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Original Research Article

Formulation, *In vitro* and *In vivo* Evaluation of Sustained released Artemether-Lumefantrine-Loaded microstructured solid Lipid Microparticles (SLMs)

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ABSTRACT

Owing to the low solubility of artemether which reduces its bioavailability, the study aimed at improving the solubility of artemether, in combination with lumefantrine loaded in a solid lipid microparticles (SLMs) using *Capra hircus* as the delivery carrier. Artemether-lumefantrine-loaded SLMs [(AL-loaded; AL0, AL1, AL2 and AL3 are 0, 3, 5 and 7% AL loaded respectively)] were prepared using hot homogenization, thereafter lyophilized and characterized. *In vitro* release of A-L was performed in simulated intestinal fluid (SIF) and simulated gastric fluid (SGF) while the *in vivo* study was carried out using Peter's Four days protocol after which the mice were subjected to histological studies. A pH stable SLMs, particle size of 9.15 ± 0.18 - 23.67 ± 0.45 µm with high entrapment efficiency of lumefantrine than artemether was obtained. The release profile of the SLMs were pH-dependent, SGF (90%) and SIF (50%). The optimized SLMs had percentage parasitemia reduction (87.01%) that is significantly different from the commercial samples (80%) in *Plasmodium berghi* infected mice albeit not to a significant extent. Post-treatment haematological parameters showed that there was a significant difference ($p < 0.05$) only in the PCV of group A (SLMs containing 3% of AL treated) and group B (commercial sample treated). The histological studies revealed that the SLMs formulations had no deleterious effects on the kidney and liver of the mice. Therefore, SLMs formulation might be an alternative means of delivering artemisinin combinations as stability and therapeutic efficacy were achieved without any significant harmful effect on the vital organs.

***Keywords*:** Solid Lipid Microparticles, *Plasmodium berghi*, artemether-lumefantrine, parasitemia, haematological parameters, histological studies.

Introduction

Mosquitoes have been associated with the transmission of many diseases like malaria, filariasis, yellow fever as well as many other diseases especially in tropical and subtropical regions. Over the years, mosquitoes have been known to be a basis for various diseases affecting human beings, especially in the tropical and subtropical regions. There are about 3500 species of mosquitoes, but the chief genera which serve as vectors for disease-causing pathogens in humans includes Anopheles (malaria, filariasis), Aedes (yellow fever, dengue fever) and Culex (filariasis).[1] As a result of this, various methods have been developed to control mosquitoes.

Malaria is one of the world's leading human killers, with greater morbidity and mortality than any other infectious diseases of the world.[2] The mortality is more in children and pregnant women and the malaria parasite has continued to developed resistance to drugs used in the therapy of malaria including the artemisinins.[3]

Artemisinins (ARTs) are particularly more active than any other antimalarial, reducing the number of parasites by approximately 10^4 per cycle[2] but despite the potent antimalarial action of ARTs, they suffer from poor pharmacokinetic characteristics, short half-life and this has necessitated the development of ART-based combination therapies (ACTs) to be used in a 3-day therapy comprising ART together with a slowly eliminated companion blood-stage schizonticide.[4] Artemether (A) is a potent and rapidly acting antimalarial agent, which is enlisted in the WHO List of Essential medicines (WHO, 2015) for the treatment of severe multi-resistant malaria. It is active against *P. vivax* as well as chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum* and is also indicated in the treatment of cerebral malaria, however, its poor aqueous solubility significantly hampers its therapeutic efficacy.[5] At present, artemether is available as tablets for oral therapy and as an intramuscular (IM) oily injection for the treatment of severe malarial infections. The oral bioavailability of artemether is low (≈40%) due to its poor aqueous solubility and degradation in stomach acids,[6] whereas the current oily intramuscular injection suffers from disadvantages such as pain on injection site, slow and erratic absorption when administered.[7] Lumefantrine (L), a highly lipophilic (BCS, Class II drug) flourene derivative, is a blood schizonticide. It acts by inhibiting detoxification of heme which in combination and free radicals induce parasite death.[8] Balancing the irregular dosage regimen and insufficient availability of these APIs through improvement in the solubility is done with co-administration of milk or any other fatty meal. WHO recommended these five ACTs; artemether plus lumefantrine, artesunate plus amodiaquine, artesunate plus mefloquine, artesunate plus sulfadoxine-pyrimethamine, and dihydroartemisinin plus piperaquine and the choice of the ACT is based on the therapeutic outcome of the combination in the country or region of intended use.[9]

Studies have demonstrated favourable results using *Capra hircus* (goat - fat extracted from adipose tissues of goat)-based lipid matrices in

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formulating solid lipid microparticles (SLM) and solid lipid nanoparticles (SLN) formulations owing to its safety and outstanding qualities as a good solid lipid material.[10-12]

There is need to develop new strategies urgently as there are increasing cases of resistance to current antimalarial agents even the ARTs especially in zones in which *P. falciparum* is endemic, and calls for combined therapy approaches.[13] Owing to the challenge of delivering accurate doses of these therapeutic agents to a specific site at the right time in safe and reproducible manner; various mechanisms have been devised to provide control release including transdermal patches, implants, inhalation systems, bioadhesive systems, and nanoencapsulation but few of these have been specific.[14] Owing to the peculiarities of *Plasmodium*-infected RBCs (pRBCs), lipid-based nano/microcarriers have been one of the most promising approaches for the targeted delivery of antimalarial drugs for example, formulation of halofantrine-loaded solid lipid microparticles (SLMs), and chloroquine-loaded solid lipid microparticles (SLMs) have been developed by our research group where we observed that SLMs are suitable carriers for anti-malarial formulations.[11,15] These lipid-based strategies have eliminated the co-administration of milk or any other fatty meal with these APIs thereby reducing the cost of treatment.

P. berghei which shows high similarity both in structure and gene content with the genome of the *P. falciparum* is the model malaria parasite used in research studies for the development and screening of antimalarial drugs and the development of an effective vaccine against malaria. It also shows pathological symptoms which are comparable to symptoms of cerebral malaria in patients infected with the human malaria parasite, however *P. falciparum* can generate genetically attenuated parasites (GAP) and they are used to induce protective immune responses by vaccination with GAP.[16]

In the present investigation, AL-loaded SLMs has been formulated using biocompatible excipients and evaluated for its potential both *in vitro* and *in vivo* for improving antimalarial efficacy in *P. berghei* infected mice and their activity compared with commercial sample tablets (reference tablets).

Materials and Methods

Materials

The materials used were goat fat (obtained from a batch processed in our Laboratory), Artemether pure powder, lumefantrine pure powder (Juhel Pharmaceutical, Awka, Nigeria), Coartem® (Glaxosmithkline, Nigeria), Phospholipon® 90H (Phospholipid GmbH, Köln, Germany), sorbic acid, sorbitol, Polysorbate 80 (Merck, Darmstadt, Germany), distilled water (Lion Water, Nigeria). All other reagents and solvents were of analytical grade and were used without further purification.

Parasites

The in vivo antimalarial evaluation was carried out with *P. berghei* NK-65, a strain free of contamination with *Eperythrozoon coccoides* and sensitive to chloroquine. This strain is sensitive to all currently used antimalarial drugs and is known to induce high mortality in mice, providing a good model to estimate antimalarial efficacy in reducing parasitemia. It was supplied from the Nigerian Institute of Medical Research (NIMR), Yaba, Lagos.

Animals

Eperythrozoon-free Swiss albino mice (CD1) weighing 20 to 25 g were obtained from the Department of Pharmacology and Toxicology, University of Nigeria, Nsukka. Animal experiments were done according to the Principles of Laboratory Animal Care and legislation in force in Nigeria.

Extraction of goat fat

The lipid used for the experiment was extracted from goat fat (*Capra hircus*) using wet rendering method,[17] after which fusion technique was used to prepare the lipid matrix. Briefly, extraneous materials were removed from the adipose tissues of the *C. hircus*, which were then cut into smaller pieces and subjected to moist heat by boiling in about half its weight of water on a water bath for 45 min. The molten fat was separated from the aqueous phase by filtering with a muslin cloth and the extracted lipid purified using 2% suspension of activated charcoal and bentonite (1:19) at 80°C for 1 h. The mixture was then filtered using a Buchner funnel.

Preparation of lipid matrix

The lipid matrix was prepared according to Friedrich *et al* and Attama *et al*.[18,19] Briefly, about 70 g of the prepared goat fat was weighed and melted in a beaker placed in a water bath at a temperature of 60°C. A 30 g of Phospholipon® 90H, was added to the melted goat fat and stirred using a magnetic stirrer and hot plate (Jenway 400, EU), until an even mix was obtained. The molten lipid matrix was then placed in a cold-water bath for 30 min at room temperature until solidification to obtain the solidified reverse micellar solutions (SRMS). The lipid matrix therefore consisted of goat fat and Phospholipon® 90H.

Preparation of SLMs

The solid lipid microparticles were prepared to contain: lipid matrix (17% w/w), artemether-lumefantrine in the ratio of 1:6 respectively (0, 3, 5, 7% w/w), polysorbate 80 (1.5%), sorbic acid (0.05%), sorbitol (4% w/w) and water (to 100% w/w) (Table 1) according to Ogbonna *et al* and the SLMs lyophilized according to Jaspart *et al*.[11,20]

Characterization of the formulated SLMs

Morphology and particle size analysis

Particle size analysis was carried out on the SLMs after formulation according to Ogbonna *et al*.[11] A specified quantity of sample from each batch was placed on a glass slide and viewed with a photomicroscope (Hund®, Weltzlar, Germany) attached with an electronic image analyzer (Moticam, Xiamen, China) at a magnification of 100 times. The size of particles of the SLM from each batch were measured (n =100), and the mean value was determined. The morphologies were also observed from the photomicrographs. Triplicate readings were taken.

Determination of the percentage yield of the SLMs

Each of the batches of the water-free SLMs was weighed after lyophilization. The yield of SLMs (% w/w) was calculated according to the following formula (17).

$$\% \text{ recovery} = \frac{W1}{(W2+W3)} \times 100 \quad \text{..... Eqn 1}$$

Where W_1= weight of water free SLMs formulated (g), W_2 = weight of added drug (g), W_3 = weight of the excipients (lipid matrix + polysorbate 80 + sorbitol + sorbic acid) (g).

Time-dependent pH stability studies

The pH of dispersions of the SLMs were determined over time (at day 0, 30, 60 and 90), using a pH meter (pH ep® Hanna instrument, Padova, Italy).

Determination of the loading capacity and encapsulation efficiency

Loading capacity (LC) expresses the ratio of the entrapped active pharmaceutical ingredient (API) to the total weight of the lipids.[22] The LC of the SLMs was determined using the formula;

$$\text{Loading capacity} = \frac{W\alpha}{W1} \times 100 \quad \text{..... Eqn 2}$$

Where W1= weight of lipid in the formulation and Wα = amount of API entrapped by the lipid

The determination of encapsulation efficiency (EE) was done according to Ogbonna *et al* with little modifications.[22] A 200 mg each of the lyophilized SLMs batches AL1, AL2 and AL3 containing 3%, 5% and 7% of AL respectively was crushed and soaked in 10 mL methanol for 24 h. Thereafter, the following procedures were carried out. A 0.5 mL of the filtrate in each batch was diluted to 5 mL using methanol. The dilutions were filtered through Whatman No. 1 filter paper and analyzed spectrophotometrically at a predetermined wavelength of 296 nm for lumefantrine using methanol as blank. Also, 2 mL of the filtrate from each batch was diluted to 10 mL using 0.2% NaOH in a 25 mL volumetric flask.

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Table 1: Composition and drug content of artemether-lumefantrine SLMs

Codes	A (g)	L (g)	LM (%)	P80 (%)	S (%)	SA (%)	W q.s (%)	DC of A (%)	DC of L (%)	MPS (µm) *,†
AL0	-	-	17	1.5	4	0.05	100			9.15 ± 0. 18
AL1	0.429	2.571	17	1.5	4	0.05	100	76	64	14.20 ± 0.38
AL2	0.714	4.286	17	1.5	4	0.05	100	71	55	15.35 ± 0.72
AL3	1.000	6.000	17	1.5	4	0.05	100	69	97	23.67 ± 0.45

Key: MPS is Mean particle size, A-L is artemether-lumefantrine, SA is sorbic acid, DC is drug content, P80 is Polysorbate 80, LM is Lipid matrix, S is sorbitol, W is water, *Mean SD. *,†.

The solution was heated in water bath at 50°C for 30 min after which the content was cooled and diluted further to 20 mL using 2 mL of methanol and 8 mL of 0.2 M acetic acid. The content of the 20 mL volume in the flask was filtered using Whatman No. 1 filter paper and analyzed spectrophotometrically using a UV-Vis spectrophotometer (Unico 2102 PC UV/Vis Spectrophotometer, East Norwalk, CT, USA) at a predetermined wavelength of 242 nm using methanol and 0.2 M acetic acid mixture in the ratio of 1:4 respectively as the blank to determine the drug content of artemether. Triplicate readings were taken. The encapsulation efficiency EE % was calculated using the formula.[23]

The procedures were repeated for all the unlyophilized batches except that 2 mL in each case was centrifuged and the supernatant used for the encapsulation efficiency of lumefantrine and artemether.

$$EE = \frac{actual\ drug\ content}{theoretical\ drug\ content} \times 100 \quad \text{..... Eqn 3}$$

In vitro drug release

A 400 mL of the dissolution medium comprised Tween 80 and simulated gastric fluid (SGF pH = 1.2 ± 0.2) in the ratio 1:49 respectively maintained at 37 ± 1°C.The 9 cm x 4.5 cm, polycarbonate dialysis membrane (MWCO 6000–8000, Spectrum Labs, Breda, The Netherlands) was pre-treated by immersing it in the dissolution medium for 24 h, before the *in vitro* release study commenced. For each batch of the formulations (AL1 and AL2 containing 3% and 5% of the artemether-lumefantrine respectively), 50 mg of the formulations was enclosed in the dialysis membrane containing 3 mL of the dissolution medium. The dialysis membrane was securely tied with a thermo-resistant thread, immersed in the dissolution medium and agitated by a stirrer at 100 rpm. At intervals of 15, 20, 30 min, and subsequent 1 h intervals for 8 h, 5 mL aliquots of the dissolution medium were collected and immediately replaced with 5 mL of fresh medium. The withdrawn samples were analyzed spectrophotometrically using a UV-Vis spectrophotometer (Unico 2102 PC UV/Vis Spectrophotometer, East Norwalk, CT, USA) at predetermined wavelength of 296 nm for lumefantrine using Tween 80 and SGF in the ratio 1:49 as blank. The amount of drug released at each time interval was determined using the standard Beer's plot for lumefantrine. According to Ogbonna *et al*;[22] Niesko *et al*.[24] with little modification, the artemether was derivatized and the drug released determined. The same procedure was repeated using SIF and Tween 80.[22,24]

Kinetics and mechanism of drug release

The kinetics and mechanism of drug release from the SLMs were studied using the following models: zero order kinetics, first order kinetics, Higuchi, Korsmeyer and Hixson-Crowell cube root models. The zero-order kinetics describes the cumulative percentage of the amount of drug released from formulated SLMs (Q) versus time 'Q vs t',[25] first-order kinetics model using log cumulative of percentage of drug remaining versus time or 'log (100-Q) vs t',[26] Higuchi model using the cumulative percentage of drug release versus square root of time or Q vs $t^{1/2}$.[27-28] Korsmeyer model was plotted using the log cumulative % drug release versus log time or 'log Q vs log t' while Hixson-Crowel cube root model was plotted using cube root of percentage drug remaining in polymer matrix versus time or '$(100-Q)^{1/3}$ vs t'. The Korsmeyer plot characterizes drug release from cylindrical shaped matrices.[29] Hixson-Crowel cube root model describes the release from systems where there is a change in surface area and diameter of particles.[30] The linearity of these plots was determined by their R^2 values and the plot (model) with the highest linearity was taken as that which describes the kinetics and mechanism of drug release.

The release exponent n ≤0.5 for Fickian diffusion release from slab (non-swellable matrix), 0.5 < n < 1.0 for non-Fickian release (anomalous). This means that drug release followed both diffusion and erosion-controlled mechanisms and n = 1 (zero order release), where drug release is independent of concentration and n >1.0 indicates a super case-IItransport relaxational release.[31] Also, 0.45 < n < 1.0 is for non-Fickian release (anomalous) from cylinders (non-swellable matrix) and 0.43 < n < 1.0 for non-Fickian release (anomalous) from non-swellable spherical samples.

In vivo release studies using Peter's Four days suppressive protocol

Preparation of the animals

Twelve healthy, non-pregnant adult Swiss albino mice were selected and divided into three groups of four mice per cage. The mice were allowed water and food ad libitum and allowed to acclimatize for seven days. The experimental protocols were in accordance with the guidelines for conducting animal experiments stipulated by our Institution's Animal Ethics Committee with ethical approval number FPSRE/UNN/20/0037. The protocols were also in compliance with the Federation of European Laboratory Animal Science Association and the European Community Council Directive of November 24, 1986 (86/609/EEC) (EEC, 1986).[32]

In vivo studies

The 4-day test was performed according to Peters *et al*.[33] Briefly, each mouse was inoculated intraperitoneally (i.p) with 0.2 mL of infected blood containing about 10,000,000 *P. berghei* parasitized erythrocytes. The animals were left for four days. On day 4 after parasitic inoculation, parasitemia levels were measured and average parasitemia determined for each group. Group A received optimized SLMs (AL1 containing 3% artemether-lumefantrine), group B received a commercial sample of artemether-lumefantrine (reference tablet), group C received no treatment at all and the treatment doses were based on body weight. Group D was not infected with *P.berghei* and therefore left untreated. The base line of the following parameters [packed cell volume (PCV), haemoglobin content (Hb), white blood cell content (WBC) and red blood cell content (RBC)] were determined and their values also were taken before treatment, after parasite inoculation and post treatment. Four days after infection and also post treatment the parasite counts were taken from thin blood smears of the tail blood of mice, fixed with methanol and stained with Giemsa's stain. The efficacy of the SLMs and reference tablets was determined by monitoring the mean percentage parasitemia suppression activity against time as well as the animal survival period. Percentage parasitemia was calculated based on the parasite count pre-treatment and post-treatment using the formula;

$$\%\ parasitemia = \frac{(Av\ pretreatment - Av\ posttreatment)parasitemia}{Average\ pretreatment\ parasitemia} \times 100 \quad \text{--------- Eqn 4}$$

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Histological studies

The mice were sacrificed seven days post treatment and histological studies carried out on the liver and kidney of a mouse from each group. From each group (A- C), tissue sections of the liver and kidney of the mouse were taken, fixed in 10% normal saline and dehydrated in ascending grades of ethanol. The tissues were thereafter, cleared in chloroform overnight, infiltrated and embedded in molten paraffin wax. The blocks were later trimmed, sectioned at 5-6 µm, deparaffinized in xylene and rinsed with water. Subsequently, the sections were stained with Haematoxylin and Eosin (H and E) and fixed for viewing under a moticam fitted to the polarized Photomicroscope.

Statistical analysis

Statistical analysis was done using Graphpad Software (Model). All values were expressed as mean ± SD. Data was analyzed by one-way ANOVA. Differences between Means were assessed by a two-tailed Student's t-test. $P < 0.05$ was considered statistically significant.

Results and Discussion

Owing to the emergence of chloroquine-resistant Plasmodium strains, there is a need to formulate ACTs as novel SLMs and evaluate the *in vitro-in vivo* activity. This novelty is necessitated because of the reported cases of resistance even in the ACTs presently marketed in Nigeria. Furthermore, most of the marketed artemether-lumefantrine requires the use of fatty meal as an instruction to increase oral absorption and bioavailability. By formulating the drugs as SLMs such caution and advice which the patients most often ignore will no longer be required as the drug is already present in lipid core. In the present study, control release SLMs delivery system of A-L was developed and evaluated both *in vitro* and *in vivo* for oral delivery of artemether-lumefantrine. SLMs are a suitable carrier for A-L with high drug content especially AL1 with entrapment efficiency of 55, 42, 74, and 69 for lyophilized artemether, unlyophilized artemether, lyophilized lumefantrine and unlyophilized lumefantrine respectively.

Morphology and particle size

Figure 1 shows the morphological characteristics of the drug unloaded SLMs, SLMs containing 3, 5 and 7% of AL SLMs were mostly irregular in shape and the particle size ranged from 9.15 ± 0.18µm (unloaded SLMs) to 23.67 ± 0.45 µm (7 % drug loaded SLMs). It can be deduced that as the concentration of drug increased the particle size increased which collaborated the works of Uronnachi *et al* and Ogbonna *et al*.[11,34] Differences in sizes of the individual particles may be related to the orientation of the particles during imaging. Successful SLMs were prepared with the lipid matrices. They were discrete, polydispersed, poorly flowable and mostly irregularly shaped with varying size ranges might have been adduced to the surface of viewing which could have been 'edge on' or 'side on'. The particle sizes of the unloaded SLMs (AL0) were significantly lower than those of the AL-loaded SLMs ($p < 0.05$).

The particle sizes of the SLMs were within the micrometer limits for SLMs. Particle size of SLMs determines the site of applicability and the bioavailability of the drug. Large particle sizes (< 50 μm) are much more reactive owing to attractive forces such as Van der Waals whereas small particle sizes of lipid formulations (< 20 μm) are theorized to be well tolerated by a single cell contact.[35] Therefore, owing to the size ranges, the SLMs might be administered through parenteral reconstitution.

Determination of encapsulation efficiency and loading capacity

The results in Table 2 showed that the batch, AL1 containing 3% of the active pharmaceutical ingredient had the highest encapsulation efficiency, (74% and 55% of lumefantrine and artemether respectively). Suffice to say that after lyophilization, the quantity of drugs entrapped for the batch containing 3% of the active pharmaceutical ingredients increased while for the batches AL2 and AL3 containing 5% and 7% of the drugs, only the artemether showed increase in entrapment efficiency while the entrapment efficiency of lumefantrine decreased. The decrease EE of lumefantrine in batches AL2 and AL3 might be owing to its poor solubility in both water and oil, forcing some of the molecularly dispersed drug to be precipitated out from the formulation. Owing to that, the batch of SLMs containing 3% of the drugs was optimized for both *in vitro* and *in vivo* antimalarial studies, 5% SLMs was evaluated only for the *in vitro* studies while owing to the abysmal low entrapment of the drugs in AL3 batch, it was not used for further studies. This one finding on the entrapment efficiency is not in collaboration with our earlier work, Ogbonna *et al*, where an increase in the concentration of the drug (halofantrine) in the SLMs, increased its entrapment efficiency.[11]

Short term dependent pH stability studies

The pH stability of the SLMs is shown in Table 2. The pH of all the SLMs formulations was in the acidic region throughout the stability study period. Degradation of the drugs and or the excipients in a dosage formulation is the factor which decisively affects the change in pH of a liquid formulation. A drug that is *ab initio* stable may be affected by degradation of excipients with storage through the generation of an unfavorable pH (increase or decrease) or reactive species for the drug.[18] Therefore, the slight difference in pH values of the AL-loaded SLMs was not attributed to drug degradation since there was also a fall in the pH of the unloaded SLMs. The pH decrease may be due to release of the fatty acids from the lipid matrix. However, the little decrease in pH of the AL-loaded SLMs indicated that the formulations would need a buffering agent to keep the pH more stable.[36] The results (Table 2) showed there was no significant change in pH ($p > 0.05$) over three months which implies little or no degradation of the drug or excipients used in the formulation. The AL-loaded SLMs, therefore showed slight pH stability over the study period. However, the change in pH from its post-formulation pH in AL-loaded SLMs is very minimal to alter the expected activity of the API, as the degradation might probably be only on the lipid matrix.

Calibration plots indicated linear relationships between absorbance and concentration of artemether and lumefantrine with the solvents used. The following relationships were obtained; Tween + SGF for artemether: A293 = 0.333C (r^2 = 0.9520); Tween + SGF for lumefantrine, A296 = 0.416C (r^2 = 0.9630); Tween 80 + SIF (pH, 7.40) for artemether, A293 = 0.326C (r^2 = 0.9740); Tween + SIF (pH, 7.4) for lumefantrine A296 = 0.195C (r2 = 0.9510). High linearity ($r^2 > 0.9$) was obtained for the calibration plot. With $r^2 > 0.9$ for the solvents, proper analysis of artemether and lumefantrine could be performed at the wavelengths of absorption maxima of the drugs in these solvents combined.

In vitro release studies

The *in vitro* release results presented in Figures 2-3 show the *in vitro* release profile in SIF and SGF of AL-loaded SLMs and commercial sample (reference tablet). In SIF, AL-loaded SLMs containing 3 % of AL exhibited 16, 17, 17.5 and 18% lumefantrine release at $T_{0.5}$, T_1, T_2 and T_3 h, respectively while in SGF exhibited 1, 4, 10 and 12%, respectively.

Also in SIF, the reference tablet exhibited 0.9, 1, 1.1 and 1.3% lumefantrine release at $T_{0.5}$, T_1, T_2 and T_3 h, respectively while in SGF exhibited 1, 2, 2.5 and 4%, respectively. Therefore, after 8 h the cumulative percentage lumefantrine release from batch AL1 (SLMs containing 3% of the drug) and from the reference tablet were both higher in SGF than SIF. In SIF, AL-loaded SLMs containing 3% of AL exhibited 42, 46, 49 and 50% artemether release at $T_{0.5}$, T_1, T_2 and T_3 h, respectively while in SGF exhibited 4, 10, 20 and 26% respectively. Also in SIF, reference tablet exhibited 20, 20.1, 22 and 29% artemether release at $T_{0.5}$, T_1, T_2 and T_3 h, respectively while in SGF exhibited 4, 10, 18 and 24%, respectively. Consequently, after 8 h the cumulative percentage artemether release of batch AL1 (SLMs containing 3% of the drug) and from the reference tablet were both higher in SGF than SIF too. The *in vitro* release of the drugs in both the optimized SLMs (AL1) and reference tablets showed that the releases were all pH-dependent. Also, the SLMs containing 3% of the artemether exhibited dose dumping owing to initial burst effect.

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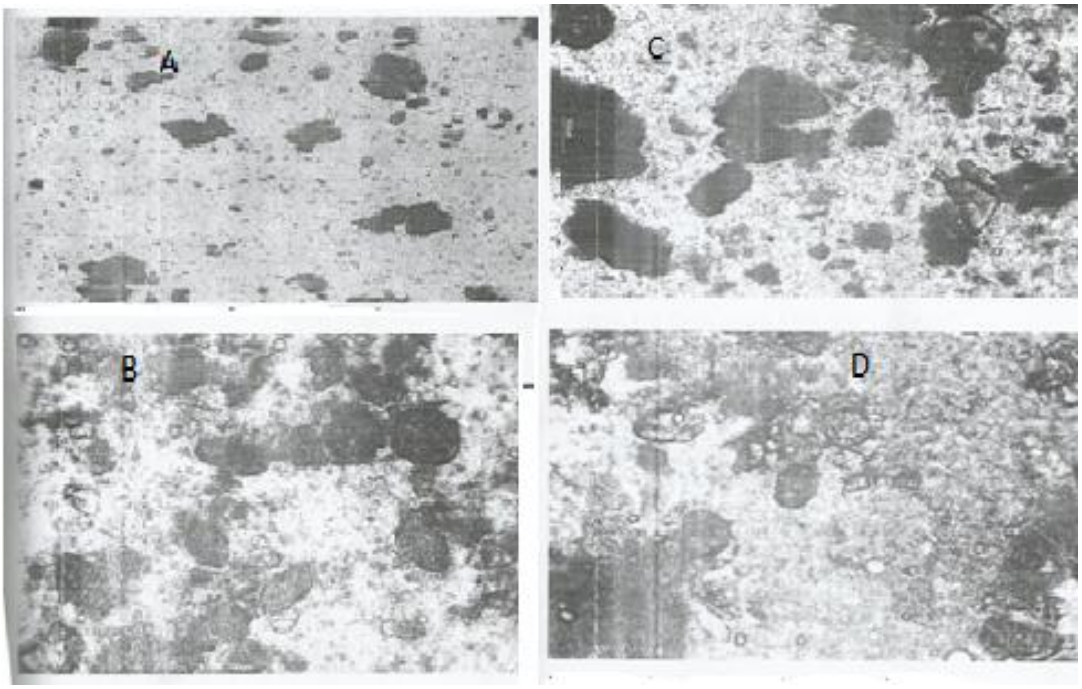

Figure 1: Photomicrographs of SLMs formulations ie drug unloaded SLMs **(A)**, SLMs containing 3% **(B)**, 5% **(C)** and 7 % **(D)** of AL respectively (Mag. x 400)

Table 2: Encapsulation efficiency and pH stability studies

	A	UnA	L	UnL	pH	Stability	study	
Codes	**EE (%)**	**EE (%)**	**EE (%)**	**EE (%)**	**Day 0**	**Day 30**	**Day 60**	**Day 90**
AL0					5.34 ± 0.31	5.74 ± 0.01	4.79 ± 0.10	5.00 ± 0.13
AL1	55	42	74	69	5.44 ± 0.05	5.24 ± 0.00	5.23 ± 0.10	5.23 ± 0.01
AL2	34	27	38	67	5.62 ± 0.50	5.33 ± 0.17	5.78 ± 0.02	5.50 ± 0.17
AL3	29	18	15	63	4.80 ± 0.07	4.97 ± 0.01	4.84 ± 0.18	4.90 ± 0.05

Key: A EE% = percentage entrapment efficiency of lyophilized artemether, UnA EE% = percentage entrapment efficiency of unlyophilized artemether, L EE% = percentage entrapment efficiency of lyophilized lumefantrine, UnL EE% = percentage entrapment efficiency of unlyophilized lumefantrine

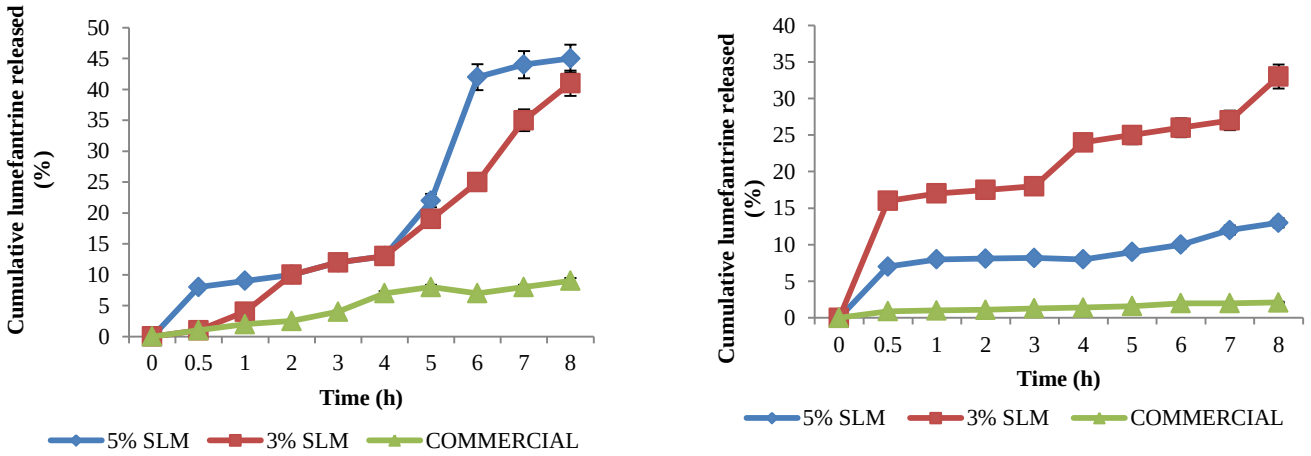

Figure 2: Release profile of the SLMs formulation of lumefantrine and commercial sample in SGF and SIF

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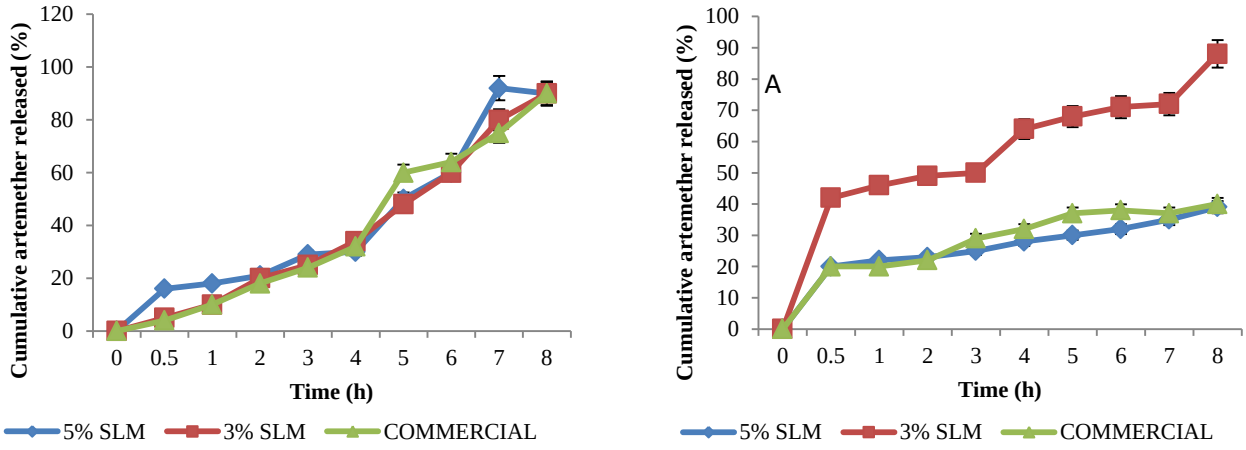

Figure 3: Release profile of the SLMs formulation of artemether and commercial sample in SGF and SIF.

The *in vitro* release profile of AL-loaded SLMs showed that the batch (AL1) formulation exhibited an initial high release of the artemether and continued with a higher release of the drug. Such initial high release according to Chinaeke *et al* it might be due to presence of an encapsulated drug in the periphery of the formulation, owing to adsorption of the drugs on the surface of the SLMs and/ or precipitation from the superficial lipid matrix.[37] The lumefantrine exhibited gradual but incremental release in SGF. In addition, the SLMs (AL1 and AL2) showed higher sustained release characteristics significantly different from the reference tablets ($p < 0.05$). The findings from the SGF dissolution medium showed that AL-loaded SLMs could be used once daily at the recommended dose for the treatment of malaria. Conceptually, f_2 is a measure of similarity in the percent dissolution between two curves while f_1 is a function of the absolute difference between the two curves and is a measure of the relative error between the two curves. F_2 ranges between 0 and 100 and a higher value indicating more similarity between the two profiles. In SGF the similarity factor was 98.31 for artemether and 97.44 for lumefantrine while in SIF, artemether was 100.23 and lumefantrine was 100.33. From the results, the optimized batch is accepted as similar to reference batch according to Vinod *et al*, as an average difference of not more than 10 % at any sample time point or the dissolution profile difference between the two batches is not more than dissolution profile difference between the two reference batches.[38]

In vitro release kinetics

The *in vitro* release kinetics was studied using five kinetic models as shown in Table 3. In SGF, the results of the plots showed linearity by all the 5 models (lumefantrine in reference tablets), 4 models (lumefantrine in AL1) and 3 models (artemether in AL1) with r^2 value > 0.9 (Table 3).

Korsmeyer-Peppas model was predominant among the kinetics models. this implied that drug release followed both diffusion and erosion-controlled mechanisms.[31]

In vivo studies

The *in vivo* studies show changes in the hematological properties (PCV, Hb, RBC and WBC) of the mice in the groups as illustrated in Figure 4. There were signs of rough hair coat, weakness, dullness and paleness in all the mice infected with *P. berghei* in collaboration with the study by Saganuwan *et al*.[39] Post-treatment hematological parameters showed that there were significant reductions ($p < 0.05$) in PCV, RBC and WBC % of the untreated Group C as compared to Groups A (SLMs containing 3% of the drugs) and B (commercial sample treated). Figure 4 showed that the PCV, RBC and WBC of baseline and pre-treatment were significantly higher ($p < 0.05$) than their values after post-treatment for the untreated Group C. Also, the post-treatment PCV result showed that there was a significant difference between the Group A (SLMs containing 3% of the drugs) and group B (commercial sample treated). These results correlated with the earlier work by Ogbonna *et al* where it was observed that the values of PCV, RBC and WBC were significantly reduced post-treatment when compared to the treated groups and for the baseline and pre-treatment period.[11]

The data in Figure 5 showed that mice in group A which were administered with SLMs containing 3% artemether-lumefantrine had 87.10% parasite clearance; group B which were administered with a commercial formulation of artemether-lumefantrine had 80.00% parasite clearance while group C which were infected but received no treatment had 14.89% parasite clearance. Therefore, the AL-loaded SLMs showed the highest percentage reduction in parasitemia (87.10%) while the commercial sample ie reference tablet (+ control) had 80.00% parasitemia reduction. The lipid carrier might have improved the solubility of the AL in SLMs and might have provided a prolonged, controlled and sustained delivery of the artemether-lumefantrine.[25] The percentage parasitemia reduction exhibited by the optimized SLMs formulation (AL1) was comparable to that of the reference tablet. The *in vivo* pharmacodynamic properties of AL-loaded SLMs collaborated to the *in vitro* release of the drugs from SLMs as both the *in vitro* and *in vivo* effects of the AL-loaded SMLs were greater than that of the commercial reference sample.

Increased surface area of the SLMs through globules formulation r the absorption of the drug molecule, led to enhanced intestinal permeability thus resulting in improved bioavailability and subsequent reduction in parasitemia. These findings in improved bioavailability collaborated the work of Bindhani *et al*.[40]

Literatures have shown that the bioavailability of artemether-lumefantrine increases with the administration of milk or fatty meals.[8] The formulation of artemether-lumefantrine in fatty microparticles could have improved its bioavailability as the solid lipid microparticles could have protected the entrapped artemether-lumefantrine from degradation. In most Sub-Saharan African communities, the greater percentage of people live below two dollars per day and will find it cumbersome and overburdening to adhere to the instruction of taking ACT combinations with milk and or fatty meal.

This poses a great challenge in treating malaria in the rural communities owing to an increase in expenses to be incurred by purchasing the milk. Therefore, an SLMs will probably mitigate against such expenses.

The physicochemical properties of the SLMs such as small particle size, charge or surface modifications are very important as they might have also imparted on the penetration behavior of the formulations thereby supporting the work of Tchoryk *et al*.[41] It can be ascertained that the SLMs were able to deliver the drugs to microparticulate core efficiently in therapeutic model ensuring their clinical use as demonstrated by Parvathaneni *et al*.[42]

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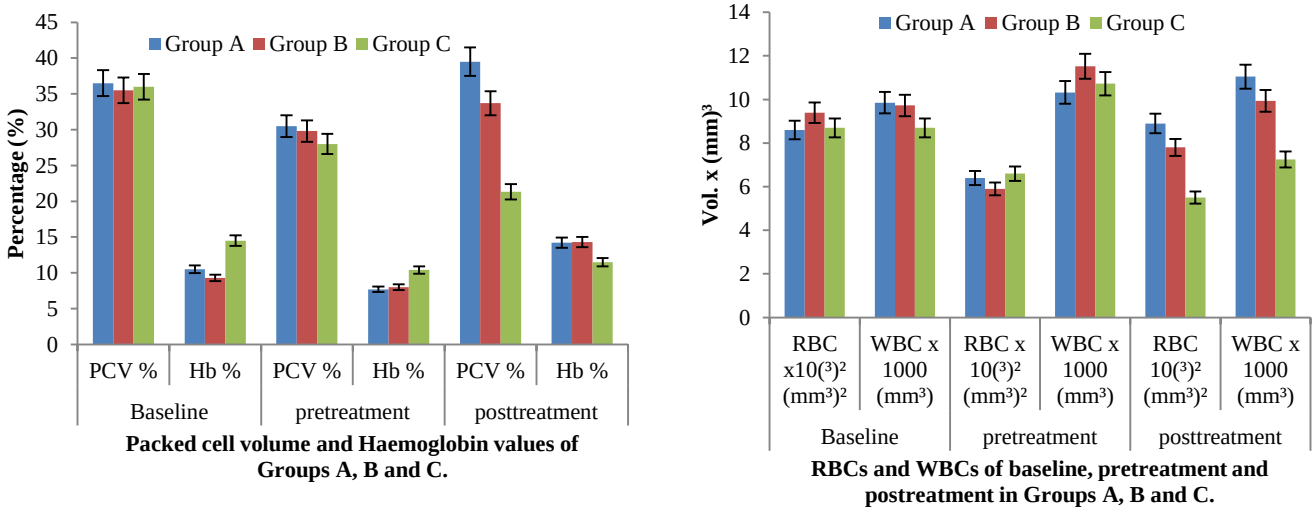

Figure 4: Percentage composition of PCV, Hb and volume of RBCs and WBCs in Groups A, B and C at baseline, pretreatment and posttreatment

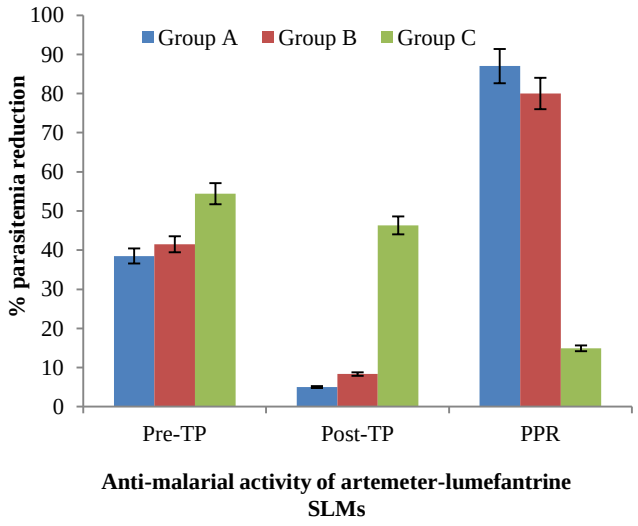

Figure 5: Antimalarial activity showing the percentage parasitemia reduction of Group A which received SLM containing artemether-lumefantrine, group B which received commercial sample of artemether-lumefantrine and group C which received no treatment.

Histological studies

Figure 6 shows photomicrographs of liver sections of mice from experimental groups A. B, C and D. Groups A and B showed varying degrees of periportal hepatitis (mainly mononuclear cell infiltration of the portal area-P) with the infiltrating cells (arrow) while group Cshowed varying degrees of periportal mononuclear infiltrationof cells-periportal hepatitis (arrow) and group D showed normal portal area and hepatocytes. Figure 7 shows photomicrographs of kidney section of mice from experimental groups A, B, C and D. Group A showed tubular dilatation and mild areas of tubular degenerations (black arrow) but not in B. Also, groups Aand B showed normal glomerulus (GM) while group D showed normal glomerulus (GM) and renal tubules (white arrow) but mild tubular degeneration in C (black arrow). The interaction of uptake, biotransformation and elimination of potentially harmful agents *in vivo* results from the capability of a drug to produce liver damage,[43] as a change in either the relative or absolute weight of an organ post-administration of a drug is a sign of the deleterious effects of that drug.[44]

The study showed that administration of optimized AL-loaded SLMs for short-term did not cause any noticeable significant variation in the body regarding the relative vital organs' morphology (shape or size or weight) of the treated mice. This implies that short-term administration of this combination therapy in any type of dosage forms like injectables, oral, etc will probably have no negative effects on somatic growth. The micrographs of the red blood cells (erythrocytes) of the mice are presented in Figure 8 (groups A-C) - where black dots in the red blood cells are signs of parasitic infection. In groups A and B (Figure 8), the red blood cells of the mice showed that blood cells have been infected with the parasites (black spots), but the number of infected blood cells decreased owing to treatment with AL-loaded SLMs formulation and the reference tablets. The converse is the case ingroup C that has parasite-infected but untreated mice (Figure 8C) where more black spots on red blood cells demonstrated an increase in the number of infected cells. This work collaborated the work of Ogbonna *et al* where antimalarial drug administration led to a decrease in the number of red blood cells infected with the parasite. Small granules observed in the plasma of infected blood could be neutrophiles and white blood cells responding to infection.[15]

Conclusion

It can be inferred that SLMs are good candidates for the administration of artemether-lumefantrine as the formulations were relatively stable in pH value throughout the study period as the changes in pH were not significant. The formation of submicron globules provides increased surface area for the absorption of the A-L molecules, enhancing intestinal permeability of the drugs thus resulting in improved bioavailability. The parasitemia reduction on the AL-loaded SLMs was comparably higher than the reference sample, though not significantly. The histological studies also established the safety of the SLMs formulations as they showed no evidence of disastrous side effect on the vital organs of mice.

Conflict of interest

The authors declare no conflict of interest.

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Table 3: Kinetic models and mechanisms of drug release studies

Batches	Zero order		First Order		Higuchi		Korsmeyer	Peppas	Hixson-Crowell	
	r^2	K_0	r^2	K_1	r^2	K_h	r^2	n	r^2	N
Art (3% SLMs) in SIF	0.279	12.460	0.864	-0.081	0.839	22.400	0.858	0.245	0.868	-0.209
Art (3% SLMs) in SGF	0.978	10.620	0.858	-0.109	0.856	29.900	0.991	1.085	0.916	-0.287
Lum (3% SLMs) in SIF	0.304	4.621	0.823	-0.015	0.825	8.305	0.808	0.243	0.810	-0.050
Lum (3% SLMs) in SGF	0.952	4.555	0.931	-0.026	0.829	12.940	0.967	1.215	0.942	-0.087
Art (5% SLMs) in SIF	0.253	5.806	0.839	-0.020	0.835	10.310	0.865	0.234	0.821	-0.065
Art (5% SLMs) in SGF	0.941	11.410	0.805	-0.125	0.809	29.430	0.846	0.670	0.867	-0.311
Lum (5% SLMs) in SIF	0.010	1.866	0.709	-0.004	0.758	3.095	0.740	0.183	0.704	-0.016
Lum (5% SLMs) in SGF	0.890	5.635	0.868	-0.033	0.739	14.730	0.752	0.664	0.876	-0.106
Art (Coartem) in SIF	0.344	6.271	0.845	-0.022	0.872	11.690	0.903	0.289	0.828	-0.073
Art (Coartem) in SGF	0.974	10.360	0.844	-0.105	0.847	29.290	0.987	1.081	0.727	-0.245
Lum (Coartem) in SIF	0.560	0.313	0.866	0.000	0.895	0.614	0.905	0.320	0.865	-0.003
Lum (Coartem) in SGF	0.902	1.260	0.922	-0.005	0.918	3.228	0.961	0.807	0.922	-0.018

*key: r^2 = regression coefficient, K_0= Zero order rate constant, K_1= First order rate constant, K_h = Higuchi rate constant, n = Korsmeyer- Peppas constant, N = Hixson-Crowell constant

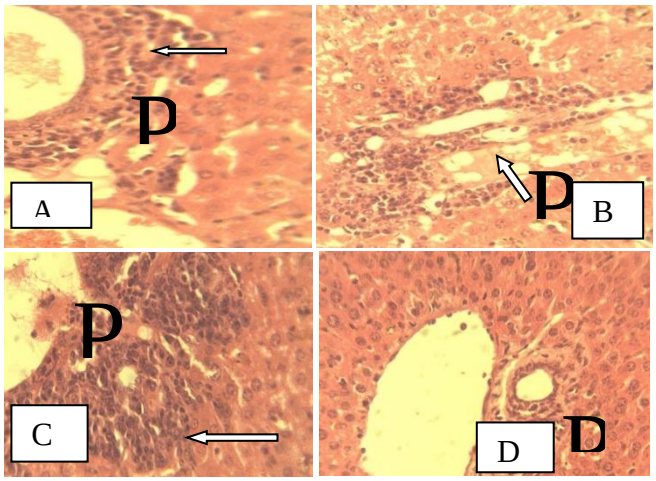

Figure 6: Photomicrograph of the liver section of mice in groups, A, B, C and D that is optimized SLMs formulation of AL, reference tablet, no treatment group and uninfected animals respectively. (Mag. H and E x 400).Key: Tubular degenerations (black arrow) while white arrow shows normal glomerulus (GM) and renal tubules

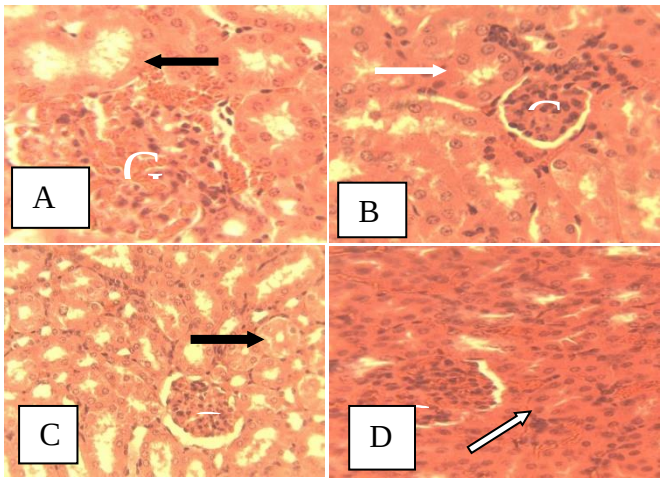

Figure 7: Photomicrograph of the kidney section of mice in groups A, B, C and D that is optimized SLMs formulation of AL, reference tablet, no treatment group and uninfected animals respectively. (Mag. H and E x 400). Key: Tubular degenerations (black arrow) whilewhite arrow shows normal glomerulus (GM) and renal tubules

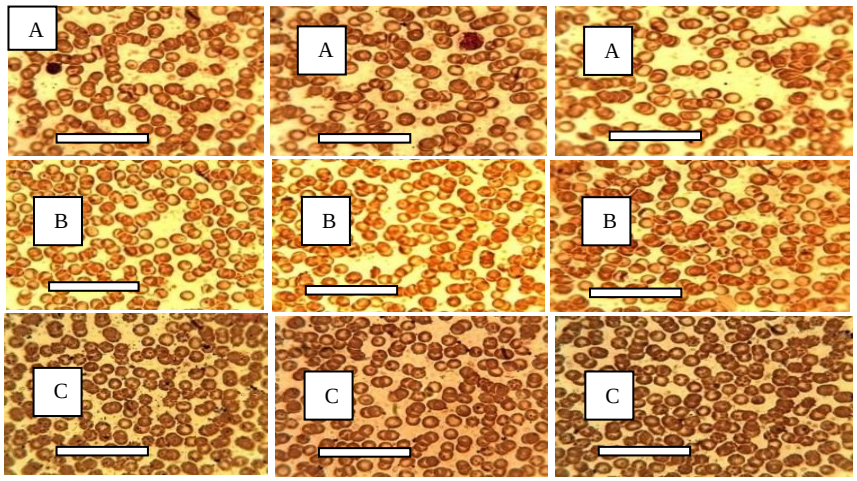

Figure 8: Micrographs of the blood cells (erythrocytes) of mice in groups A, B and C (Bar represents 40 μm).

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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