NOVEL DRUG DELIVERY SYSTEMS CONTAINING ARTESUNATE AND AMODIAQUINE

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TITLE

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CERTIFICATION

This is to certify that Ozioma Blessing Onokala, a postgraduate student in the Department of Pharmaceutics, with the registration number PG/M.Pharm./11/59497, has satisfactorily completed the requirements for the award of Master of Pharmacy (M.Pharm) degree in Physical Pharmaceutics. The work embodied in this project is original and has not been submitted in part or full for any other diploma or degree of this or any other University.

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Sign/ Date

Head of Department: Prof. K.C. Ofokansi

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Sign/Date

DEDICATION

This work is dedicated to God Almighty for His goodness and mercies; and to my mother, Prof. (Mrs.) P.C. Onokala, for her motherly care and support.

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I acknowledge the grace and mercies of God Almighty, who made possible the successful completion of this project work, to Him, be all the glory.

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TABLE OF CONTENTS

APPENDICES

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Globally, an estimated 3.4 billion people are at risk of malaria, including Nigerians. The current World Health Organization (WHO) Guideline for the treatment of malaria recommends the use of artemisinin combination therapy (ACT) for the treatment of uncomplicated malaria. Presently, the available formulations of ACTs are administered through the oral route. Rectal administration, which formed the basis of this work, is an alternative route of administration which overcomes the limitations of oral and parenteral administration of drugs.

Differential Scanning Calorimetry (DSC) was employed to study the compatibility between artesunate, amodiaquine and the suppository bases and to determine the optimum ratio of the excipients. Three batches of structured suppositories of artesunate and amodiaquine (ACT) were formulated by the melt moulding (fusion) technique, evaluated for various parameters including physical characterization, weight variation, drug content, morphology and *in vitro* drug release. The drug release data were analyzed by zero order and Higuchi models. The suppositories were further evaluated in mice against virulent rodent malaria parasite *Plasmodium berghei* using Peterôs 4 days suppressive protocol. Data were expressed as mean ± SD. The parasitemia of the different groups were statistically assessed by ANOVA.

The compatibility results proved that artesunate and amodiaquine are compatible. The optimum excipient ratio as determined by DSC was 4:4:3 for Phospholipon®90G-Softisan®154 matrix, Castor oil, and PEG 4000 respectively. The colours of the prepared suppositories ranged from light yellow to deep yellow; no fissures, fat blooming, exudation or migration of active ingredients was observed. The weight variation of all the suppositories met the BP Standards, with uniform drug content. The release profile for artesunate ranged from 99.21 % to 81.31 % and from 99.89 % to 28.81 % for amodiaquine. The developed suppositories reduced parasitemia to undetectable levels 28 days after drug administration. The survival rate of animals treated with E and PS suppositories was 100 %, while the survival of animals treated with P was 80 %, 55 days after drug administration. The results of the hemoglobin and hematocrit tests 55 days after drug administration revealed that the blood levels were normal for animals in the treatment groups. Stability studies revealed that the suppositories stored at 4 ^{o}C were more stable than the suppositories stored at 27 ^{o}C

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 General Introduction

Administration of rectal artesunate as a pre-referral treatment has been evaluated in Africa. It was observed that pre-referral rectal artesunate reduced death or permanent disability in patients with severe malaria who experienced delays greater than six hours, reaching a health care facility [1-3]. The observations from these studies led the World Health Organization to recommend the use of rectal artesunate as pre-referral treatment in severe malaria [4]. However, there are concerns that the frequent use of artesunate monotherapy may lead to the resistance of plasmodium to artesunate, hence, the need to formulate an artemisinin combination therapy (ACT) suppository.

In this work, artesunate and amodiaquine were formulated into structured rectal suppositories with polyethylene glycol 4000, Phospholipon® 90G, Softisan® 154, and castor oil.

1.2 Malaria

Malaria is a protozoan infection and is spread from one person to another by female mosquitoes of the genus Anopheles or through transfusion of infected blood or by mother ï to-child transmission. Malaria is caused by five species of parasite that affect humans, and all of these species belong to the genus *Plasmodium : Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae* and *Plasmodium knowlesi*. The most important of these are *Plasmodium falciparum and Plasmodium vivax*.

1.2.1 Epidemiology of malaria

In 2013, there were 97 countries and territories with ongoing malaria transmission, and 7 countries in the prevention of re-introduction phase, making a total of 104 countries and territories in which malaria is presently considered endemic.

Globally, an estimated 3.4 billion people are at risk of malaria. The World Health Organization estimates that 207 million cases of malaria occurred globally in 2012 (uncertainty range 135-287 million) and 627,000 deaths (uncertainty range 473,000-789,000).

Most cases (80 %) and deaths (90 %) occurred in Africa and most deaths (77 %) were in children under 5 years of age [5].

In Nigeria, the disease is a major health problem with stable transmission throughout the country. It accounts for about 50 % of out-patient consultation, 15 % of hospital admission and is the prime amongst the top three causes of death in the country [4].

1.2.2 *Plasmodium* species responsible for malaria

(A) *Plasmodium falciparum*

Plasmodium falciparum differs from other human malarial species in that the infected red blood cells do not remain in the circulation for the entire life cycle. After 24-32 hours, when young parasites mature from the ring to the trophozoite stage, the infected red blood cells adhere to endothelial cells in the microcirculation of various organs. This phenomenon, termed ñ sequestrationò, is believed to occur mainly to avoid splenic removal of the infected red blood cells. Sequestration causes microcirculatory obstruction, impaired tissue perfusion and inflammatory cells activation and it is linked to the severity of the disease [6]. High parasitaemia is undoubtedly a risk factor for death from falciparum malaria, but the relation between parasitaemia and prognosis varies according to the level of malaria transmission. In low-transmission areas, mortality from acute falciparum malaria begins to increase with parasite densities over 100 000/µl (approximately 2.5 % parasitaemia), whereas in areas of higher transmission much higher parasite densities may be well tolerated. Parasitaemia > 20 % is associated with a high risk in any epidemiological context [7].

Malaria due to *Plasmodium falciparum* (*P. falciparum*) is the most deadly form, and it predominates in West Africa [5] . The majority of the malaria infections in Nigeria, West Africa are caused by *Plasmodium falciparum* (Figure 1). *P. falciparum* causes malignant tertian malaria, the most dangerous form of human malaria. If treated early, the infection usually responds within 48 hours to appropriate chemotherapy [8].

(B) *Plasmodium vivax*

Plasmodium vivax (*P. vivax*) infection is much less likely to progress to severe malaria than *P. falciparum* infection. Severe vivax malaria may present with some symptoms similar to those of severe *P. falciparum* malaria and can be fatal. Severe anemia and respiratory distress occur in all ages, although severe anemia is particularly common in young children and people with co-morbid conditions [7]. *P. vivax* has a wider distribution than *P. falciparum* because it is able to develop in the *Anopheles* mosquito vector at lower temperatures, and to survive at higher altitudes and in cooler climates. Although *P. vivax* can occur throughout Africa, the risk of *P. vivax* infection is considerably reduced in the region by the high frequency of the Duffy negativity trait among many African populations; in individuals without the Duffy antigen, red blood cells are resistant to infection with *P. vivax*. In many areas outside Africa, infections due to *P. vivax* are more common than those due to *P. falciparum* [5].

P. vivax causes benign tertian malaria. Like the other benign malaria, it produces milder clinical attacks than does *P. falciparum*, because the erythrocytes it infects are not sequestered in the peripheral microvasculature. *P. vivax* infection has a low mortality rate in untreated adults and is characterized by relapses caused by latent tissue forms [8].

(C) *Plasmodium ovale*

Plasmodium ovale (*P. ovale*) causes a rare malarial infection with a periodicity and relapses similar to those of *P. vivax*, but it is even milder and more readily cured [8].

(D) *Plasmodium malariae*

Plasmodium malariae causes quartan malaria, an infection that is common in localized areas of the tropics. Clinical attacks may occur years after infection but are much rarer than after infection with *P. vivax* [8].

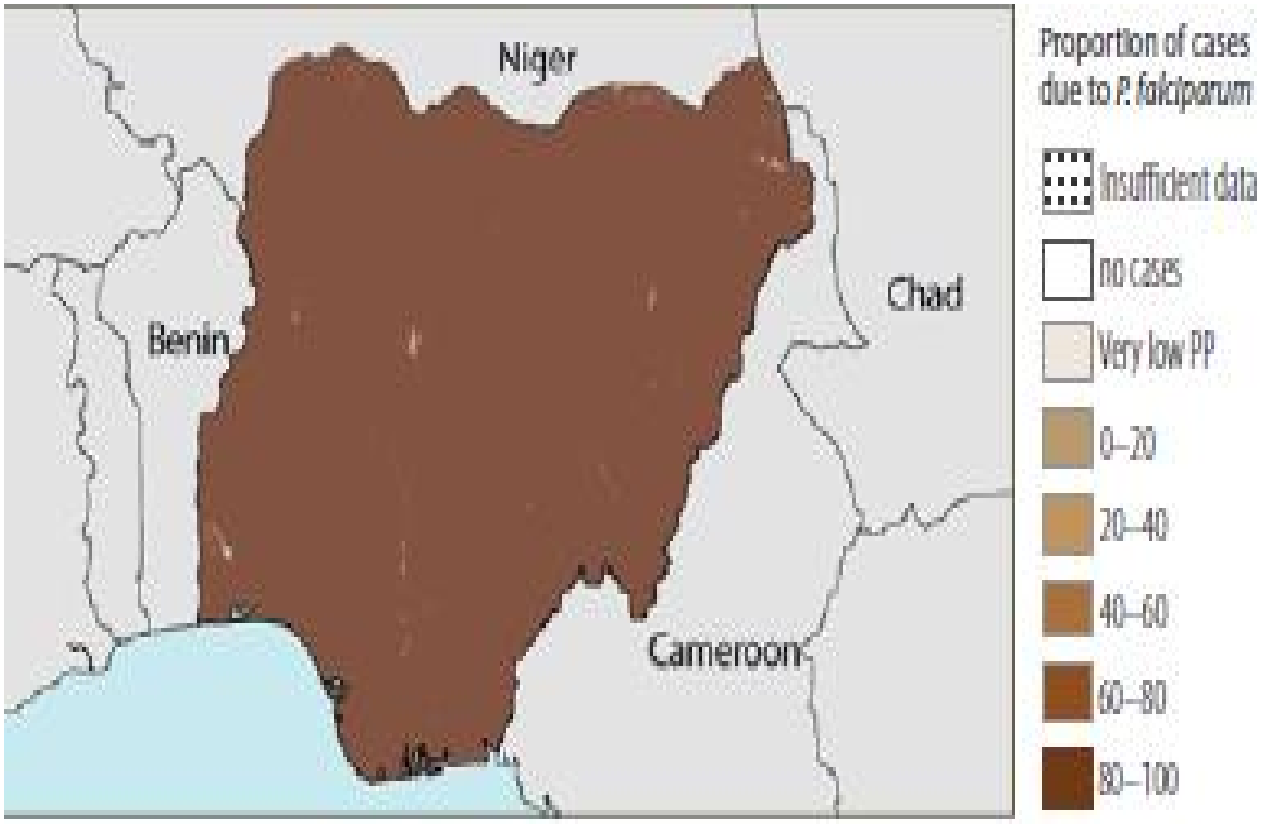

Figure 1: Proportion of malaria cases due to *Plasmodium falciparum* in Nigeria

Source: World Malaria Report 2013 by the World Health Organization

(E) *Plasmodium knowlesi*

The monkey parasite *Plasmodium knowlesi* (*P. knowlesi*) can cause malaria in humans living in close proximity to macaque monkeys (particularly on the island of Borneo and in other South-East Asian countries). It is transmitted mainly in forests and along forest fringes. *P. knowlesi* replicates every 24 hours, which can result in rapidly increasing parasite densities, severe disease and death in some individuals. The severe manifestations are similar to those of severe falciparum malaria, with the exception of coma. Early diagnosis and treatment are therefore essential. A definitive diagnosis is made by polymerase chain reaction [7].

1.2.3 Malaria infection and the lifecycle of the mosquito

Human beings may be infected by sporozoites injected by an infected female mosquito (genus *Anopheles*). These parasite forms rapidly leave the circulation and localize in hepatocytes, where they transform, multiply, and develop into tissue schizonts. This primary asymptomatic tissue (preerythrocytic or exoerythrocytic) stage of infection lasts 5 to 15 days, depending on the *Plasmodium* species. Tissue schizonts then rupture, each releasing thousands of merozoites that enter the circulation, invade erythrocytes, and initiate the erythrocytic stage of cyclic infection. Once the tissue schizonts burst in *Plasmodium falciparum* and *Plasmodium malariae* infections, no form of the parasite remains in the liver. But in *P. vivax* and *P. ovale* infections, there persist tissue parasites that can produce relapses of erythrocytic infection months to years after the primary attack. Once plasmodia enter the erythrocytic cycle, they cannot invade other tissues; thus, there is no tissue stage of infection for human malaria contracted by transfusion. In erythrocytes, most parasites undergo asexual development from young ring forms to trophozoites and finally to mature schizonts. Schizont-containing erythrocytes rupture, each releasing 6 to 24 merozoites, depending on the *Plasmodium* species. It is this process that produces febrile clinical attacks.

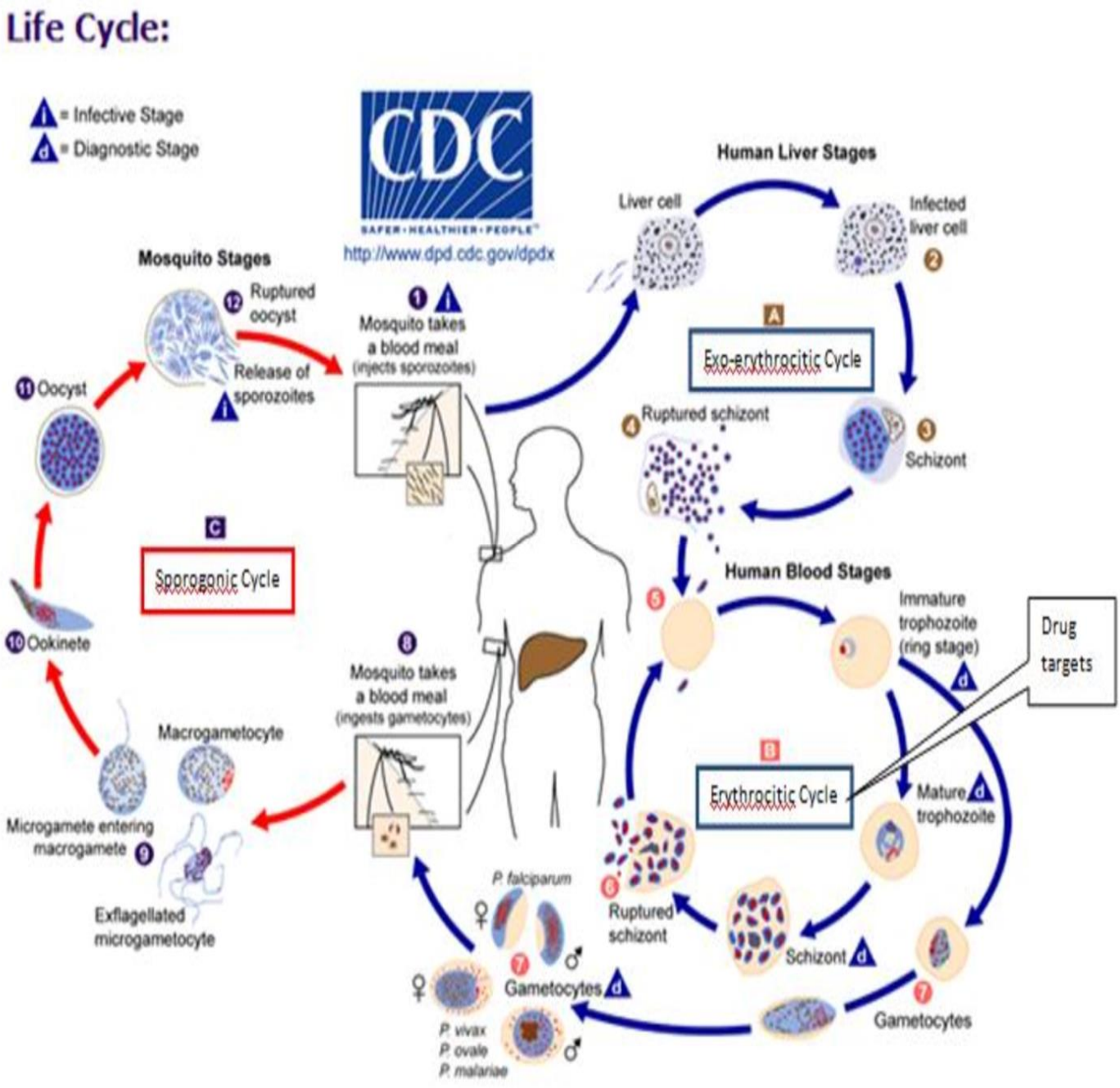

Figure 2: Lifecycle of the mosquito (Source: The United States Centers for Disease Control).

The released merozoites invade more erythrocytes to continue the cycle, which proceeds until death of the host, modulation by drugs or acquired partial immunity. The periodicity of parasitemia and febrile clinical manifestations thus depend on the timing of schizogony of a generation of erythrocytic parasites. For *P. falciparum, P. vivax*, and *P. ovale*, it takes about 48 hours to complete this process. Synchronous rupture of infected erythrocytes and release of merozoites into the circulation lead to typical febrile attacks on days 1 and 3, hence the designation ñtertian malaria.ò. Repeated cycles of erythrocyte invasion and rupture lead to chills, fever, headache, fatigue, other nonspecific symptoms, and, with severe malaria, signs of organ dysfunction. The periodic febrile pattern is less regular in falciparum malaria due to a combination of asynchronous release of parasites and segregation of infected erythrocytes in the periphery. In *P. malariae* infection, schizogony requires about 72 hours, resulting in malarial attacks on days 1 and 4, or ñquartan malaria.ò

Some erythrocytic parasites differentiate into sexual forms known as gametocytes. After infected human blood is ingested by a female mosquito, exflagellation of the male gametocyte is followed by male gametogenesis and fertilization of the female gametocyte in the insectôs gut. The resulting zygote, which develops as anoocyst in the gut wall, eventually gives rise to the infective sporozoites, which invades the salivary gland of the mosquito. The insect then can infect another human host by taking a blood meal [8], thus the cycle continues.

1.2.4 Severe malaria

Severe malaria is most commonly caused by *Plasmodium falciparum*, although *P. vivax* and *P. knowlesi* can also cause the severe disease [9,10]. Severe malaria can be defined by clinical (Table 1) or laboratory criteria (Table 2).

1.2.4.1 Pathogenic characteristics of severe malaria [12]

(A.) Cytoadherence

Cytoadherence is the ability of parasites to adhere to the vascular endothelium. It was recognized as early as 1892 by Marchiafava and Bignami [13]. Mature forms of parasites (asexual stage and gametocytes) can adhere to the vascular endothelium of several organs

(lung, heart, brain, liver, and kidney), the subcutaneous adipose tissues and the placenta. This feature of the disease *in vivo* has been related exclusively to *P. falciparum* [14,15]. However, sequestration *in vitro* to some endothelial cell lines and placental cryosections has also been seen in reticulocytes infected with *P. vivax* [16].

Parasite sequestration is thought to be the pathological base of the severe manifestation of malaria, including cerebral malaria [17]. It causes blood flow impairment leading to local hypoxia. It enhances parasite replication and the sticking of infected red blood cells to non-infected red blood cells (rosetting). Moreover, when parasites sequester, the effects of parasite toxins are more localized; the host immune response is stimulated, which may cause a focused production of inflammatory mediators and tissue damage. As a consequence, both healthy red blood cells and infected red blood cells become more rigid and less deformable [18].

Sequestration is mostly mediated by mature parasite forms, approximately 20 hours after red blood cell invasion. The parasites produce new proteins that are exported to the infected red blood cell surface and increase the adhesiveness of the infected red blood cells to the endothelium. During their 48-hour life cycle, the parasites can remain sequestered for 24 hours in the deep microvasculature. In this manner, they evade clearance by the spleen, and make the diagnosis more difficult since they are not seen in the peripheral blood [12].

Sequestration of *P. falciparum* has been attributed to a different class of molecules of parasite origin and ligands present on the human endothelium. Among those, the *P. falciparum* histidine-rich protein (PfHRP) and the erythrocyte membrane protein 1 (PfEMP1), have received significant attention. PfHRP is related to the establishment of knobs, symmetric membrane arrangements which appear on the surface of infected red blood cells, while PfEMP1, a multimeric protein encoded by the var (variant) gene [14,15] protrudes from the knobs and plays a major role in sequestration and thus virulence. To adhere to the endothelium, the parasites first adhere, roll and then become firmly attached to the endothelium adhesion molecules.

Table 1: Clinical features defining severe malaria [11]

Clinical features defining severe malaria
• Impaired consciousness or unrousable coma • Prostration, i.e. generalized weakness so that the patient is unable to walk or sit up without assistance • Failure to feed • Multiple convulsions ï more than two episodes in 24 hours • Deep breathing, respiratory distress (acidotic breathing) • Circulatory collapse or shock, systolic blood pressure < 70 mmHg and <50 mm Hg in children • Clinical jaundice plus evidence of other vital organ dysfunction • Haemoglobinuria • Abnormal spontaneous bleeding • Pulmonary edema (radiological)

Table 2: Laboratory findings defining severe malaria [11]

Laboratory findings defining severe malaria
• Hypoglycemia (blood glucose < 2.2 mmol/l or < 40 mg/dl) • Metabolic acidosis (plasma bicarbonate < 15 mmol/l) • Severe normocytic anemia (Hb < 5 g/dl, packed cell volume < 15 %) Haemoglobinuria • Hyperparasitemia (> 2 % or 100 000/µl in high stable malaria transmission areas) • Hyperlactatemia (lactate > 5 mmol/l) • Renal impairment (serum creatinine > 265 µmol/l)

.

Among these molecules, ICAM-1, a major sequestration receptor which is involved in cerebral sequestration serves as a rolling receptor. On the other hand, CD 36 gives stationary and stable adherence under flow [19,20] . Sequestration is also seen during gestational malaria, when parasites adhere to the placenta. PfEMP1 is again the main adhesion receptor which adheres to the trophoblastic villous endothelium mainly through chondroitin-4-sulfate (CSA) and other sugars such as glycosaminoglycans and possibly hyaluronic acid (HA). Malaria in pregnancy can be severe for mothers and induce fetal death especially during the first pregnancy, when women usually lack sufficient immunity against CSA-binding parasites [21-24].

(B) Rosetting

Rosetting is one of the forms of cytoadherence of late stages of infected red blood cells to non-parasitized red blood cells and /or platelets [25]. The infected red blood cells ligand involved in rosette formation is PfEMP1. The three receptors associated with resetting include: Complement Receptor 1 (CR1), Heparin Sulfate (HS), and the ABO blood group [25-26]. PfEMP1 has been shown to bind to CR1, specifically at the C3b-binding site. The lectin-like DBL-domain of PfEMP1 can make strong adhesion with carbohydrate structures particularly A blood group antigen, favoring rosettes formation [27] . For this reason, non-O blood groups are considered risk factors for life-threatening malaria, through the mechanism of enhanced rosette formation [28,29].

P. falciparum, P. vivax, and *P.ovale* are all able to form rosettes [30-31], but only those caused by *P. falciparum* have been associated with severe malaria. In African children, they may enhance microcirculatory obstruction [32]. Recently, it has been shown that 4-HNE, a biomembrane lipid peroxidation product driven by haem iron of the malarial pigment can be transferred from the infected red blood cells to normal red blood cells in rosettes, favouring their removal by macrophages [28]. This could partly explain the rapid loss of normal non- parasitized red blood cells in severe malaria anemia.

(C) Innate immune response

The immune response to the malaria parasite is both species and stage specific [34]. The activation of components of the innate immune system is crucial to control parasite

replication, contributing to the subsequent elimination and resolution of the infection [35]. Neutrophils, monocytes/ macrophages, dendritic cells, natural killer (NK) cells, and gamma T cells are all the cells of the innate immune system in charge of controlling the early progression of the malaria disease through phagocytosis and/ or production of inflammatory mediators. Much of the symptoms of malaria attacks such as fever, nausea, headaches, and others are the consequences of the inflammatory response orchestrated by the cells of the innate immune system, stimulated by parasites of their products at the rupture of the late stage infected erythrocytes [34,36].

(D) Specific immune response

Malaria is an important cause of morbidity, but not everyone infected with the malaria parasite becomes seriously ill or dies. In areas of stable endemicity, repeated exposure to the parasite leads to the acquisition of specific immunity, which restricts serious problems to young children; malaria in older subjects causes a relatively mild febrile illness. However, individuals with no previous experience of malaria become ill on their first exposure to the plasmodium parasite. They develop a febrile illness which may become severe and in some cases may lead to death [37,38].

Immunity to malaria is provided by innate mechanisms, and subsequently by the development of acquired immunity. Following repeated infections, people living in malaria endemic areas gradually acquire mechanisms, which help limit the inflammatory response to the parasite that causes the acute febrile symptoms. Sterilizing immunity is never achieved. Parasites have evolved to maintain a balanced relationship with their human hosts. In this sense they can partially escape from the host effector mechanisms, while the hosts are able to develop partial immunity against the parasite. This type of immunity requires repeated infections, takes years to develop and usually lasts shortly. Natural acquired immunity is called premonition since low parasite burden often persists in the presence of circulating antibodies to the various stages, in the absence of the clinical disease.

1.2.4.2 Specific complications of severe malaria infection

(A) Anemia

Anemia is one of the most common causes of morbidity and mortality in malaria infection particularly in pregnant women and in children [39]. Malarial anemia could be acute or chronic; in holoendemic areas, chronic malarial anemia is more common. Acute malarial anemia could be drug- induced, or occur after massive erythrocytes lysis due to elevated parasitemia or due to immune haemolysis [40]. Dyserythropoiesis plays an important role in the pathogenesis of anemia; examination of bone marrow from children with severe anemia showed hypercellularity, mild to normal erythroed hyperplasia and abnormal features of late erythroid progenitors. Hemozoin and its phagocytosis by bone marrow macrophages has been proposed to cause dyserythropoiesis either through direct accumulation in the bone marrow and generation of reactive toxic species or activation of the innate immune response [41] .

(B) Thrombocytopenia

The pathogenesis of malaria thrombocytopenia is complex and may be related to coagulation disturbances, spleenomegaly and platelet destruction by macrophages, bone marrow alterations, antibody-mediated platelet destruction, oxidative stress and platelets aggregation [42] .

(C) Acute respiratory distress syndrome (ARDS)

Different pathological presentations of ARDS were observed among the different species of human malaria: the greatest severity and frequency of cases are due to *Plasmodium falciparum* and could be partially attributed to the sequestration and rosetting of infected red blood cells in the pulmonary microcirculation [43], heavy parasitaemia and white blood cell agglutinates were associated with acute respiratory distress syndrome in *P. vivax* [44].

(D) Liver dysfunction

The pathogenesis of malarial hepatic dysfunction is not completely known; reduction in portal venous flow as a consequence of micro-occlusion of portal venous branches by

parasitized erythrocytes, intra-hepatic cholestasis due to reticuloendothelial blockage and hepatic microvilli dysfunction, suppression of bilirubin excretion due to effect of parasitemia or endotoxemia or metabolic acidosis, apoptosis and oxidative stress are all mechanisms involved in hepatic damage [45,46] .

(E) Kidney dysfunction

P. falciparum acute renal failure is more common in adults; its pathogenesis is complex and it includes mechanical and immunologic factors, volume depletion, hypoxia, hyperparasitemia and other factors [47-49]. Black water fever, a rare but severe complication of severe malaria, is characterized by fever, intravascular haemolysis with haemoglobinuria, dark urines and often acute renal failure [50-52] .

1.2.5 Diagnosis of malaria

Malaria can be diagnosed through clinical or laboratory diagnosis.

1.2.5.1 Laboratory diagnosis of malaria

Malaria can be diagnosed in the laboratory through the following techniques:

(A) Thick film microscopy (TFM)

The gold standard for malaria parasite identification and quantification has been the microscopic examination of thick and fixed thin blood smears using Giemsa stain. In cases of anticipated low malaria parasite densities, care should be taken to maintain the pH of the stain around 7.0 ; in addition, freshly prepared stain achieves better results [53]. This method is cost-effective relative to other methods of diagnosis. It has a threshold sensitivity of 5 to 50 parasite/µL (depending on the laboratory scientistôs expertise) [54]. Microscopy can also characterize the infecting species and also determine their relative densities [55].

Limitations of microscopy

This method is also time-consuming for optimal blood film preparation, examination and interpretation [56].

(B) Rapid diagnostic technique (RDT)

Rapid Diagnostic Technique, an immuno-chromatographic capture procedure was developed to improve the timeless sensitivity and objectivity of malaria diagnosis through less reliance on expert microscopy [56] . Several studies have evaluated the effectiveness of RDTs compared with microscopy and the results have consistently shown higher sensitivity and specificity but inability to differentiate mixed infections [57-59].

Limitations of RDT

However, several factors in the manufacturing process as well as environmental conditions may affect RDT performance, and these include : sub-optimal sensitivity at low parasite densities, inability to accurately identify parasites to the species level or quantify infection density, and a higher unit cost relative to microscopy [60] .

(C) Polymerase chain reaction

Polymerase chain reaction (PCR) , detects specific nucleic acid sequence and its values lie in its sensitivity, with the ability to detect >5 parasites/μL of blood [61]. PCR is useful both for initial parasite diagnosis and for follow-up during drug efficacy studies [62]. PCR techniques have been utilized to enhance malaria diagnosis in mixed infections and especially in patients with low parasite densities [63] . Nested PCR and Real-Time PCR techniques have been used for detection of *Plasmodium vivax* [64]. It is also useful as a sensitive standard against which other non-molecular methods can be evaluated [65].

Limitations of PCR

However, it is expensive and time-consuming because of the amount of resources needed for the running of the PCR laboratory, it is used more for research purposes [66].

1.2.5.2 Clinical diagnosis of malaria

Clinical diagnosis is also referred to as presumptive diagnosis. It is the basis for therapeutic care for the majority of febrile patients in malaria endemic areas where laboratory support is often out of reach. Overlap of malaria symptoms with other tropical diseases like typhoid, respiratory tract infections and viral infections impairs the specificity of clinical diagnosis thereby encouraging indiscriminate use of anti-malarials in endemic areas. Accuracy of clinical diagnosis varies with the level of endemicity, and age group; therefore no single clinical algorithm can be regarded as a universal predictor [67] .

A study conducted in Nigeria revealed the need for complete shift from symptom-based diagnosis to parasite-based diagnosis. This can bring significant improvement to tropical fever management, reduce drug wastage and also help to curtail development of malaria drug resistance [66].

1.2.6 Pharmacological treatment of malaria

The correct management of clinical malaria cases is a complex issue that has taken into account different targets that may be differently prioritized according to the various clinical and epidemiological situations:

- To prevent progression of uncomplicated malaria to severe life threatening complications (*P. falciparum* but also *P. vivax*);
- To prevent mortality of patients with severe malaria (*P. falciparum* but also *P. vivax* and *P. ovale*);
- To limit the spreading of the infection / disease in the population;
- To limit as much as possible the emergence of plasmodium resistant strains.

1.2.7 Properties of the ideal anti-malarial drug

The ideal antimalarial drug should possess the following properties:

- Ability to act rapidly against replicating blood erythrocytic asexual forms, primarily schizonts, that are responsible for the clinical manifestation of the disease (parasitological cure);
- Ability to act against liver hypnozoites, when appropriate (radical cure).
- In endemic areas, the ideal anti-malarial drug should act against the sexual forms (gametocytes) that are responsible for the transmission of the infection in the population via the vector mosquitoes; also, in endemic areas, the ideal anti-malarial drug should prevent the selection of plasmodia resistant strains (high resistance barrier) [6].

1.2.8 The artemisinin-based combination therapy

The artemisinin- based combination therapy (ACT) involves the use of combinations of an artemisinin derivative and another structurally unrelated and more slowly eliminated antimalarial to reduce emergence of resistant strains of plasmodium as well as increase the efficacy of anti-malarial therapy, hence, the artemisinin combination therapy exhibits the properties of the ideal antimalarial drug.

Artesunate-Sulfadoxine-Pyrimethamine

Sulfadoxine-pyrimethamine is a fixed combination of a long-acting sulfonamide and the anti-folate pyrimethamine. These are synergistic against sensitive parasites. Serious sulfonamide toxicity is unusual with a single-dose treatment of malaria. The anti-folate properties of pyrimethamine rarely produce toxicity. The combination with artesunate is available as separate tablets containing 500 mg of sulfadoxine and 25 mg of pyrimethamine. The total recommended treatment is 4 mg/kg BW of artesunate, given once for 3 days and a single administration of sulfadoxine-pyrimethamine 1.25/25 mg base/kg BW on admission. This Sulfadoxine-Pyrimethamine dose was developed in adults but in the main target group (children aged 2-5

years), the weight adjusted dose produced blood concentrations of both components that are approximately half those in adults [57].

Artemether-Lumefantrine

This was the first fixed dose combination of an artemisinin derivative with a second unrelated antimalarial compound. Lumefantrine (formerly benflumetol) is an aryl amino-alcohol the same general group as mefloquine and halofantrine. It was discovered and developed in the Peopleôs Republic of China. Lumefantrine is active against all human malaria parasites, including multi-drug resistant *P.falciparum*. Artemether-lumefantrine is dispensed as tablets containing 80/480 mg of artemether and lumefantrine respectively [69].

Artesunate-Mefloquine

Mefloquine is a quinolone methanol compound related to quinine [72]. Where adherence can be assured, the dose should be split at 15 mg/kg initially followed 8-24 hours later by a second 10 mg/kg or as 8.3 mg/kg daily for 3 days. This improves bioavailability and reduces vomiting [70]. There is no formulation of mefloquine for children.

Combining artesunate or artemether (4 mg/kg/day for 3 days) with mefloquine has the benefit of efficacy, decrease in manifestation of resistance, as well as the additional benefit that if mefloquine is split as 8.3 mg/kg/day for 3 days or not given until the second day of treatment then absorption is increased and gastrointestinal adverse effects are lessened [70-71].

Artesunate-Chlorproguanil-Dapsone

Chlorproguanil-dapsone is an antifol- sulfonamide combination with a similar mode of action and synergistic properties to sulfadoxine-pyrimethamine. Chorproguanil can be considered as a prodrug for the active antifol chlorcycloguanil to which it is metabolized by the polymorphic CYP $_{450}$2C19 [73]. The advantages of this combination are good tolerability and rapid elimination [74] providing less selective pressure on the spread of resistance and greater activity than Sulfadoxine-Pyrimethamine against moderately resistant *Plasmodium falciparum*. Disadvantages include concerns over the safety of dapsone (for dapsone-induced hemolytic anemia), and lack of a post-treatment prophylactic effect.

Artesunate-Atovaquone-Proguanil

Atovaquone-proguanil has a different (and synergistic) mode of action to other antimalarials affecting parasite respiration at the level of the cytochrome chain. Proguanil is acting itself in the combination, and not via the antifol triazine metabolite cycloguanil- so it remains effective against antifol-resistant parasites. Atovaquone absorption (like that of lumefantrine and halofantrine) is augmented by co-administration with fats. Its elimination half-life of 1-2 days provides for an effective 3-day treatment regimen [75]. It is remarkably well tolerated with no serious adverse effects. The main impediment to its use is the high cost of atovaquone manufacture.

Artesunate- Pyronaridine

Pyronaridine is a synthetic antimalarial developed in China and used originally as a monotherapy. It bears closest structural similarity to amodiaquine, although pyronaridine is much more active against resistant malaria parasites. It is extensively distributed and eliminated slowly. The mechanism of action of the drug and mechanisms of potential resistance have not been well characterized.

Dihydroartemisinin- Piperaquine

Piperaquine is a bisquinoline compound related to chloroquine and other 4-aminoquinolines. Piperaquine replaced chloroquine as the first-line treatment of falciparum malaria in China in 1978. The fixed dose combination formulated in tablets contains dihydroartemisinin (40 mg) and piperaquine (320 mg). The most common adverse effects are gastrointestinal (nausea, vomiting, abdominal pain, and diarrhea), but they are usually mild and self-limiting. The main determinant of the parasitological efficacy is the slow elimination of piperaquine. This also determines the ñ post-treatment prophylactic effect, ò which is important if the drug is going to be used as Intermittent Preventive Treatment (IPT) [76].

The simplicity of administration, the excellent efficacy even against multi-drug resistant strains and the favorable toxicity profile, make the DHA-piperaquine combination one of the more promising of the currently available artemisinin combination therapy [80].

1.2.8.1 Amodiaquine [80]

1.2.8.1.2 General characteristics of amodiaquine

Name: Amodiaquine hydrochloride (BANM, rINNM).

4-(7-Chloro-4-quinolylamino)-2-(diethylaminomethyl) phenol dihydrochloride dehydrate [77].

4-[(7-Chloro-4-quinolyl) amino]-Ŭ-(diethylamino)-o-cresoldihydrochloride dehydrate [78, 79].

The compound has a molecular weight of 464.8 g/mol and a melting point of 158 ŬC [77, 82, 83].

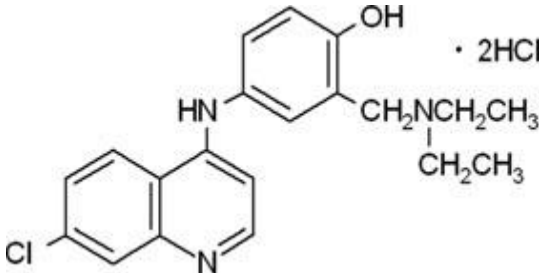

Figure 3: Chemical structure of amodiaquine dihydrochloride [78]

1.2.8.1.3 Therapeutic indicators

Amodiaquine hydrochloride is a Mannich base 4-aminoquinoline antimalarial recommended in the treatment of uncomplicated malaria caused by *Plasmodium falciparum* [77, 84]. After absorption, it is metabolized chiefly to desethylamodiaquine (ADQm), which also shows antimalarial activity. The WHO recommends the use of amodiaquine together with artesunate for the treatment of uncomplicated falciparum malaria, to reduce the risk of drug resistance compared with monotherapy [84].

1.2.8.1.4 Mechanism of action

In vitro studies suggest that amodiaquine inhibits the digestion of hemoglobin as the antimalarial mode of action [166]. The drug also inhibits the glutathione-dependent destruction of ferriprotoporphyrin IX in the malaria parasite, resulting in the accumulation of this peptide, which is toxic for the parasite [167].

1.2.8.1.5 Therapeutic index and toxicity

No data on the toxicity of amodiaquine after repeated oral administration to animals were available in the open literature. Single-dose toxicity studies reported a median lethal dose (LD_{50}) of 225 mg/kg (mouse intraperitoneal), LD_{50} of 550 mg/kg (mouse oral), and maximum tolerable dose (LD_0) of 137 mg/kg (mouse intraperitoneal) [86, 87].

The adverse effects of amodiaquine are similar to those of chloroquine. Symptoms of an overdose include headache, dizziness, visual disorders, cardiovascular collapse, and convulsions followed by early respiratory and cardiac arrest [88-90].

1.2.8.1.6 Physicochemical properties

Solubility

The United States Pharmacopoeia lists amodiaquine as water soluble at room temperature [78]. Literature research revealed a solubility of one part in 22 parts of water for amodiaquine. However, the temperature at which the tests were performed was not reported [83, 90].

Polymorphism and partition coefficient

No polymorphs of amodiaquine have been reported. Experimental studies on the partitioning of amodiaquine between 1-octanol and 0.1 M phosphate buffer and 0.1 M acetate buffer showed a log D of 2.61 and ī 1.4 at pH 7.4 and 5.0, respectively [91].

pKa

Amodiaquine is a diprotic weak base with a pKa of 7.08 and 8.14 at 25 ŭC [91, 92].

1.2.8.1.7 Pharmacokinetic properties

Absorption and Bioavailability

The absolute bioavailability of amodiaquine is not known; it is not recommended for intravenous (i.v.) use as slow i.v. injection causes a decrease in the systolic pressure [94]. Amodiaquine is rapidly absorbed from the gastrointestinal tract and undergoes extensive first pass metabolism in the liver to ADQm. Administration of amodiaquine /artesunate FDC tablets along with a high-fat

meal in healthy human volunteers slightly delayed and increased the C_{max} and AUC of amodiaquine and its main metabolite [95]. Because bioequivalence could not be demonstrated when the drug was administered in fed and fasted state, the product leaflets recommend against the administration of combination amodiaquine and artesunate formulations after a high-fat meal [97, 97].

Permeability

Amodiaquine hydrochloride is reported to be readily absorbed from the gastrointestinal tract [77]. However, literature searches on the absolute bioavailability or fraction absorbed retrieved no results.

Distribution, Metabolism, and Elimination

Amodiaquine is distributed extensively in the body. Using ^{14}C-labeled amodiaquine, it has been demonstrated that the drug accumulated specifically in the liver, kidneys, spleen, and bone marrow in rats, which are also sites of observed toxicity in man [98].

Amodiaquine and ADQm, both demonstrate protein binding to the extent of 90ï 95 %.The main metabolic pathway in the liver is through the cytochrome P450, CYP2C8 isoenzyme [86, 100].

1.2.8.1.8 Dosage form

Dosage form strengths

Doses of amodiaquine are generally expressed in terms of its free base. Two hundred milligrams of amodiaquine is equivalent to 153 mg of amodiaquine base. The WHO treatment guidelines for malarial therapy recommend administration of artesunate along with amodiaquine for effective therapy of uncomplicated *P. falciparum* malaria [84]. Because amodiaquine is generally administered along with artesunate, it is available in coblistered packs as kits and additionally as fixed dose combinations (FDCs). The FDCs are formulated as bilayered tablets with amodiaquine in one layer and artesunate in the other layer. Coblistered amodiaquine tablets are available in dose strengths of 153 and 300 mg base [93]. The total recommended treatment is 10 mg base /kg body weight (BW) of amodiaquine in combination with a second antimalarial

drug once a day for 3 days. Amodiaquine, when combined with artesunate has proved to be efficacious in areas where 28-day cure rates with amodiaquine monotherapy exceed 80 %.

1.2.8.2 Artesunate

Artesunate is an antimalarial agent and a hemi-succinate derivative of dihydroartemisinin. It is activated *in vivo* by hydrolysis, to dihydroartemisinin, the active metabolite of the drug [101-103].

1.2.8.2.1 Chemistry and synthesis

Artemisinin is comparatively easily purified by crystallization after extraction from *Artemisia annua* plants but is extremely difficult to synthesize *de novo*. Artemisinin is the parent compound for semisynthetic derivatives that have been chemically modified at the C_{10} position to produce artesunate, artemether, arteether, dihydroartemisinin, and artelinic acid. Artesunate has variously been formulated for oral, rectal, and parenteral administration [105].

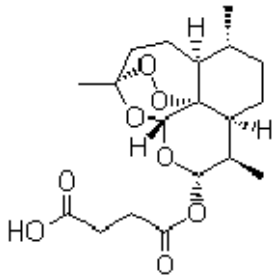

Figure 4: Chemical structure of artesunate [104]

[Chemical name: 3R, 5aS, 6R, 8aS, 9R, 10S, 12R, 12aR)-Decahydro-3, 6, 9-trimethyl-3, 12-epoxy-12H-pyrano (4, 3-j)-1, 2-benzodioxepin-10-ol hydrogen succinate; Molecular formula: $C_{19}H_{28}O_8$; Molecular weight: 384.42]

1.2.8.2.2 Therapeutic indications

Artesunate and other artemisinin derivatives are used for treatment of uncomplicated and severe malaria in both adults and children.

Uncomplicated malaria

Most physicians currently use an oral dose of artesunate of 4 mg/kg per day for three days for patients with uncomplicated malaria when in combination with a second antimalarial.

Severe malaria

Intrarectal administration

Patients with malaria presenting in rural areas may be obtunded or vomiting and unable to take oral medications, leading to significant delay in treatment if facilities for parenteral treatment are unavailable. In such circumstances the rectal route of administration is attractive because in areas where this route is culturally acceptable, rural healthcare workers can be trained to identify moderate and severe malaria and administer rectal drugs before transfer of patients to hospital. The wider therapeutic index of artemisinin derivatives means that they are excellent choices for rectal administration despite the inevitable variability of absorption from this route.

1.2.8.2.3 Mechanism of action

Artesunate exerts a cidal effect on all species of plasmodium that infect humans [116-119]. *In vitro P. falciparum* IC^{50} values (median and range) have been reported as 4.2 (0.5ï 34.6) nM for artesunate [120]. The asexual stages of infection are the most susceptible, with artemisinins inducing up to a 10, 000-fold reduction in parasite biomass per asexual cycle [121]. In common with other antimalarials, artesunate and other artemisinins are particularly active against the large ring stage of infection when parasites are beginning to become most metabolically active.

However, in contrast with other currently useful antimalarials, artesunate and other artemisinins also target tiny ring stages of infection [122,123] (present only a few hours after red cells are invaded by merozoite stages). Artesunate and other artemisinins also inhibit metabolism of parasites more quickly than other antimalarials used to treat severe malaria, [122-124] a pharmacodynamic property that is of potential benefit given that most deaths in African children occur in the first 12 to 24 hours after admission. They also reduce cytoadherence of infected red cells, a recognized virulence determinant [125]. Artesunate and other artemisinins do not interfere with hepatic stages of parasite development and therefore have no causal prophylactic value. They do kill early gametocyte stages of development and have the potential to interfere

with mosquito transmission [126].This property may be useful in areas where transmission rates for malaria are comparatively low [127].

For several decades, the antimalarial action of artesunate and other artemisinins has been attributed to their chemical capability to generate free radicals. This mechanism of action has been suggested partly on the grounds that well recognized sources of free radicals (such as tert-butylperoxide) can themselves kill malaria parasites, albeit in comparatively high (mM) concentrations [128].

More recently, an alternative mechanism of action for artemisinins based on inhibition of the malarial parasiteôs calcium ATPase (sarcoplasmic endoplasmic reticulum calcium ATPase, SERCA) has been suggested [129].

1.2.8.2.4 Therapeutic index and toxicity

Artemisinin and its derivatives are safe and remarkably well tolerated. There have been reports of mild gastrointestinal disturbances, dizziness, tinnitus, and bradycardia [130].

1.2.8.2.5 Pharmacokinetic properties

Absorption and bioavailability

Once absorbed, artesunate is rapidly (within minutes) converted primarily to dihydroartemisinin (DHA) and thence to inactive metabolites via hepatic cytochrome $P\text{-}_{450}$ and other enzyme systems [106]. DHA is itself a potent antimalarial with an elimination half-life of about 45 minutes [107,108]. DHA is mostly (90 %) bound to plasma proteins [109]. The absolute bioavailability of antimalarial activity after a single dose of oral artesunate in uncomplicated adult malaria is about 60 % [107,110]. Parenteral artesunate is pharmacokinetically superior to artemether for the treatment of severe malaria, whether given intravenously [111,112] or by the intramuscular route (to children) [111]. Rectal artesunate in African children with moderate malaria (defined as being unable to take oral medications or prostration/obtundation) shows rapid but variable absorption with peak plasma DHA concentrations appearing in about two hours and bioavailability of between 20 % and 60 % [113-115].

1.3 Rectal drug administration

1.3.1 Anatomy and physiology of the rectum [131]

Rectal dosage forms are introduced into the body through the anus and are thus brought into contact with the most caudal part of the gastrointestinal tract i.e. the rectum. Anatomically, the rectum is part of the colon, forming the last 150-200 mm of the gastrointestinal tract. The rectum can be subdivided into the anal canal and the ampulla, the latter forming approximately 80 % of the organ. The rectum can be considered as a hollow organ with a relatively flat wall surface, without villi and with only three major folds- the rectal valves. The rectal wall is composed of epithelium, which is one cell layer thick, and contains cylindrical cells and goblet cells which secrete mucus. The total volume of mucus is estimated as approximately 3 mL, spread over a total surface area of approximately 300 cm^2. The pH of the mucous layer is reported as approximately 7.5.

1.3.2 Absorption of drugs from the rectum

There are three major separate veins in the rectum. The lower and middle haemorrhoidal veins drain directly into the general circulation, while the upper one drains into the portal vein which flows to the liver. This means that drug molecules can enter the general circulation directly or by passing through the strongly metabolizing liver. In the latter case, only a proportion of the drug molecules (if they are of the high clearance type) will enter the general circulation intact. Thus the bioavailability may be less than 100 %. Compared to the small intestine, this situation is still more favorable [131].

Depending on the character of its vehicle, a suppository will either dissolve in the rectal fluid or melt on the mucous layer. Since the volume of the rectal fluid is too small, complete dissolution in the vehicle will be difficult and require extra water. Independent of the vehicle type, drugs dissolved in the suppository will diffuse out towards the rectal membranes. Suspended drugs will first have to leave the vehicle (if it is water immiscible) under the influence of either gravity or motility movements and then can start dissolving in the rectal fluid. The dissolved drug molecules will have to diffuse through the mucous layer and then into and through the epithelium forming the rectal wall.

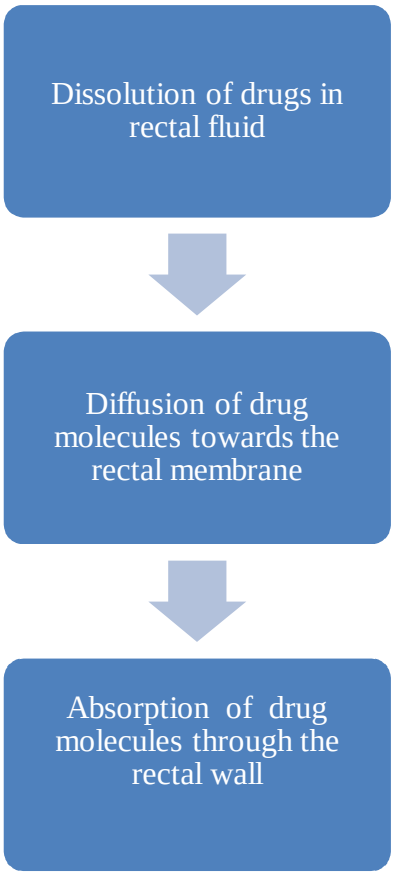

Figure 5: The process of drug release from a suppository

Factors affecting drug absorption from the rectum

- Quantity of fluid available

  The quantity of fluid available for drug dissolution is very small- approximately 3 mL, spread in a layer of approximately 100 µm thick over the organ. Only under non-physiological circumstances is this volume enlarged, e.g. by osmotic attraction by water ï soluble vehicles or during diarrhea. Thus, the dissolution of slightly water-soluble drugs can easily be the slowest step in the absorptive process [131].

- Properties of rectal mucus

  Properties of the rectal fluid, such as composition, viscosity and surface tension, may affect absorption from the rectum.

- Motility of the rectal wall

  The rectal wall may exert pressure on a suppository present in the lumen by two distinct mechanisms. First, the abdominal organs may simply press on to the rectum, especially when the body is in an upright position. This may stimulate spreading and thus promote absorption. The second source of pressure is the motility of the muscles of the rectal wall, which may originate from the normally occurring colonic motor complexes. There are waves of contractions running over the wall of the colon in a caudal direction and are associated with the presence of food residues in the colon.

- pH of rectal secretions

  The principal method of drug absorption is diffusion through lipid regions of cell membranes and, therefore, unionized drugs, which are more soluble in lipids than the ionized forms, are absorbed more readily. The state of ionization of a drug depends on the pH of the environment, acidic and basic drugs being the most ionized and, hence, least well absorbed at high and low pH respectively [132].

- Physical state of medicament

  It is advantageous to use the fine powder of the medicament to increase the surface area and enhance dissolution and absorption of the medicament from the suppository. This precaution is particularly relevant to rectal dosage forms because the rectum lacks the large surface area and considerable movement of contents that aid absorption in the gut [132].

- Presence of adjuncts in the base

  The presence of adjuncts like emulsifying agents and surfactants in suppository bases could either increase or decrease the rate and extent of drug absorption from suppositories.

1.3.3 Types of rectal preparations

Rectal preparations for pharmaceutical use include suppositories, rectal tablets, rectal capsules, rectal ointments, and enemas.

1.3.4 Suppositories

Suppositories are mainly used for the administration of drugs via the rectal route [131]. Suppositories are conical, ovoid, or torpedo ï shaped solid preparations for insertion into the rectum where they melt, dissolve, or disperse and exert a local or systemic effect [132].

1.3.4.1 Properties of an ideal suppository [132]

The ideal suppository should possess the following properties:

- It should melt at body temperature or dissolve or disperse in body fluids.
- It should release any medicament readily.
- It should keep its shape when being handled.
- It should be non- toxic and non-irritant to the mucous membrane.
- It should be stable on storage.
- It should be compatible with any added medicament.
- It should be stable if heated above its melting point.

- It should be easily moulded and should not adhere to the mould.
- It should be mouldable by pouring or cold compression.

1.3.4.2 Advantages of suppositories as pharmaceutical dosage forms

Suppositories possess the following advantages as pharmaceutical dosage forms:

- Suppositories are often sought as alternative route of administration to overcome the gastric irritation, nausea, and vomiting that may be associated with oral administration.
- Suppositories may be administered when the oral route is not convenient, as in infants, and elderly patients.
- Administration of drugs in form of suppositories is easy and safe.
- Drugs administered in form of suppositories are absorbed into the systemic circulation without passing through the portal circulation, thereby avoiding first pass effect.
- Rectal administration of unpalatable medicament overcomes the discomfort associated with such medicament.
- Rectal administration of anti-malarial drugs, especially in the third world countries, allow effective treatment to be instituted early in outlying rural areas, thus, providing the valuable time to seek appropriate medical care [132].

1.3.4.3 Limitations of rectal dosing

Due to the limited rectal area for absorption (in total approx. 300 cm^3) and the limited amount of water (3 ml), the rectum is thought to be unsuitable for absorption of very hydrophilic or very soluble compounds. Rectal administration of suppositories is sometimes associated with leakage of the suppositories from the rectum. The erratic nature of absorption in the rectum may lead to a decrease to 10 % of the oral availability of a given drug. Hence, switching from oral to rectal dosing without proper information, should be discouraged [131].

1.3.4.4 Types of suppository bases and excipients

Phospholipon®90 G

Phospholipon®90 G is an excipient used in the manufacturing of pharmaceuticals, cosmetics, personal care products, and dietetic products. It contains phosphatidylcholines, soya, ascorbyl palmitate and Ŭ-tocopherol. It is manufactured in granules, has a light beige colour and a characteristic smell. The density of Phospholipon®90G is between 0.9 and 1.0 g/cm ^{3} at 20 ^{o}C. Its pH value (at 20 ^{o}C) is 6 ± 1 at 10 g/L. It is considered non-toxic for humans and animals and is biologically degradable [187].

Castor oil

Castor oil consists chiefly of the triglyceride of ricinoleic acid (12-hydroxyoleic acid), which is present to the extent of about 80 per cent. It also contains small amounts of other glycerides, the fatty acid constituents of which include oleic, linoleic, stearic, and 9,10-dihydroxystearic acids. It is soluble at 20 ^{o}C, in 2.5 parts of alcohol (90 per cent); miscible with dehydrated alcohol, with ether, and with glacial acetic acid. It is a nearly colourless or faintly yellow viscous oil with a slight odour. The weight per ml of castor oil at 20 ^{o}C is 0.953 g to 0.964 g. It gives a clear solution with half its volume of light petroleum ether (boiling range, 40 to 60 ^{o}C); it is only partially soluble in two volumes. On cooling to 0 ^{o}C, it remains bright, but on cooling to -18 ^{o}C it congeals to a yellowish mass. The most distinctive features of the oil are its high density, the highest of any natural oil, its behaviour with light petroleum, its solubility in alcohol (90 per cent), its high acetyl value, and its high viscosity. Castor oil is used as a purgative, emollient, and oily vehicle for pharmaceutical formulations.

Softisan® 154

Softisan® types (100,133,134,138,142,154) are specialty hard fats based on triglycerides of blends of natural, saturated, even-numbered un-branched vegetable fats with a chain length of C_{10} ï C_{18}. They are exceptionally resistant to oxidation, so there is no risk of rancidity.

As a result, the Softisan® products are free of any antioxidants and stabilizers. Softisan®154 is prepared with hydrogenated palm oil. It is a white mass with a neutral odor and taste.

It is characterized, along with other Softisan® types by its exceptional hardness at room temperature and its sharp melting range. The narrow interval between the melting and solidification points allows for rapid and economical processing. It can be heated far above its melting point, without alteration of its fast solidification time and its good contractibility. Softisan® is readily soluble in diethyl ether, toluene and acetone; almost insoluble in methylene chloride, ethanol (96 %) and water, and miscible in fats and oils.

Polyethylene glycol

Poly ethylene glycol (PEG) is used in the formulation of suppositories. PEG bases are water-soluble which do not melt at body temperature but dissolve slowly in body fluids, releasing the drug. PEG suppository bases are polymers of ethylene glycol and may be liquids or solids depending on the molecular weight. They are available in a wide range of molecular weights from the low-molecular-weight liquid PEG 300 to the high-molecular-weight solid PEG 8000. An individual PEG can be used as a base itself or two or more PEGs can be used in combination to alter the hardness of the suppository [85].

Table 3: Characteristic physical and chemical values of Softisan ® 154

Tests	Values
1. Acid value (mg KOH/g)	Max. 1
2. Iodine value (mg I_2/100g)	Max. 3
3. Saponification (mg KOH/g)	195-210
4. Hydroxyl value (mg KOH/g)	Max.10
5. Unsaponifiable matter (%)	Max.1
6. Melting Point (^oC)	53-58
7. Water (%)	Max.0.1
8. Iodine color value (mgI_2/100ml)	Max.3

Source: Sasol Germany, GmbH, Product Information: Softisan® 100, 133,134,138,142,154 (Hard fats) 26.13.042e/04.00.

1.3.4.5 Methods of preparation of suppositories [81]

Suppositories are prepared by rolling (hand-shaping), cold compression and melt moulding (fusion).

Rolling (hand-shaping) Technique

This is the oldest and the simplest method of preparing suppositories. It requires considerable skill, yet avoids the complications of heat and mold preparation. The medicament is mixed with the base in a mortar till a smooth mass is formed. A suppository cylinder is formed by rolling the mass on a pill tile with a flat board, partially aided by the palm of the hand.

Cold Compression Technique

This method of suppository preparation also avoids heat. The suppository mass (a mixture of the base and drug), is forced into a mould under pressure, using a wheel-operated press.

Melt Moulding (Fusion) Technique

In this method, a suitable base is melted then, the prescribed amount of finely powdered medicament is incorporated in the base (manually or with the use of mixers, homogenizers or vortex machines). The resulting mixture is then poured into suppository moulds [132]. The suppository moulds are allowed to cool, and the formed suppositories removed by opening the mould. More than one medicament may be formulated in the same suppository.

1.3.4.6 Compatibility and stability studies of suppositories

A proper choice of the pharmaceutical excipients, which improve physico-chemical properties and bioavailability of drugs, determines the successful formulation of a stable and effective solid dosage form. Even though excipients are considered as pharmaceutically inert, physical and chemical interactions with an active component are possible. Potential interactions between drugs and excipients can affect the chemical nature, the stability and bioavailability of medicines and as a result their therapeutic efficacy and safety. Therefore, very important phase in the pre-formulation stage of all dosage forms is examination of drug-excipient compatibility [134-139].

Differential scanning calorimetry (DSC) is a thermo-analytical method described in the European Pharmacopoeia (EP), United States Pharmacopoeia (USP) and Japanese Pharmacopoeia (JP) and is widely used to evaluate physical properties of drugs as well as to study compatibility and stability of the components in pharmaceutical preparations. A possible interaction might be identified by revealing changes in appearance, shift or disappearance of endothermic or exothermic peaks, and/or variations in the corresponding enthalpies of reactions [140-142]. Such information is very helpful for analyzing any instability issues during the design and development of new formulations. Differential scanning calorimetry has been used to analyze the drug-excipient compatibility and stability of suppository formulations [180]. Other methods of determination of the compatibility and stability of suppositories include Fourier-Transform Infra-red (FTIR) analysis, accelerated stress test among others [179].

1.4 Rationale and objectives of the study

Malaria can progress from fever to life-threatening disease within hours, and some people who survive severe malaria sustain substantial permanent damage to the central nervous system. The only way to give effective antimalarial treatment to patients in the community who cannot be treated orally is to take them to a health-care facility (e.g., hospital, dispensary, or other clinic) at which injections can be given. From an isolated rural village, however, reaching such a clinic can take many hours, or even days, by which time the disease may have progressed too far to be treated successfully. Anti-malarial suppositories could be given rectally at, or near, home by a parent, neighbour, or community health worker when severe malaria is suspected, reducing time to treatment (referral to a medical facility may still be needed for diagnosis and further treatment). The suppositories could also replace some anti-malarial injections in clinics (which might require medical expertise to administer) [1,135].

Artemisinins have been shown to act more rapidly than other classes of anti-malarial drugs. Hospital-based studies have shown that a single dose of artesunate, given rectally, can be effective in reducing parasitemia load [142]. However, the use of combinations of an artemisinin derivative and another structurally unrelated and more slowly eliminated antimalarial (the artemisinin combination therapy) will reduce emergence of resistant strains of plasmodium as well as increase the efficacy of anti-malarial therapy [84].

The efficacy and tolerability of oral formulations of artesunate and amodiaquine has been tested formally in several clinical trials in different epidemiological African settings, including Nigeria [174-178], yielding positive results. Presently, there are marketed rectal suppositories of artesunate. Studies have shown the efficacy of rectal suppositories of artesunate when used as pre-referral treatment in rural settings [189]. However, literature searches revealed that there is presently no research recorded on rectal suppositories of a combination of artesunate and amodiaquine.

The present study was therefore designed to formulate and evaluate artesunate and amodiaquine suppositories.

The objectives of the present study were:

1. To determine the compatibility between artesunate, amodiaquine hydrochloride and the excipients to be used in the suppository formulation.
2. To determine the optimum ratio for the combination of the excipients to be used in the suppository formulation.
3. To design and develop artesunate and amodiaquine suppositories with excipients (stable at tropical temperatures).
4. To evaluate the formulated suppositories through validated quality control procedures to ensure they meet the required standard.
5. To assess the stability of the formulated suppositories after storage at different temperatures for 6 months.

CHAPTER TWO

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Drugs

The following were the active pharmaceutical ingredients (APIs) used in the formulation of the suppositories: pure samples of artesunate and amodiaquine hydrochloride (kindly provided by Emzor Pharmaceutical Industries, Lagos, Nigeria).

2.1.2 Excipients

The following excipients were used in the formulation of the suppositories: Phospholipon® 90 G (Phospholipid, GmbH Nattermannallee), Softisan® 154 (Fa. Condea Chemie GmbH, Germany), polyethylene glycol 4000 (PEG 4000, AR grade from Carl Roth GmbH, Karlsruhe, Germany), castor oil (PAXS Pharmaceuticals, Onitsha, Nigeria), paraffin oil (Nomagbon Pharmaceuticals Limited, Benin City, Nigeria).

2.1.3 Chemicals

The following chemicals were utilized in the basic decomposition of artesunate: absolute ethanol (Sigma ï Aldrich, Europe) glacial acetic acid (BDH, Germany), sodium hydroxide pellets (BDH, England). The following chemicals were utilized in the preparation of the phosphate buffer solution pH 7.0: sodium dihydrogen phosphate 1-hydrate was purchased from Kermel, India, di-sodium hydrogen phosphate was purchased from Qualikems, India, and distilled water was purchased from the National Centre for Energy, Research and Development (NCERD), University of Nigeria, Nsukka, Nigeria.

2.1.4 Reagents

The following reagents were used in the course of the experiment: Geimsa stain, for staining of thick blood smear for microscopic malaria parasite count, and Drabkins solution, prepared with 2 % glacial acetic acid (BDH, Germany) in a pint of gentian violet, for hemoglobin determination.

2.2. METHODS

2.2.1 Ultra-violet spectrophotometric analysis of amodiaquine hydrochloride and artesunate

2.2.1.1 Ultra-violet spectrophotometric analysis of amodiaquine hydrochloride

(A) Preparation of standard solution of amodiaquine hydrochloride

Amodiaquine base (10 mg) was dissolved in 100 ml of phosphate buffer solution (pH 7.0). The stock solution was filtered with Whatman No. 1 filter paper. Suitable dilutions of the stock solution were done to obtain the following concentrations: 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 µg/ml. Exactly 5 ml of the stock solution (concentration: 1 µg/ml) was scanned with a UV-Visible spectrophotometer (JENWAY 6405) to establish the wavelength of maximum absorption (λ_{max}). The wavelength of maximum absorption was determined to be 335 nm. Then the absorbance values of 5 ml of the different dilutions were recorded at the established wavelength of maximum absorption. The blank solution was phosphate buffer solution pH 7.0.

2.2.1.2 Ultra-violet spectrophotometric analysis of artesunate

(A) Basic decomposition of artesunate followed by acid reaction.

Artesunate being an artemisinin derivative is a sesquiterpene lactone with an unusual peroxide bridge. It is difficult to detect and identify by standard spectrophotometric methods. It absorbs light only at low wavelengths, has a relatively low molar extinction coefficient, and has no distinct ultra-violet ï visible spectrum or fluorescent properties [8,9]. In order to assay artesunate by ultra-violet method, it is necessary to involve it in a reaction process that would break the endo-peroxide ring and introduce at least one double bond in the molecule [10] .

The UV assay method applied in this experiment was adopted from an original research work [134].

(B) Preparation of working solutions:

- Sodium hydroxide (0.1N) was prepared thus: 0.42 g of sodium hydroxide was weighed and dissolved in distilled water and made up to 100 ml with distilled water.
- Acetic acid (0.1M) in 20 % ethanol was prepared thus: 1.44 ml of glacial acetic acid was measured and diluted to 200 ml with 20 % ethanol.

(C) Preparation of standard solution of artesunate

The pure sample of artesunate (10 mg) was weighed, dissolved in absolute ethanol and made up to 10 ml to give a concentration of 1.0 mg/ml. The solution (1 ml) was transferred to a 10 ml volumetric flask containing 4 ml of 0.1 N sodium hydroxide. The flask was placed in a water bath at 50 ± 0.2 ^{o}C for 60 minutes. The solution was allowed to cool and made up to volume with 0.1 M acetic acid in 20 % ethanol [134]. The wavelength of maximum absorption was determined to be 315 nm, therefore the spectrophotometric analysis was done at 315 nm. The stock solution was diluted to the following concentrations with 0.1M acetic acid in 20 % ethanol: 10, 20, 30, 40 and 50 µg/ml. The blank solution was 0.1 M acetic acid in 20 % ethanol.

2.3 Solubility studies of artesunate and amodiaquine hydrochloride in the excipients

The solubility of artesunate and amodiaquine hydrochloride in different excipients was determined by the shake flask method [135] as follows:

An excess of artesunate was added individually to 500 mg of polyethylene glycol 4000, Softisan® 154, Phospholipon® 90G and 3 ml of castor oil in screw-capped tubes to obtain supersaturated solutions.

An excess of amodiaquine hydrochloride was added individually to 500 mg of polyethylene glycol 4000, Softisan® 154, Phospholipon® 90G and 3 ml of castor oil in screw-capped tubes to obtain supersaturated solutions.

The mixtures in the screw-capped tubes were then shaken for 24 hours using a hotplate and stirrer (JENWAY 1000) at 20 rpm in water maintained at 37 ± 0.5^{o}C. After 24 hours, the supernatant (0.5 ml) was suitably diluted and analyzed with a spectrophotometer (6405 JENWAY).

2.4 Simultaneous ultra-violet spectrophotometric analysis of amodiaquine hydrochloride and artesunate

Artesunate and amodiaquine hydrochloride were co-formulated in suppository dosage form. There is currently no official method for the simultaneous assay of artesunate and amodiaquine hydrochloride. The procedures reported in literature for simultaneous assay of artesunate and amodiaquine hydrochloride include indirect colorimetric assay (ICA) [169] and high performance liquid chromatography [116,170,188]. Unfortunately, these methods have their demerits which greatly impair their functionality especially in sub-Saharan Africa. It is either the problem of non-availability of specific reagents (like fast TR red salt used in the indirect colorimetric assay) and the equipment needed for the assay procedure, uncertainty of outcome of assay or that of excessive cost of the method (s)[171].

The author established through several research procedures that artesunate and amodiaquine could be simultaneously analyzed by ultra-violet spectroscopic methods without interferences. Artesunate was assayed in the presence of amodiaquine base in the ratio of 1: 3 respectively (the same ratio they appeared in the suppository dosage form) using a modification of the basic decomposition of artesunate described earlier [134]. Likewise, amodiaquine was assayed in the presence of artesunate in the ratio of 3: 1 respectively. The method was reproducible, accurate and exhibited intra-and inter-day precision.

2.4.1 Ultra-violet spectrophotometric analysis of amodiaquine hydrochloride in the presence of artesunate.

Preparation of standard solution of amodiaquine hydrochloride in the presence of artesunate

Amodiaquine base (10 mg) and artesunate (3.3 mg) were dissolved in 100 ml of phosphate buffer solution (pH 7.0). The stock solution was filtered with Whatman No. 1 filter paper. Suitable dilutions of the stock solution were done to obtain the following concentrations of amodiaquine: 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 µg/ml. Then the absorbance values of 5 ml of the different dilutions were recorded at the established wavelength of maximum absorption (335 nm) of amodiaquine. The blank solution was phosphate buffer solution pH 7.0.

2.4.2 Ultra-violet spectrophotometric analysis of artesunate in the presence of amodiaquine hydrochloride

The UV assay method applied in this experiment was a modification of an original research work [134].

Preparation of standard solution of artesunate in the presence of amodiaquine HCl

Artesunate (10 mg) and amodiaquine HCl (30 mg base equivalent) were weighed, dissolved in absolute ethanol and made up to 10 ml to give a concentration of 1.0 mg/ml of artesunate. The solution (1 ml) was transferred to a 10 ml volumetric flask containing 4 ml of 0.1 N sodium hydroxide. The flask was placed in a water bath at $50 \pm 0.2\ ^{o}C$ for 60 minutes. The solution was allowed to cool and made up to volume with 0.1 M acetic acid in 20 % ethanol. The spectrophotometric analysis was done at 315 nm. The stock solution was diluted to the following concentrations of artesunate with 0.1 M acetic acid in 20 % ethanol: 10, 20, 30, 40 and 50 µg/ml. The blank solution was 0.1 M acetic acid in 20 % ethanol [134].

2.5 Pre-formulation studies –Preparation of lipid matrices (suppository bases)

It has been established from previous studies that the optimal ratio for the combination of Phospholipon 90® G and other fatty bases was 3:7, therefore Phospholipon ®90 was combined with Softisan®154 in 3:7 ratio respectively [181]. Softisan® 154 (14 g) was melted in a water bath. Phospholipon 90® G (6 g) was gradually added to the molten Softisan® 154 while the mixture was stirred continuously. Different quantities of castor oil and polyethylene glycol 4000 were gradually added to different quantities of the Softisan®154 and Phospholipon 90®G mixture. The melting point and the physical nature of the different ratios of the components were determined with a melting point apparatus and by placing the mixture between the thumb and the index fingers respectively.

The physical nature of different ratios of the components ranged from liquid, semi-solid, and fine solid and hard solid. The components that exhibited a fine solid nature were analyzed by differential scanning calorimetry (DSC).

2.6 Differential scanning calorimetric analysis

2.6.1 Differential Scanning Calorimetric Analysis (DSC) of different ratios of excipients for the suppository formulations

The optimum ratio of the polyethylene glycol 4000, Softisan® 154, Phospholipon® 90G and castor oil was determined by experimenting with different ratios till a firm, consistent blend emerged. Seven ratios of the excipients (Table 4) which produced a firm, consistent blend, suitable for use in formulation of suppositories, were subjected to DSC (thermal) analysis.

DSC (thermal) analysis is useful for the physical characterization of compounds. Thermal analysis was used to evaluate changes in thermodynamic properties that occur when the material was supplied with heat energy. These thermodynamic properties include changes in melting point values and changes in enthalpy, among others.

Procedure for the differential scanning calorimetry (DSC) analysis

Approximately 3 mg quantity of each sample was placed in an aluminum pan (25 εl), covered with a perforated lid and subjected to thermal treatment using a differential scanning calorimeter (DSC) (DSC 204 F1 Phoenix®, NETZSCH, 6.240.10, Germany) which was initially calibrated for the temperature and enthalpy of the signal using the melting transition of a standard, indium (156.8 ^{o}C). Dry nitrogen was used as the purge gas (purge 20 ml min^{-1}). The probes were heated from a start temperature of 25 to 300 °C at a rate of 10 °C min^{-1}. The melting points (Tm) and enthalpies were evaluated with the Proteus thermal analysis software attached to the DSC.

Table 4: Ratios of excipients used in formulating lipid matrices

S/N	Phospholipon ® 90G/Softisan® 154 (g)	Castor Oil (ml)	PEG 4000 (g)
1	0.1	0.05	0.1
2	0.1	0.1	0.2
3	0.3	0.1	0.2
4	0.4	0.1	0.2
5	0.4	0.3	0.2
6	0.4	0.2	0.2
7	0.4	0.4	0.3
8	0.4	0.3	0.4

2.6.2 Differential scanning calorimetric analysis for compatibility studies

Compatibility between artesunate and amodiaquine hydrochloride, as well as artesunate and amodiaquine hydrochloride and the lipid matrices was investigated by DSC. The samples for DSC thermal analysis were prepared thus:

Pure samples of artesunate, amodiaquine HCl and the lipid matrix (made up of Phospholipon 90®G, Softisan® 154, castor oil, and PEG 4000) were weighed out in a ratio of 0.5:0.5: 9 and melted to form sample 1. Pure sample of artesunate and the lipid matrix (made up of Phospholipon® 90G, Softisan ®154, castor oil, and PEG 4000) were weighed out in a ratio of 1:9 and melted to form sample 2. Pure sample of amodiaquine hydrochloride and the lipid matrix (made up of Phospholipon® 90G, Softisan® 154, castor oil, and PEG 4000) were weighed out in a ratio of 1:9 and melted to form sample 3. The lipid matrix (made up of Phospholipon® 90G, Softisan® 154, castor oil, and PEG 4000) formed sample 4.

Approximately 3 mg quantity of each sample was placed in an aluminum pan (25 εl), covered with a perforated lid and subjected to thermal treatment using a differential scanning calorimeter (DSC) (DSC 204 F1 Phoenix®, NETZSCH, 6.240.10, Germany) which was initially calibrated for the temperature and enthalpy of the signal using the melting transition of a standard, indium (156.8 ^{o}C). Dry nitrogen was used as the purge gas (purge 20 ml min^{-1}). The probes were heated from a start temperature of 25 to 300 °C at a rate of 10°C min^{-1}. The melting point (Tm) and enthalpies were evaluated with the Proteus thermal analysis software attached to the DSC.

2.7 Preparation of artesunate and amodiaquine suppositories

2.7.1 Calibration of Suppository Moulds

The suppository moulds used in the experiment were metallic and had six cavities each. They had screws which when loosened opened the mould longitudinally. The theoretical capacity of the moulds was 1g for each mould. Since the capacity of suppository moulds could vary depending on the age of the mould and the type of suppository base used, each mould was calibrated thus:

Polyethylene glycol 4000 (6 g) was weighed and melted in a thermostatic water-bath. Castor oil (8 ml approx. 7.672 g) was added to the PEG 4000. The molten polyethylene glycol 4000 was

poured into the cavities of a clean suppository mould lubricated with pure paraffin oil and was left for 2 minutes until the mass was set. The excess PEG 4000 was removed from the mould with a stainless steel spatula. The suppository mould was then placed in a refrigerator at 4 ^{o} C for 30 minutes. The suppositories were gently removed from the mould and weighed. The average weight was used as the true capacity of the mould. The mean weight, 1.35 g, was taken as the true capacity of the mould.

2.7.2 Calculation of displacement values

The volume of a suppository from a particular mould is uniform but its weight will vary because the densities of medicaments usually differ from the density of the base with which the mould was calibrated. Therefore, allowances were made for the change in density of the suppository mass due to added medicaments. The displacement value (the number of parts by weight of the medicament that displaces one part by weight of the base) was calculated for all the batches of suppositories formulated.

2.7.3 Artesunate and amodiaquine suppositories prepared with polyethylene glycol 4000 and castor oil

2.7.3.1 Determination of displacement value of polyethylene glycol 4000 /castor oil in artesunate and amodiaquine HCl

Preparation of blank suppositories

Polyethylene glycol 4000 (6 g) was weighed and melted in a thermostatic water-bath. Castor oil (8 ml approx. 7.672 g) was added to the PEG 4000.The molten polyethylene glycol 4000 was poured into the six cavities of a clean suppository mould lubricated with pure paraffin oil and was left for 2 minutes until the mass was set. The excess PEG 4000 was removed from the mould with a stainless steel spatula. The suppository mould was then placed in a refrigerator at 4 ^{o}C for 30 minutes. The six suppositories were gently removed from the mould and weighed.

Preparation of medicated suppositories

Medicated suppositories containing approximately 40 per cent of the medicament were prepared as follows:

Polyethylene glycol 4000/castor oil mixture (6 g), artesunate (1 g) and amodiaquine bases (3 g) were weighed. The polyethylene glycol 4000/castor oil mixture was melted in a water-bath. The medicaments were gradually dissolved in the melted mass by vortexing (Vortex- Genie Ê Scientific Industries, Inc USA). The melted mass was poured into the six cavities of a clean suppository mould lubricated with pure paraffin oil and was left for 2 minutes until the mass was set. The excess base was removed from the mould with a stainless steel spatula. The suppository mould was then placed in a refrigerator at 4 ^{o}C for 30 minutes. The six suppositories were gently removed from the mould and weighed. The displacement value was then calculated as stated below.

Calculation of displacement value of artesunate and amodiaquine in polyethylene glycol 4000 /castor oil

Weight of 6 un-medicated suppositories = 7.62 g

Weight of 6 medicated suppositories (containing 40 % of artesunate and amodiaquine base in 1:3 ratio) = 7.74 g

Quantity of base in this $= \frac{\square\square}{\square\square\square} \times 1.30 = 4.64$ g

Artesunate and amodiaquine content $= \frac{\square\square}{\square\square\square} \times 1.30 = 3.10$ g

Base displaced by 3.1 g of artesunate and amodiaquine $\square 7.62 - 4.64\square g = 2.98$ g

Therefore the displacement value of artesunate and amodiaquine in the PEG 4000/ castor oil mixture $= \frac{\square.\square}{\square.\square\square} = 1.04$ approximately 1.00

The displacement value of artesunate and amodiaquine in the PEG 4000/ castor oil is 1.00 , that is, 1 mg of the lipid matrix is displaced by 1.0 mg of artesunate and amodiaquine.

To prepare 30 suppositories, each containing 25 mg of artesunate and 98 mg amodiaquine HCl (equivalent to 75 mg amodiaquine base).

Total amount of artesunate = 30 × 25 mg = 750 mg

Total amount of amodiaquine HCl = 30× 98 mg = 2940 mg

Total amount of medicament = 3,690 mg = 3.69 g

This displaces $\frac{3.690}{1.0}$ = 3.69 g of PEG 4000/castor oil matrix

Therefore, the amount of PEG 4000/ castor oil mixture required to prepare 30 suppositories using a mold with capacity of 1.35 g = (30 × 1.35) − 3.69 = 36.81 g

2.7.3.2 Preparation of artesunate and amodiaquine suppositories with PEG 4000 and castor oil

The artesunate and amodiaquine suppositories containing PEG 4000 and castor oil were prepared thus: PEG 4000/ castor oil mixture (36.81 g) was weighed and melted in a water-bath. The medicaments (750 mg of artesunate and 2,940 mg of amodiaquine hydrochloride) were gradually dissolved in the melted mass by vortexing (Vortex-Genie Ê Scientific Industries, Inc USA). The melted mass was poured into the six cavities of a clean suppository mould lubricated with pure paraffin oil and was left for 2 minutes until the mass was set. The excess base was removed from the mould with a stainless steel spatula. The suppository mould was then placed in a refrigerator at 4 ^{o}C for 30 minutes. The six suppositories were gently removed from the mould, packaged in air-tight containers sealed with aluminum foil and stored at 4 ^{o}C.

2.7.4 Artesunate and amodiaquine suppositories prepared with Phospholipon® 90G, Softisan® 154 and castor oil

2.7.4.1 Determination of displacement value of Phospholipon® 90 G/ Softisan® 154 /castor oil in artesunate and amodiaquine HCl

Preparation of blank suppositories

Phospholipon® 90 G (1.8 g), Softisan® 154 (4.2 g), castor oil (6 ml approx. 5.754 g) were weighed and melted in a thermostatic water-bath. The molten mixture was poured into the six cavities of a clean suppository mould lubricated with pure paraffin oil and was left for 2 minutes until the mass was set. The excess base was removed from the mould with a stainless steel spatula. The suppository mould was then placed in a refrigerator at 4 ^{o}C for 30 minutes. The six suppositories were gently removed from the mould and weighed.

Preparation of medicated suppositories

The previous extensive research on Phospholipon® 90G and Softisan® 154 proved that the optimal ratio for the excipients was 3:7 respectively; therefore, the same ratio was used throughout this research work [133].

Medicated suppositories containing approximately 40 per cent of the medicament was prepared thus: Phospholipon 90® G/ Softisan® 154 /castor oil matrix (6 g), artesunate (1 g) and amodiaquine base (3 g) were weighed on a weighing balance. The medicaments were gradually dissolved in the melted mass by vortexing (Vortex-Genie Ê Scientific Industries, Inc USA). The melted mass was poured into the six cavities of a clean suppository mould lubricated with pure paraffin oil and was left for 2 minutes until the mass was set. The excess base was removed from the mould with a stainless steel spatula. The suppository mould was then placed in a refrigerator at 4 ^{o}C for 30 minutes. The six suppositories were gently removed from the mould and weighed.

Calculation of displacement value of artesunate and amodiaquine in Phospholipon® 90 G /Softisan® 154 /castor oil

Weight of 6 un-medicated suppositories = 7.62 g

Weight of 6 medicated suppositories (containing 40 % of artesunate and amodiaquine base in 1:3 ratio) = 7.92 g

Quantity of base in this

$$= \frac{\square\square}{\square\square\square} \times 7.92 = 4.75 \text{ g}$$

Artesunate and amodiaquine content $= \frac{\square\square}{\square\square\square} \times 7.92 = 3.17$ g

Base displaced by 3.17 g of artesunate and amodiaquine = 7.62 – 4.75 g = 2.87 g

Therefore the displacement value of artesunate and amodiaquine in the Phospholipon® 90 G/Softisan® 154/castor oil $= \frac{\square.\square\square}{\square.\square\square} = 1.105$ approximately 1.10.

The displacement value of artesunate and amodiaquine in the Phospholipon® 90G / Softisan® 154/ castor oil matrix is 1.1 , that is, 1 mg of the lipid matrix is displaced by 1.10 mg of artesunate and amodiaquine.

To prepare 30 suppositories, each containing 25 mg of artesunate and 98 mg amodiaquine HCl (equivalent to 75 mg amodiaquine base).

Total amount of artesunate = 30 × 25 mg = 750 mg

Total amount of amodiaquine HCl = 30× 98 mg = 2940 mg

Total amount of medicament = 3,690 mg = 3.69 g

This displaces = $\frac{3.35}{1.0}$ = 3.35 g of Phospholipon® 90G/Softisan®154/castor oil mix

Therefore, the amount of Phospholipon®90G/Softisan®154/ castor oil matrix required using 1.35 g mold capacity = (30 × 1.35) – 3.35 = 37.15 g

2.7.4.2 Preparation of artesunate and amodiaquine suppositories with Phospholipon®90G, Softisan® 154 and castor oil

Artesunate and amodiaquine suppositories containing Phospholipon®90G, Softisan® 154 and castor oil were prepared thus:

Phospholipon®90G/ Softisan® 154/ castor oil matrix (37.15 g) was weighed and melted in a water-bath. The medicaments (750 mg of artesunate and 2,940 mg of amodiaquine hydrochloride) were gradually dissolved in the melted mass by vortexing (Vortex-Genie Ê Scientific Industries, Inc USA). The melted mass was poured into the cavities of a clean suppository mould lubricated with pure paraffin oil and was left for 2 minutes until the mass was set. The excess base was removed from the mould with a stainless steel spatula. The suppository mould was then placed in a refrigerator at 4 ^{o}C for 30 minutes. The six suppositories were gently removed from the mould, packaged in air-tight containers sealed with aluminum foil and stored at 4 ^{o}C.

2.7.5 Artesunate and amodiaquine suppositories prepared with Phospholipon ® 90G, Softisan® 154, polyethylene glycol 4000 and castor oil

2.7.5.1 Determination of displacement value of Phospholipon® 90 G/ Softisan® 154 /PEG 4000 /castor oil in artesunate and amodiaquine HCl

Preparation of blank suppositories

The blank suppositories were prepared thus: Phospholipon® 90 G (1.02 g), Softisan® 154 (2.38 g) and PEG 4000 (2.6 g) were weighed and melted in a thermostatic water bath. Castor oil (3 ml approx. 2.877 g) was added to the mixture. The molten mixture was poured into the six cavities of a clean suppository mould lubricated with pure paraffin oil and was left for 2 minutes until the

mass was set. The excess base was removed from the mould with a stainless steel spatula. The suppository mould was then placed in a refrigerator at 4 ^{o}C for 30 minutes. The six suppositories were gently removed from the mould and weighed.

Preparation of medicated suppositories

Medicated suppositories containing approximately 40 per cent of the medicament were prepared thus: Phospholipon® 90 G/ Softisan® 154/ PEG 4000/ castor oil matrix (6 g), artesunate (1 g) and amodiaquine base (3 g) were weighed. The medicaments were gradually dissolved in the melted mass by vortexing (Vortex-Genie Ê Scientific Industries, Inc USA). The melted mass was poured into the six cavities of a clean suppository mould lubricated with pure paraffin oil and was left for 2 minutes until the mass was set. The excess base was removed from the mould with a stainless steel spatula. The suppository mould was then placed in a refrigerator at 4 ^{o}C for 30 minutes. The six suppositories were gently removed from the mould and weighed.

Calculation of displacement value of artesunate and amodiaquine in PEG 4000, Phospholipon® 90 G, Softisan® 154 and castor oil

Weight of 6 un-medicated suppositories = 7.68 g

Weight of 6 medicated suppositories (containing 40 % of artesunate and amodiaquine base in 1:3 ratio) = 8.10 g

Quantity of base in this

$$= \frac{\square\square}{\square\square\square} \times 8.10 = 4.86 \text{ g}$$

Artesunate and amodiaquine content

$$= \frac{\square\square}{\square\square\square} \times 8.10 = 3.24 \text{ g}$$

Base displaced by 3.24 g of artesunate and amodiaquine = (7.68 ï 4.86) g

= 2.82 g

Therefore, the displacement value of artesunate and amodiaquine in the entire lipid matrix = $\frac{\square.\square\square}{\square.\square\square} = 1.15$ approximately 1.2.

The displacement value of artesunate and amodiaquine in the entire lipid matrix is 1.20; that is, 1 mg of the lipid matrix is displaced by 1.2 mg of artesunate and amodiaquine.

To prepare 30 suppositories, each containing 25 mg of artesunate and 98 mg amodiaquine HCl (equivalent to 75 mg amodiaquine base).

Total amount of artesunate = 30 × 25 mg = 750 mg

Total amount of amodiaquine HCl = 30× 98 mg = 2940 mg

Total amount of medicament = 3,690 mg = 3.69 g

This displaces = $\frac{3.690}{1.2}$ = 3.08 g of entire lipid matrix

Therefore, the amount of the entire lipid matrix required using 1.35 g mold capacity =

(30 × 1.1) ï 3.08 = 37.42 g of the entire lipid matrix.

2.7.5.2 Preparation of artesunate and amodiaquine suppositories containing PEG 4000, Phospholipon® 90 G ,Softisan® 154 and castor oil

Artesunate and amodiaquine suppositories containing PEG 4000, Phospholipon® 90 G, Softisan® 154 and castor oil were prepared thus:

Phospholipon® 90G/ Softisan® 154/ PEG 4000/castor oil matrix (37.42 g) was weighed and melted in a water-bath. The medicaments (750 mg of artesunate and 2,940 mg of amodiaquine hydrochloride) were gradually dissolved in the melted mass by vortexing (Vortex-Genie Ê Scientific Industries, Inc USA). The melted mass was poured into the six cavities of a clean suppository mould lubricated with pure paraffin oil and was left for 2 minutes until the mass was set. The excess base was removed from the mould with a stainless steel spatula. The suppository mould was then placed in a refrigerator at 4^{o}C for 30 minutes. The six suppositories were gently removed from the mould, packaged in air-tight containers sealed with aluminum foil and stored at 4 ^{o}C.

The formulation compositions of the artesunate and amodiaquine suppositories are shown in Table 5.

2.8 Evaluation of artesunate and amodiaquine suppositories

2.8.1 Visual characterization

Color

The intensity, nature and homogeneity of the randomly selected suppositories (six from each batch) were assessed with the naked eye (subjective evaluation) [173].

Surface characteristics

The surface characteristics of the suppositories are relatively easy to assess. It is important to check for the absence of fissuring, pitting, fat blooming, exudation, and the migration of the active ingredients [182] .

The randomly selected suppositories (six from each batch) were cut longitudinally and examined with the naked eye (subjective evaluation) to assess the visual characteristics [182].

2.8.2 Weight uniformity test

Twenty suppositories were weighed using a digital weighing balance (Model no: YP-1002N) [183]. Each suppository was then weighed and the average weight was calculated.

2.8.3 Content uniformity test

Uniformity is required in some monographs to ensure the consistency of dosage units. These dosage units should have drug substance content within a narrow range around the label claim. Content uniformity test was determined by spectrophotometric method according to the BP procedure [172] as follows:

The suppository (three from each batch) was individually melted and dissolved in 20 ml of phosphate buffer solution (pH 7.0) in separate volumetric flasks. The solution was filtered with Whatman filter paper after manual agitation of the solution to ensure proper dissolution of the pharmaceutical active ingredients (artesunate and amodiaquine). For amodiaquine, suitable dilutions were made to give the following concentrations of amodiaquine: 18.75 µg/ml, 37.5 µg/ml. The absorbance values were then measured using a Jenway 6405 UV/VIS

spectrophotometer. For artesunate, the solution was made to undergo basic decomposition as described earlier [134]. Suitable dilutions were made to give the following concentrations of artesunate: 25 µg/ml, 50 µg/ml. The absorbance values were measured using the spectrophotometer. The tests were performed in triplicates.

2.8.4 Softening time test

The softening time was determined as follows: One suppository was placed in a beaker containing 200 ml of phosphate buffer pH 7.0, maintained at 37 ± 0.5 ^{o}C with a magnetic stirrer rotated at 20 rpm. The time taken for the suppository to form a soft mass was noted. The above steps were repeated for other batches of the suppositories. The test was done in triplicates.

2.8.5 *In vitro* drug release studies

Dissolution testing is used to measure the rate and extent of a drug dissolving in a deýned medium under deýned conditions.

In the present study, a modified basket method was adopted in which a Hot plate (with a thermostat) with a magnetic stirrer (JENWAY 1000 Hotplate and Stirrer) was employed for all *in vitro* drug release studies. Phosphate buffer pH 7.0 was used as the dissolution medium and the rate of stirring was 50 rpm. The suppositories were placed in a basket made of wire mesh, and immersed in 50 ml of phosphate buffer pH 7.0, maintained at 37 ± 0.5 ^{o} C . The tests were done in triplicates.

Table 5: Formulation compositions of the suppositories

Code	Artesunate (mg)	Amodiaquine Base (mg)	PEG 4000 (g)	Castor Oil (g)	Phospholipon® 90 G (g)	Softisan® 154 (g)
P	25	75	0.520	0.710	-	-
PS	25	75	-	0.620	0.186	0.434
E	25	75	0.341	0.460	0.136	0.319

The weight per ml of castor oil is 0.959 g; therefore the volume was converted to grams.

P= Artesunate/amodiaquine/PEG 4000/ castor oil suppository

PS= Artesunate/amodiaquine/ castor oil/ Phospholipon® 90G/ Softisan® 154 suppository

E= Artesunate/amodiaquine/PEG 4000/ castor oil/ Phospholipon® 90G/ Softisan® 154 suppository

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2.8.5.1 *In vitro* release of artesunate and amodiaquine from the suppositories prepared with polyethylene glycol 4000

From the disintegration time test ,artesunate and amodiaquine suppositories prepared with PEG 4000 disintegrated within 20 minutes therefore, 5 ml aliquots were withdrawn and replaced with equal volume of fresh media (to maintain sink conditions) after 0, 5, 10, 15, and 20 minutes.

For Amodiaquine, suitable dilutions of the aliquots were prepared using phosphate buffer pH 7.0. The absorbance values were then measured using a Jenway 6405 UV/VIS Spectrophotometer. The tests were performed in triplicates.

For artesunate, the aliquots were made to undergo basic decomposition as described earlier [130]. Suitable dilutions were made and the absorbance values were measured using a Jenway 6405 UV/VIS Spectrophotometer. The tests were performed in triplicates.

2.8.5.2 *In vitro* release study of artesunate and amodiaquine from suppositories prepared with Phospholipon® 90 G and Softisan® 154

From the disintegration time test, artesunate and amodiaquine suppositories prepared with Phospholipon® 90G and Softisan® 154 disintegrated within 240 minutes therefore, 5 ml aliquots were withdrawn were withdrawn and replaced with equal volume of fresh media (to maintain sink conditions) after 0, 30, 60, 90, 120, 150, 180, 210 and 240 minutes.

For amodiaquine, suitable dilutions of the aliquots were prepared using phosphate buffer pH 7.0. The absorbance values were then measured using a Jenway 6405 UV/VIS Spectrophotometer at 335 nm. The tests were performed in triplicates.

For artesunate, the aliquots were made to undergo basic decomposition as described by earlier researches [134]. Suitable dilutions were made and the absorbance values were measured using a Jenway 6405 UV/VIS Spectrophotometer at 315 nm. The tests were performed in triplicates.

2.8.5.3 *In vitro* release study of artesunate and amodiaquine from the suppositories prepared with Phospholipon® 90 G, Softisan® 154 and polyethylene glycol 4000

From the disintegration time test, artesunate and amodiaquine suppositories prepared with Phospholipon® 90G, Softisan® 154 and PEG 4000 disintegrated between 210 and 270 minutes therefore, 5 ml aliquots were withdrawn and replaced with equal volume of fresh media (to maintain sink conditions) after 0, 30, 60, 90, 120, 150, 180, 210, 240 and 270 minutes as applicable.

For amodiaquine, suitable dilutions of the aliquots were prepared using phosphate buffer pH 7.0. The absorbance values were then measured using the spectrophotometer at 335nm. The tests were performed in triplicates.

For artesunate, the aliquots were made to undergo basic decomposition described by earlier researchers [134]. Suitable dilutions were made and the absorbance values were measured using the spectrophotometer at 315 nm. The tests were performed in triplicates.

2.8.5.4 Determination of the kinetics and mechanism of drug release

In order to understand the kinetics of drug release, the results of the *in vitro* drug release study were fitted into kinetic equations of zero order and Higuchi models. The kinetic model that best fitted the dissolution data was evaluated by comparing the regression coefficient (r^2) values from each model. The model that gave higher 'r^2' value was considered as best fit model.

2.8.6 Morphology Study

The morphology study was done with the aid of a polarizing light microscope (Motic, Japan, Model NP-400). Minute quantities of the pure samples of artesunate, amodiaquine, the blank and medicated suppositories were placed on separate microscope slides, gently heated, cooled and then viewed under the microscope. The pictures of the materials on the slides were captured with a digital camera attached to the microscope.

2.8.7 Pharmacodynamic Evaluation: *In vivo* antimalarial efficacy testing in *Plasmodium berghei*- infected mice

2.8.7.1 Experimental animals

All the animal experiments in this study were conducted according to the guidelines established by the Institutional Animal Care and Use Committee of the University of Nigeria, Nsukka, and adhered to the European Community Guidelines for the Use of Experimental Animals (86/609/EEC) [162]. The experimental animals (35 male mice weighing 30-40 g) were obtained from the animal house of the Department of Pharmacology, University of Nigeria, Nsukka. The animals were housed in well-aerated animal care facility maintained at 12 ï h light/dark cycle. The animals were fed with standard mouse diet (manufactured by Grand Oil/ Cereals Limited, Jos) and supplied with clean drinking water *ad libitum*. The animals were acclimatized to the laboratory conditions for three weeks prior to the experiment.

2.8.7.2 Parasite inoculation

Plasmodium berghei was kindly provided by the Nigerian Institute for Medical Research, Yaba, Lagos, Nigeria. The parasites were maintained through serial blood passage in mice wherein the mice previously infected with *Plasmodium berghei* and with high parasitemia level (38 %) served as the donor. Blood samples were taken from the donor mouse and diluted with normal saline (Dana Pharmaceuticals) such that 0.2 ml injected intraperitoneally into the experimental animals contained 1×10^{7} infected erythrocytes.

The *in vivo* anti-malarial efficacy test was performed according to Peterôs 4 day suppressive test [135]. On day 0, 2-4 hr. post-infection, the treatment groups received a single dose of the different batches of artesunate and amodiaquine suppositories (4 mg/kg and 10 mg/kg respectively) according to their groups. The control groups were either left without treatment or given blank suppositories according to the values of their body weight. From days 1 to 3 (24, 48 and 72 hr. post-infection), the treatment groups of mice received the same dose of different batches of the formulation and by the same route as on day 0. The same procedure was repeated for the control groups.

2.8.7.3 Experimental protocols

The experimental animals were divided into groups based on their body weight. They were divided into seven groups, each group comprising of five animals. The mice were randomly assigned to a given treatment or control group. The animals were distributed thus:

Group 1

Animals in this group were infected with *Plasmodium berghei*. They were treated with artesunate and amodiaquine suppositories formulated with polyethylene glycol 4000 /castor oil. The dose administered was 4 mg/kg/day and 10 mg/kg/day for artesunate and amodiaquine respectively for days 0 to 4.

Group 2

Animals in this group were infected with *Plasmodium berghei*. They were treated with artesunate and amodiaquine suppositories formulated with polyethylene glycol 4000, Softisan® 154, castor oil and Phospholipon® 90G. The dose administered was 4 mg/kg/day and 10 mg/kg/day for artesunate and amodiaquine respectively for days 0 to 4.

Group 3

Animals in this group were infected with *Plasmodium berghei*. They were treated with artesunate and amodiaquine suppositories formulated with Softisan® 154, castor oil and Phospholipon® 90G. The dose administered was 4 mg/kg/day and 10 mg/kg/day for artesunate and amodiaquine respectively for days 0 to 4.

Group 4

Animals in this group were infected with *Plasmodium berghei*. They were administered with blank suppositories formulated with polyethylene glycol 4000, and castor oil. The suppository was administered from days 0 to 4.

Group 5

Animals in this group were infected with *Plasmodium berghei*. They were administered with blank suppositories formulated with polyethylene glycol 4000, Softisan®154, castor oil and Phospholipon® 90 G. The suppository was administered from days 0 to 4.

Group 6

Animals in this group were infected with *Plasmodium berghei*. They were administered with blank suppositories formulated with Softisan®154, castor oil and Phospholipon® 90G. The suppository was administered from days 0 to 4.

Group 7

Animals in this group were infected with *Plasmodium berghei*. They were not administered with any suppositories. Animals in this group served as the control.

2.8.7.4 The Parasite count

The course of malaria infection (induced by *Plasmodium berghei*) following intraperitoneal inoculation of the mice was studied in each experimental mouse that received 1×10^{7} parasitized red blood cells in 0.2 ml inoculums [135]. Thin blood films were prepared from the tail vein of the infected mice, fixed with methanol and stained with 10 % Geimsa stain. The parasite count was done on days 1, 3, 4, and 28 after infection.

Parasitemia was reported as mean percentage parasitemia after counting 100 red blood cells from each slide ten times.

Percent anti-malarial activity was calculated using the following formula [135]:

$$\text{Activity} = 100 - \left[\frac{\text{Mean parasitemia of treated}}{\text{Mean parasitemia of control}}\right] \times 100$$

2.8.7.5 Survival trend of experimental animals

The animals in all groups were observed for the survival trend for a period of 55 days [135].

2.8.7.6 Hemoglobin estimation

This was carried out using a standard procedure [81] as follows:

Well-mixed venous blood (20 µl) was added to 5 ml of Drabkins solution in a test tube to give a dilution of 1:250. The mixture was then allowed to stand for 10 minutes at room temperature. The absorbance was colorimetrically determined (with a digital photocolorimeter by EI, Model no.:312) at 540 nm using Drabkins solution as blank. The absorbance reading was multiplied by a factor of 36.8 to give the actual hemoglobin value.

2.8.7.7 Hematocrit test

This was carried out using standard procedure [81] as follows:

Well-mixed anti-coagulated blood was aspirated into a capillary tube with one end sealed with plasticin. The tube was spun in a hematocrit centrifuge (Mode 800D, New Life Medical Instrument, England) for 5 minutes and then read off a PCV reader.

2.9 Stability studies

Stability tests were done on the suppositories six months post ï formulation. Suppositories (three from each batch) were stored at 27 ^{o}C for six months. Suppositories (three from each batch) were stored at 4 ^{o}C for six months [182].

The suppositories were analyzed by differential scanning calorimetry to determine the effect of storage temperature on the stability of the suppositories.

2.9.1 Statistical analysis

All experiments were performed in triplicates for validity of statistical analysis. Results were expressed as mean ± SD. The mean parasite count was expressed as mean ± SEM and the parasitemia of the different groups were statistically assessed by Analysis of Variance followed by LSD *post hoc* using statistical package for social sciences (SPSS) Version 16.0, Illinois, Chicago, USA software. Differences were considered significant at $\boldsymbol{p} < 0.05$.

CHAPTER THREE
RESULTS AND DISCUSSION

3.1 Ultra-violet spectrophotometric analysis result of amodiaquine hydrochloride and artesunate

Amodiaquine hydrochloride and artesunate were assayed by ultra-violet spectrophotometric analysis. The basis for using spectrophotometric measurements to quantitatively analyze a light-absorbing chemical species, in solution is the Beer-Lambert law: Aλ = Ůλbc where Aλ is absorbance at a given wavelength, Ůλ is the molar absorptivity at that wavelength (formerly known as molar extinction coefficient), b is the distance the light travels through the solution (called the pathlength), and c is the concentration of the analyte in solution. The Beer- Lambert law simply states that absorbance is directly proportional to the concentration of analyte (in this case, artesunate and amodiaquine) in the sample.

The standard Beer-Lambert plot of amodiaquine hydrochloride in phosphate buffer pH 7.0 at 335 nm is shown in Figure 6. The linear plot obtained for amodiaquine showed that the Beer-Lambertôs law was obeyed by amodiaquine hydrochloride in phosphate buffer at 335 nm, and concentration range of 2 to 12 µg/ml (r^2 = 0.994).

The standard Beer- Lambertôs plot of artesunate (after basic decomposition) at 315 nm is shown in Figure 7. The linear plot obtained for artesunate showed that the Beer-Lambertôs law was obeyed by artesunate at 315 nm, and concentration range of 0.01 to 0.06 µg/ml (r^2 = 0.9149).

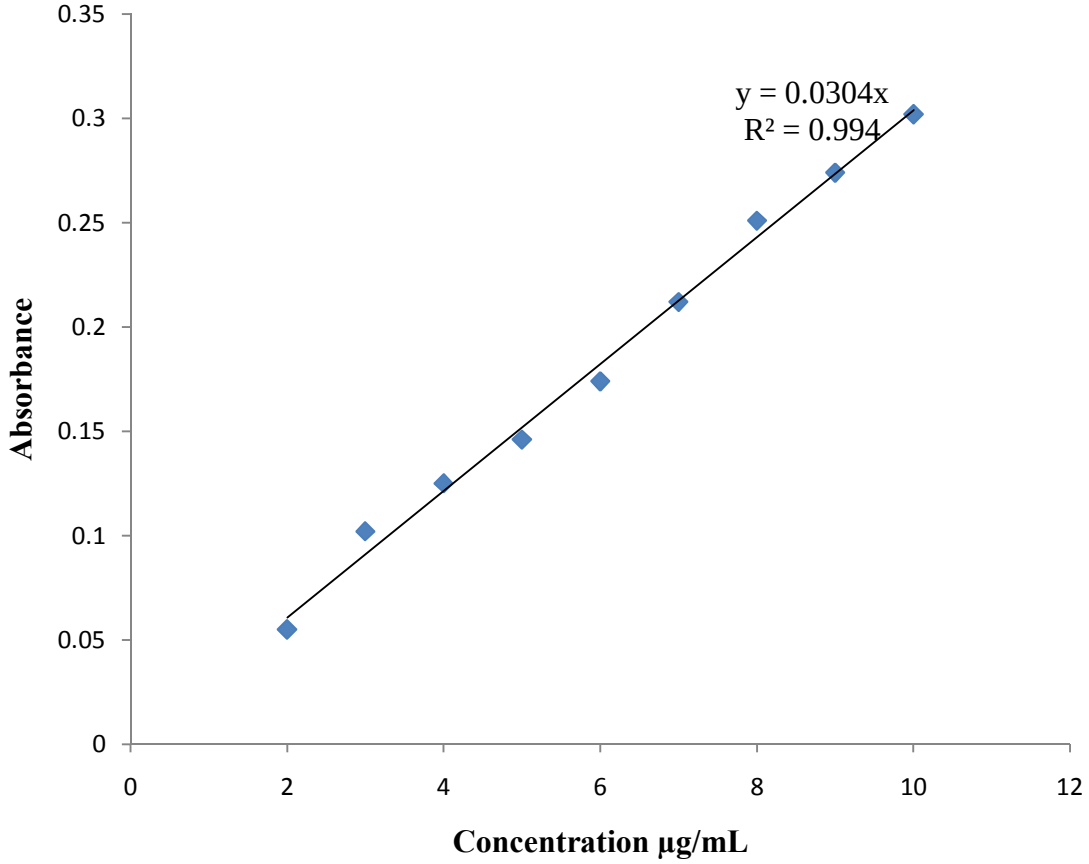


Figure 6: Standard plot of amodiaquine hydrochloride in phosphate buffer pH 7.0 at 335nm

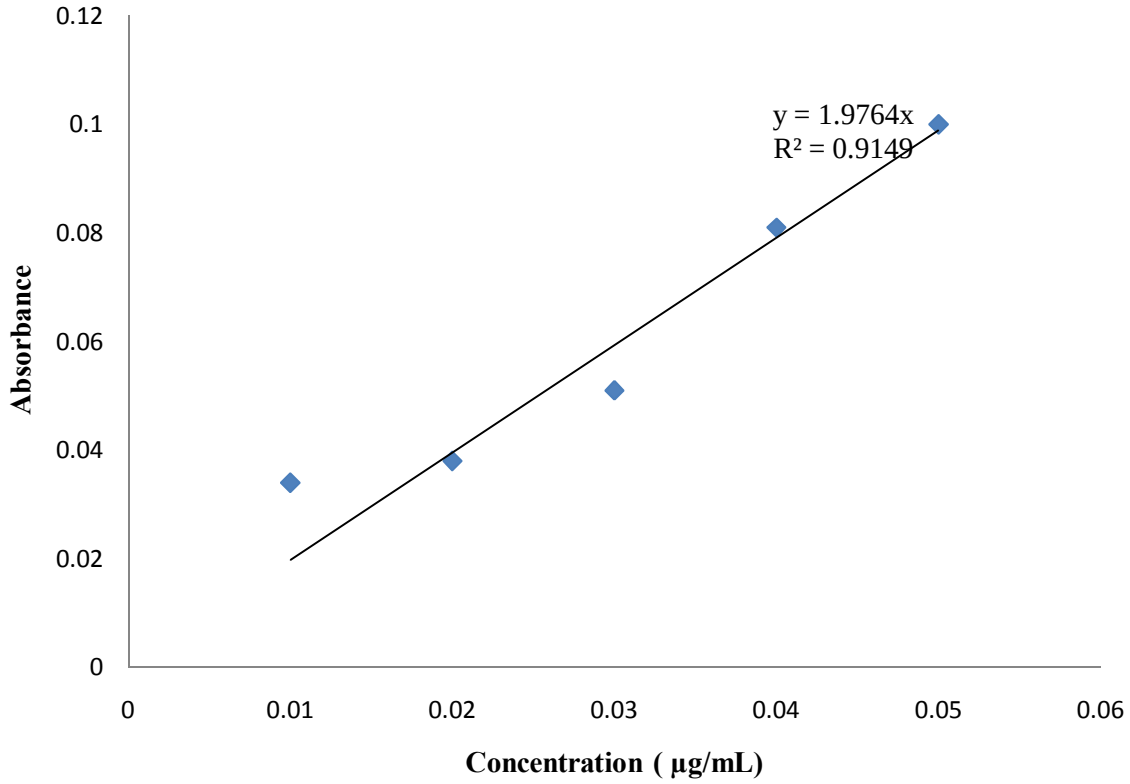


Figure 7: Standard Beer's plot of artesunate at 315nm (after basic decomposition)

Table 6: Solubility of amodiaquine hydrochloride in the excipients

Excipient	Solubility (mg/g)
PEG 4000	22.00
Softisan ®154	9.80
Castor oil	2.97
Phospholipon® 90 G	2.06

Table 7: Solubility of artesunate in the excipients

Excipient	Solubility (mg/g)
PEG 4000	1.00
Softisan ®154	1.09
Castor oil	2.85
Phospholipon® 90 G	4.25

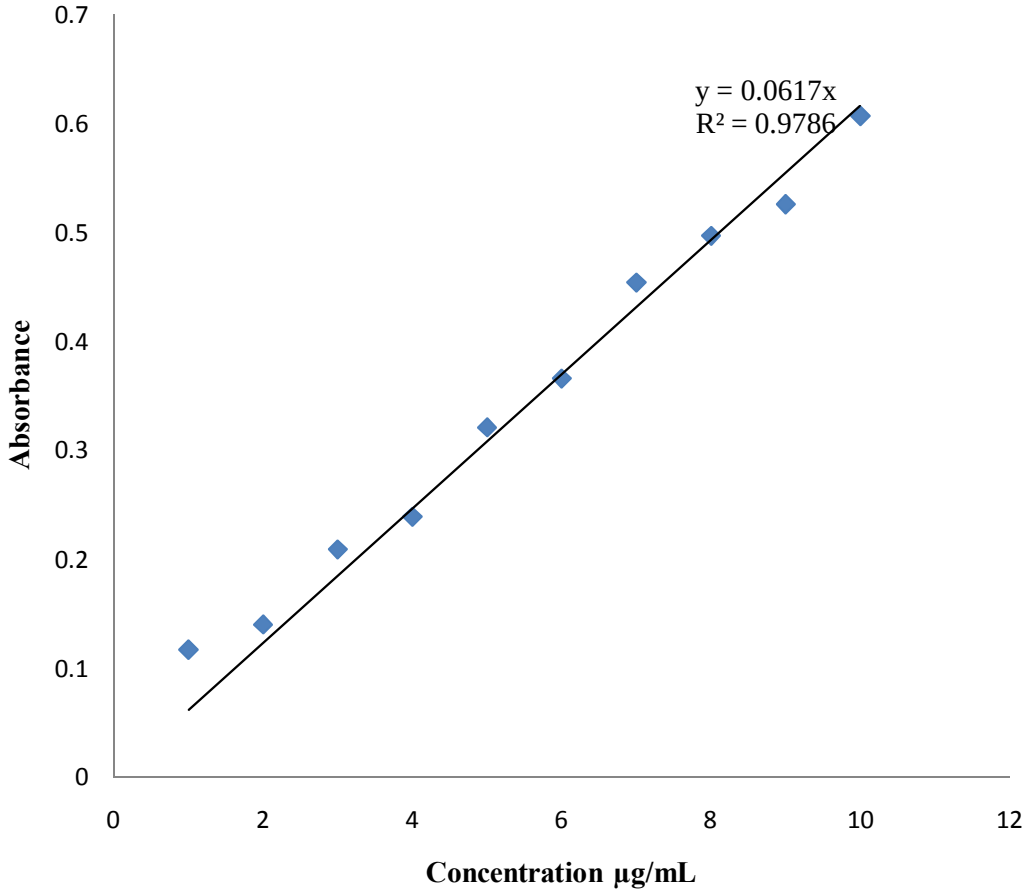


Figure 8: Beer-Lambert's plot of amodiaquine hydrochloride in the presence of artesunate

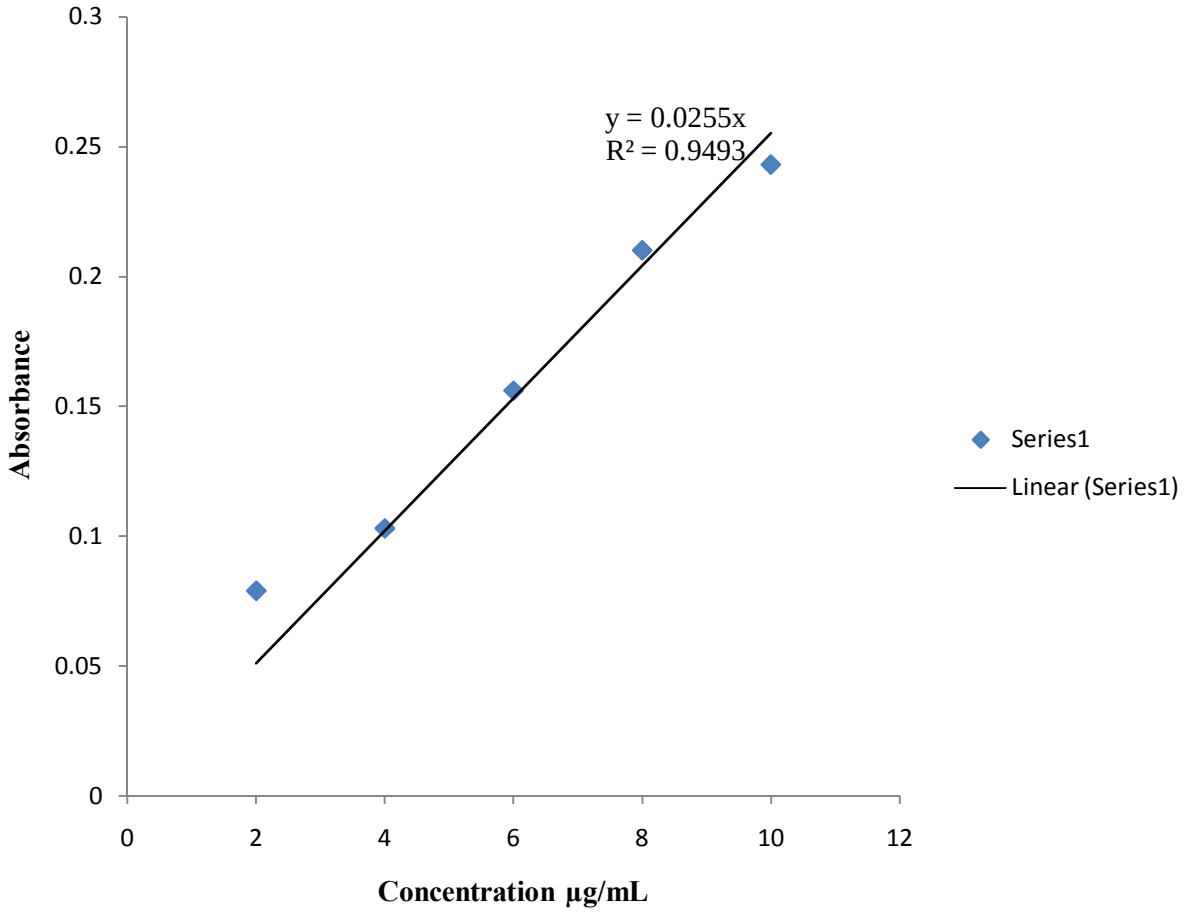


Figure 9: Beer- Lambert's plot of artesunate in the presence of amodiaquine hydrochloride

3.2 Result of solubility test of amodiaquine and artesunate in the excipients

The results of the solubility studies of the pure samples of amodiaquine and artesunate in the PEG 4000, Softisan® 154, Phospholipon® 90 G, and castor oil are shown in Tables 6 and 7 respectively. In the excipients tested , amodiaquine presented the following solubility order: PEG 4000> Softisan® 154> castor oil > Phospholipon® 90 G while artesunate presented the following solubility order: Phospholipon® 90G > castor oil>Softisan® 154> PEG 4000. Amodiaquine hydrochloride is more hydrophilic than artesunate [78, 101-103]. Hence, it was most soluble in PEG, the most hydrophilic excipient. This implies that the extent of the release of amodiaquine from PEG suppositories will be close to 100 %. Artesunate was most soluble in Phospholipon® 90 G, a hydrophobic excipient. This confirms the poor water solubility property of artesunate.

3.3 Result of ultra-violet spectrophotometric analysis of drug combination

The procedure for the determination of the combination of amodiaquine hydrochloride and artesunate has been described in Chapter Two.

The standard Beer-Lambert plot of amodiaquine hydrochloride (in the presence of artesunate) in phosphate buffer pH 7.0 at 335 nm is shown in Figure 8. The linear plot obtained for amodiaquine showed that the Beer-Lambertôs law was obeyed by amodiaquine hydrochloride in phosphate buffer at 335 nm, and concentration range of 2 to 12 µg/ml (r^2 = 0.9786).

The standard Beer- Lambertôs plot of artesunate, after basic decomposition (in the presence of amodiaquine) at 315 nm is shown in Figure 9. The linear plot obtained for artesunate showed that the Beer-Lambertôs law was obeyed by artesunate at 315 nm, and concentration range of 2 to 12 µg/ml (r^2 = 0.9493).

3.4 Result of differential scanning calorimetry (DSC) for the choice of ratios of excipients

The DSC thermograms of different ratios of the excipients are shown in figures 10-17.

Figure 10, the DSC curves of the lipid matrix (made up of 0.1g /0.05g/0.1g of Phospholipon® 90G and Softisan®154 mixture/ castor oil /PEG 4000 respectively) showed an endothermic peak at 61.7 ^{o}C and an enthalpy value of -28.08 mW/mg.

Figure 11, the DSC curves of the lipid matrix (made up of 0.1g /0.1g/0.2g of Phospholipon® 90G and Softisan®154 mixture/ castor oil /PEG 4000 respectively) showed an endothermic peak at 61.6 ^{o}C and an enthalpy value of -30.85 mW/mg.

Figure 12, the DSC curves of the lipid matrix (made up of 0.3g /0.1g/0.2g of Phospholipon® 90G and Softisan® 154 mixture/ castor oil /PEG 4000 respectively) showed an endothermic peak at 64.3 ^{o}C and an enthalpy value of -28.41 mW/mg.

Figure 13, the DSC curves of the lipid matrix (made up of 0.4g /0.1g/0.2g of Phospholipon® 90G and Softisan®154 mixture/ castor oil /PEG 4000 respectively) showed an endothermic peak at 61.5 ^{o}C and an enthalpy value of -11.82 mW/mg.

Figure 14, the DSC curves of the lipid matrix (made up of 0.4g /0.3g/0.2g of Phospholipon® 90G and Softisan®154 mixture/ castor oil /PEG 4000 respectively) showed an endothermic peak at 65.3 ^{o}C and an enthalpy value of -24.72 mW/mg.

Figure 15, the DSC curves of the lipid matrix (made up of 0.4g /0.2g/0.2g of Phospholipon® 90G and Softisan®154 mixture/ castor oil /PEG 4000 respectively) showed an endothermic peak at 59.8 ^{o}C and an enthalpy value of -23.51 Mw/mg.

Figure 16, the DSC curves of the lipid matrix (made up of 0.4g /0.4g/0.3g of Phospholipon® 90G and Softisan®154 mixture/ castor oil /PEG 4000 respectively) showed an endothermic peak at 58.9^{o}C and 63.3 ^{o}C and enthalpy values of -17.97 mW/mg and -20.61 mW/mg respectively.

Figure 17, the DSC curves of the lipid matrix (made up of 0.4g /0.3g/0.4g of Phospholipon® 90G and Softisan®154 mixture/ castor oil /PEG 4000 respectively) showed an endothermic peak at 59.9 ^{o}C and an enthalpy value of -31.09 mW/mg.

Implication of DSC results

The samples analyzed by DSC were eight, of which seven had singular sharp endothermic peaks. The DSC curves of the lipid matrix (made up of 0.4g /0.4g/0.3g of Phospholipon® 90G and Softisan®154 mixture/ castor oil /PEG 4000 respectively) showed endothermic peaks at 58.9 and 63.3 ^{o}C. This implies that this particular ratio of the excipients was the least crystalline as it produced an imperfect matrix which melted over a range of temperatures.

It has been proven from previous studies on suppositories that highly ordered crystalline lipid matrices (with sharp melting points) lead to drug expulsion from the suppositories. The formation of highly ordered structures, particularly during storage, leaves little space for drug molecules. This leads to drug expulsion, and consequently leads to the formation of dry crystals in the solid dosage forms [152-154].

Matrices with low enthalpy values favor drug loading of poorly soluble drugs [186]. The enthalpy value obtained during the thermal analysis of the lipid matrix (made up of 0.4g /0.4g/0.3g of Phospholipon® 90G and Softisan®154 mixture/ castor oil /PEG 4000 respectively) showed low enthalpy values (-17.97 and ï 20.6 mW/mg) when compared with the other six ratios. Although the ratio (made up of 0.4g /0.1g/0.2g of Phospholipon® 90G and Softisan®154 mixture/ castor oil /PEG 4000 respectively) had the least enthalpy value (-11.82 mW/mg), it was not chosen because it exhibited a sharp melting point.

Therefore, the excipient ratio of 4: 4:3 (Phospholipon® 90G and Softisan ®154 mixture/ castor oil /PEG 4000 respectively) was chosen as the optimal ratio for the formulation of artesunate and amodiaquine suppositories.

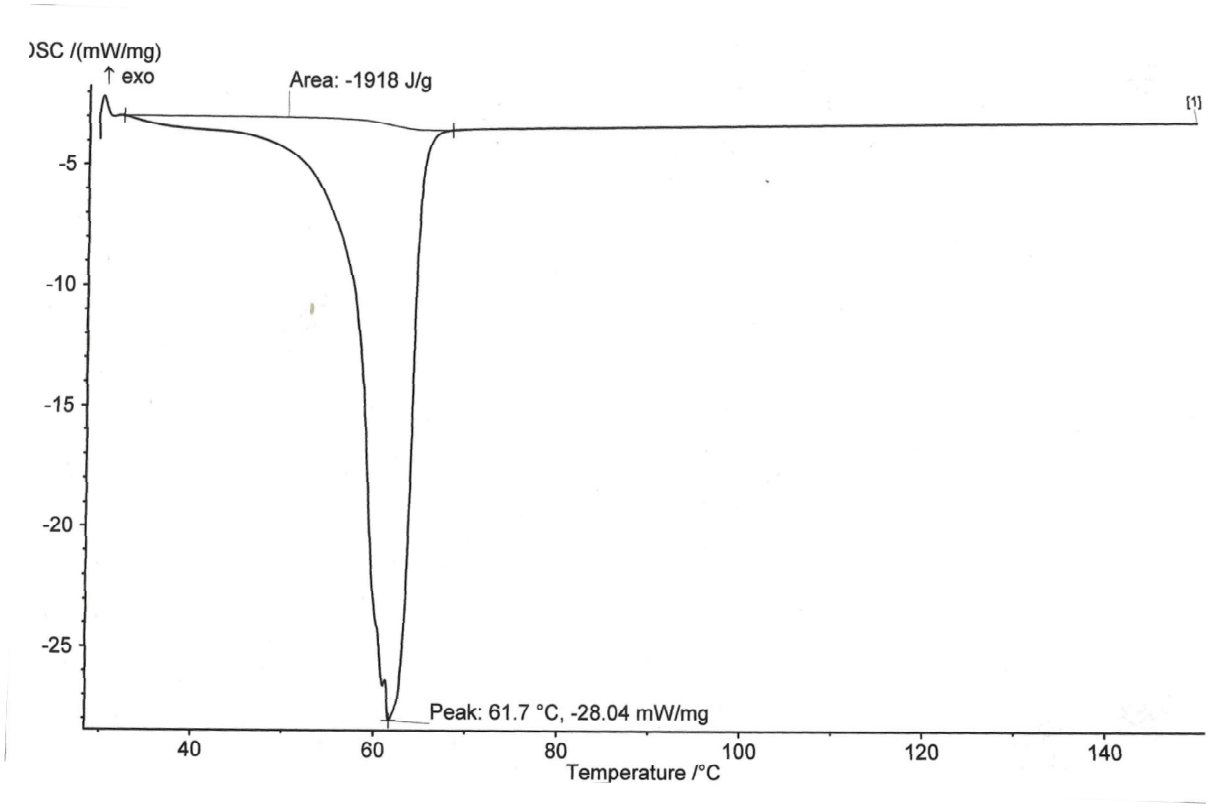


Figure 10: DSC thermogram of 0.1g /0.05g/0.1g of Phospholipon ®90G and Softisan®154 mixture/ castor oil /PEG 4000 respectively

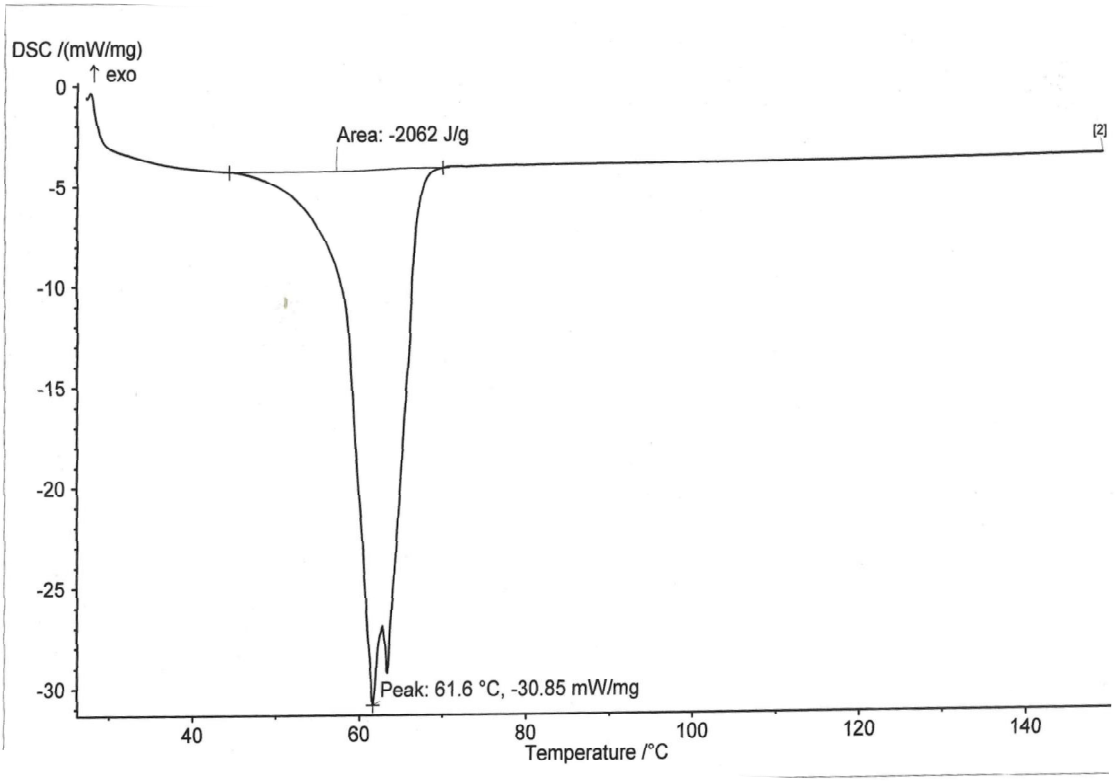


Figure 11: DSC thermogram of 0.1g /0.1g/0.2g of Phospholipon®90G and Softisan® 154 mixture/ castor oil /PEG 4000 respectively

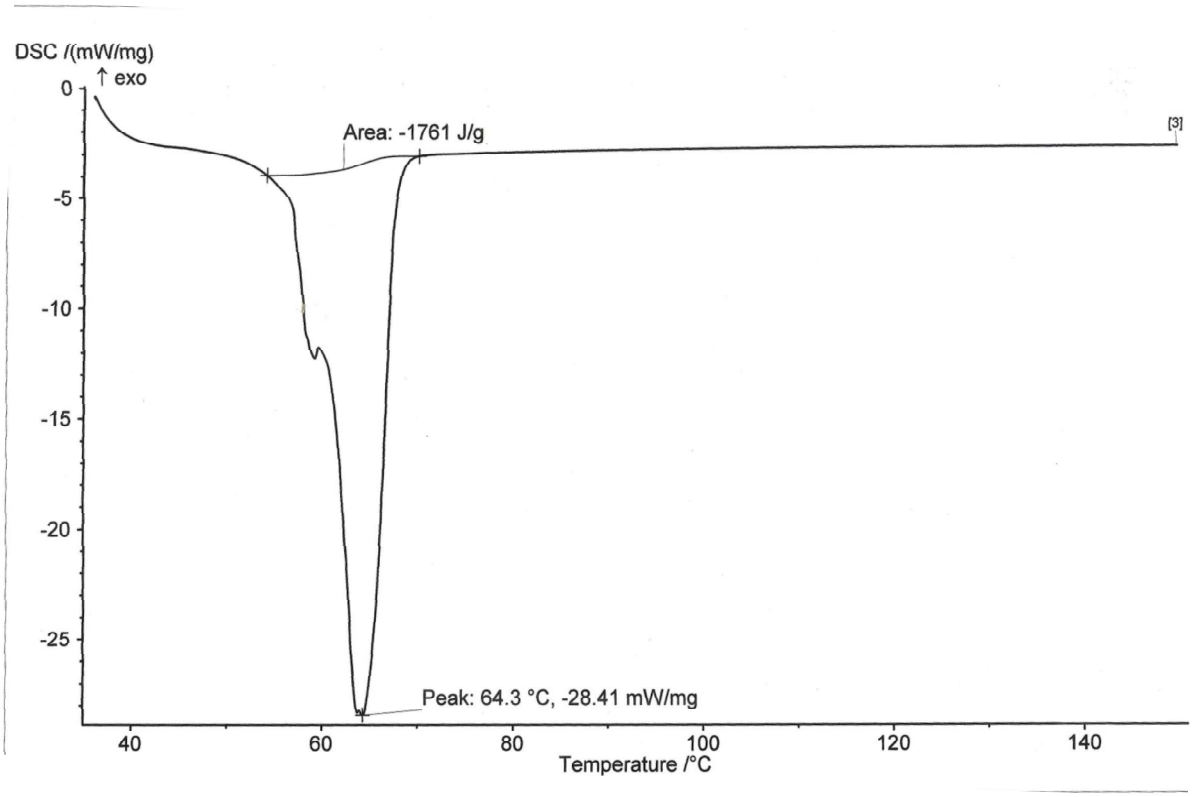


Figure 12: DSC thermogram of 0.3g /0.1g/0.2g of Phospholipon® 90G and Softisan®154 mixture/ castor oil /PEG 4000 respectively

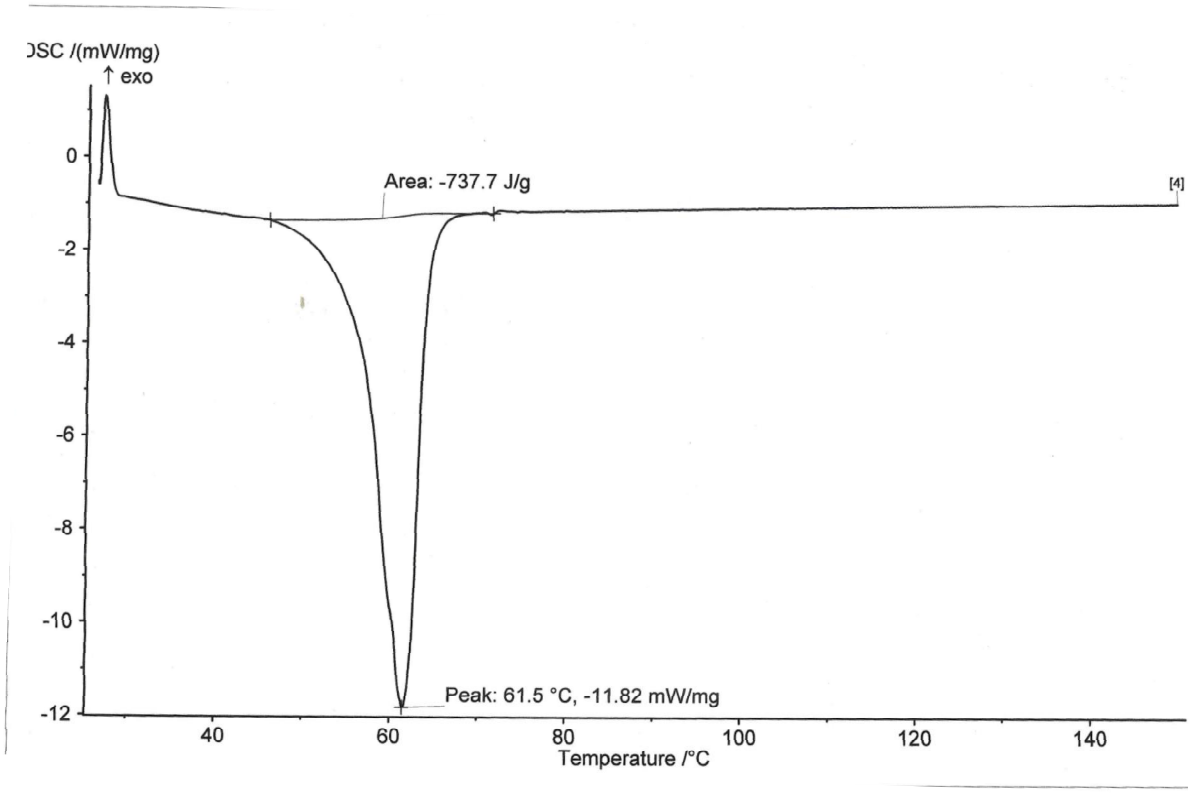


Figure 13: DSC result of 0.4g /0.1g/0.2g of Phospholipon ®90G and Softisan®154 mixture/ castor oil /PEG 4000 respectively

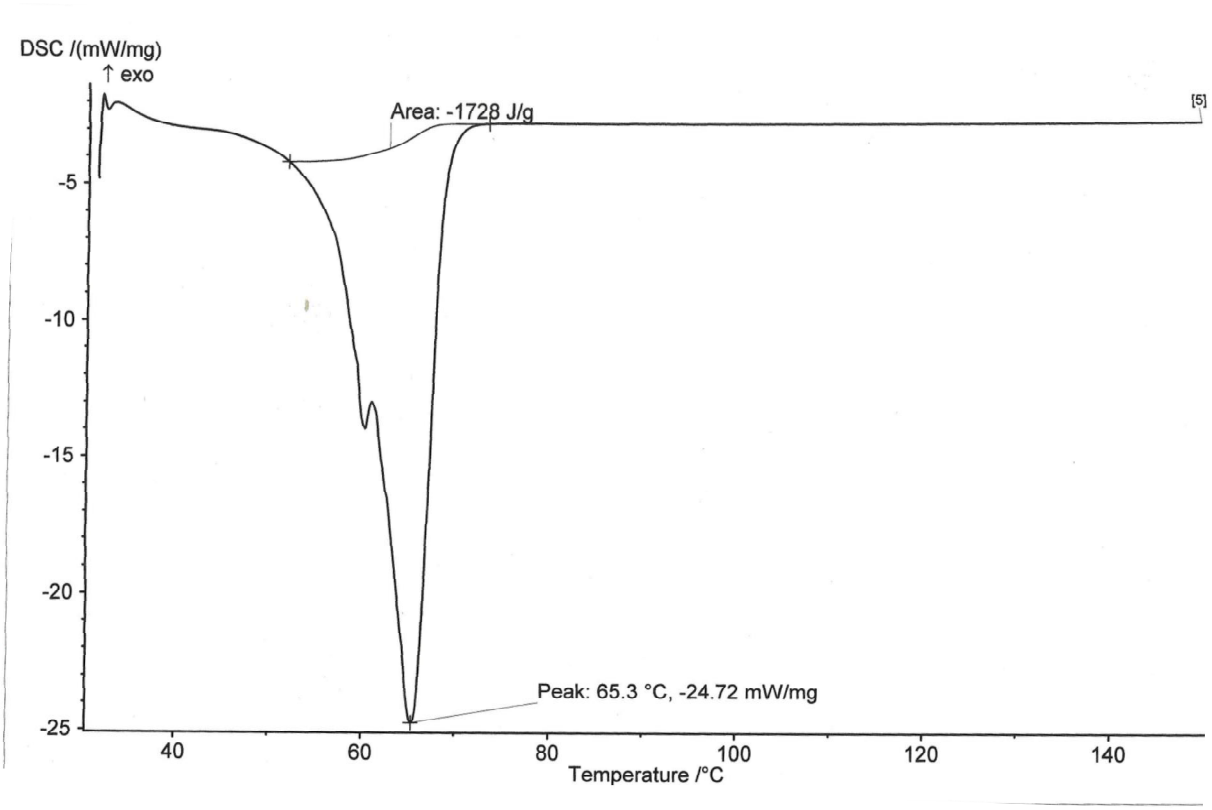


Figure 14: DSC result of 0.4g /0.3g/0.2g of Phospholipon®90G and Softisan®154 mixture/ castor oil /PEG 4000 respectively

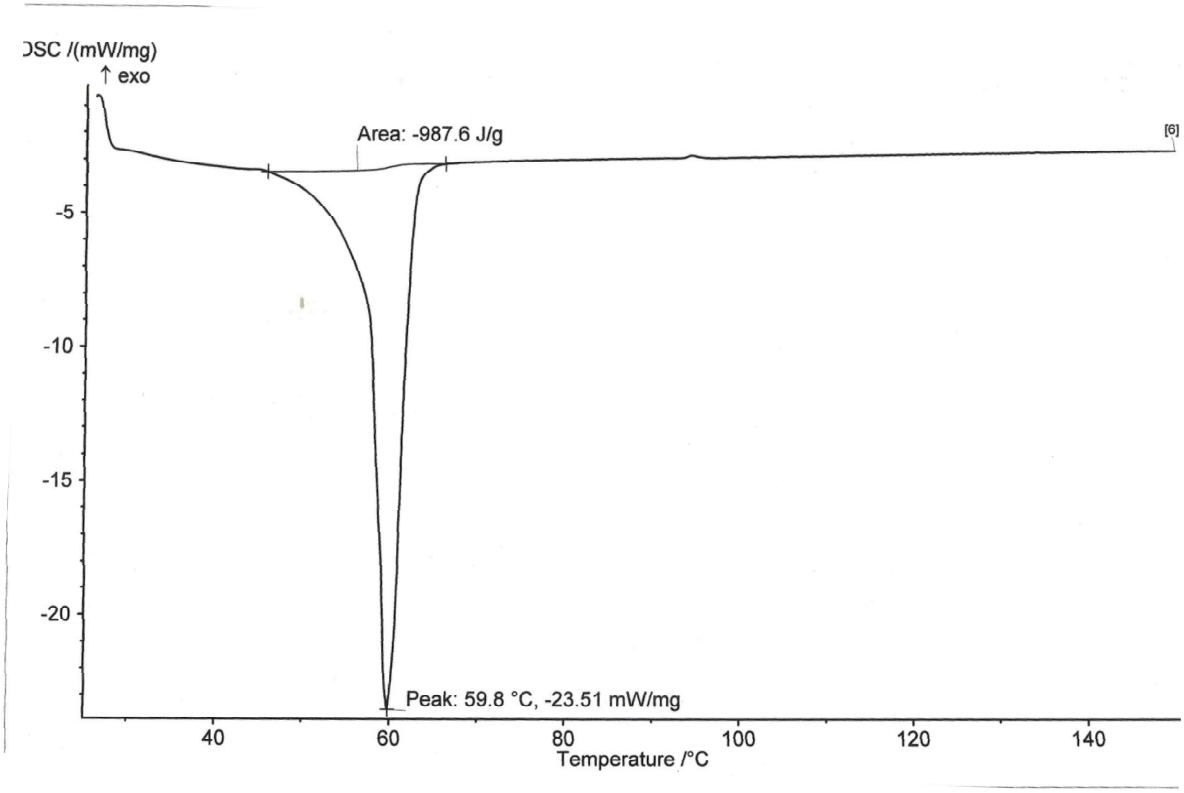


Figure 15 : DSC result of 0.4g /0.2g/0.2g of Phospholipon® 90G and Softisan ®mixture/ castor oil /PEG 4000 respectively

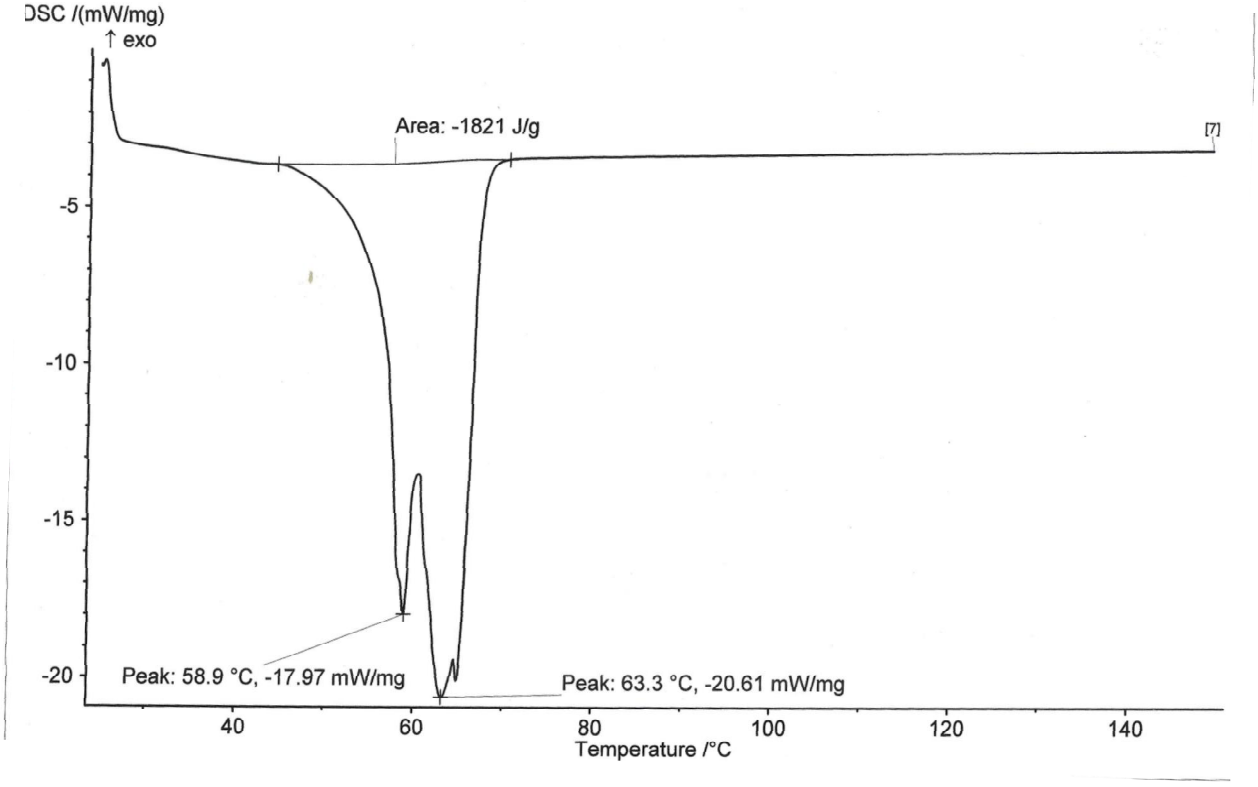


Figure 16: DSC result of 0.4g /0.4g/0.3g of Phospholipon ®90G and Softisan®154 mixture/ castor oil /PEG 4000 respectively

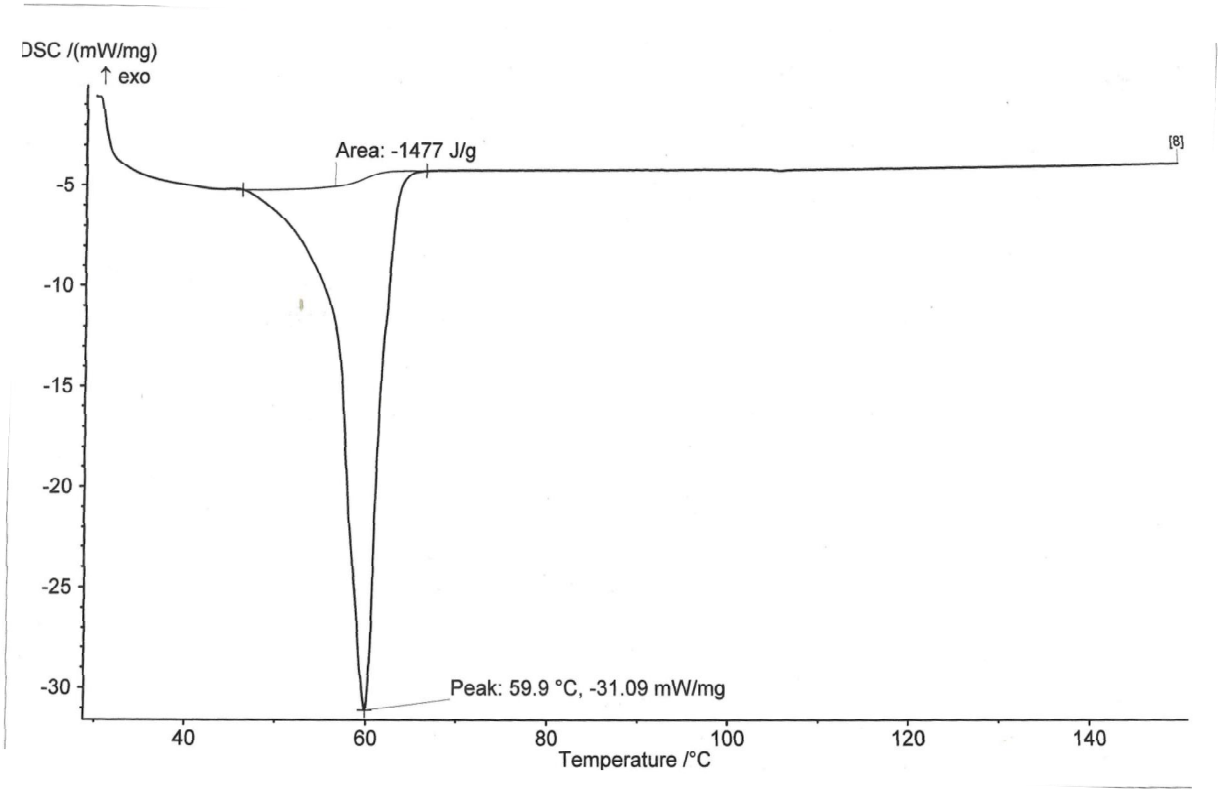


Figure 17 : DSC result of 0.4g /0.3g/0.4g of Phospholipon® 90G and Softisan®154 mixture/ castor oil /PEG 4000 respectively

3.5 Result of compatibility studies of amodiaquine hydrochloride, artesunate and the lipid matrix

The results of the compatibility studies are presented in F igures 18 to 22.

The DSC curves of the lipid matrix in Figure 18, showed an endothermic reaction with a very sharp peak at 60.5 ^{o}C. This implies that Phospholipon® 90G and Softisan®154 are compatible and formed stable matrices, which is consistent with results from previous researches [151].

The DSC curves of the lipid matrix and amodiaquine hydrochloride in Figures 19, showed an endothermic reaction with a sharp peak at 60.0 ^{o}C and a weak peak at 166.5 ^{o}C. This is consistent with the results of a previous research on amodiaquine [184].

The DSC curves of the lipid matrix and artesunate in Figure 20, showed two endothermic reactions with a sharp peak at 62.8 ^{o}C and an exothermic broad peak at 175.3 ^{o}C.

The DSC curves of the lipid matrix, artesunate and amodiaquine in Figure 21 showed an endothermic reaction with a sharp peak at 64.5 ^{o}C . The sharp peaks of 166.5 ^{o}C and 175.3 ^{o}C exhibited by amodiaquine and artesunate respectively were hidden. This phenomenon could be said to be due to the dissolution of the drugs into the excipients which took place during the thermal analysis, thereby masking the sharp peaks of the drugs as described by earlier researches on the thermal properties of the artesunate and amodiaquine combination [185].

Therefore, the DSC traces of the compatibility studies show that artesunate, amodiaquine, Softisan®154, Phospholipon®90G, poly ethylene glycol 4000 and castor oil are compatible and formed a homogenous matrix.

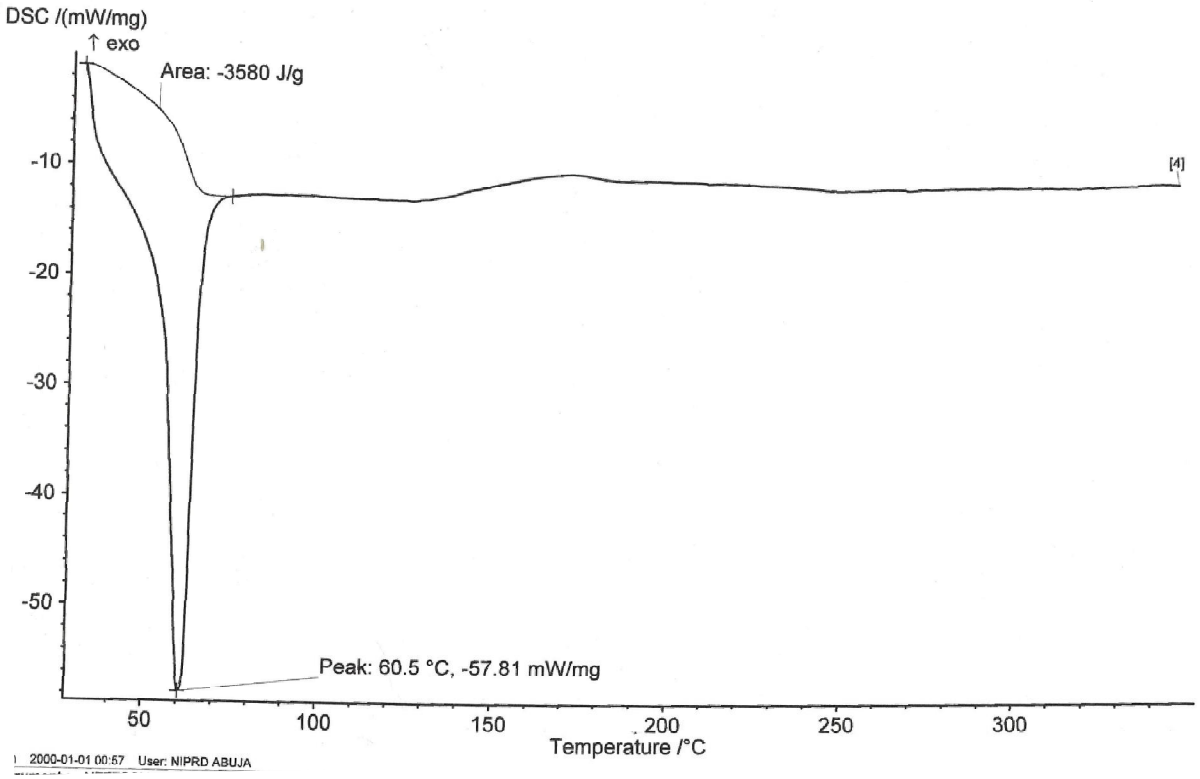


Figure 18: DSC thermogram of the lipid matrix

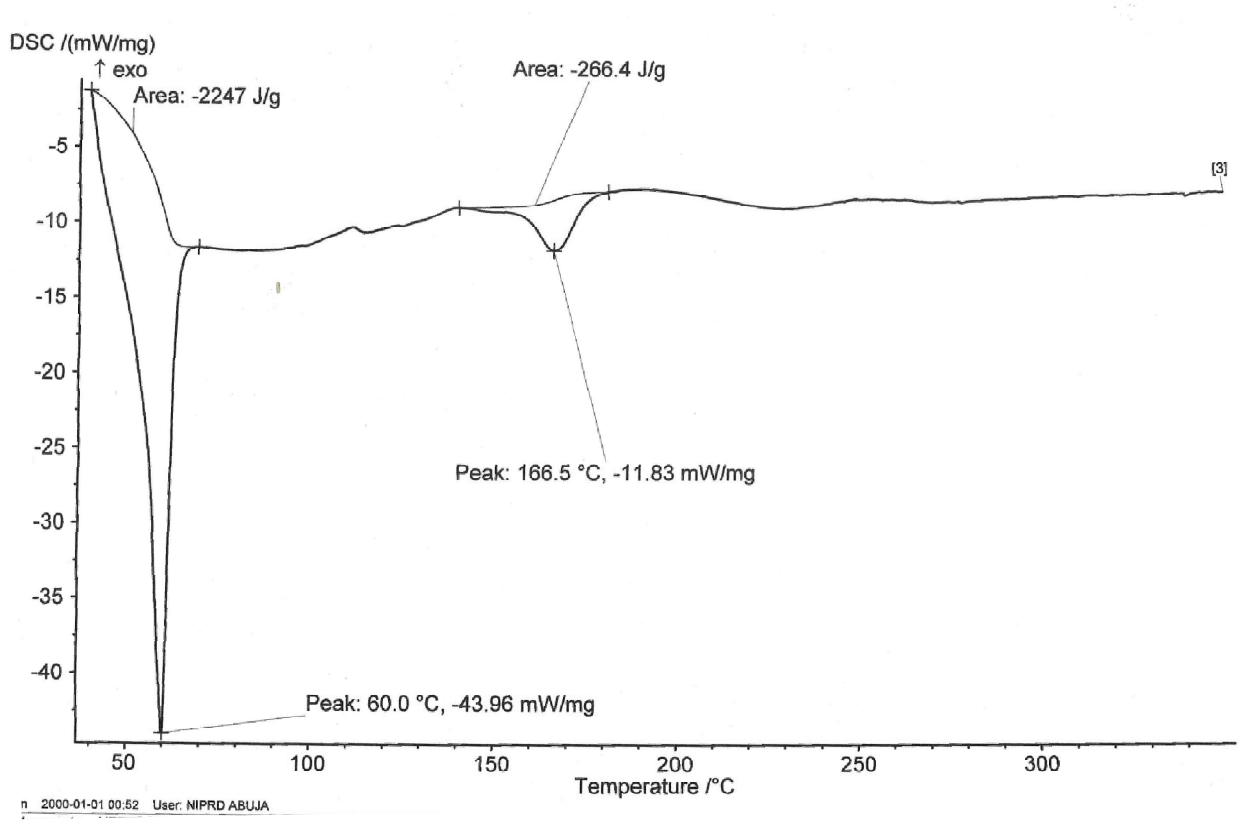


Figure 19: DSC Thermogram of amodiaquine and the lipid matrix in the ratio 2:8 respectively

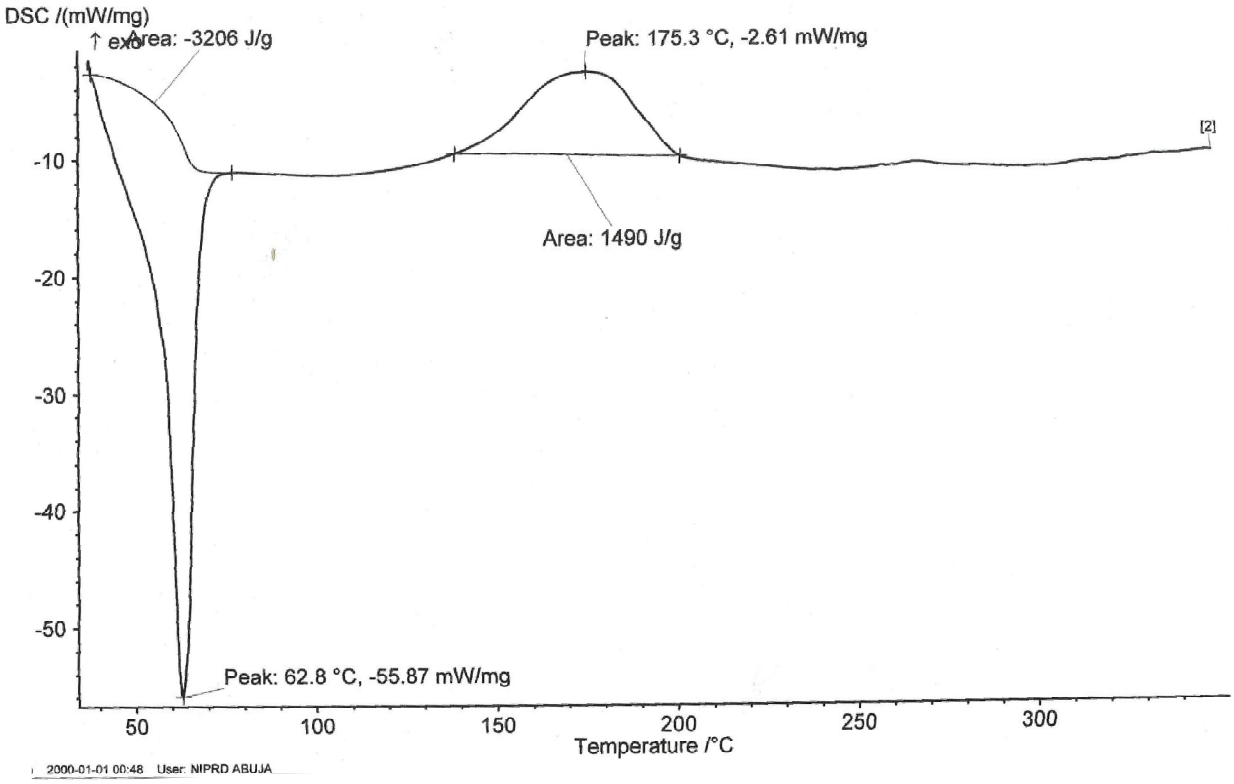


Figure 20: DSC Thermogram of artesunate and the lipid matrix in the ratio 2:8 respectively

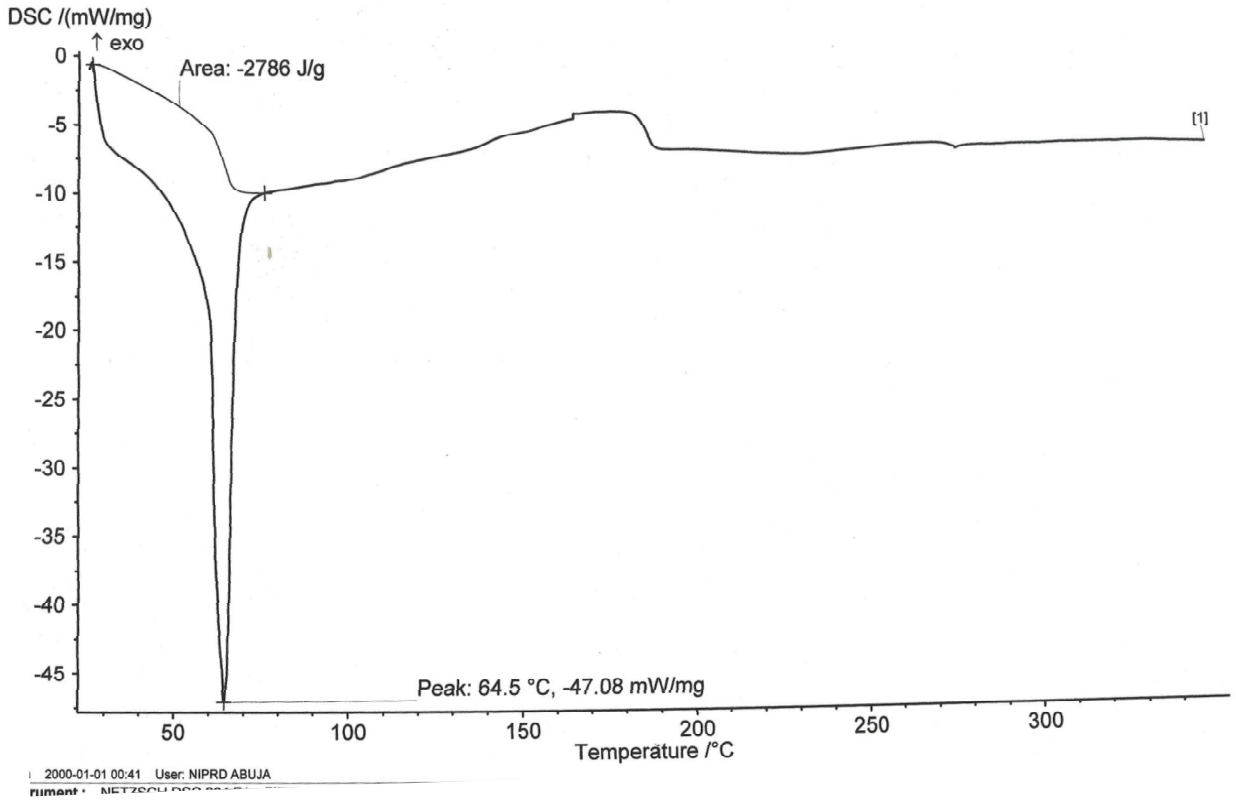
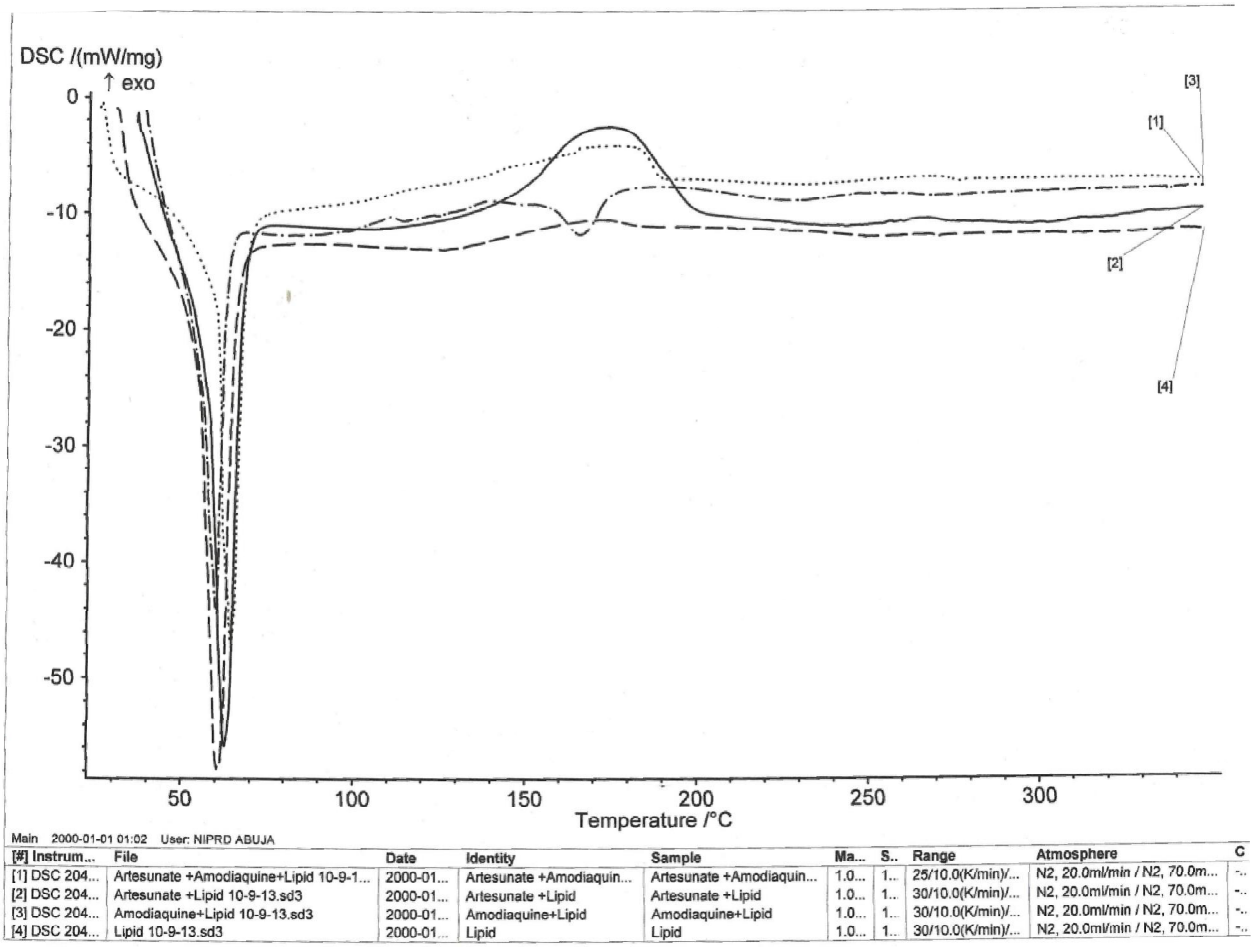


Figure 21: DSC thermogram of artesunate, amodiaquine and the lipid matrix in the ratio 1:1:8 respectively



Main 2000-01-01 01:02 User: NIPRD ABUJA

[#] Instrum...	File	Date	Identity	Sample	Ma...	S..	Range	Atmosphere	C
[1] DSC 204...	Artesunate +Amodiaquine+Lipid 10-9-1...	2000-01...	Artesunate +Amodiaquin...	Artesunate +Amodiaquin...	1.0...	1..	25/10.0(K/min)/...	N2, 20.0ml/min / N2, 70.0m...	-..
[2] DSC 204...	Artesunate +Lipid 10-9-13.sd3	2000-01...	Artesunate +Lipid	Artesunate +Lipid	1.0...	1..	30/10.0(K/min)/...	N2, 20.0ml/min / N2, 70.0m...	-..
[3] DSC 204...	Amodiaquine+Lipid 10-9-13.sd3	2000-01...	Amodiaquine+Lipid	Amodiaquine+Lipid	1.0...	1..	30/10.0(K/min)/...	N2, 20.0ml/min / N2, 70.0m...	-..
[4] DSC 204...	Lipid 10-9-13.sd3	2000-01...	Lipid	Lipid	1.0...	1..	30/10.0(K/min)/...	N2, 20.0ml/min / N2, 70.0m...	-..

Figure 22: Super-imposed DSC thermogram of compatibility studies of artesunate, amodiaquine HCl and the lipid matrix

Figure 23 **Figure 24** **Figure 25**

Figure 23: Formulated suppository of artesunate (25mg) and amodiaquine (75mg) prepared with PEG 4000/Phospholipon®90G/Softisan®154/castor oil

Figure 24: Formulated suppository of artesunate (25mg) and amodiaquine (75mg) prepared with Phospholipon®90G/ Softisan ®154/ castor oil

Figure 25: Formulated suppository of artesunate (25mg) and amodiaquine (75mg) prepared with PEG 4000/castor oil

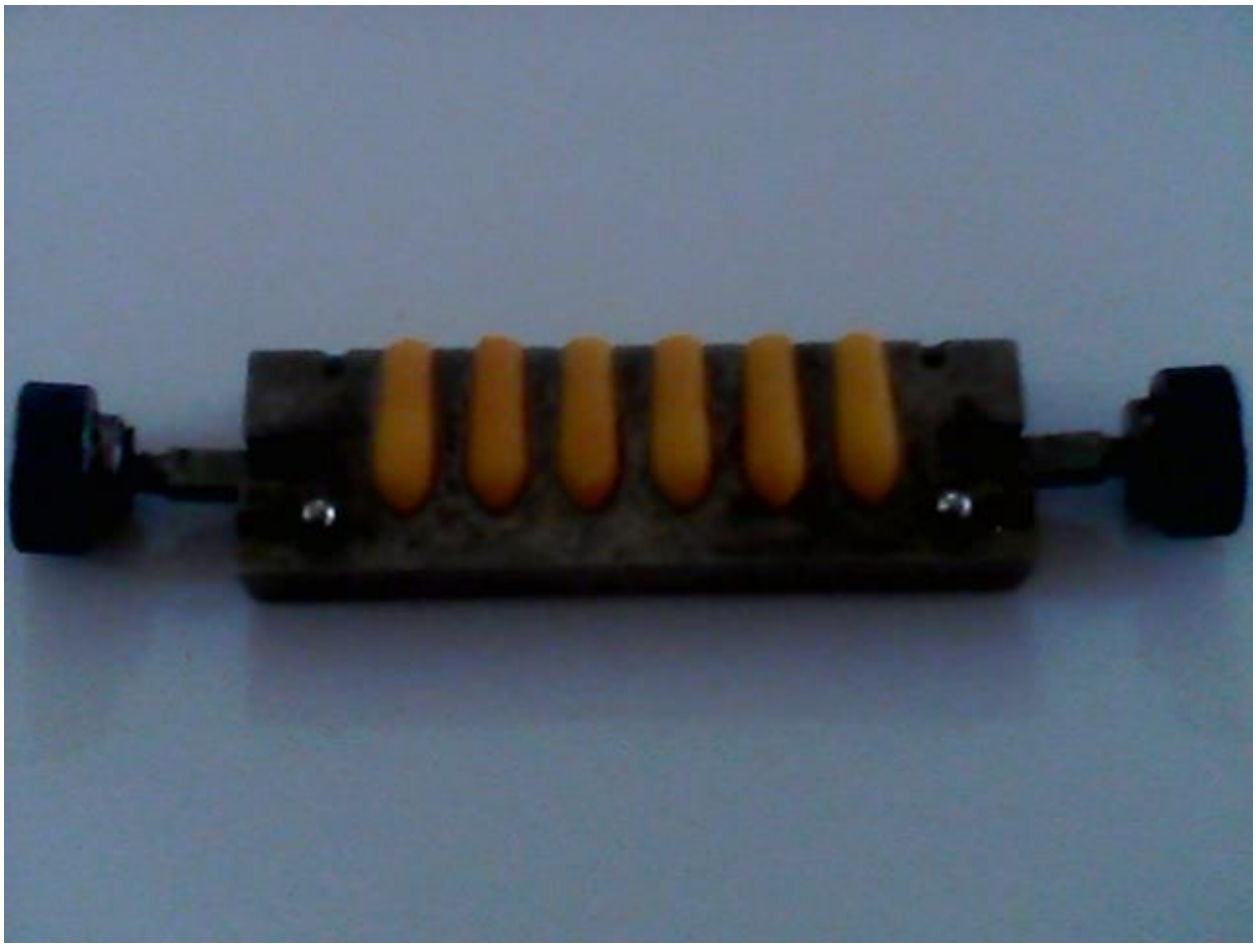


Figure 26: Formulated suppository of artesunate (25mg) and amodiaquine (75mg) prepared with PEG 4000/castor oil in the suppository mold before removal

3.6 Results of the physical characterization of formulated amodiaquine and artesunate suppositories

3.6.1 Colour

The colours of the formulated artesunate and amodiaquine suppositories ranged from bright yellow to deep yellow for suppositories prepared with Phospholipon® 90 G/ Softisan® 154/ PEG 4000/ castor oil; Phospholipon® 90 G/ Softisan® 154 / castor oil, and PEG 4000/ castor oil respectively (Table 6). The yellow colours were probably as a result of the inclusion of yellow ï colored amodiaquine hydrochloride [78], as one of the active pharmaceutical ingredients in the suppository formulation. The varying degrees and shades of yellow could be as a result of the unique characteristics of the different ratios of the suppository excipients in the different formulations.

3.6.2 Surface characteristics

The surface characteristics of the formulated artesunate and amodiaquine suppositories are presented in Table 7. There was absence of fissuring, pitting, exudation and fat blooming in artesunate and amodiaquine suppositories prepared with Phospholipon®90G/Softisan®154/PEG 4000/castor oil, Phospholipon®90G/ Softisan®154/castor oil, and PEG 4000/castor oil; this implies that the different excipients remained physically stable after formulation into suppositories. There was also no migration of active ingredients in the artesunate and amodiaquine suppositories formulated with PEG 4000 and castor oil ; Phospholipon®90G / Softisan®154 / PEG4000 / castor oil and Phospholipon®90G/ Softisan®154 /castor oil.

3.7 Result of weight uniformity test

The results of the weight uniformity test are presented in Table 8. All the suppositories had acceptable results with regard to the uniformity of weight described in BP 2004 [155] . Not more than two of the individual weights deviated from the average weight by more than 5 % and none deviated by twice that percentage; the standard deviations of the prepared formulations ranged from ± 0.01 to ± 0.02.

3.8 Result of drug content uniformity test

The results of the content uniformity test are presented in Table 9. The amodiaquine content of suppositories containing PEG 4000/ castor oil, Phospholipon ® 90G/ Softisan® 154/ castor oil, Phospholipon® 90G/ Softisan® 154/ PEG 4000/ castor oil was 108 %, 106.75 %, 106.67 % respectively. This complied with the USP Criteria for Suppositories which states that the requirements are met if the range of the drug content is within the range of 85.0 % to 115.0 % of the label claim. The artesunate content of suppositories containing PEG 4000/ castor oil, Phospholipon® 90G/ Softisan ®154/ castor oil, Phospholipon ®90G/ Softisan ®154/ PEG 4000/ castor oil was 132.24 %, 132.24 %, 131.84 % respectively. This did not comply with the USP Criteria for Suppositories which states that the requirements are met if the range of the drug content is within the range of 85.0 % to 115.0 % of the label claim.

3.9 Result of softening time test

The results of the softening time test are presented in Table 12. The artesunate and amodiaquine suppositories formulated with PEG 4000 and castor oil softened and completely disintegrated within 20 minutes. The artesunate and amodiaquine suppositories formulated with Phospholipon®90 G/Softisan® 154/PEG 4000/ castor oil softened between 210 and 270 minutes while the artesunate and amodiaquine suppositories formulated with Phospholipon ®90 G/ Softisan® 154/ castor oil softened within 240 minutes. This suggests that suppositories prepared with PEG 4000 are more likely to release the active ingredients faster than the suppositories without PEG 4000.

Table 8: Colors of formulated artesunate and amodiaquine suppositories (n=6)

Code	Color
P	Deep yellow
PS	Yellow
E	Bright yellow

Code:

P: Formulated PEG 4000 suppositories of artesunate and amodiaquine

PS: Phospholipon®90 G/ Softisan®154 suppositories of artesunate and amodiaquine

E: Formulated PEG 4000/ Softisan®154/Phospholipon®90 G suppositories of artesunate and amodiaquine

Table 9: Surface characteristics of formulated artesunate and amodiaquine suppositories (n=6)

Code	Fissuring	Pitting	Exudation	Fat blooming	Migration of Active ingredient
P	Absent	Absent	Absent	Absent	Absent
PS	Absent	Absent	Absent	Absent	Absent
E	Absent	Absent	Absent	Absent	Absent

Code:

P: Formulated PEG 4000 suppositories of artesunate and amodiaquine

PS: Phospholipon®90 G/ Softisan ®154 suppositories of artesunate and amodiaquine

E: Formulated PEG 4000/ Softisan® 154/Phospholipon ®90G suppositories of artesunate and amodiaquine

Table 10: Weight and length variation of formulated artesunate and amodiaquine suppositories (n= 30; ± S.D.)

Code	Weight Variation (g) ± S.D	Length Variation (cm)± S.D
P	1.36 ± 0.02	2.58 ± 0.03
PS	1.27 ± 0.01	2.59 ± 0.04
E	1.26 ± 0.01	2.59 ± 0.01

Code:

P: Formulated PEG 4000/castor oil suppositories of artesunate and amodiaquine

PS: Phospholipon®90G/ Softisan ®154/castor oil suppositories of artesunate and amodiaquine

E: Formulated PEG 4000/ Softisan®154/Phospholipon®90G/castor oil suppositories of artesunate and amodiaquine

Table 11: Drug content uniformity (n=3)

Code	Drug	Drug content
P	Artesunate	100.24 %
PS	Artesunate	100.01 %
E	Artesunate	100.84 %
P	Amodiaquine	99 %
PS	Amodiaquine	98.75 %
E	Amodiaquine	99.67 %

Code:

P: Formulated PEG 4000/castor oil suppositories of artesunate and amodiaquine

PS: Phospholipon® 90G/ Softisan ®154/castor oil suppositories of artesunate and amodiaquine

E: Formulated PEG 4000/ Softisan®154/Phospholipon® 90G/castor oil suppositories of artesunate and amodiaquine

Table 12: Softening time (n=3)

Code	Time (minutes)
P	20
PS	240
E	270

Code:

P: Formulated PEG 4000/castor oil suppositories of artesunate and amodiaquine

PS: Phospholipon®90 G/ Softisan®154/castor oil suppositories of artesunate and amodiaquine

E: Formulated PEG 4000/ Softisan ®154/Phospholipon®90 G/castor oil suppositories of artesunate and amodiaquine

3.10 *In vitro* drug release results

The *in vitro* release of amodiaquine and artesunate from the formulated suppositories are shown in Figures 27-30.

3.10.1 *In vitro* release of artesunate and amodiaquine from artesunate and amodiaquine suppositories prepared with PEG 4000 and castor oil

The *in vitro* release graphs of artesunate and amodiaquine from artesunate and amodiaquine suppositories prepared with PEG 4000 and castor oil are shown in Figures 27 and 29. The rate and extent of the release of artesunate and amodiaquine from suppositories formulated with PEG 4000 and castor oil (P),was more than that observed in the other suppository formulations (PS and E). The faster rate of drug release from the PEG base may be due to the high water solubility of the base, which allows the drug to be, released by both diffusion and erosion mechanisms [141]. The release of amodiaquine was 99.82 % while the release of artesunate was 99.21 %, after 20 minutes.

3.10.2 *In vitro* release of artesunate and amodiaquine from artesunate and amodiaquine suppositories prepared with Phospholipon® 90 G / Softisan® 154/ castor oil (PS)

The *in vitro* release graphs of artesunate and amodiaquine from artesunate and amodiaquine suppositories prepared with Phospholipon® 90 G / Softisan® 154/ castor oil (PS) are shown in Figures 28 and 30.The release of amodiaquine (34.57 %, after 270 minutes) was lower than the release of artesunate (98.73 %, after 210 minutes). This corresponds with the results of previous studies on amodiaquine suppositories in which it was proven that the release of amodiaquine from fatty bases was sustained and incomplete [156].

3.10.3 *In vitro* release of artesunate and amodiaquine from artesunate and amodiaquine suppositories prepared with Phospholipon® 90G / Softisan® 154/ PEG 4000/castor oil (E)

In vitro release of artesunate and amodiaquine in artesunate and amodiaquine suppositories prepared with Phospholipon®90G / Softisan®154/ PEG 4000/castor oil (E).The release of amodiaquine (28.81 %, after 240 minutes) was lower than the release of artesunate (81.31 %, after 240 minutes). The delayed and incomplete release of amodiaquine from the suppositories could possibly result from the low affinity of the hydrophilic amodiaquine for the fatty bases used in the formulation.

It has been posited that Phospholipon 90®G shields the surface area of the lipid matrix which causes a delay in the diffusion of drugs out of the core lipids, hence the slow and incomplete rate and extent of artesunate and amodiaquine release from Batches PS and E when compared with Batch P. This result is in agreement with the findings of previous researchers who confirmed Phospholipon 90®G as a retarding agent [163,164].

In summary, the slow release of amodiaquine hydrochloride from PS and E suppositories could be attributed to the high lipophilicity of the lipid matrices, the high water solubility of amodiaquine hydrochloride, and the non-miscibility of the lipid matrix with the dissolution media [165].

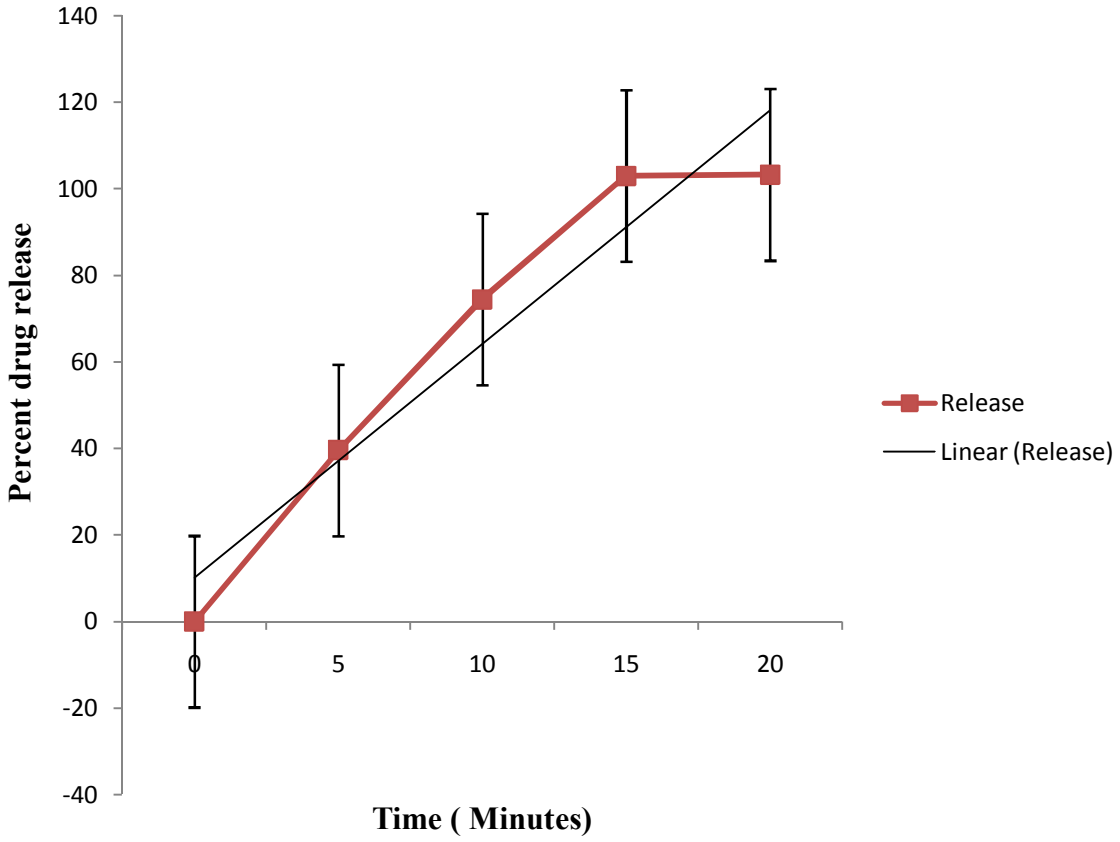


Figure 27: *In vitro* release of amodiaquine from artesunate and amodiaquine suppositories prepared with PEG 4000 and castor oil (E)

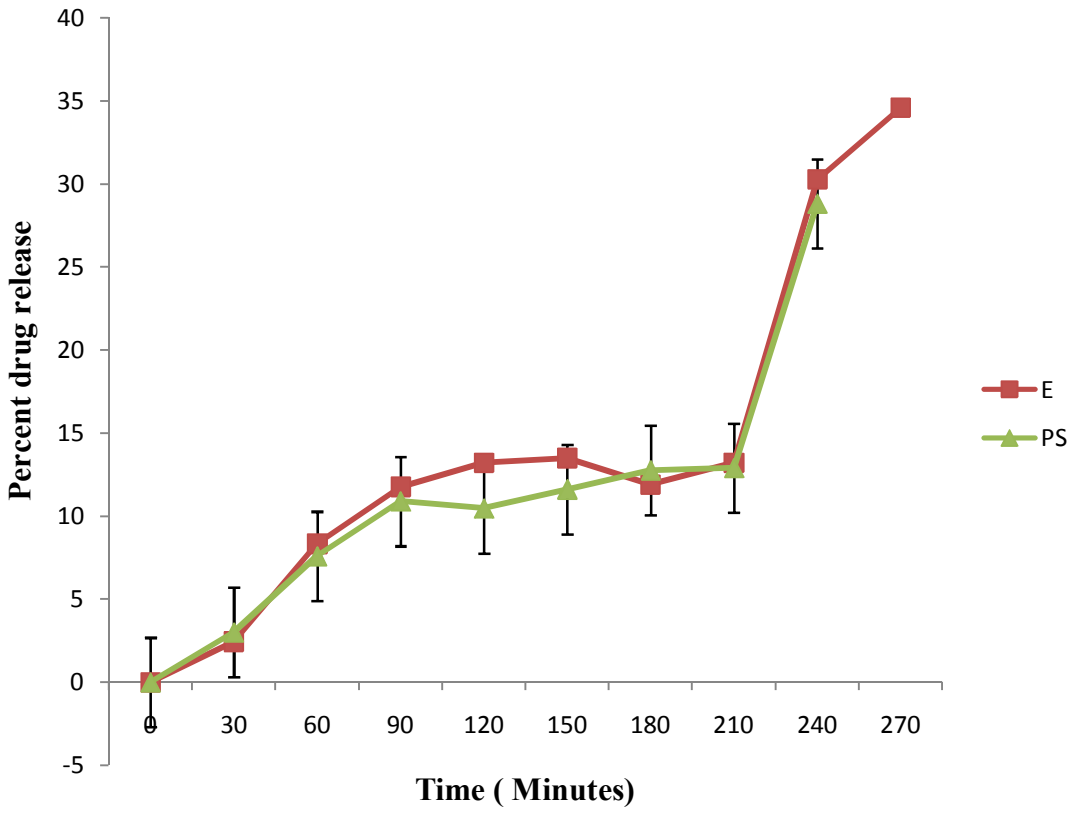


Figure 28: *In vitro* release of amodiaquine from artesunate and amodiaquine suppositories prepared with Phospholipon®90G/Softisan® 154/castor oil (PS) and PEG 4000 / Phospholipon®90 G / Softisan®154/ castor oil (E)

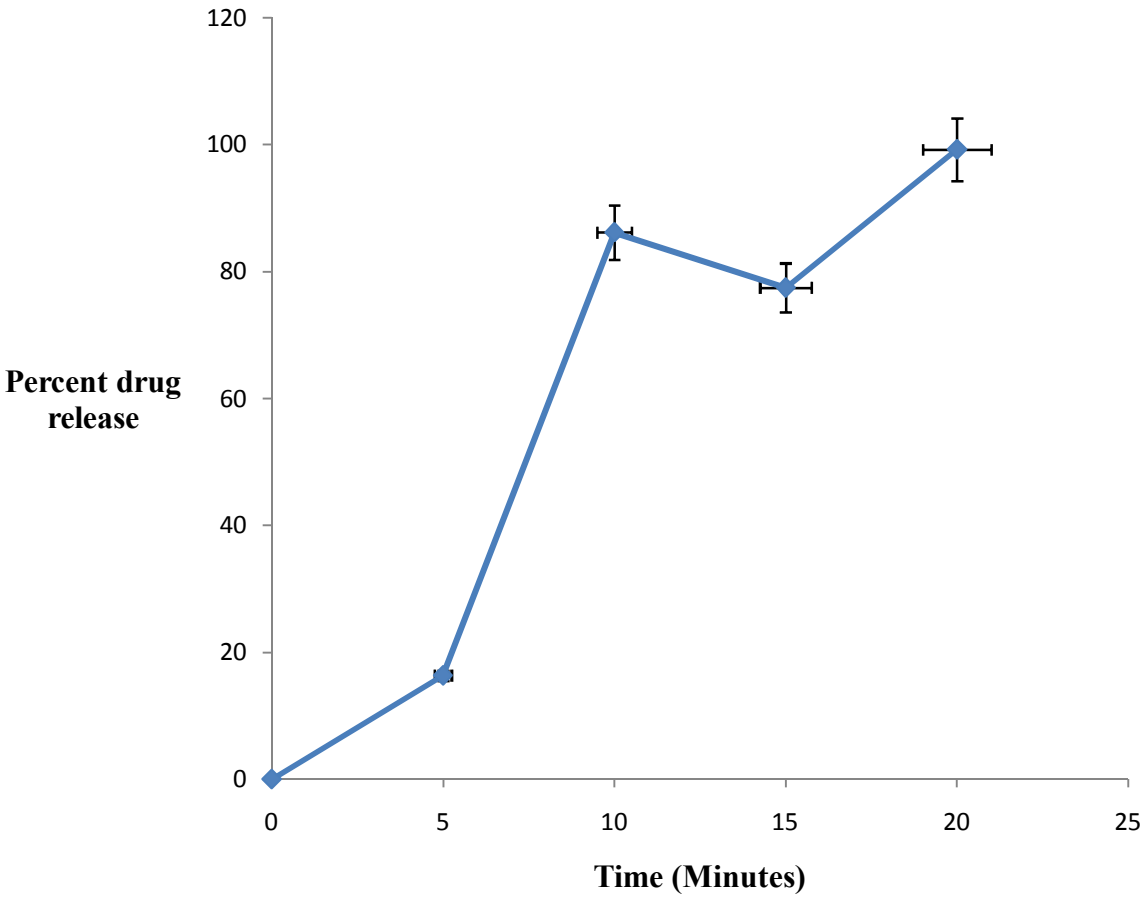


Figure 29: *In vitro* release of artesunate from artesunate and amodiaquine suppositories prepared with PEG 4000 and castor oil (P)

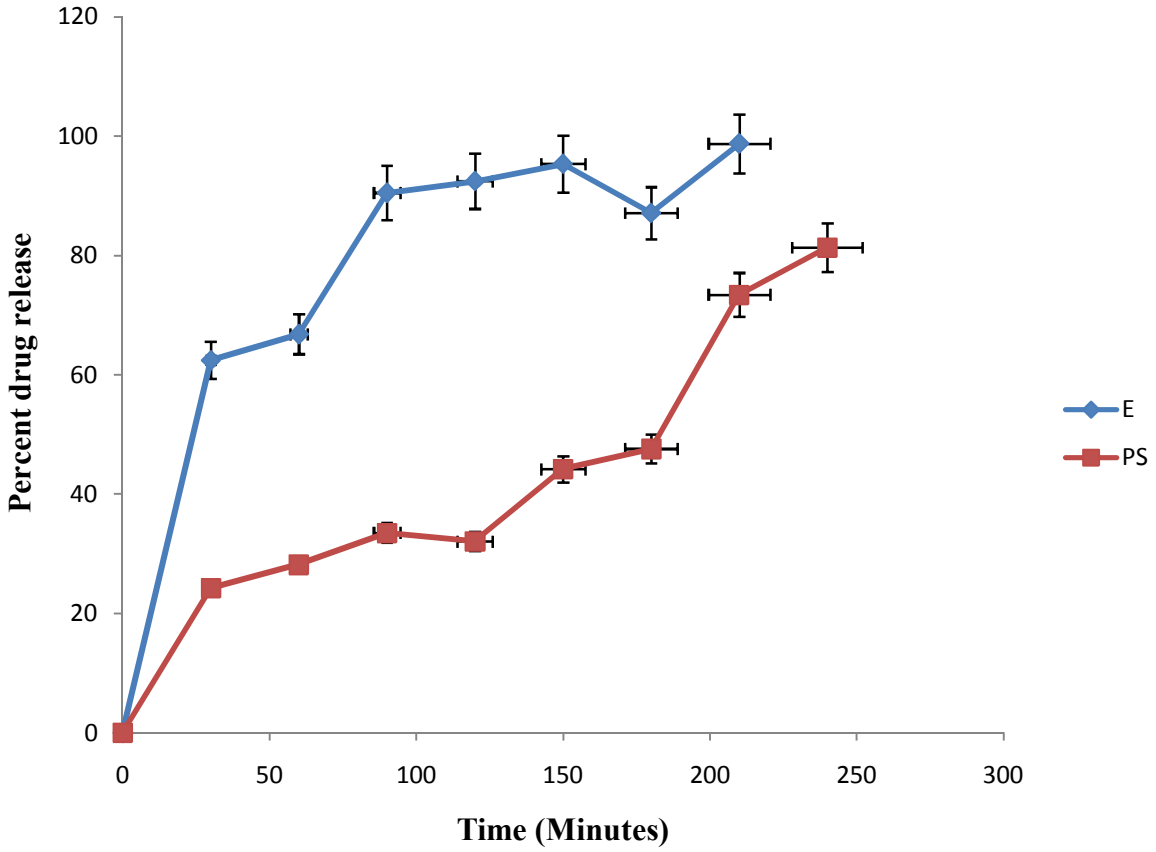


Figure 30: ***In vitro*** **release of artesunate from artesunate and amodiaquine suppositories prepared with Phospholipon®90 G / Softisan®154/castor oil (PS) and PEG 4000 / Phospholipon®90 G / Softisan®154/ castor oil (E)**

3.11 Kinetics of drug release from formulated amodiaquine and artesunate suppositories

Figures 31-42 represent the kinetics of the release of amodiaquine and artesunate from the suppositories.

The release of amodiaquine from suppositories prepared with PEG 4000 / Phospholipon®90 G / Softisan®154/ castor oil (E) is best described as following zero order, while the release of amodiaquine from Phospholipon®90 G / Softisan®154/castor oil (PS) and PEG 4000 and castor oil (P) suppositories is best described by the Higuchi diffusion model.
The release of artesunate from P and PS formulations is best described as following zero order. The release of artesunate from E formulation is best described by the Higuchi diffusion model. This implies that the blood levels of amodiaquine and artesunate from E and P, PS respectively, follow the zero order kinetics, wherein blood levels of the drugs would remain constant throughout the delivery period; this is ideal for both controlled and sustained delivery of drugs [158]. This also implies that the release of amodiaquine from PS and P formulations, and the release of artesunate from E formulation is diffusion- controlled.

Higuchi plots were found to be of highest linearity with regression coefficient (r^2) (0.959) greater than that of zero order (0.907) [157].

It has been reported that an ideal matrix formulation should contain polymers and diluents at amounts as little as possible, as well as releasing its content in a sustained release profile over a reasonable length of time preferably with zero order kinetics [159-160]. The results of the kinetics of drug release from the suppositories are therefore acceptable.

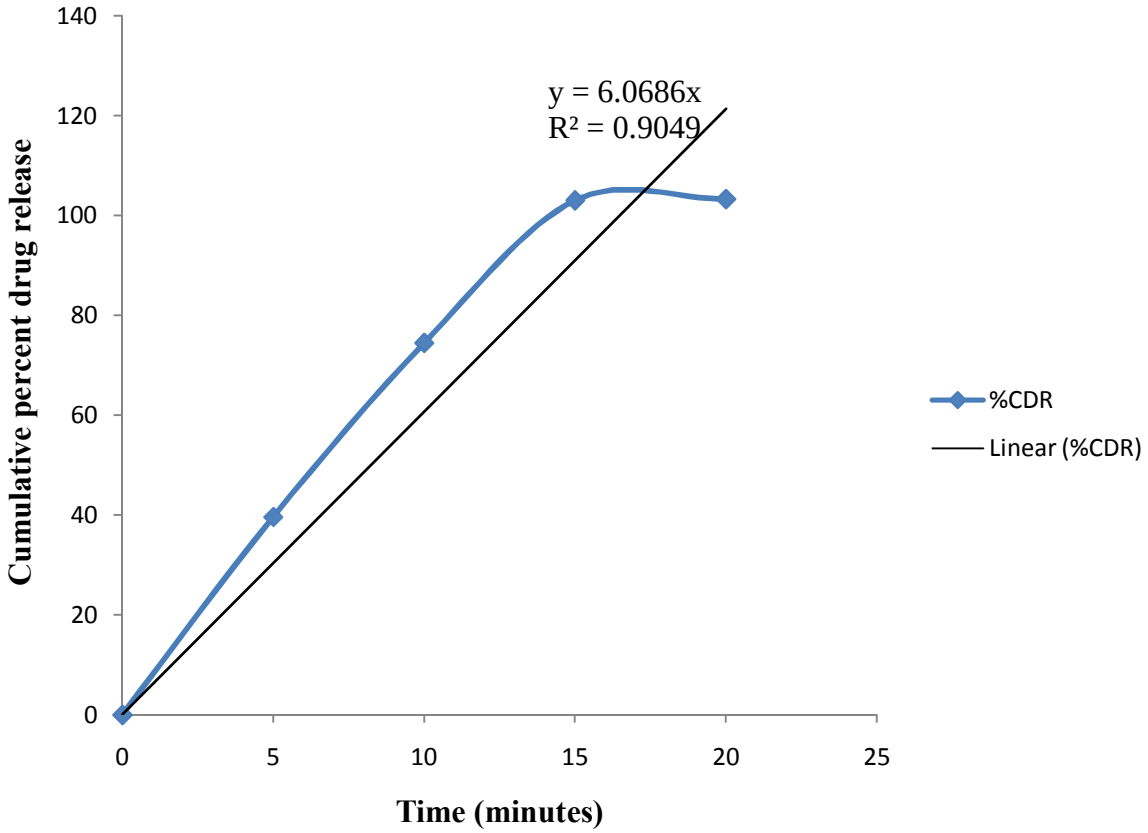


Figure 31: Zero order release profile of amodiaquine from artesunate and amodiaquine suppositories formulated with PEG 4000 and castor oil

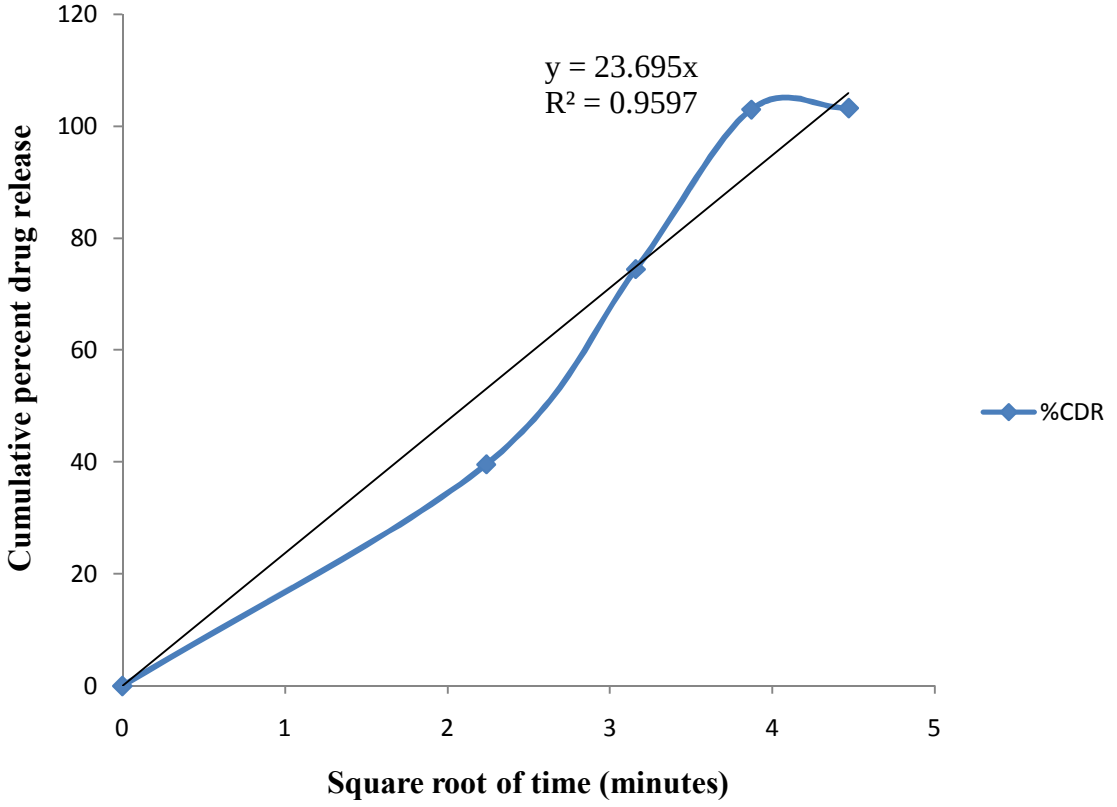


Figure 32: Higuchi release profile of amodiaquine from artesunate and amodiaquine suppositories formulated with PEG 4000 and castor oil

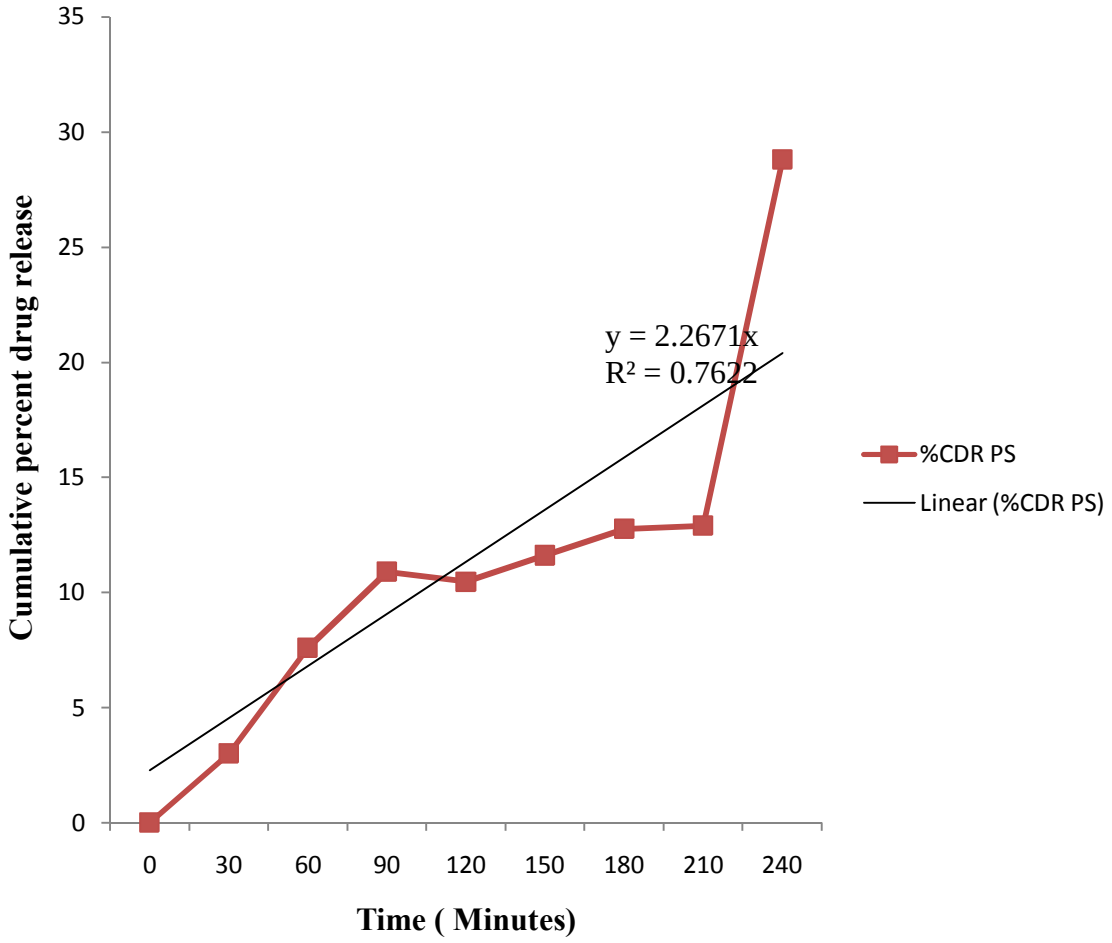


Figure 33: Zero order release profile of amodiaquine from artesunate and amodiaquine suppositories formulated with Phospholipon®90G, Softisan®154 and castor oil (PS)

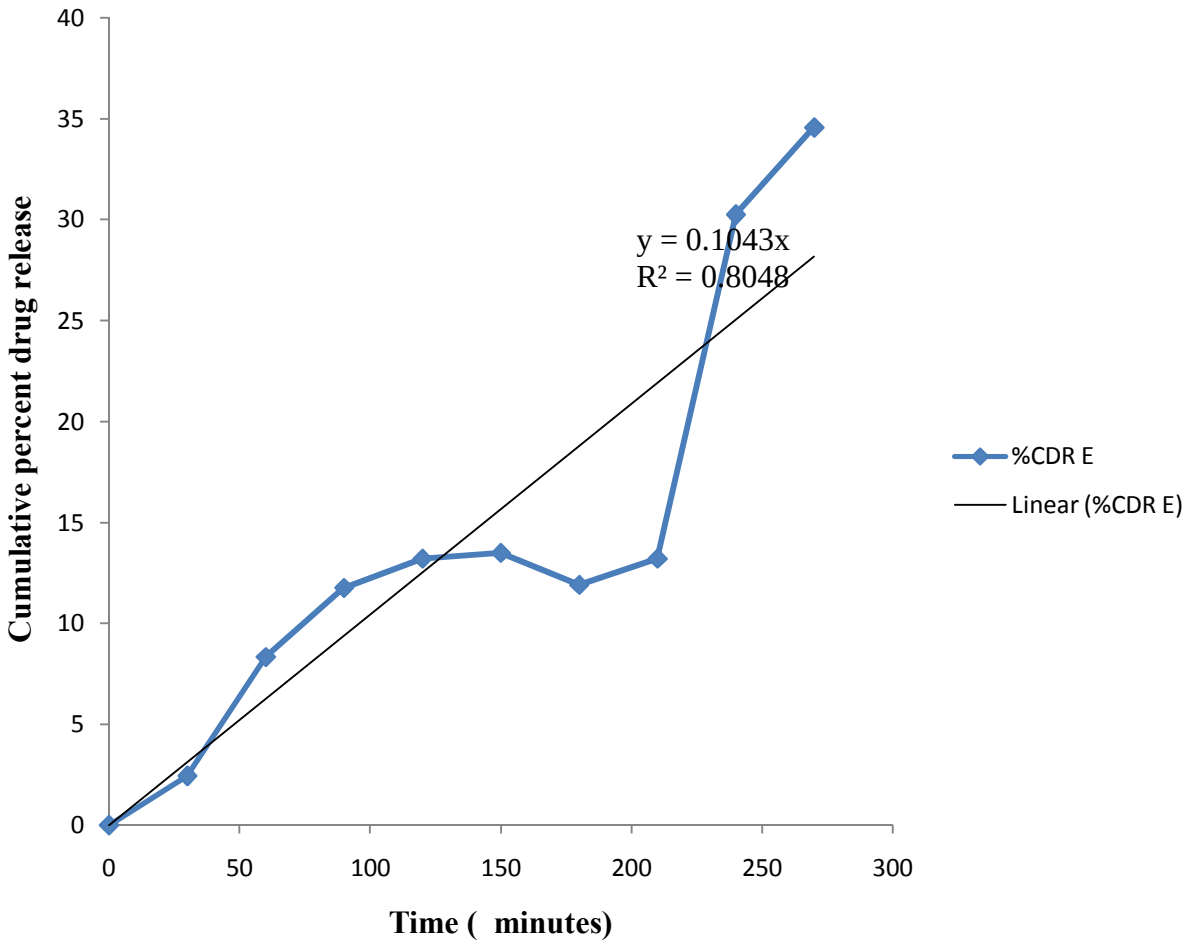


Figure 34: Zero order release profile of amodiaquine from artesunate and amodiaquine suppositories formulated with PEG 4000, Phospholipon®90G, Softisan®154 and castor oil (E)

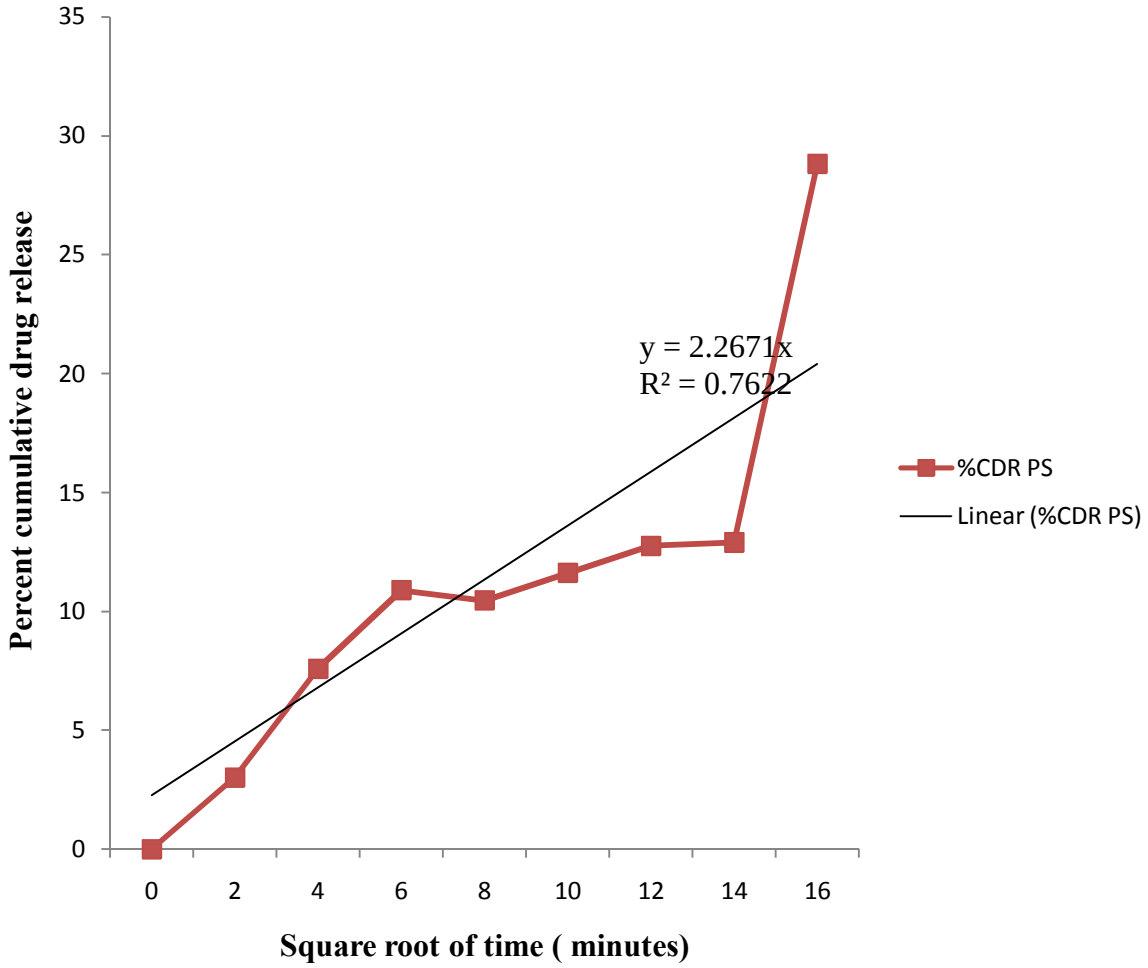


Figure 35: Higuchi release profile of amodiaquine from artesunate and amodiaquine suppositories formulated with Phospholipon®90G, Softisan®154 and castor oil (PS)

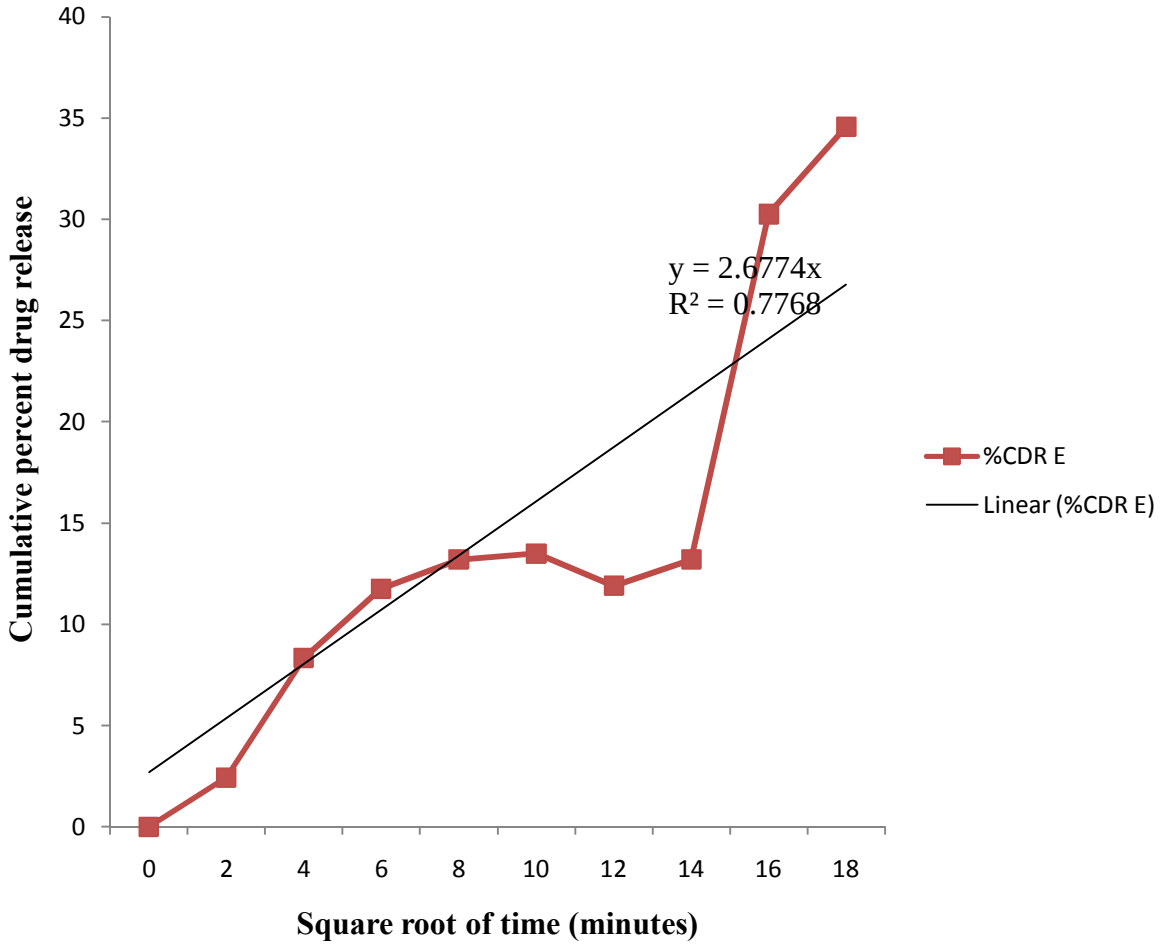


Figure 36: Higuchi release profile of amodiaquine from artesunate and amodiaquine suppositories formulated with PEG 4000, Phospholipon® 90G, Softisan ®154 and castor oil (E)

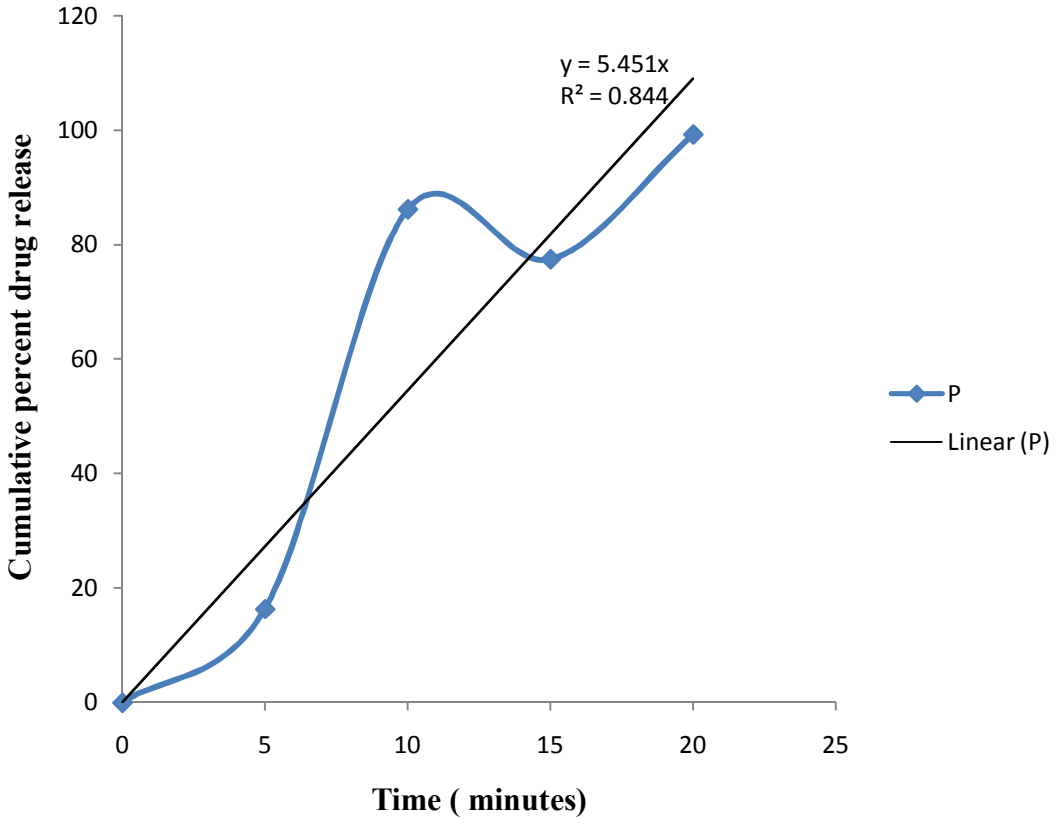


Figure 37: Zero order release profile of artesunate from artesunate and amodiaquine suppositories formulated with PEG 4000 and castor oil

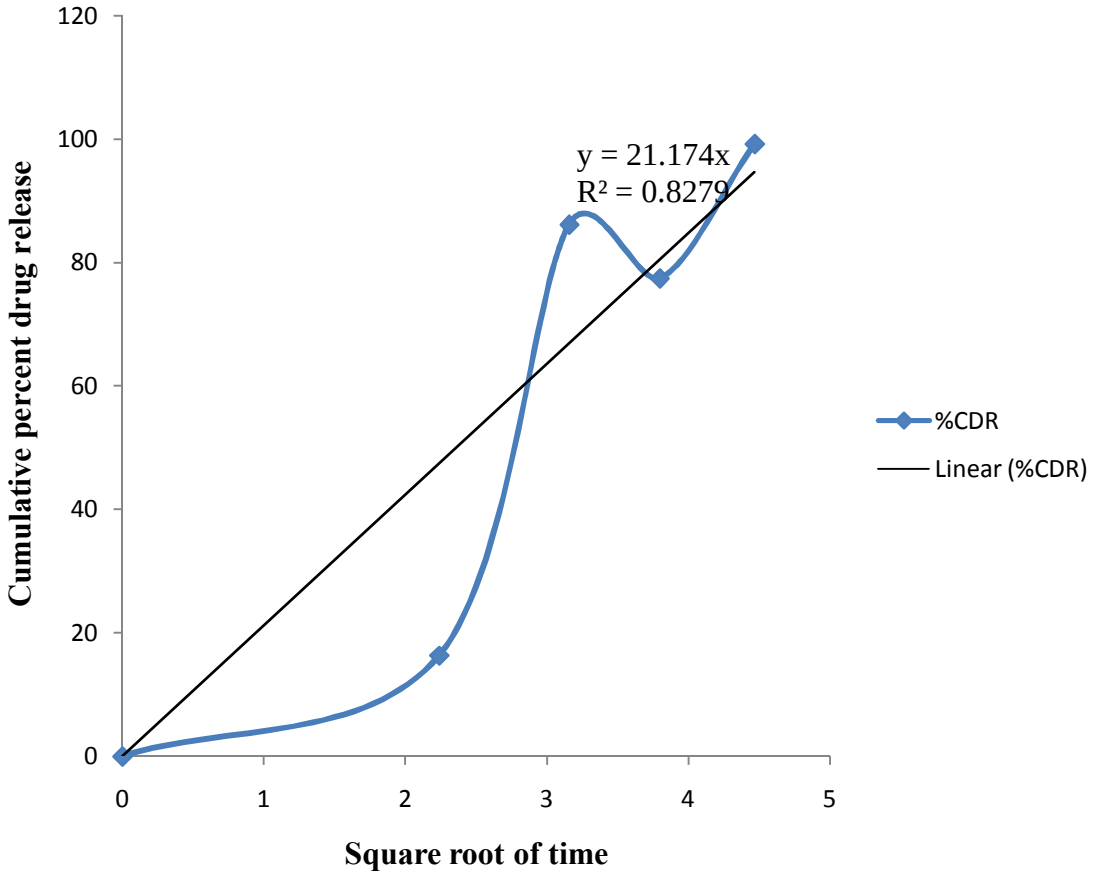


Figure 38: Higuchi release profile of artesunate from artesunate and amodiaquine suppositories formulated with PEG 4000 and castor oil

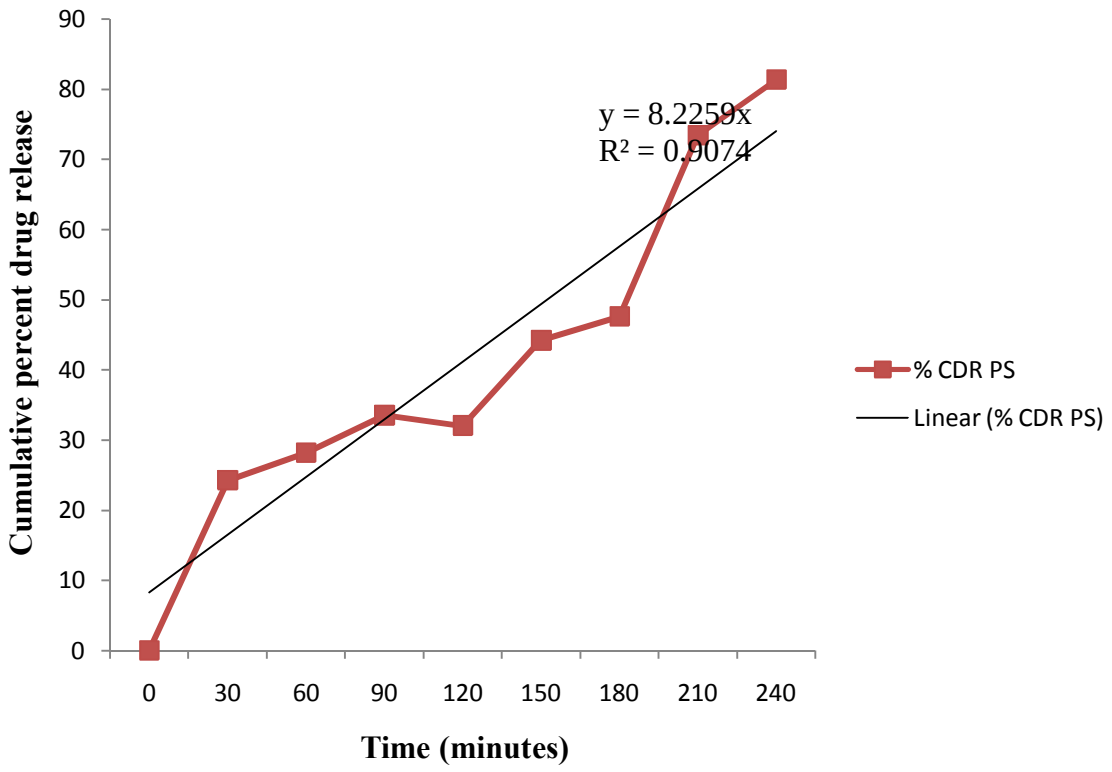


Figure 39: Zero order release profile of artesunate from artesunate and amodiaquine suppositories formulated with Phospholipon®90G, Softisan®154, and castor oil (PS)

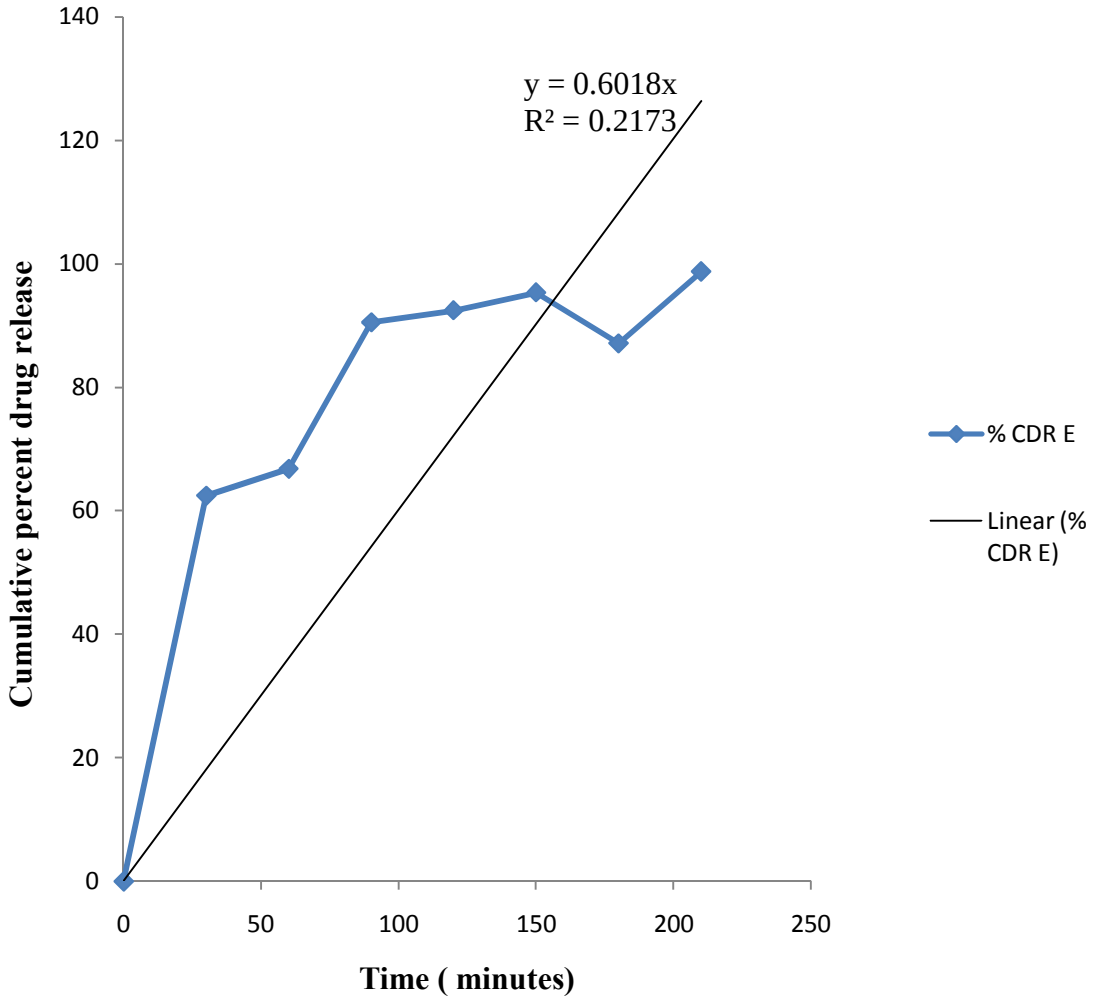


Figure 40: Zero order release profile of artesunate from artesunate and amodiaquine suppositories formulated with PEG 4000, Phospholipon®90G, Softisan®154 and castor oil (E)

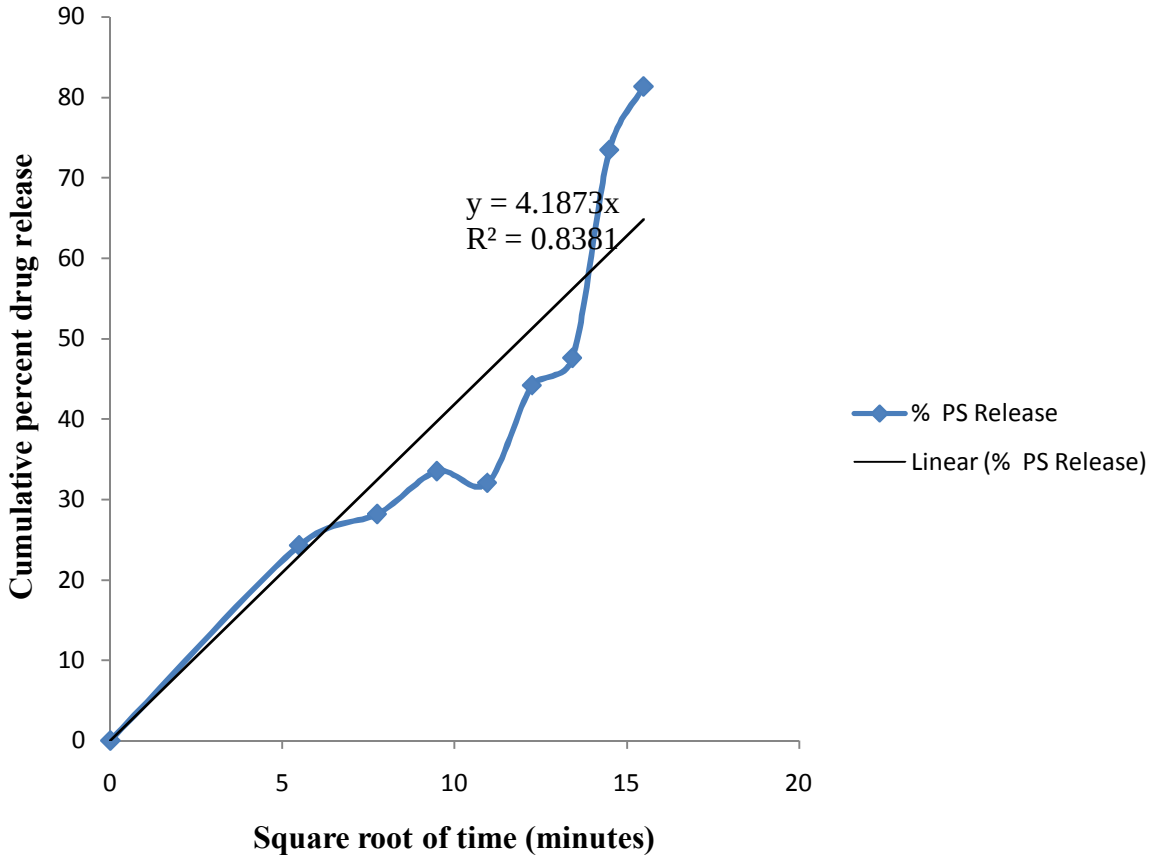


Figure 41: Higuchi release profile of artesunate from artesunate and amodiaquine suppositories formulated with Phospholipon®90G, Softisan®154 and castor oil (PS)

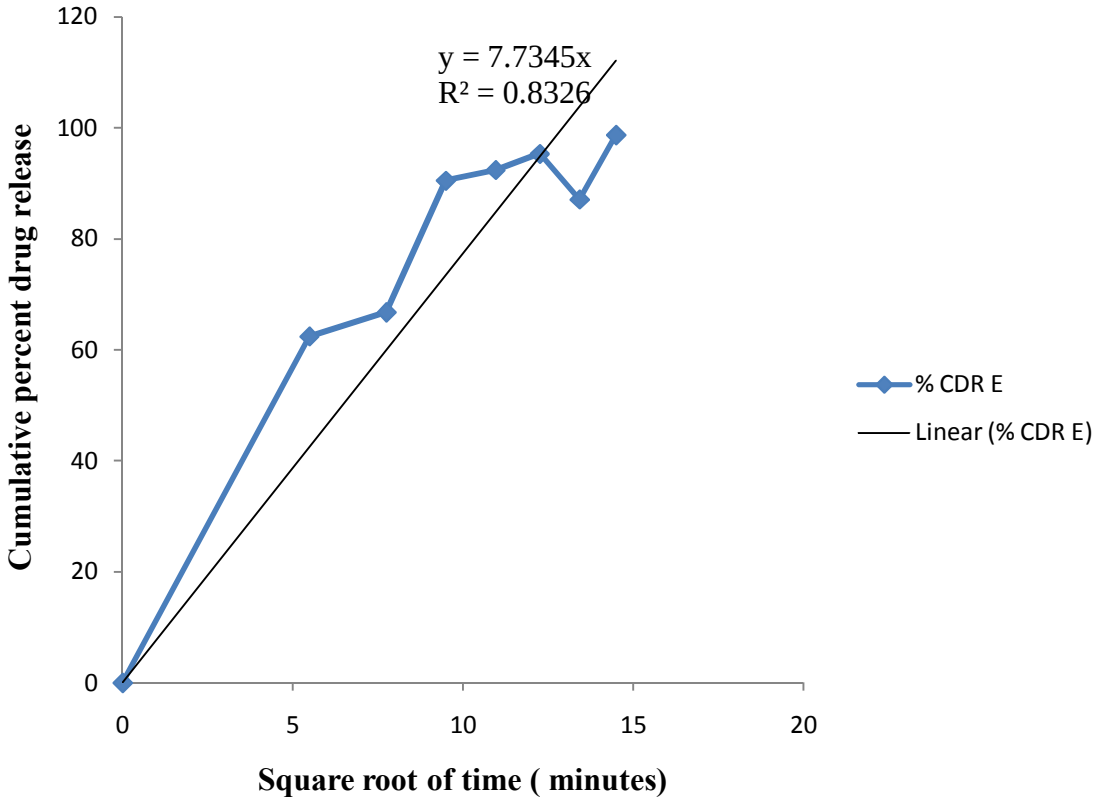


Figure 42: Higuchi release profile of artesunate from artesunate and amodiaquine suppositories formulated with PEG 4000, Phospholipon®90G, Softisan ®154 and castor oil (E)

3.12 Result of morphology analysis

The results of the morphology of artesunate, amodiaquine, the excipients and the formulated suppositories are presented in Figures 43 to 50. The crystal structures of amodiaquine particles were needle-shaped and had streaks of yellow, pink, green, and blue colours embedded in them. The crystalline structures of artesunate particles were circular and chain-like in shape. The photomicrograph of the formulated suppositories showed that there was a proper blend between the artesunate, amodiaquine and excipients used in the formulation.

3.13 Results of pharmacodynamic evaluation: *In vivo* antimalarial efficacy testing in *Plasmodium berghei*- infected mice

3.13.1 Suppressive effect of suppositories on *Plasmodium berghei*

The results of the suppressive effect of the artesunate and amodiaquine ï loaded suppositories against *P. berghei*- infected mice are presented in Tables 13 and 14.

On day 1 post infection, the suppressive effect of the suppositories of the treatment groups relative to the non- treatment groups was significant (p ’ 0.05) for PEG 4000/ castor oil suppositories, Phospholipon ®90 G/Softisan® 154/ castor oil suppositories and Phospholipon® 90G/ Softisan ®154/ PEG 4000/ castor oil suppositories. The suppressive effect of the suppositories on the treatment groups relative to the groups given the blank suppositories was also significant (p ’ 0.05) for PEG 4000/castor oil suppositories and Phospholipon ®90 G/ Softisan ®154/ PEG 4000/ castor oil suppositories.

On day 3 post infection, the suppressive effect of the suppositories of the treatment groups relative to the non-treatment groups was significant (p ’ 0.05) for PEG 4000/castor oil suppositories, Phospholipon ®90G/ Softisan ®154/ castor oil suppositories and, Phospholipon 90® G/ Softisan® 154/PEG 4000 / castor oil suppositories. The suppressive effect of the suppositories on the treatment groups relative to the groups given the blank suppositories was also significant (p ’ 0.05) for PEG 4000/ castor oil suppositories, Phospholipon®90 G/ Softisan®154/ castor oil suppositories and, Phospholipon® 90G/ Softisan® 154/PEG 4000 / castor oil suppositories.

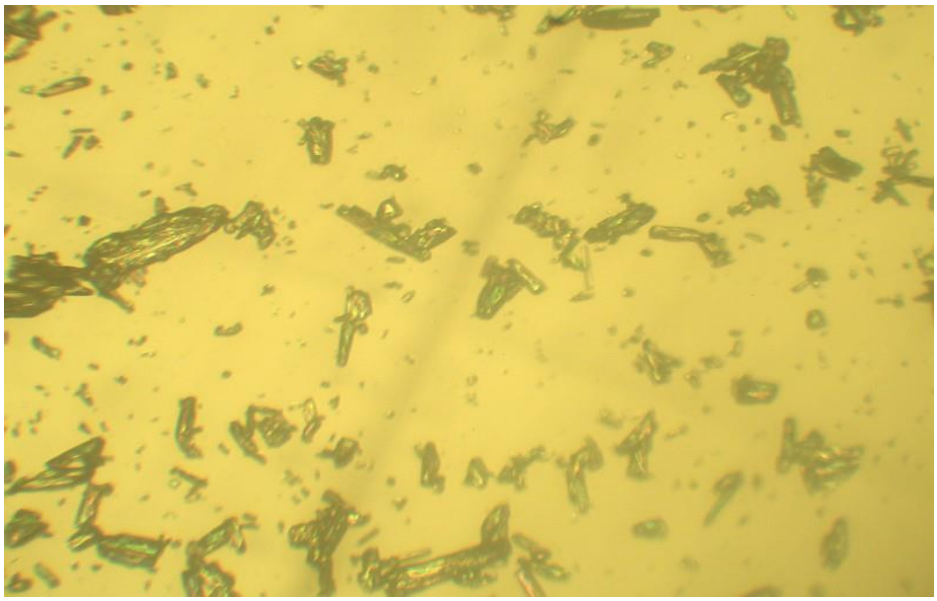


Figure 43: Photomicrograph of amodiaquine hydrochloride

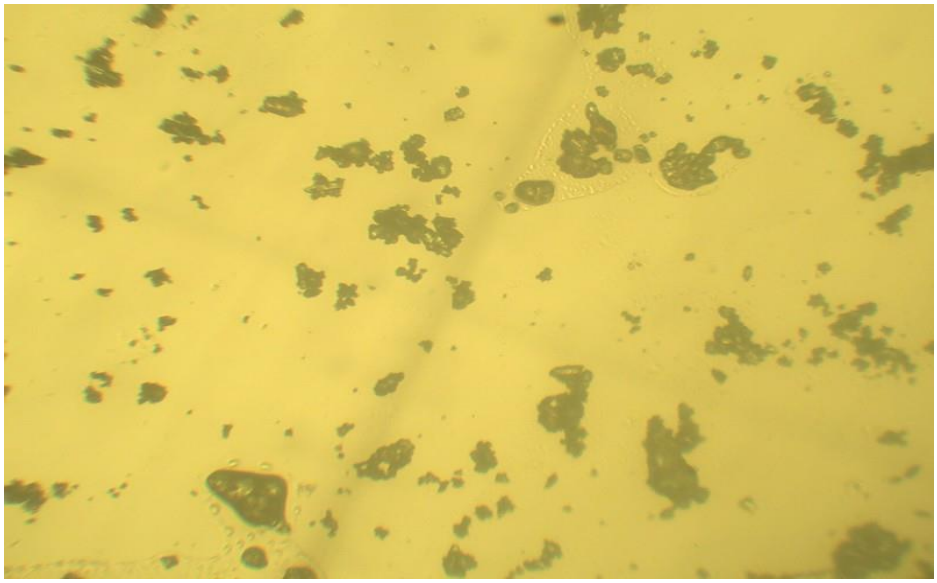


Figure 44: Photomicrograph of artesunate

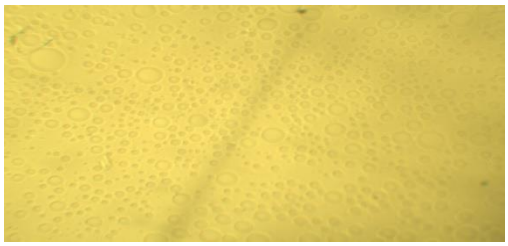


Figure 45

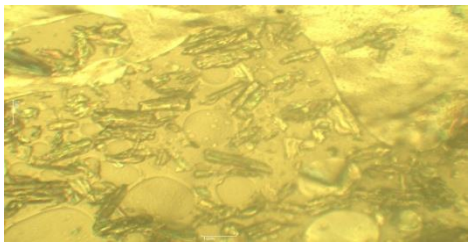


Figure 46

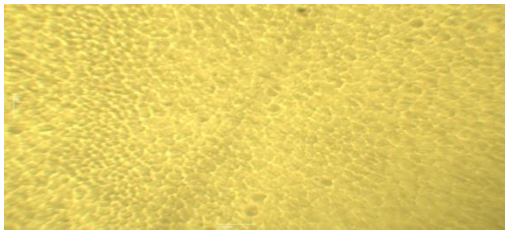


Figure 47

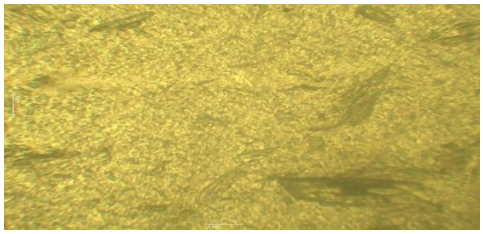


Figure 48

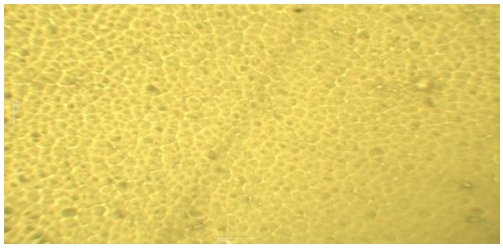


Figure 49

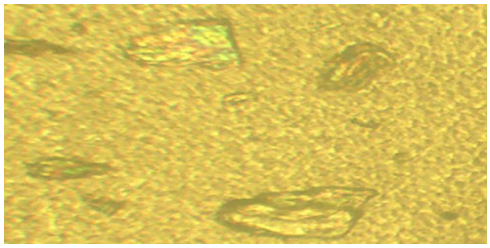


Figure 50

Figure 45: Photomicrograph of blank suppository prepared with PEG 4000/ castor oil

Figure 46: Photomicrograph of artesunate and amodiaquine suppository prepared with PEG 4000 / castor oil

Figure 47: Photomicrograph of blank suppository prepared with Phospholipon®90G/ Softisan ®154/castor oil

Figure 48: Photomicrograph of artesunate and amodiaquine suppository prepared with Phospholipon®90G/ Softisan®154/castor oil

Figure49: Photomicrograph of blank suppository prepared with PEG 4000/ Phospholipon®90G/ Softisan ®154/ castor oil

Figure 50: Photomicrograph of artesunate and amodiaquine suppository prepared with PEG 4000/Phospholipon®90G/ Softisan®154/castor oil

On day 4 post infection, the suppressive effect of the suppositories on the treatment groups relative to the non-treatment groups was significant (p ’ 0.05) for PEG 4000/ castor oil suppositories, Phospholipon®90G/Softisan®154/castor oil suppositories and, Phospholipon 90G /Softisan ® 154 /PEG4000/castor oil suppositories. The suppressive effect of the suppositories on the treatment groups relative to the groups given the blank suppositories was also significant (p ’ 0.05) for PEG 4000/ castor oil suppositories, Phospholipon® 90 G/ Softisan ®154/ castor oil suppositories and Phospholipon®90 G/ Softisan®154/PEG 4000 / castor oil suppositories. On day 28 post-infection, zero parasite parasitemia was observed for animals that survived. This implies that the suppositories cleared the parasites and prevented recrudescence as well.

Figure 51 shows the slide of the blood smear of the donor mouse from which the parasite was obtained. The dark spots around the erythrocytes show the extent of the destruction of the erythrocytes by the *Plasmodium berghei*.

The spleen is an important blood filter that removes abnormal erythrocytes. There are phagocytic cells in the spleen which act during the invasion of the erythrocytes by the malaria parasite. Abnormal erythrocytes are removed if they are not as flexible as the normal erythrocytes and consequently are unable to squeeze through the slits between the endothelial cells that line the splenic sinuses. As a result of this, several structural changes occur in the spleen, which may include an increase in the size of the spleen (splenomegaly) [188,189]. However, if the malaria parasite is suppressed effectively during treatment, there may be no manifestation of splenomegaly.

Figure 52(a) shows the marked splenomegaly which occurred in the untreated mouse compared to the normal spleen size observed in the treated mouse (Figure 52 (b)). This implies that the formulated artesunate and amodiaquine suppositories suppressed the *Plasmodium berghei* effectively.

3.13.2 Percent activity of formulated artesunate and amodiaquine suppositories

The percent activity of the formulated suppositories is shown in Figure 53.

The percent activity was determined by comparison of the mean parasitemia of the treated group to the mean parasitemia of the respective control groups, on days 1,3,and 4 after infection [135].

There was a progressive increase in the percent activity of the artesunate and amodiaquine suppositories prepared with PEG 4000 and castor oil (35.71%, 50.00%, and 71.76% on days 1, 3 and 4 respectively). There was also a progressive increase in the percent activity of the artesunate and amodiaquine suppositories prepared with Phospholipon® 90 G, Softisan® 154 and castor oil (32.45 %, 61.59%, 82.47% on days 1, 3, and 4 respectively). However, the percent activity of the artesunate and amodiaquine suppositories prepared with Phospholipon® 90 G, Softisan® 154 , PEG 4000 and castor oil showed an initial increase, followed by a slight decline (8.33, 68.80,68.70 % on days 1,3,and 4 respectively). The slight decline could have been as a result of the prolonged release of artesunate and amodiaquine from the suppositories. Also, the slight decline in activity may be attributed to the erratic absorption of drugs from the rectum.

These *in vivo* findings further reiterate the efficacy of the three batches of the formulated artesunate and amodiaquine suppositories (PEG 4000/ castor oil suppositories, PEG 4000/ Softisan® 154/ Phospholipon® 90G / castor oil suppositories, and Phospholipon® 90G / Softisan® 154/castor oil suppositories) in reducing the parasite load in *Plasmodium berghei*-infected mice.

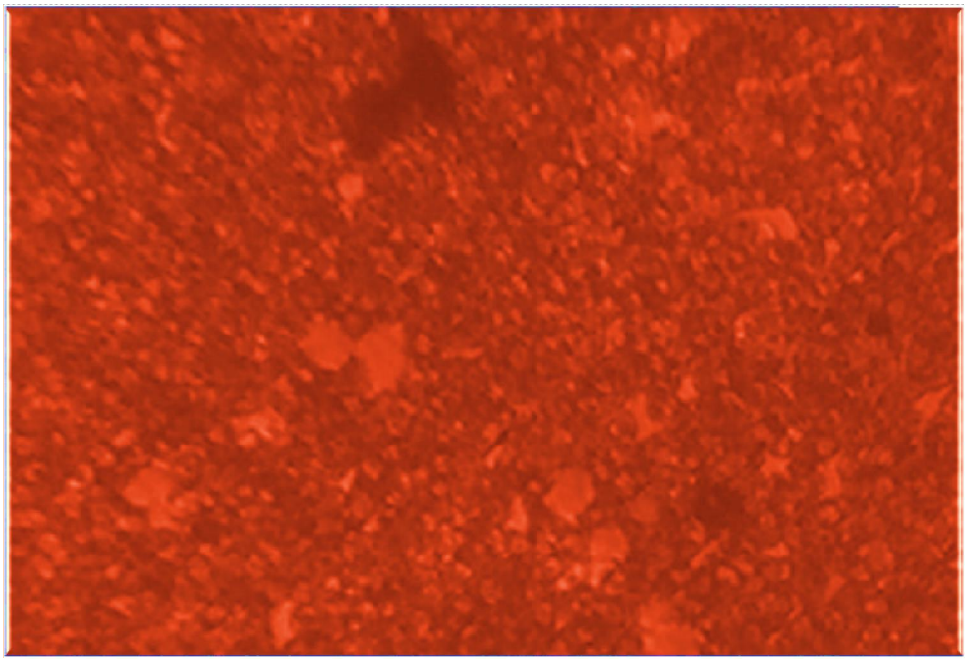


Figure 51: Photomicrograph of slide of blood smear of donor mouse

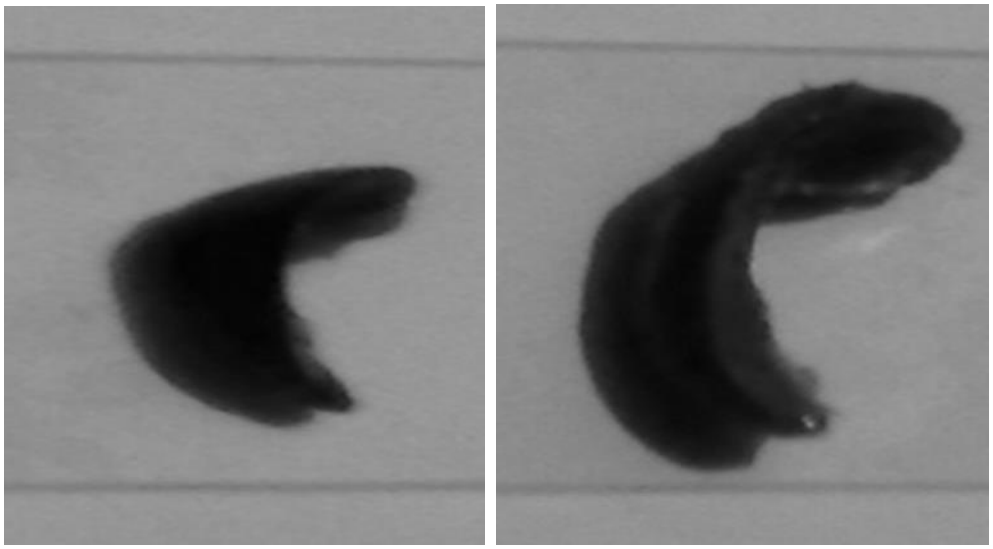


Figure 52 (a) **Figure 52 (b)**

Figure 52: Marked splenomegaly in *Plasmodium berghei* –infected mice (in non-treatment group) - Figure 52 (a) when compared with *Plasmodium berghei* –infected mice (in treatment group) – Figure 52 (b) 15 days after infection.

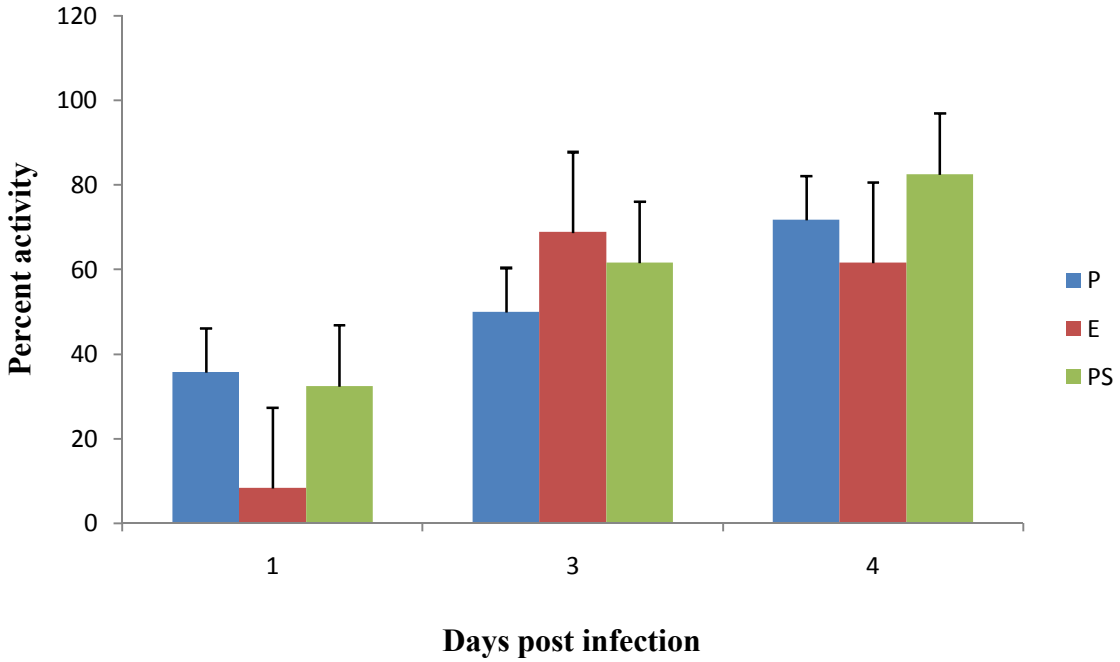


Figure 53: Percent activity of the percent activity of the three batches of artesunate and amodiaquine suppositories against *Plasmodium berghei*- infected mice

Code:

P: Animals administered with PEG 4000 /castor oil suppositories of artesunate and amodiaquine

E: Animals administered with PEG 4000/Softisan® 154/Phospholipon® 90G/ castor oil suppositories of artesunate and amodiaquine

PS: Animals administered with Phospholipon® 90G/ Softisan® 154/castor oil suppositories of artesunate and amodiaquine

Table 13: Suppressive effect of artesunate-amodiaquine loaded suppositories against *Plasmodium berghei* in mice

Suppository treatment (n=5)	Mean parasite count ± SEM		
	Day 1	**Day 3**	**Day 4**
Nil treatment	25.60±4.00	31.40±2.82	19.00±2.97
PEG 4000 loaded artesunate-amodiaquine	18.00±2.26[a,b]	11.80±2.13[a,b]	6.00±0.82[a,b]
PEG 4000+Softisan®154+Phospholipon® 90G loaded artesunate-amodiaquine	17.60±1.18[a,b]	7.80±1.20[a,b]	5.20±0.58[a,b]
Softisan® 154+Phospholipon®90G loaded artesunate-amodiaquine	20.40±2.71[b]	10.60±0.41[a,b]	4.00±0.77[a,b]
PEG 4000 / Softisan ®154 / Phospholipon® 90G only	19.20±0.92[b]	25.00±3.37	16.60±1.83
Softisan®154 / Phospholipon®90G only	30.20±1.74	27.60±3.88	19.4±2.52
PEG 4000 only	28.00±3.59	23.60±3.87	17.00±2.86

[a]P<0.05 vs. nil treatment group (Analysis of Variance followed by LSD *post hoc* test)

[b]P<0.05 vs. treatment with PEG only suppositories (Analysis of Variance followed by LSD *post hoc*)

n=number of mice per group

Table 14: Suppressive effect of non-drug loaded suppositories against *P. berghei* in mice

Suppository treatment (n= 5)	**Mean parasite count ± SEM**		
	Day 1	**Day 3**	**Day 4**
Nil treatment	25.60±4.00	31.40±2.82	19.00±2.97
PEG 4000 loaded artesunate-amodiaquine	18.00±2.26	11.80±2.13	6.00±0.82
PEG 4000 /Softisan® 154/ Phospholipon® 90G only	19.20±0.92	25.00±3.37	16.60±1.83
Softisan®154 / Phospholipon®90G only	30.20±1.74	27.60±3.88	19.4±2.52
PEG 4000 only	28.00±3.59	23.60±3.87	17.00±2.86

[a]P<0.05 vs. nil treatment group (Analysis of Variance followed by LSD *post hoc*)

[b]P<0.05 vs. treatment with PEG only suppositories (Analysis of Variance followed by LSD *post hoc*)

n=number of mice per group

3.13.3 Result of the survival trend test of *Plasmodium berghei* –infected mice

The result of the survival trend of the experimental animals is presented in Figure 54. On day 55 post infection day, the survival rate was 100 %, 100 %, 80 % for mice administered with, artesunate-amodiaquine loaded PEG 4000/ castor oil suppositories, Phospholipon®90 G/ Softisan®154/ PEG 4000/castor oil suppositories, Phospholipon®90 G/Softisan® 154 /castor oil suppositories, respectively. The survival rate for the control groups were 20, 0, 20, and 0 %, for mice administered with, blank PEG 4000/castor oil suppositories, blank Phospholipon 90 G/ Softisan®154/ PEG 4000/castor oil suppositories, blank Phospholipon®90 G/ Softisan®154 / castor oil suppositories and the nil treatment group respectively. The mice that survived after being administered with blank suppositories post- infection may have acquired immunity against *P. berghei*.

3.13.4 Result of hemoglobin estimation

The results of the hemoglobin test conducted on the surviving animals 55 days post infections are presented in Figure 55. It was earlier stated clearly that anemia is one of the complications of severe malaria, since severe malaria causes massive destruction of red blood cells. Therefore, the hemoglobin and hematocrit tests were done to evaluate the efficacy of the suppressive effect of the artesunate and amodiaquine-loaded suppositories and also to detect recrudescence. The animals that survived were compared with non-infected mice.

The normal range of hemoglobin concentration for mice is 12.1 to 15.0 g/dL [186]. The hemoglobin concentration of the animals treated with PEG 4000 /castor oil, and those treated with Phospholipon®90G/ Softisan®154/castor oil suppositories were within the normal concentration (12.8 and 13.73 g/dL respectively). However, the hemoglobin concentration of the animals treated with PEG 4000, Phospholipon® 90G/Softisan®154/castor oil was slightly lower than the normal (11.6).

The hemoglobin concentration of the infected mice administered with blank PEG 4000/castor oil suppositories was lower than that of the treatment group (9.4 g/dL). However the hemoglobin

concentration of the infected mice administered with blank PEG 4000/Phospholipon® 90G/Softisan®154/castor oil suppositories was as high as that of the treatment group (12.6 g/dL). The hemoglobin concentration of the non-infected mice (control group) was higher than that of the treatment groups.

The non-treatment group that survived could have acquired immunity against *Plasmodium berghei.* However, the results show that artesunate and amodiaquine suppositories were efficacious in the treatment of malaria although further studies need to be done on them. The mice that survived and maintained a normal hemoglobin concentration after being administered with blank suppositories post infection may have acquired immunity against *P. berghei.*

3.13.5 Result of hematocrit Estimation

The drop in the PCV that is responsible for malarial anemia occurs both through an increase in the rate at which old red blood cells are broken and a decrease in the rate at which new ones are produced. Plasmodium not only causes the rupture of parasitized red blood cells, but also stimulates the activity of macrophages in the spleen, which then destroys both parasitized and un-parasitized red blood cells [161].

The result of the hematocrit test is presented in Figure 56. The normal value of PCV for mice is between 39.5 and 50.6% [186]. The hematocrit value of the animals treated with PEG 4000 /castor oil, and those treated with Phospholipon® 90G/ Softisan® 154/castor oil suppositories were within the normal values (41.6 and 45.5 % respectively). However, the hematocrit value of the animals treated with PEG 4000, Phospholipon® 90G/Softisan® 154/castor oil was slightly lower than the normal (38.4%). The hematocrit values of the infected mice administered with blank PEG/Castor oil suppositories and blank PEG 4000/Phospholipon® 90G/Softisan® 154/castor oil suppositories were as high as that of the treatment group (44 and 51 % respectively). The hemoglobin concentration of the non-infected mice (control group) was as high as that of the treatment groups (43.2%). The non-treatment group that survived could have acquired immunity against *Plasmodium berghei.* However, more research work could be done to investigate the hematological effects of artesunate and amodiaquine suppositories.

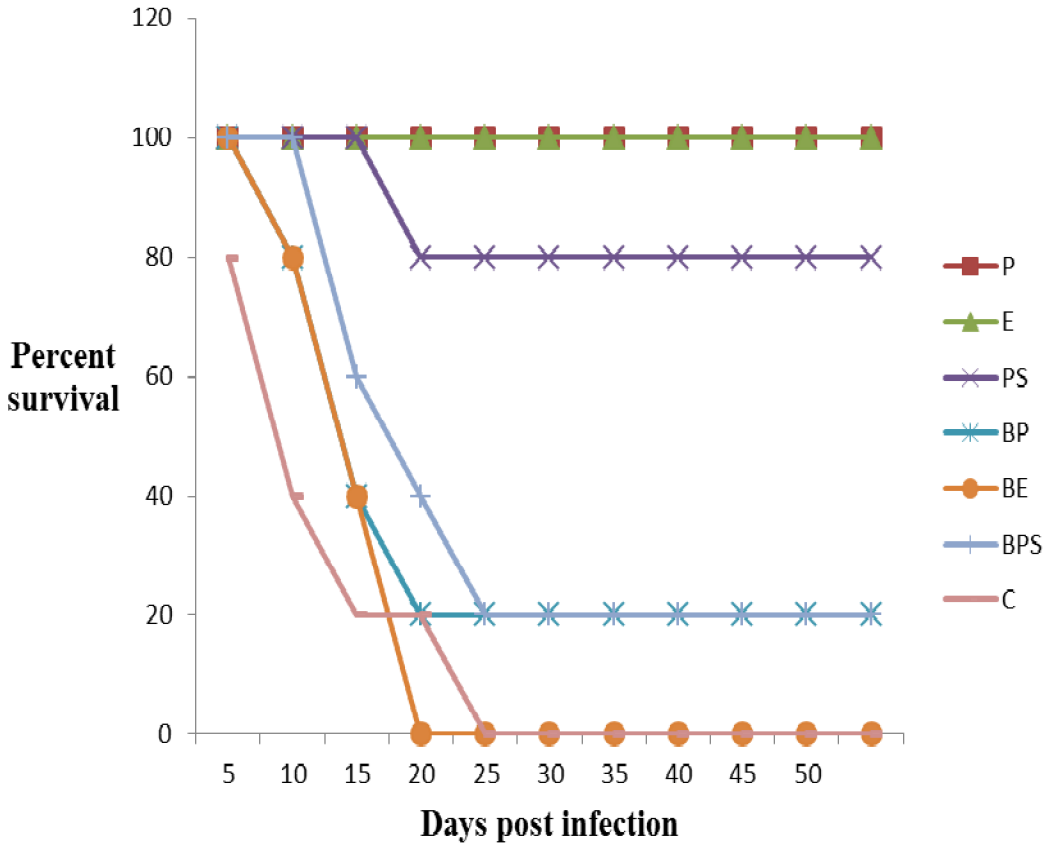


Figure 54: Survival plot for the various treatment and control groups of experimental animals

Code:

P: Animals administered with PEG 4000 /castor oil suppositories of artesunate and amodiaquine

E: Animals administered with PEG 4000/Softisan® 154/Phospholipon ®90G/castor oil suppositories of artesunate and amodiaquine

PS: Animals administered with Phospholipon® 90G/ Softisan®154/castor oil suppositories of artesunate and amodiaquine

BP: Animals administered with blank PEG 4000/castor oil suppositories

BE: Animals administered with blank PEG 4000/Softisan ®154/Phospholipon ®90G/castor oil suppositories

BPS: Animals administered with blank Phospholipon® 90G/ Softisan ®154/castor oil suppositories

C: Control group

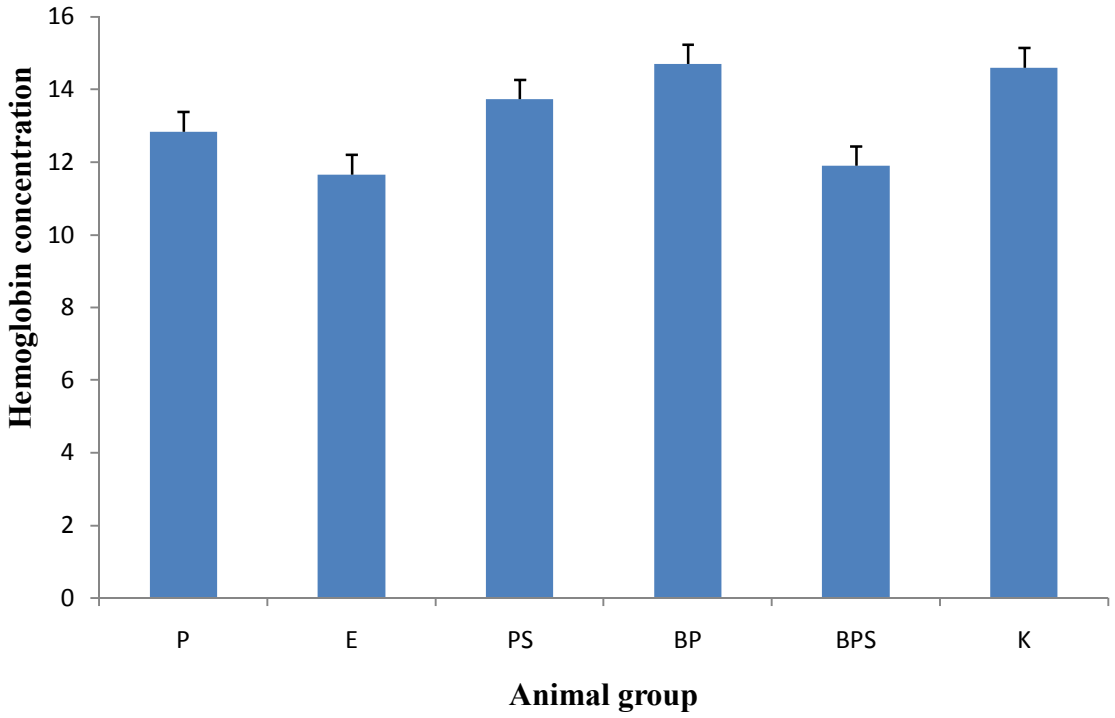


Figure 55: Hemoglobin estimation of mice 55 days post- infection

Code:

P: Animals administered with PEG 4000 /castor oil suppositories of artesunate and amodiaquine

E: Animals administered with PEG 4000/Softisan® 154/Phospholipon®90G/castor oil suppositories of artesunate and amodiaquine

PS: Animals administered with Phospholipon® 90G/ Softisan®154/castor oil suppositories of artesunate and amodiaquine

BP: Animals administered with blank PEG 4000/castor oil suppositories

BPS: Animals administered with blank Phospholipon® 90G/ Softisan®154/castor oil suppositories

K: Control group

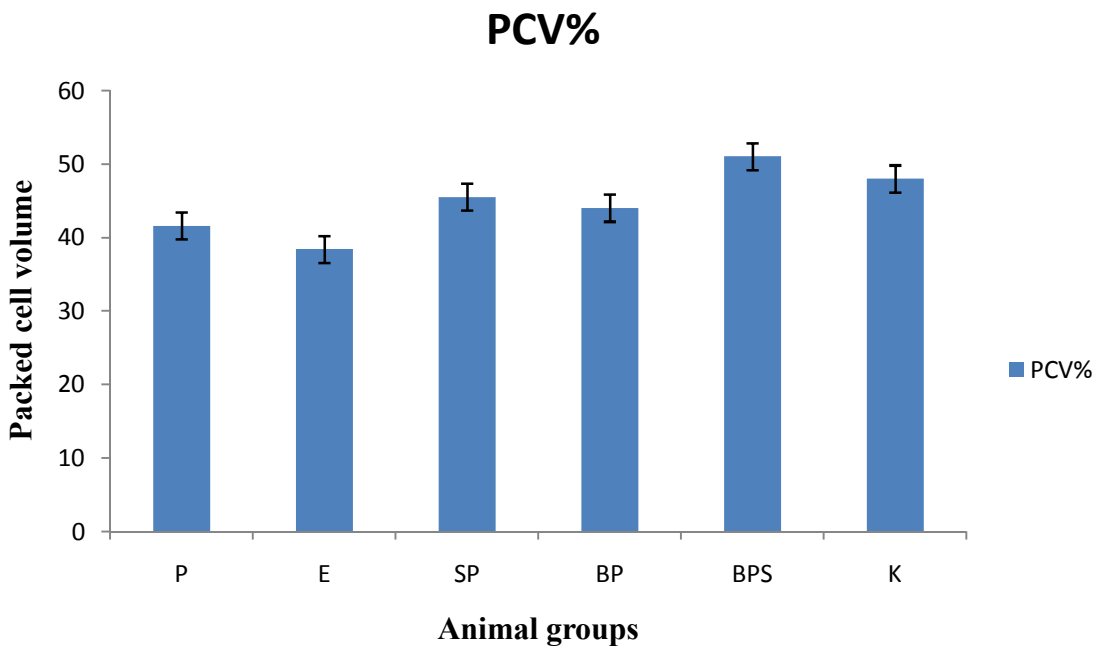


Figure 56: Hematocrit estimation of mice 55 days post infection

Code:

P: Animals administered with PEG 4000 /castor oil suppositories of artesunate and amodiaquine

E: Animals administered with PEG 4000/Softisan® 154/ Phospholipon®90G/Castor oil suppositories of artesunate and amodiaquine

PS: Animals administered with Phospholipon 90®G/ Softisan® 154/castor oil suppositories of artesunate and amodiaquine

BP: Animals administered with blank PEG 4000/castor oil suppositories

BPS: Animals administered with blank Phospholipon® 90G/ Softisan® 154/castor oil suppositories

K: Control group

3.14 Result of stability studies

The DSC curves of the stability studies are presented in Appendix 7.

Figures 7(a)-7(c) and 7(d)-7(f) show the DSC curves of the 3 batches of the formulated artesunate and amodiaquine suppositories stored at 27 ^{o}C and 4 ^{o}C respectively for 6 months. Figure 7(a) shows two peaks at 65.3 ^{o}C and 166.7 ^{o}C. This could be as a result of the storage of this particular suppository at 27 ^{o}C, resulting in the crystallization of amodiaquine hydrochloride at 166.7 ^{o}C [180]. All the other DSC curves of the stability study (Figures 7(b),7(c),7(d),7(e),7(f)) showed singular peaks. This could be said to be due to the dissolution of the drugs into the excipients which took place during the thermal analysis, thereby masking the sharp peaks of the drugs as described in earlier researches on the thermal properties of the artesunate and amodiaquine combination [185]. However, it was observed that the peak temperatures of the suppositories stored at 27 ^{o}C (Figures 7(a)-7(c)) showed higher peaks at (65.3 ^{o}C,62.6 ^{o}C, 64.1 ^{o}C) respectively when compared with the suppositories stored at 4 ^{o}C (Figures 7(d)-7(e),which showed lower peaks at (61.7 ^{o}C,59.6 ^{o}C,64.0 ^{o}C), respectively.

This implies that the optimum temperature for the storage of the suppositories is 4 ^{o}C. Hence, the shelf-life of the formulated suppositories could be at least 6 months.

CHAPTER FOUR
CONCLUSION

Artesunate and amodiaquine suppositories were successfully prepared by melt- moulding (fusion) method using Phospholipon®90G, Softisan® 154, PEG 4000 and castor oil.

There was absence of fissuring, pitting, exudation and fat blooming in the formulated artesunate and amodiaquine suppositories. All the suppositories had acceptable results with regard to the uniformity of weight standards. The artesunate and amodiaquine suppositories formulated with PEG 4000 and castor oil softened and completely disintegrated within 20 minutes. The artesunate and amodiaquine suppositories formulated with Phospholipon®90 G/Softisan® 154/PEG 4000/ castor oil softened between 210 and 270 minutes while the artesunate and amodiaquine suppositories formulated with Phospholipon®90 G/ Softisan® 154 /castor oil softened within 240 minutes.

The release of amodiaquine from E formulation showed zero order kinetics, while the release of amodiaquine from PS and P formulations showed Higuchi kinetics. The release of artesunate from PEG 4000/castor oil and Phospholipon®90 G/ Softisan® 154 /castor oil suppositories followed zero order. The release of artesunate from Phospholipon®90 G/Softisan® 154/PEG 4000/ castor oil suppositories followed the Higuchi diffusion model. Higuchi plots were found to be of highest linearity with regression coefficient (r^2) of 0.959 greater than that of zero order (0.907).

The crystal structures of amodiaquine particles were needle-shaped and had streaks of yellow, pink, green, and blue colours embedded in them. The crystalline structures of artesunate particles were circular and chain-like in shape.

The percent activity of the formulated artesunate and amodiaquine suppositories ranged from 68.70 to 82.47% three days after infection. This confirms the efficacy of the suppositories.

On day 28 post-infection, zero parasitemia was observed for the animals that survived the test. This implies that the suppositories cleared the parasites and prevented recrudescence as well.

On day 55 post infection, the average survival rate was 93.3 % for mice administered with artesunate-amodiaquine suppositories while the average survival rate for the nil- treatment groups was 8 %. The large difference between the survival rate of the treatment and non-treatment group further emphasizes the efficacy of the formulated artesunate and amodiaquine suppositories.

It was observed during a six-month stability study that 4 ^{o} C was the optimum temperature for the storage of the artesunate and amodiaquine suppositories. The suppositories were relatively stable after storage for 6 months.

In conclusion, artesunate and amodiaquine suppositories were efficacious in the treatment of malaria; however, there is need for further studies in animals and humans.

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APPENDICES

Appendix 1(a): Spectrophotometric data for Beer-Lambert's plot of amodiaquine hydrochloride in phosphate buffer p H 7.0 at 335nm

Concentration (µg/ml)	Absorbance
2	0.055
3	0.102
4	0.125
5	0.146
6	0.174
7	0.212
8	0.251
9	0.274
10	0.302

Appendix 1(b): Spectrophotometric data for Beer-Lambert's plot of artesunate (after basic decomposition) at 315 nm

Concentration (µg/ml)	Absorbance
0.01	0.034
0.02	0.038
0.03	0.051
0.04	0.081
0.05	0.100

Appendix 1(c): Spectrophotometric data for Beer-Lambert's plot of amodiaquine hydrochloride in the presence of artesunate in phosphate buffer p H 7.0 at 335nm

Concentration (µg/ml)	Absorbance
1	0.117
2	0.140
3	0.209
4	0.239
5	0.321
6	0.366
7	0.454
8	0.497
9	0.526
10	0.607

Appendix 1(d): Spectrophotometric data for Beer-Lambert's plot of artesunate in the presence of amodiaquine hydrochloride at 315nm

Concentration (µg/ml)	Absorbance
2	0.079
4	0.103
6	0.156
8	0.210
10	0.243

Appendix 2(a): *In vitro* drug release studies of amodiaquine in artesunate – amodiaquine suppositories formulated with PEG 4000 and castor oil (P)

Time (minutes)	Percent Drug Release
0	0.00
5	39.59
10	74.44
15	102.98
20	103.22

Appendix 2(b): *In vitro* drug release studies of amodiaquine in artesunate – amodiaquine suppositories formulated with, Phospholipon ®90 G, Softisan ®154, PEG 4000 and castor oil (E)

Time (minutes)	Percent Drug Release of E
0	0.00
30	2.44
60	8.33
90	11.76
120	13.20
150	13.49
180	11.90
210	13.20
240	30.26
270	34.57

Appendix 2(c): *In vitro* drug release studies of amodiaquine in artesunate – amodiaquine suppositories formulated with, Phospholipon® 90 G, Softisan ®154, and castor oil (PS)

Time (minutes)	Percent release of PS
0	0.00
30	3.01
60	7.59
90	10.89
120	10.46
150	11.61
180	12.76
210	12.90
240	28.81

Appendix 2(d): *In vitro* drug release studies of artesunate in artesunate – amodiaquine suppositories formulated with PEG 4000 and castor oil (P)

Time (minutes)	Percent Drug Release of P
0	0.00
5	16.33
10	86.15
15	77.43
20	99.21

Appendix 2(e): *In vitro* drug release studies of artesunate in artesunate – amodiaquine suppositories formulated with, Phospholipon®90 G, Softisan® 154, PEG 4000 and castor oil (E)

Time (minutes)	Percent Drug Release of E
0	0.00
30	62.43
60	66.79
90	90.50
120	92.44
150	95.34
180	87.11
210	98.73

Appendix 2 (f): *In vitro* drug release studies of artesunate in artesunate – amodiaquine suppositories formulated with, Phospholipon ®90 G, Softisan ®154, and castor oil (PS)

Time (minutes)	Percent Drug Release of PS
0	0.00
30	24.27
60	28.16
90	33.50
120	32.04
150	44.17
180	47.57
210	73.42
240	81.31

Appendix 3(a): Parasite count of *P. berghei* –infected mice administered with artesunate – amodiaquine suppositories prepared with PEG 4000 and castor oil (group A).

		Parasite count		
Group A	Animal mark	Day 1	Day 3	Day 4
	1	18%	13%	4%
	2	13%	9%	5%
	3	13%	5%	8%
	4	22%	16%	3%
	5	24%	16%	4%
Mean		18%	11.8%	4.8%

Appendix 3(b): Parasite count of *P. berghei* –infected mice administered with artesunate – amodiaquine suppositories prepared with PEG 4000, Softisan® 154, Phospholipon® 90G and castor oil (group B).

		Parasite count		
Group B	Animal mark	Day 1	Day 3	Day 4
	1	21%	11%	5%
	2	16%	9%	4%
	3	15%	5%	6%
	4	17%	5%	4%
	5	19%	9%	7%
Mean		17.6%	7.8%	5.2%

Appendix 3 (c):Parasite count of *P. berghei* –infected mice administered with artesunate – amodiaquine suppositories prepared with Softisan® 154, Phospholipon® 90G and castor oil (group C).

		Parasite count		
Group C	Animal mark	Day 1	Day 3	Day 4
	1	22%	4%	4%
	2	18%	5%	3%
	3	30%	5%	3%
	4	18%	18%	3%
	5	14%	21%	7%
Mean		20.4%	10.6%	3.4%

Appendix 3(d): Parasite count of *P. berghei* –infected mice administered with non-medicated suppositories prepared with PEG 4000, and castor oil (group D)

		Parasite count		
Group D	Animal mark	Day 1	Day 3	Day 4
	1	19%	16%	16%
	2	36%	38%	12%
	3	28%	24%	13%
	4	36%	22%	16%
	5	21%	18%	28%
Mean		28%	23.6%	17%

Appendix 3(e): Parasite count of *P. berghei* –infected mice administered with non-medicated suppositories prepared with PEG 4000, Softisan® 154, Phospholipon® 90G and castor oil (group E)

		Parasite count		
Group E	Animal mark	Day1	Day 3	Day 4
	1	21%	36%	18%
	2	19%	25%	18%
	3	21%	18%	22%
	4	16%	18%	12%
	5	19%	28%	13%
Mean		19.2%	25%	16.6%

Appendix 3(f): Parasite count of *P. berghei* –infected mice administered with non-medicated suppositories prepared with softisan® 154, phospholipon® 90G and castor oil (group F)

		Parasite count		
Group F	Animal mark	Day 1	Day 3	Day 4
	1	32%	29%	28%
	2	34%	38%	17%
	3	24%	32%	22%
	4	32%	15%	14%
	5	29%	24%	16%
Mean		30.2%	27.6%	19.4%

Appendix 3 (g): Parasite count of *P. berghei* –infected mice without treatment (group G)

		Parasite count		
Group G	Animal mark	Day 1	Day 3	Day 4
	1	22%	38%	17%
	2	36%	32%	17%
	3	32%	36%	29%
	4	25%	22%	11%
	5	13%	29%	21%
Mean		25.6%	31.4%	19%

Appendix 4: Percent activity of artesunate and amodiaquine suppositories (formulated with PEG 4000, Phospholipon ®90G, Softisan ®154, and castor oil) against *Plasmodium berghei*- infected mice.

Days Post-infection	% Activity of P	%Activity of E	%Activity of PS
1	35.71	8.33	32.45
3	50	68.80	61.59
4	71.76	68.70	82.47

Code:

P: *Plasmodium berghei*-infected mice group treated with PEG 4000 suppositories of artesunate and amodiaquine

E: *Plasmodium berghei*-infected mice group treated with PEG 4000/ Softisan ®154/Phospholipon® 90G suppositories of artesunate and amodiaquine

PS: *Plasmodium berghei*-infected mice group treated with Phospholipon ®90G/ Softisan® 154 suppositories of artesunate and amodiaquine

Appendix 5: Percentage of survival for the treatment and control groups

Days post infection	% of survival of P	% of survival of E	% of survival of PS	% of survival of BP	% of survival of BE	% of survival of BPS	% of survival of C
0	100	100	100	100	100	100	100
5	100	100	100	100	100	100	80
10	100	100	100	80	80	100	40
15	100	100	100	40	40	60	20
20	100	100	80	20	0	40	20
25	100	100	80	20	0	20	0
30	100	100	80	20	0	20	0
35	100	100	80	20	0	20	0
40	100	100	80	20	0	20	0
45	100	100	80	20	0	20	0
50	100	100	80	20	0	20	0
55	100	100	80	20	0	20	0

Code:

P: *Plasmodium berghei*-infected mice group treated with PEG 4000 suppositories of artesunate and amodiaquine

E: *Plasmodium berghei*-infected mice group treated with PEG 4000/ Softisan ®154/Phospholipon® 90G suppositories of artesunate and amodiaquine

PS: *Plasmodium berghei*-infected mice group treated with Phospholipon ®90G/ Softisan® 154 suppositories of artesunate and amodiaquine

BP: *Plasmodium berghei*-infected mice group treated with blank PEG 4000 suppositories

BE: *Plasmodium berghei*-infected mice group treated with blank PEG 4000/Softisan ® 154/Phospholipon ®90G suppositories

BPS: *Plasmodium berghei*-infected mice group treated with blank Phospholipon®90G/ softisan ®154 suppositories

C: *Plasmodium berghei*-infected mice group given no treatment

Appendix 6 (a): Hemoglobin concentration (Hb) and packed cell volume (PCV) results of treatment groups of *Plasmodium berghei*- infected mice

Animal mark	PCV %	Hb (g/dl)
P 1	44	12.6
P 2	47	14.0
P 3	41	13.6
P 4	34	11.8
P 5	42	12.2
E 1	39	12.9
E 2	32	10.1
E 3	43	11.2
E 4	37	11.2
E 5	41	12.9
PS 1	41	12.2
PS 2	46	13.3
PS 3	47	14.7
PS 4	48	14.7

Code:

P: *Plasmodium berghei*-infected mice group treated with PEG 4000 suppositories of artesunate and amodiaquine

E: *Plasmodium berghei*-infected mice group treated with PEG 4000/ Softisan ®154/Phospholipon® 90G suppositories of artesunate and amodiaquine

PS: *Plasmodium berghei*-infected mice group treated with Phospholipon ®90G/ Softisan® 154 suppositories of artesunate and amodiaquine

Appendix 6 (b): Hemoglobin concentration (Hb) and packed cell volume (PCV) results of non-treatment groups of *Plasmodium berghei*- infected mice

Animal mark	PCV %	Hb (g/dl)
BP	44	9.4
BE	51	12.6
K 1	39	16.2
K 2	45	20.2
K 3	52	7.6
K 4	55	14.7
K 5	25	11.9

Code:

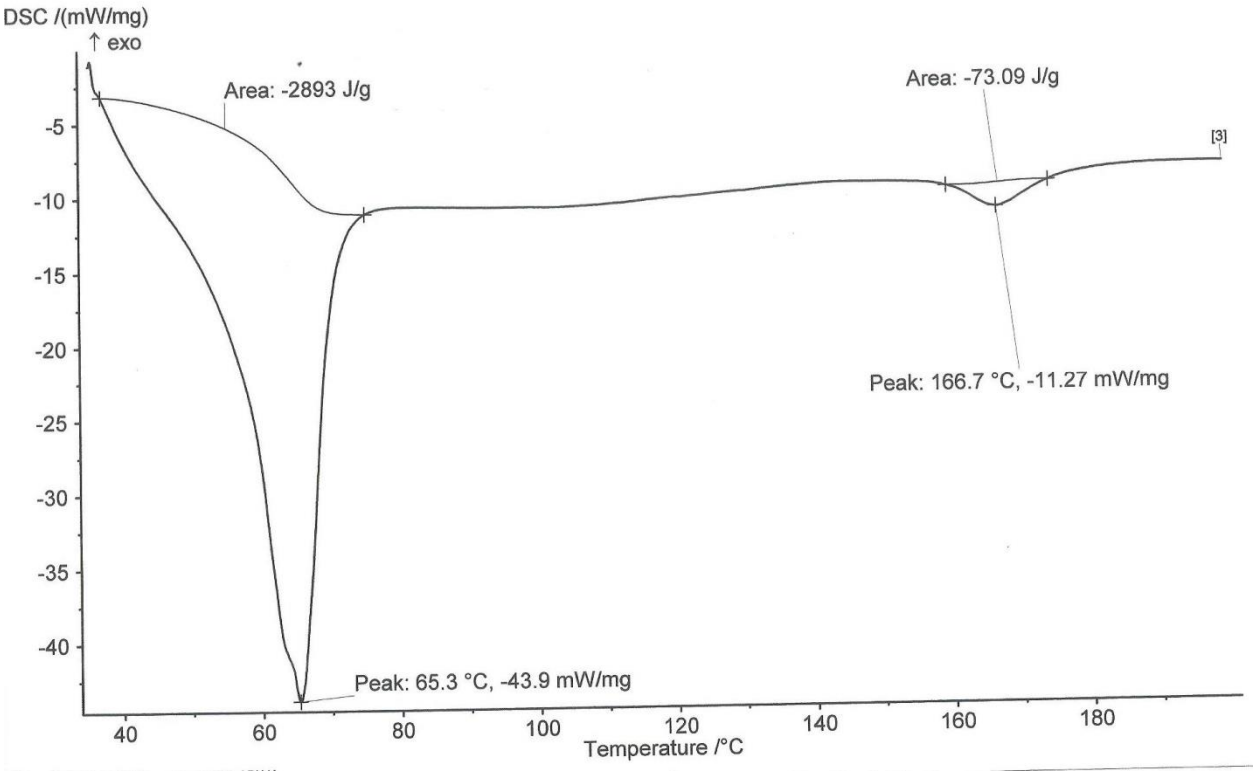
BP: *Plasmodium berghei*-infected mice group treated with blank PEG 4000 suppositories of artesunate and amodiaquine

BE: *Plasmodium berghei*-infected mice group treated with blank PEG 4000/Softisan ® 154/Phospholipon ®90G suppositories

PS: *Plasmodium berghei*-infected mice group treated with blank Phospholipon ®90G/ Softisan ®154 suppositories

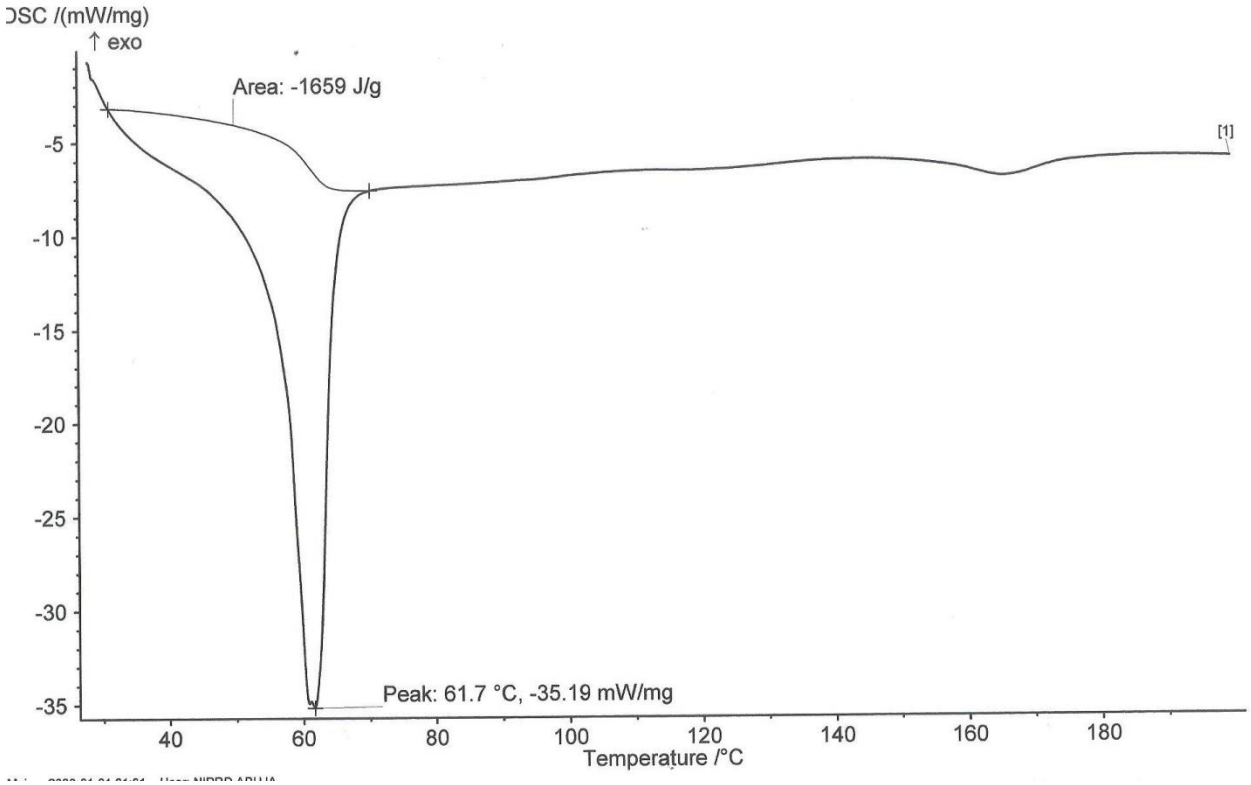
K: Non-infected mice group given no treatment

Appendix 7 (a)



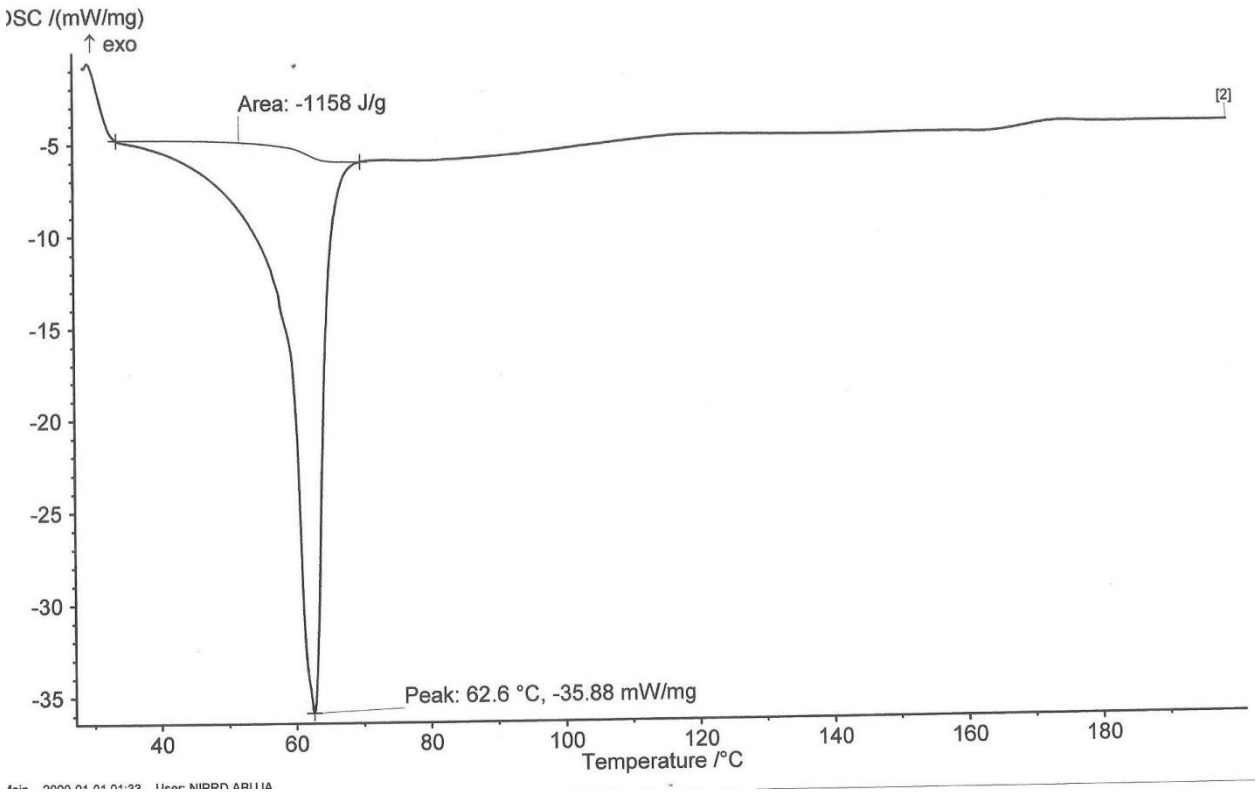
Appendix 7 (a): DSC thermogram of artesunate and amodiaquine suppositories prepared with Phospholipon®90G, Softisan®154, PEG 4000 and castor oil stored at 27 °C for six months.

Appendix 7 (b)



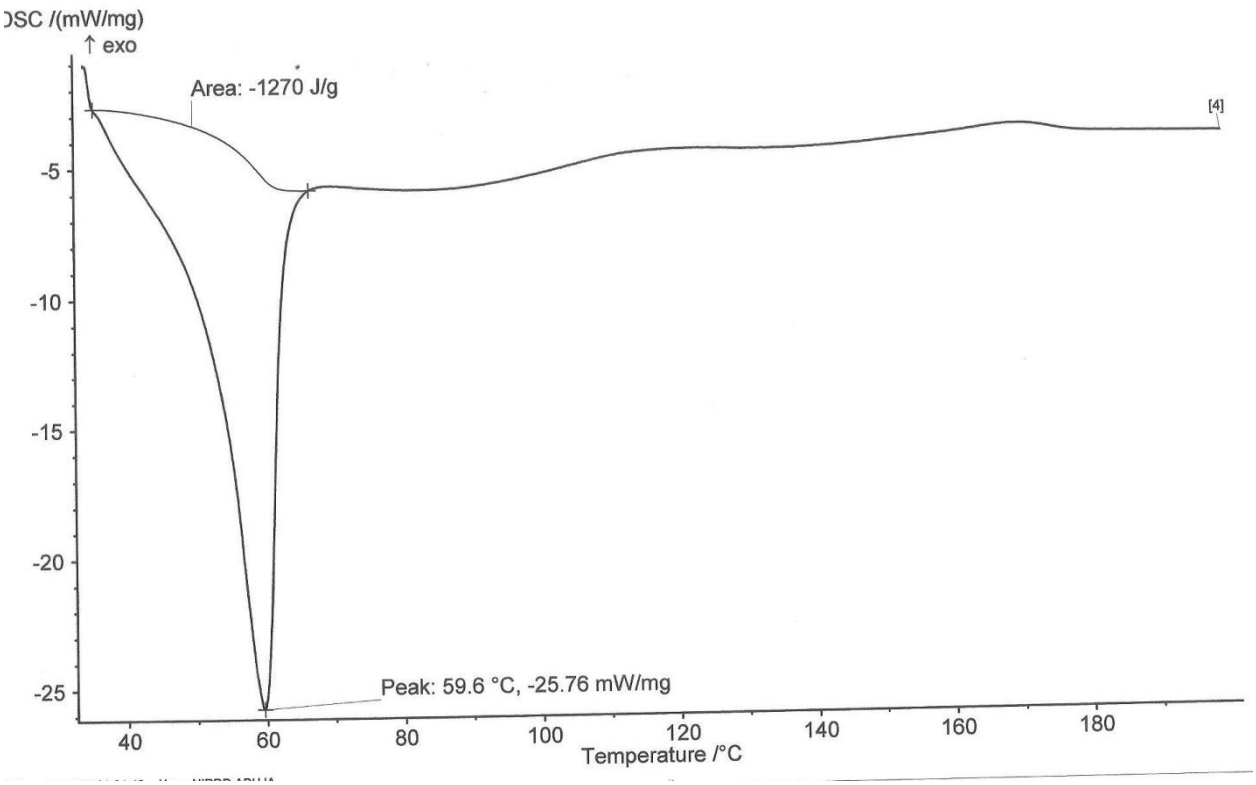
Appendix 7 (b): DSC thermogram of artesunate and amodiaquine suppositories prepared with Phospholipon®90G, Softisan®154, PEG 4000 and castor oil stored at 4 °C for six months.

Appendix 7 (c)



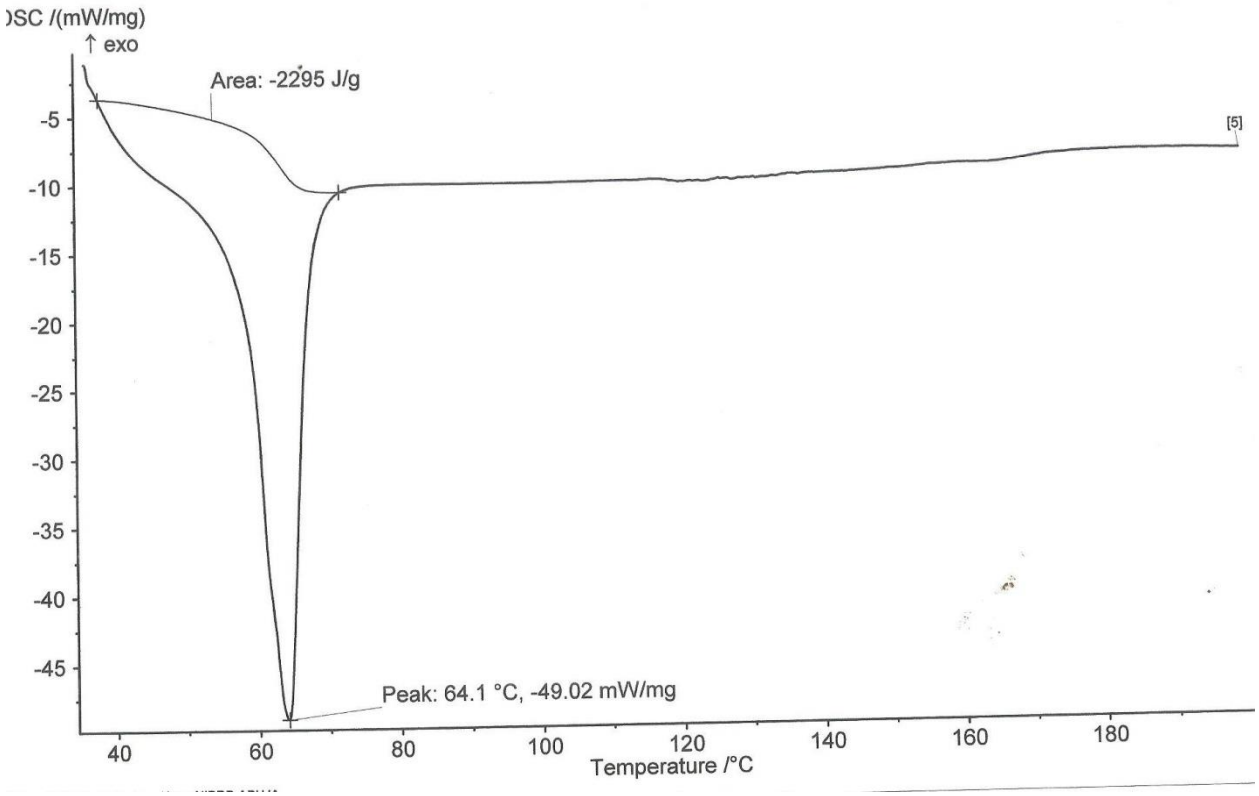
Appendix 7 (c): DSC thermogram of artesunate and amodiaquine suppositories prepared with PEG 4000 and castor oil stored at 27 ^{o}C for six months.

Appendix 7 (d)



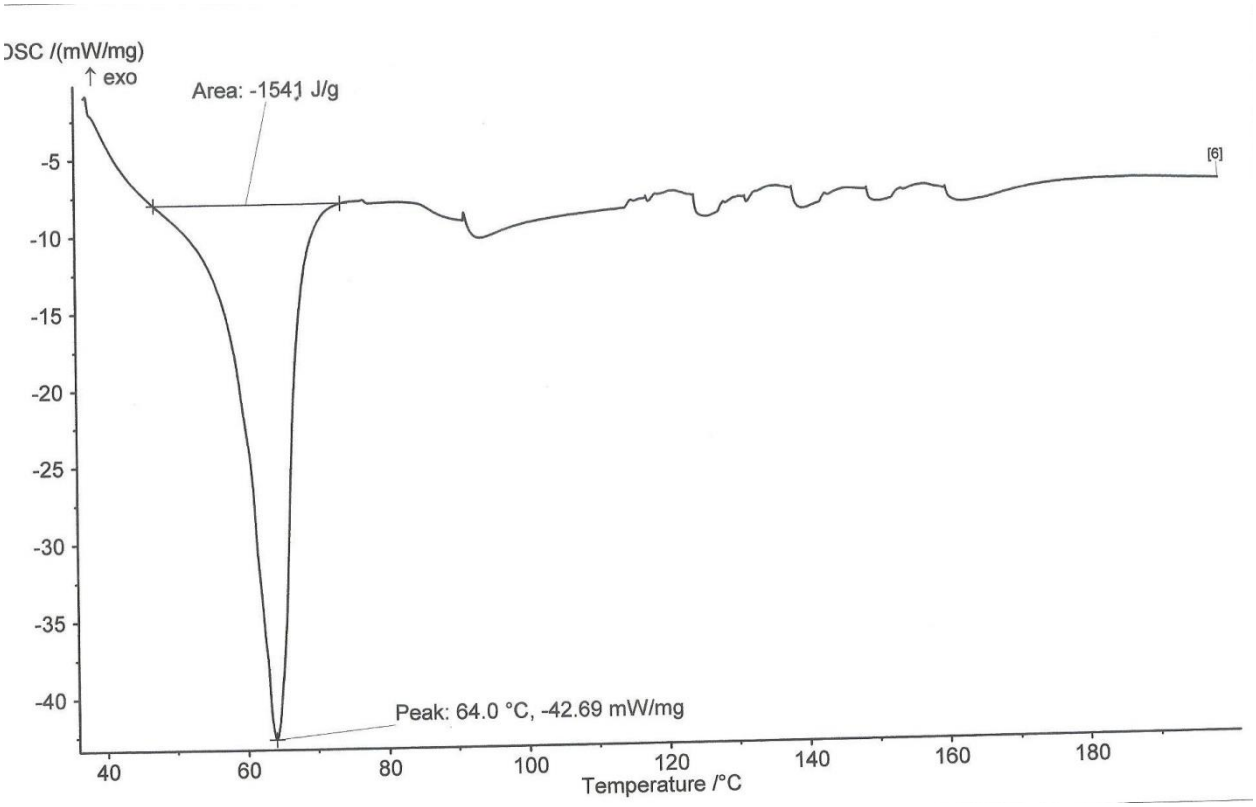
Appendix 7 (d): DSC thermogram of artesunate and amodiaquine suppositories prepared with PEG 4000 and castor oil stored at 4^{o}C for six months.

Appendix 7 (e)



Appendix 7 (e): DSC thermogram of artesunate and amodiaquine suppositories prepared with Phospholipon®90G, Softisan®154, and castor oil stored at 27 °C for six months.

Appendix 7(f)



Appendix 7(f): DSC thermogram of artesunate and amodiaquine suppositories prepared with Phospholipon ®90G, Softisan ®154, and castor oil stored at 4 °C for six months.