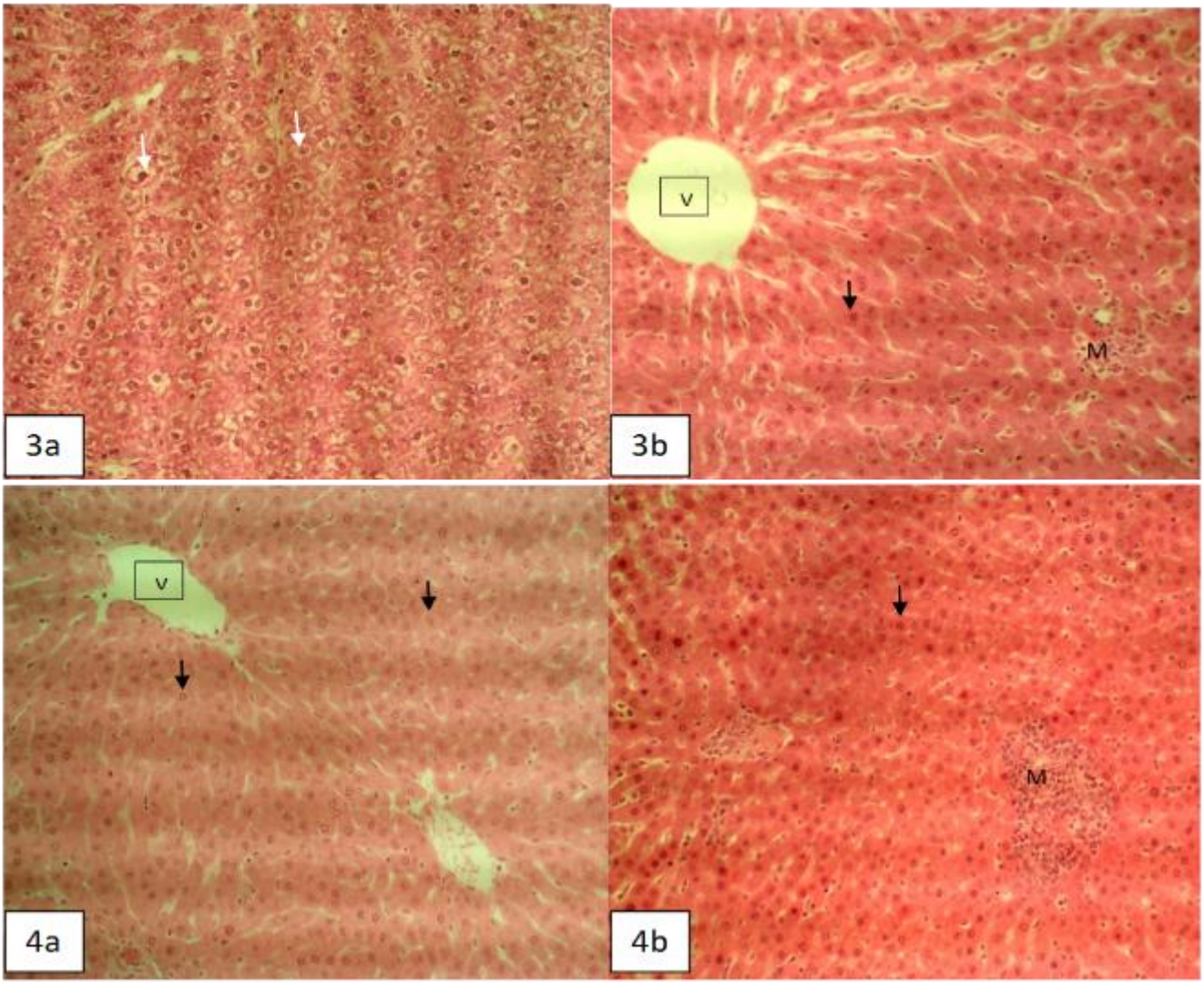

Figure 3 Sections of the liver in the different groups and indicates normal hepatic histo-architechture for albino mice in group 4a, with a normal central vein (V) and hepatocytes (black arrows). Areas of infiltration of mono-nuclear leukocytes (M, areas of cell swelling) were observed in groups 3b and 4b, while areas of hepatocellular necrosis (white arrows) were seen in group 3a. H&E x100

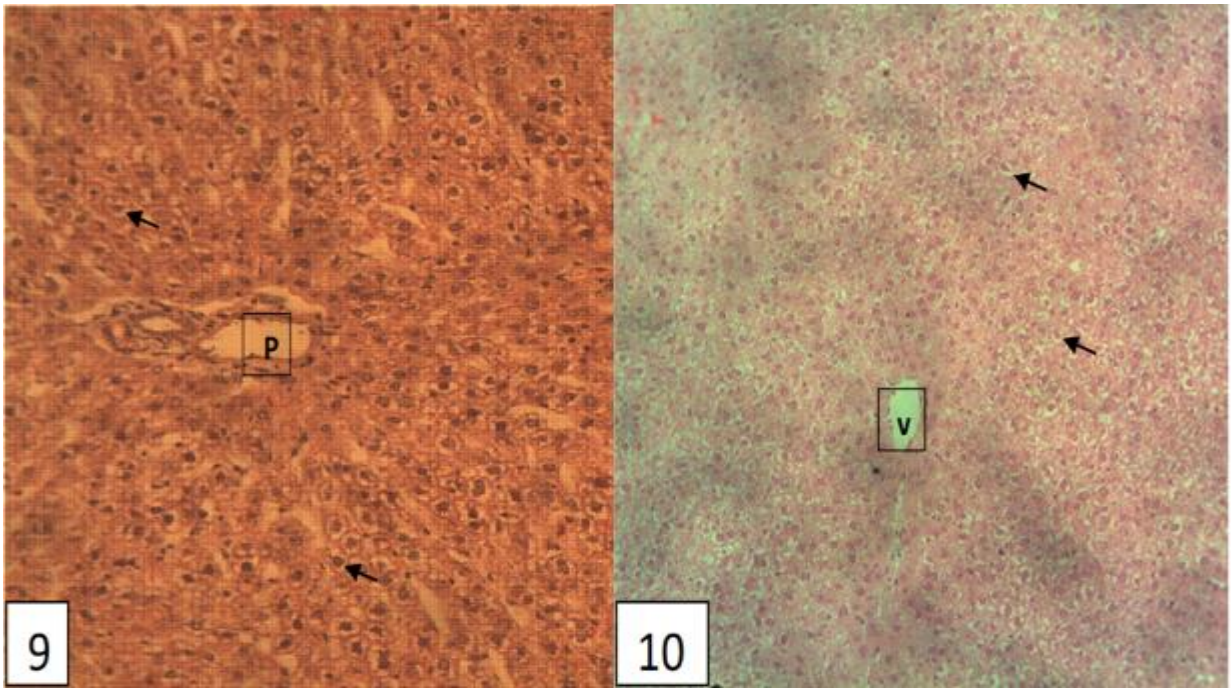

Figure 4 Sections of the liver in the different groups, and the study showed normal hepatic histo-morphology for albino mice. Normal hepatic lobules consisting of normal hepatocytes (black arrows) arranged in interconnecting cords around the central veins (V) were observed, as well as normal sinusoidal capillaries (white arrows) and normal structures of the portal triads (P). Areas of infiltration of mono-nuclear leukocytes (M, areas of cell swelling) were observed in group 7. H&E x100.

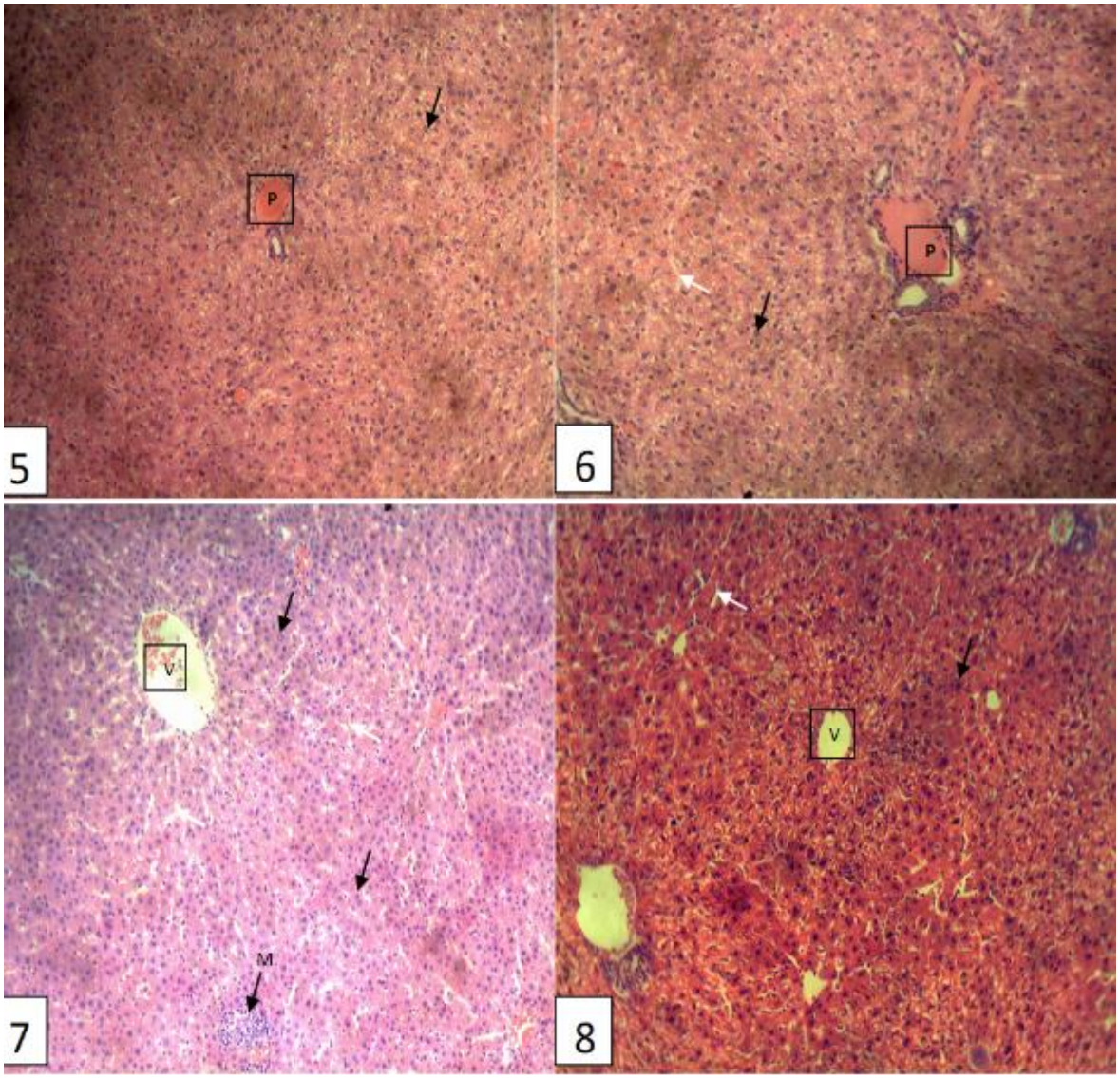

Figure 5 Sections of the liver in the different groups and reveals hepatic lobules with areas of hepatocellular necrosis (black arrows). The normal structure of the portal triad (P) and central vein (V) were also observed. H&E x100

4. Conclusion

This research developed artemether-doxycycline-loaded solid lipid nanoparticles using a hot shear homogenization technique (HHT). The LD50 of the formulated SLNS was above 5000 mg/kg. The hematological showed that the percentage parasitemia, PCV, RBC, and HB of all groups treated with artemether-doxycycline-loaded SLNs were in the range of 1.05% to 14.50%, 38.75% to 56.75%, 0.7.05 x 10^{12}/L to 7.30 x 10^{12}/L, and 12.88 g/dL to 18.03 g/dL, respectively. The percentage mean parasitemia mean of all groups were compared with mean percentage parasitemia of negative control (SLN), indicating a highly active anti-plasmodial therapeutic effect. Biochemical and histological results showed that the formulated SLNS did not increase the adverse effects of artemether and doxycycline. The artemether-doxycycline loaded solid lipid nanoparticles developed resulted in a safe and effective alternative drug with reduced side effects.

Compliance with ethical standards

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Disclosure of conflict of interest

No conflict of interest to disclosed.

Statement of ethical approval

Ethical approval was obtained from “Animal research ethics committee”.

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