

IN VITRO AND IN VIVO ACTIVITY OF
PHOSPHOLIPASE A2 ENZYME AGAINST
PLASMODIUM sp.

THESIS SUBMITTED
UNIVERSITY OF MUMBAI

FOR THE DEGREE OF
MASTER OF SCIENCE
IN
APPLIED BIOLOGY

SUBMITTED BY
SHEETAL SUDHAKAR SHETTY

UNDER THE GUIDANCE OF
DR. MRUNAL GHAG SAWANT

HAFFKINE INSTITUTE FOR TRAINING, RESEARCH & TESTING
MUMBAI

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UNDER THE GUIDANCE OF
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STATEMENT OF CANDIDATE

As required by the University Regulation No. **R.2190**, I wish to state that the work embodied in this thesis titled “**In vitro and In vivo Activity of Phospholipase A₂ Enzyme against Plasmodium sp.**” forms my own contribution to the research work carried out under the guidance of **Dr. Mrunal Ghag Sawant** at the **Department of Zoonosis, Haffkine Institute for Training, Research and Testing, Parel, Mumbai – 400012**. This work has not been submitted to any other degree of this or any other University. Whenever references have been made to previous work of others it has been clearly indicated as such and included in the Bibliography.

Signature of the candidate

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Certified by,

Signature of Guide

Name: Dr. Mrunal Ghag Sawant



**THIS THESIS IS DEDICATED TO ALL THE
MICE WHO SACRIFICED THEIR LIFE TO
MAKE THIS PROJECT A REALITY.**

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“Research is to see what everybody else has seen, and to think what nobody else has thought” – Albert Szent-Györgyi

This above quote has been my inspiration and motivation to enter into the world of research. From young curiosity to mature exploration, it has been a long and arduous journey. This Master’s degree has been an eye-opening experience with several ups and downs, but it was made possible because of all the people who held my hand through it all.

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ABBREVIATIONS

hrs: Hours

sp.: Species

μM: Micromolar

mins: Minutes

DNA: Deoxyribonucleic Acid

RNA: Ribonucleic Acid

yrs: Years

HIV: Human Immunodeficiency Virus

ACT: Artemisinin-based Combination Therapy

IV: Intravenous

CO₂: Carbon-di-oxide

O₂: Oxygen

N₂: Nitrogen

RPMI: Rosewell Park Memorial Institute

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

km: Kilometer

ELISA: Enzyme-linked Immunosorbent Assay

MRC-5: Medical Research Council cell strain 5

EGTA: Ethylene glycol-bis (β-aminoethyl ether)- N, N, N', N'- tetraacetic acid

S.E.M.: Standard Error of the Mean

Pf: *Plasmodium falciparum*

CHAPTER 1:

INTRODUCTION

Malaria remains one of the most devastating infectious diseases globally, causing significant morbidity and mortality, particularly in tropical and subtropical regions. According to the World Health Organization (WHO), an estimated 263 million malaria cases and 597,000 deaths occurred in 2023, with *Plasmodium falciparum* being the most lethal species ^[1]. The growing resistance of *Plasmodium* parasites to conventional antimalarial drugs, such as Chloroquine and Artemisinin derivatives, presents a formidable challenge to global malaria control and eradication efforts ^{[2][3]}. Consequently, the search for novel therapeutic targets and agents is more critical than ever.

The evolution of man goes hand in hand with malaria. The origin of most of the populations of human malaria may have been from West Africa (*P. falciparum*) and West and Central Africa (*P. vivax*) based on the occurrence of homozygous alleles of Haemoglobin C and RBC Duffy negativity that protects *P. falciparum* and *P. vivax* respectively. Molecular genetic evidence suggests strongly that the malaria parasite was a chloroplast-bearing, free-living protozoan that had adapted to live in the gut of a group of aquatic invertebrates ^[4].

The primary manner that malaria is spread is through the bite of an infected *Anopheles* mosquito. Additionally, it can spread congenitally and by the inoculation of contaminated blood. The majority of Anophelines' breeding grounds are in rural settings, and they feed at night. Therefore, in rural regions, the hours between nightfall and dawn are the most perilous for malaria. Urban locations in many malaria-endemic areas pose little or no danger. However, in other regions of the world, particularly Africa, urban transmission is widespread ^[5].

Malaria endemic transmission can occur seasonally or year-round. Malaria parasites are constantly present in the blood of 90 to 100 percent of children under five in various parts of Africa. Children are the main victims of malaria in endemic places because naturally acquired immunity grows with increased exposure. On the other hand, malaria affects people of all ages during epidemics because naturally acquired immunity declines in epidemic areas ^[5].

Resistance to antimalarial drugs such as 4-aminoquinolines (Chloroquine, Amodiaquine, Piperaquine), 8-aminoquinolines (Primaquine), arylamino alcohols (Quinine, Mefloquine, Lumefantrine), Antifolates and Artemisinin's have emerged and spread due to the genetic mutations adopted by the parasite, namely *pfert*, *pfmdr1* and *K13*. There is an interplay between drug resistance and parasite fitness, and factors such as drug pressure, herd immunity and ecological differences can influence the prevalence of drug-resistant parasites ^[6].

Phospholipase A₂ (PLA₂) enzymes, which hydrolyse the sn-2 acyl bond of phospholipids to release arachidonic acid and lysophospholipids, are widely distributed in various organisms, including mammals, plants, and venomous animals. These enzymes play pivotal roles in membrane remodelling, signal transduction, and inflammatory processes. Interestingly, PLA₂s derived from animal venoms – particularly snake venoms – have demonstrated potent antimicrobial, antiviral and antiparasitic properties ^{[7][8]}. Recent studies have shown that PLA₂s can disrupt parasite membranes, interfere with host-cell invasion, or modulate immune responses, making them promising candidates for antiparasitic drug development.

Although Phospholipase A₂ enzymes have been extensively studied for their roles in lipid metabolism and inflammation, their interaction with malaria parasites has not been systematically investigated, particularly in the context of both *in vitro* parasite inhibition and *in vivo* infection models. Existing studies are predominantly limited to antimicrobial effects or venom-derived enzymes, with limited emphasis on controlled evaluation using standardized enzyme systems. Furthermore, there is a lack of studies examining whether enzymatic modification of host erythrocyte membrane phospholipids can influence parasite development within the host. This represents a critical gap in understanding the role of membrane lipid dynamics in malaria parasite survival.

This thesis aims to evaluate the *in vitro* and *in vivo* anti-plasmodial activity of PLA₂ enzymes, particularly focusing on their cytotoxicity and parasite growth inhibition. By integrating biochemical assays, cell culture models and murine malaria models, the present study is therefore designed as an exploratory investigation to assess whether phospholipid-modifying enzymes can influence malaria parasite survival under controlled experimental conditions.

CHAPTER 2:

REVIEW OF

LITERATURE

2.1. MALARIA

2.1.1. Life Cycle of Parasite:

To understand malaria, it is important to understand the life cycle of the parasite. *Plasmodium sp.* goes through the life cycle in two organisms – the vector mosquito and the host. The life cycle is divided into two stages, namely sexual and asexual, with the asexual stage further divided into different developmental stages such as pre-erythrocytic stage, erythrocytic stage and schizogonic stage.

Plasmodium sp. can be classified as follows:

Domain: Eukaryota
Kingdom: Chromalveolata
Superphylum: Alveolata
Phylum: Apicomplexa
Class: Haemosporida
Order: Aconoidasida
Family: Plasmodiidae
Genus: *Plasmodium*

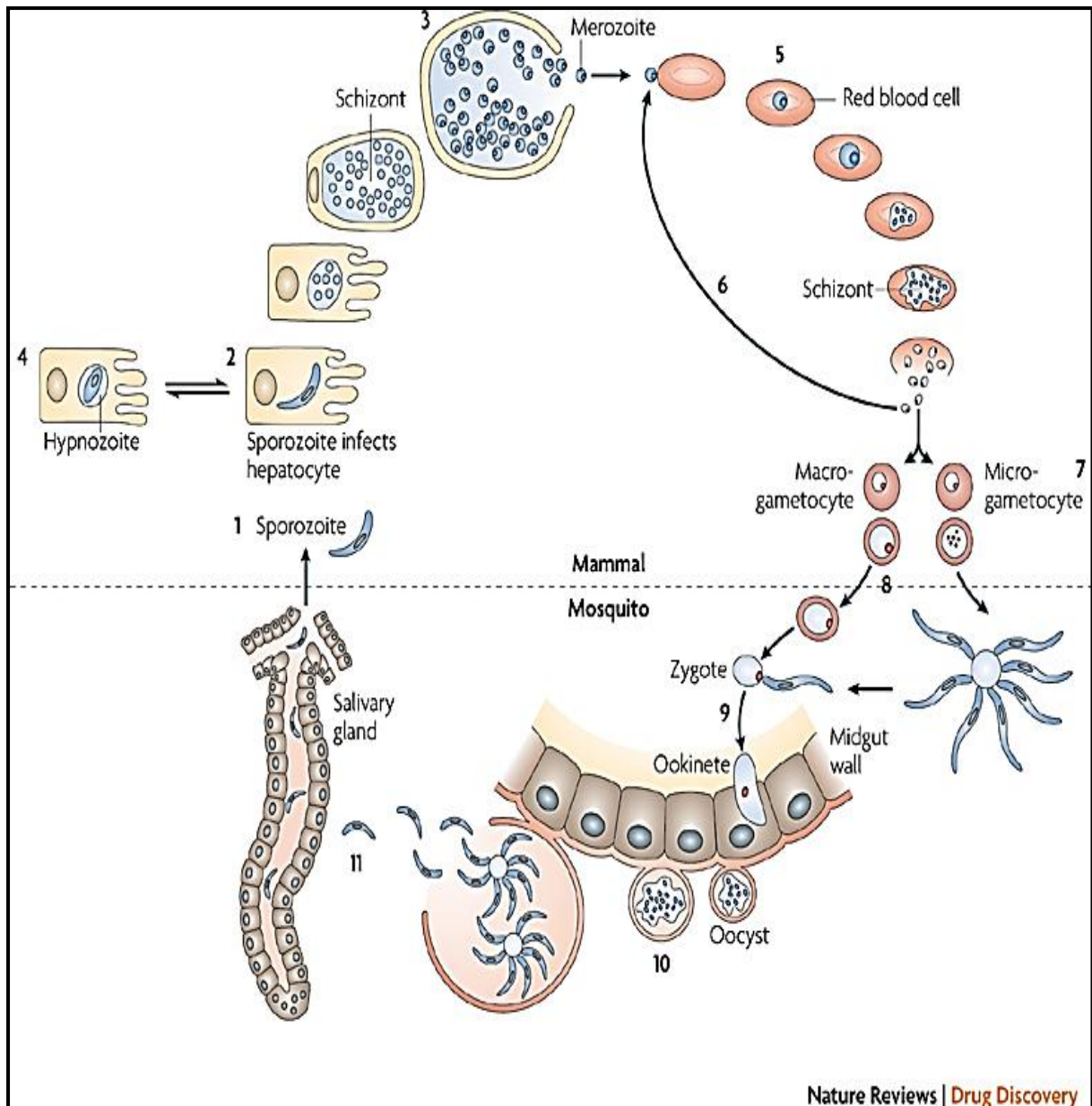


Figure 1: Malaria life cycle [Source: Nature Reviews]

Exo-erythrocytic stage

The life cycle of *Plasmodium* is complex and passes through different stages. The initiation of life cycles takes place through the bite of an infected female *Anopheles* mosquito, the sporozoites are passed through saliva into the bloodstream or lymphatic system. Upon deposition in the skin by a mosquito bite, sporozoites use gliding motility to reach blood vessels and enter the circulation. They then travel to the liver, where they cross the sinusoidal cell layer, often through Kupffer cells, before traversing through several hepatocytes. Finally, they invade a hepatocyte and form a parasitophorous vacuole for further development ^[9].

Initially, the schizonts appear in the host cytoplasm as a mononucleate round body, and further it begins to develop into mature schizonts asexually and enters the multinucleated stage of the parasite. A large number of merozoites are released. The mature schizonts have no pigment as they lack haemoglobin, are present throughout the cytoplasmic space, and are 40-70 μm large. In each of the *Plasmodium* species, the length of the parasite life cycle is constant and it can be considered a taxonomic character. The number of merozoites produced is also dependent on species. When the mature schizonts rupture the merozoites are released into the liver sinusoid, thus ending the liver cycle. These released merozoites are the ones that invade the erythrocytes.

Erythrocytic stage

The erythrocytic stage has important implications in clinical studies; this is the only stage that causes a different spectrum of symptoms that characterizes the disease also, helps in the diagnosis of infection from blood is easy, and provides a characterization of different causative agents.

The parasite species determine the time required for the parasite's life cycle to complete its erythrocytic cycle. The blood phase of the life cycle is started when the merozoites are released into the bloodstream. Merozoite consists of a single nucleus, adjacent cytoplasm, and is 1 μm in diameter. Erythrocytes are immediately invaded by merozoites to enter the trophozoite stage. The characteristic ring form is formed inside a vacuole. As the parasite grows, the cytoplasm expands and the vacuole disappears in 12-24 hrs showing a characteristic parasite pigment in the cytoplasm. The trophozoite consists of a single nucleus, no vacuole, large cytoplasm, and cytoplasmic pigment at the end of this phase. Further, as the nuclear division progresses the division produces two or more nuclei and the parasite enters the schizont stage. Until an appropriate number of merozoites are produced nuclear division continues. At the end of this phase, the schizogonic cycle is completed and erythrocytes are ruptured, releasing the merozoites into the bloodstream. The discharged merozoites enter into the blood circulation and invade other healthy erythrocytes and further continue their life cycle until there is any inhibition of the process by immune response or any drug. In the erythrocytes, the course of the schizogonic cycle may take another path and merozoites may differentiate into gametocytes- the sexual form.

The initial stage of maturation is ring form, they tend to adhere to the inner surface of the erythrocytes, and often division of the nucleus is seen in the form of two chromatin dots. The parasite disappears from the peripheral circulation as the cytoplasm becomes fleshy and the vacuole reduces in size. In *Plasmodium falciparum*-infected patients, the ring form is only an

identifiable sexual stage in the peripheral blood. The mature stages of this species mainly adhere to the endothelium of the postcapillary venules in the tissue. Similar features are seen in ring forms of *P. vivax* and *P. ovale*, but the cytoplasm may present early amoeboid tendency, and on erythrocytes some traces of stippling appear. In this species, trophozoites are seen in peripheral circulation and during the maturation of the parasite the cytoplasm expands and amoeboid pseudopods are seen on the cytoplasm, further vacuole disappears and stippling is profuse, also the pigment disappears. Regular shape is observed in *P. malariae* with a small vacuole, abundant and early pigment. In *P. ovale* and *P. vivax*, mature schizonts present a large number of irregularly arranged merozoites and occupy the whole red blood cells with more prominent stippling. In *P. malariae* smaller number of merozoites are regularly arranged around the central mass of the pigment without stippling. The two types of gametes are present in the peripheral blood the male-microgametes and female-macrogametes. They can be differentiated based on dispersed male gametocyte nuclear material and in female nuclear material is condensed.

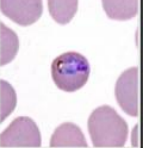
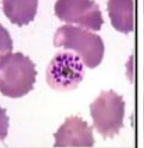
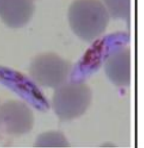
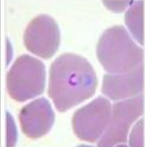
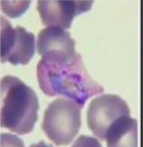
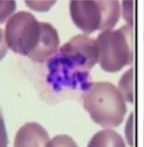
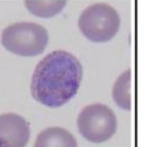
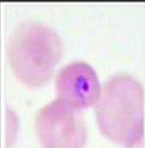
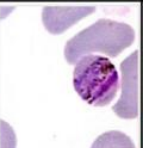
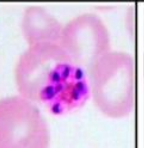
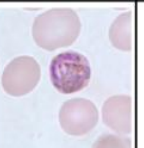
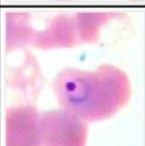
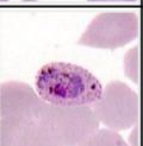
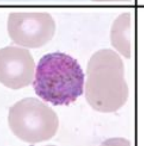
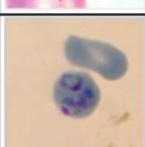
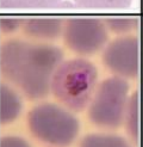
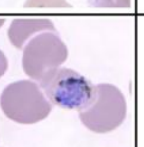
Human Malaria					
Stages Species	Ring	Trophozoite	Schizont	Gametocyte	
<i>P. falciparum</i>					- Parasitised red cells (pRBCs) not enlarged. - RBCs containing mature trophozoites sequestered in deep vessels. - Total parasite biomass = circulating parasites + sequestered parasites.
<i>P. vivax</i>					- Parasites prefer young red cells - pRBCs enlarged. - Trophozoites are amoeboid in shape. - All stages present in peripheral blood.
<i>P. malariae</i>					- Parasites prefer old red cells. - pRBCs not enlarged. - Trophozoites tend to have a band shape. - All stages present in peripheral blood
<i>P. ovale</i>					- pRBCs slightly enlarged and have an oval shape, with tufted ends. - All stages present in peripheral blood.
<i>P. knowlesi</i>					- pRBCs not enlarged. - Trophozoites, pigment spreads inside cytoplasm, like <i>P. malariae</i>, band form may be seen - Multiple invasion & high parasitaemia can be seen like <i>P. falciparum</i> - All stages present in peripheral blood.

Figure 2: Differentiation of life stages among human *Plasmodium* sp. [Source: Silamut K. and CDC]

Sporogonic cycle

As a protein source, female mosquitoes seek a blood meal for egg production. Repeated contact with the vertebrate host is required for ingestion of malaria parasite, further maturation and multiplication, and host transmission during feeding. The initiation of the sporogonic cycle occurs during the blood meal when the male gametocytes and female gametocytes are ingested by a suitable *Anopheles* species. As soon as the gametocytes reach the insect's midgut, the female gametocytes shed red blood cells and freely rain as macrogamete in the extracellular space. The male gametocytes divide into sperm-like flagellated microgametes, leave the erythrocytes, and move to fertilize the macrogametes in the midgut. The midgut of the mosquito triggers the exflagellation process, 10 mins after the blood meal. The active penetration of ookinete into the peritrophic membrane and epithelium of the midgut initiates the development of non-motile oocytes in the basal lamina of the outer gut wall where ookinetes settle, after 24-72 hrs of the blood meal. The initial oocytes have a single nucleus, a thin cyst wall, and a 6-8 μm in diameter. The mature oocytes are 40-60 μm in diameter consisting of as many as 10,000 new individual nuclei that are developed after the rapid division of the oocyte nucleus. The factors such as parasite species, the species of *Anopheles*, and the temperature determine the time required from the establishment of initial oocytes to its maturation. The products of mature oocytes are the sporozoites that are 10-15 μm in length, actively motile, narrow, and curved shaped. Through small perforation without disturbing the cell wall, sporozoites actively pass and leave the cyst, at least till most of the sporozoites exit the membrane and further enter into the hemocelomic space of the insect. The sporozoites reach the salivary gland after migration and penetrate the basal membrane, pass intracellularly through a secretory cell, and settle in the salivary gland. Oocyte-schizonts sporozoites are less motile, less infectious, and more immunogenic than gland-sporozoites. When the mosquito takes its next blood meal, the contents of saliva and sporozoites present in the saliva are injected into the host to start another asexual replicative cycle.

2.1.2. Immune Evasion:

The parasite has evolved multiple sophisticated mechanisms to evade or subvert host immunity, which include evasion of the complement system, antibody-dependent enhancement of infection, antigenic polymorphism, antigenic variation, antigenic diversion, epitope masking, and dysregulation of B cells. The parasites also exploit host protein homologies, induce

immunosuppression, and use redundant pathways for red blood cell invasion to evade host immune responses ^[10].

The merozoite surface is not a simple structure but a highly dynamic and complex array of proteins that undergo extensive processing before, during, and after invasion. The process of merozoite invasion is complex, including initial attachment, reorientation, and formation of a tight junction. This complexity has made it challenging for researchers to fully understand the invasion process and develop effective vaccines. Various proteins are present on the merozoite surface, such as GPI-anchored proteins, transmembrane-anchored proteins, peripherally-associated proteins, MSP1, MSP2, AMA1, and members of the EBA and PfRH families, which play a role in invasion and have potential as vaccine candidates. Another intriguing aspect is the parasite's ability to use different invasion pathways, which allows it to evade host immune responses and adapt to variations in host cell receptors. This phenotypic variation in invasion pathways highlights the parasite's remarkable adaptability and presents a significant challenge for vaccine development ^[11].

Superoxide dismutase 3 (SOD3) plays a role in suppressing early cellular immune responses to parasite infection. Elevated levels of SOD3 were found in both *Plasmodium falciparum* malaria patients and mice infected with various parasite species. Surprisingly, SOD3-deficient mice showed increased resistance to parasite infection, with longer survival times and lower parasitaemia compared to control mice. Conversely, SOD3-overexpressing mice were more vulnerable to infection. SOD3, primarily secreted by activated neutrophils, binds to T cells and suppresses the expression of Interleukin-2 (IL-2) and subsequent Interferon-gamma (IFN- γ) responses, which are crucial for parasite clearance, and inhibits the JNK pathway in T cells, leading to reduced IL-2 and IFN- γ production. SOD3 acts as a host factor that parasites exploit to evade immune surveillance ^[12].

2.1.3. Epidemiology:

The primary manner that malaria is spread is through the bite of an infected *Anopheles* mosquito. Additionally, it can spread congenitally and by the inoculation of contaminated blood. The majority of Anophelines' breeding grounds are in rural settings, and they feed at night. Therefore, in rural regions, the hours between nightfall and dawn are the most perilous for malaria. Urban locations in many malaria-endemic areas pose little or no danger. However, in other regions of the world, particularly Africa, urban transmission is widespread.

In the past, malaria was spread throughout the world, including as far north as North Dakota in the US. Many regions, notably the US and Europe, have eradicated endemic malaria transmission as a result of environmental changes and eradication efforts carried out in the years following World War II. In the tropics and subtropics, the disease is still widely spread. Malaria can spread in these regions either endemically, which happens annually, or epidemically, which happens occasionally under suitable conditions.

Malaria endemic transmission can occur seasonally or year-round. Malaria parasites are constantly present in the blood of 90 to 100 percent of children under five in various parts of Africa. Children are the main victims of malaria in endemic places because naturally acquired immunity grows with increased exposure. On the other hand, malaria affects people of all ages during epidemics because naturally acquired immunity declines in epidemic areas [5].

2.1.4. Symptoms:

Fever is malaria's most prevalent symptom. Myalgias, headaches, nausea, vomiting, and chills are some of the other typical symptoms. Sometimes there is coughing, diarrhoea and stomach ache. As the sickness worsens, some individuals may experience the characteristic malaria paroxysm, which alternates between periods of illness and times when they don't have any symptoms. There are three stages in a malaria paroxysm. Shivering and a cold feeling are the hallmarks of the initial cold stage, which lasts for 15-60 mins. The heated stage, which lasts for 2-6 hrs, is followed by flushed, dry skin, fever (which can reach 41°C), headache, nausea and vomiting. Lastly, there is the sweating stage, which lasts for 2-4 hrs and is characterized by a quick drop in fever and perspiration. The rupture of mature schizonts is the cause of the periodic febrile reaction in all forms of malaria. A brood of schizonts in *P. vivax* and *P. ovale* malaria matures every 48 hrs, resulting in a tertian fever ("tertian malaria"), but in *P. malariae* illness, fever happens every 72 hrs ("quartan malaria"). *P. falciparum* malaria fever can happen every 48 hrs, however it is typically erratic and lacks any clear pattern. The absence of periodic, synchronized fevers does not rule out a diagnosis of malaria because these classic fever patterns are typically not observed early in the course of the disease.

Physical signs of malaria are obscure and do not assist in diagnosis. Other than fever, there may not be any other favourable results in many cases. Although splenomegaly is common, it could not show up at the beginning of the illness. There may also be hypotension, jaundice, hepatomegaly, and pain in the abdomen. Malaria is not linked to rash and does not result in lymphadenopathy. In a case of uncomplicated malaria, a number of laboratory abnormalities

may be observed. Thrombocytopenia, leukocytosis or leukopenia, hypoglycaemia, hyponatremia, increased liver and renal function tests, normochromic, normocytic anaemia, proteinuria, and laboratory evidence of disseminated intravascular coagulation are among them (though clinically significant bleeding is rare). No eosinophilia is observed. Massive intravascular haemolysis with hemoglobinemia and haemoglobinuria is sometimes seen in patients with severe malaria.

Delays or missed diagnoses of malaria can lead to complex malaria, which can be lethal, particularly when *P. falciparum* is present. Severe anaemia and cerebral malaria are the most common deadly side effects of malaria. Any aberration in mental status in a person with malaria is referred to as cerebral malaria, which has a 15-50% case fatality rate. Other complications can include: severe hypoglycaemia; lactic acidosis; prolonged hyperthermia; shock; pulmonary, cardiac, hepatic, or renal dysfunction; seizures; spontaneous bleeding; high-output diarrhoea or vomiting; or hyperparasitaemia (more than 3-5 % of the erythrocytes parasitized) [5].

2.1.5. Diagnosis:

In most cases, direct detection of malaria parasites in thick and thin blood smears stained with Giemsa is necessary for a definitive diagnosis of malaria. As more blood is analysed, thick blood smears are far more sensitive than thin blood smears, but they are also more difficult to interpret. To identify the type of parasite causing the infection, thin blood smears are utilized, where parasites are visible inside erythrocytes. The existence of diagnostic forms might differ significantly depending on the stage of parasite, particularly in the initial stages of illness.

Due to their sequestration in the microvascular tissue of internal organs, the majority of organisms in *P. falciparum* malaria are absent from peripheral blood. Blood smears should be checked every 6-12 hrs for at least two days if malaria is suspected. New diagnostic techniques include a fluorescent stain approach for parasite detection and a quick antigen-capture dipstick test. These two tests are quick, simple, and extremely sensitive and specific.

Additional diagnostic approaches include polymerase chain reaction/DNA and RNA probe methods, as well as assays that identify malaria antibodies and antigens. Rarely are these methods utilized for individual patient diagnosis; instead, they are mostly employed in epidemiologic research and vaccination trials [5].

2.1.6. Human factors and Malaria:

Genetic factors

Certain kinds of malaria can be protected by biological traits that are present from birth. Two genetic variables linked to human red blood cells have been identified as epidemiologically significant. Sickle cell trait carriers (heterozygotes for the defective haemoglobin gene HbS) are more resistant to *P. falciparum* malaria and have a biological advantage. Because *P. falciparum* malaria has long been a primary cause of death in Africa, the sickle cell trait is now more common in Africans and people of African ancestry than in other populations. Haemoglobin-related illnesses and other blood cell dyscrasias (disease or disorder related to blood), such as Haemoglobin C, Thalassemia and G-6-PD (Glucose-6 phosphate-dehydrogenase) deficiency, are more common in malaria-endemic areas and are assumed to be linked to malaria. People who do not have the Duffy blood type have red blood cells that are resistant to *P. vivax* infection. *P. vivax* is uncommon in Africa, South of the Sahara especially in West Africa, because the majority of Africans are Duffy negative. *P. ovale*, a very similar parasite that does infect Duffy-negative individuals, has taken over *P. vivax* habitat in that location.

Malaria is influenced by other genetic factors related to red blood cells, albeit to a lesser amount. A person's chance of developing severe malaria may be influenced by a variety of genetic variables (such as the "HLA complex"- Human Leukocyte Antigen complex, which controls immunological responses).

Acquired Immunity

The way malaria affects an individual and a society is heavily influenced by acquired immunity. A person may build a partially protective immunity to malaria after several encounters. Such "semi-immune" individuals can still be infected by malaria parasites, but they are less likely to develop severe disease and are commonly asymptomatic. In regions where *P. falciparum* infection is high (much of Africa, South of the Sahara), neonates will be protected for the first few months of life by maternal antibodies passed down through the placenta. As these antibodies deteriorate over time, these young infants become more prone to malaria infection and death. They will have attained a protective semi-immune condition if they survive numerous illnesses until they reach an older age (2-5 yrs). As a result, young children are a primary risk group in high-transmission locations, and malaria control measures target them

first. In locations where transmission is limited such as Asia and Latin America, infections are less common, and a higher percentage of older children and adults lack protective immunity.

Pregnancy and Malaria

Pregnancy lowers immunity to a variety of infectious illnesses. When women establish protective immunity against *P. falciparum*, it is common for them to lose immunity once they are pregnant (especially during the first and second pregnancies). Malaria during pregnancy is dangerous to both mother and the unborn child. The latter is more likely to be born pre-term or with low birth weight, resulting in a lower likelihood of surviving during the first few months of life. As a result, malaria control initiatives in endemic nations target pregnant women along with small children for protection. Malaria infection produces different issues depending on whether the malaria transmission area is steady (high) or unstable (low).

Women in high-transmission areas have established immunity that usually prevents serious illnesses; however, parasites specifically target the placenta, associated with an increased risk during pregnancy. Women in high-transmission areas have developed immunity to malaria, but this immunity wanes somewhat during pregnancy. Malaria infection is more likely to cause maternal anaemia and the birth of low-birth-weight infants (weighing less than 2,500 gm or 5.5 pounds). It is especially problematic for women in their first and second pregnancies, as well as younger women and HIV-positive women. Females have gained no immunity to malaria in low-transmission areas. Malaria infection in mothers is more likely to result in severe malaria disease, foetal loss, maternal anaemia, or premature delivery.

Behavioural factors

Individual and community malaria risk can be influenced by human behaviour, which is often controlled by social and economic factors. In malaria-endemic areas, poor rural inhabitants generally cannot afford the housing and bed nets that would protect them from mosquito bites. These individuals frequently lack the knowledge necessary to detect malaria and treat it swiftly and effectively. Traditional, inadequate therapeutic approaches are frequently used as a result of cultural beliefs. Non-endemic travellers may choose not to use insect repellent or malaria prevention medications. Cost, hassle, or a lack of expertise are all possible reasons. Larval breeding habitats can be created by human activity (standing water in irrigation ditches, borrow pits), and agricultural activities such as harvesting (which is also impacted by the climate) may result in increased evening mosquito bite exposure. Raising domestic animals near the house may provide *Anopheles* mosquitoes with different sources of blood meals, lowering human

exposure. Non-immune people may be exposed to high malaria transmission environments as a result of war, migration (voluntary or forced), and tourism. In endemic nations, human behaviour plays a role in determining how successful malaria control efforts are in reducing transmission ^[1].

In malaria-endemic countries, the governments frequently lack financial resources. As a result, public-sector health personnel are frequently underpaid and overworked. They do not have the necessary equipment, medications, training, or supervision. When such conditions arise, the local population is aware of them and stops depending on public health facilities. The private sector, on the other hand, has its issues. Regulatory measures are frequently absent or unenforced. This promotes unlicensed, expensive health providers to give private consultations, as well as the haphazard prescription and sale of pharmaceuticals, some of which are counterfeit products. Correcting this scenario is a massive task that must be met if malaria control and eventually, elimination are to be achieved.

2.1.7. Treatment:

WHO now recommends the use of five typical ACT combinations as the initial treatment of uncomplicated *P. falciparum* malaria in endemic areas. The suggested combinations, with the exception of artesunate-sulfadoxine-pyrimethamine, are currently offered as fixed-dose therapies, which are better due to increased adherence and convenience of usage. Atovaquone-proguanil is also considered as one of the first-line treatments for travellers returning to non-endemic areas and for regions with confirmed artemisinin resistance, provided that it has not been used for prophylaxis. When ACT and atovaquone-proguanil are unavailable or contraindicated, oral quinine plus clindamycin or doxycycline may be used as a second line of treatment. However, this requires a 5-7-day course of treatment and carries a risk of poor adherence, which is primarily linked to quinine-related cinchonism (deafness, ringing in the ear, nausea). If ACTs are not available, chloroquine treatment for *P. falciparum* uncomplicated malaria is only permitted for patients returning from the few regions where *P. falciparum* is still susceptible to this medication (Haiti, Dominican Republic, Middle East, and Central America - north of the Panama Canal).

Table 1. Recommended regimens for the treatment of uncomplicated <i>P. falciparum</i> malaria (various sources) §						
Table 1A: First line regimens						
Compound TM (not exhaustive)	Manufacturers (not exhaustive)	Formulations	Dosage (adult)	Dosage (child)	Notes	Pregnancy
Artemether-lumefantrine (Riamet ® - Coartem ®)	Novartis/Chinese Academy of Medical Sciences/MMV	Tablet (adult) and Dispersible (child): 20mg/120 mg	4 tablets for 6 doses (0-8-24-36-48-60h)	5-14 kg: 1 for 6 doses 15-24kg: 2 for 6 doses 25-34kg: 3 for 6 doses (0-8-24-36-48-60h)	With food Reduced efficacy in Cambodia and border regions of Thailand	II and III trim (I trim only if no alternative regimens)
Dihydroartemisinin-piperaquine (Eurartesim ®)	Sigma-Tau/MMV/Pfizer	Tablet (adult): 320/40 mg Crushed and Dispersible (child): 160/20 mg	36-74kg: 3 tablets 74-100kg: 4 tablets once daily for 3 days	5-7 kg: ½ tablets 7-13kg: 1 tablets 24-36kg: 2 tablets once daily for 3 days	Fasting	II and III trim (I trim only if no alternative regimens)
Artesunate-anodiaquine (Coarsucam ®)	Sanofi-Aventis/DNDI/MMV	Dispersible tablet: 25/67.5 mg 50/135 mg 100/270 mg	>36 kg 200/540mg once daily for 3 days (4/10mg/kg)	4.5-9 kg: 25/67.5 mg 9-18 kg: 50/135 mg 18-36kg: 100/270 mg once daily for 3 days	Not with high fatty food	only if no alternative regimens
Artesunate-mefloquine (Artesquin ®, Mefliam-plus ®)	Mepha, ASMQ; Farmanguinhos/DNDI/Cipla	Crushed tablet: 25/55 (=50) mg 100/220 (=200) mg	200/440mg once daily for 3 days	5-8 kg: 25/55mg 9-17kg: 50/110mg 18-29kg: 100/220mg >29kg: 200/440mg once daily for 3 days	With food	II and III trim (I trim only if no alternative regimens)
Artesunate-sulfadoxine-pyrimethamine (Altinate ®, Larinate ®, Artescope ®)	Allerge, Intima, Gullin	Tablets, various co-formulations	Artesunate 4 mg/kg/day for 3 days Sulfadoxine/pyrimethamine 25/1.25 mg/kg on day 1			II and III trim
Atovaquone-proguanil (Malarone ®)	GlaxoSmithKline	Tablet (adult): 250/100 mg Tablet (child): 62.5/25 mg	4 tablets once daily for 3 days	5-8kg: 1 tablet 62.5/25 9-10Kg: 2 tablets " " 11-20Kg: 1 tablet 250/100 21-30kg: 2 tablets " " 31-40kg: 3 tablets " " >40 kg: 4 tablets " "	With fatty meal. Use for travellers	Only if no alternative regimens available

Table 1B: First line regimens (various sources)						
Compound TM (not exhaustive)	Manufacturers (not exhaustive)	Formulations	Dosage (adult)	Dosage (child)	Notes	Pregnancy
Quinine ^a + Clindamycin			10mg/kg thrice daily + 10mg/kg thrice daily for 7 days	10mg/kg thrice daily + 10mg/kg thrice daily for 7 days	Off-label use	OK
Quinine ^a + Doxycycline			10mg/kg thrice daily + 200 mg daily for 7 days	Contraindicated if <8 years	Off-label use	Contraindicated
Mefloquine (Lariam ®, Mephaquin ® or Mefliam ®)	Roche, Mepha, Cipla	Tablet: 250mg	45-60kg: 5 tablets (3+2) >60kg: 6 tablets (3+2+1) 6-8 hours apart	20-25mg/kg divided in 1-3 doses 6 hours apart: 5-10 kg: ½-1 tablet 10-20kg: 1-2 tablets 20-30kg: 2-3 tablets 30-45kg: 3-4 tablets	With food Not suitable for SE-Asia	II and III trim; I trim only if no alternative regimens

§: please refer to drug package instruction before use
^a Dosages are for Quinine dihydrochloride. Equivalent doses for available quinine salts are as follow: quinine base 100 mg= quinine bisulfate 169 mg= quinine dihydrochloride 122 mg= quinine hydrochloride 111 mg=quinine sulfate 121 mg=quinine gluconate 160 mg.

Table 3. Treatment of severe malaria §		
Parenteral Drug	Dosage / body weight	Notes
i.v. Artesunate	2.4 mg/kg at 0,12,24 then once a day	Available as single-dose vial containing drug as a sterile dry-filled powder and a single-dose vial of a buffer solution to be reconstituted in a clear colorless 10 mg per mL solution. Adding 5 ml of normal saline solution it can be administered direct i.v. over 1 to 2 minutes into an established i.v. line.
i.v. Quinine	20 mg*/kg (loading dose) then 10 mg/kg at 8-h intervals * Quinine doses are usually prescribed as quinine dihydrochloride salt (10 mg of salt = 8.3 mg of quinine base).	contra-indicated if previous blackwater fever or quinine hypersensitivity or cardiac arrhythmia are known avoid loading dose if oral quinine or mefloquine has been given within 24 hours or if QT interval at baseline ECG is >25% each dose in 10 mL/kg of saline or 5% dextrose solutions (maximum concentration 60mg/ml) rate-controlled infusion not exceeding 5 mg salt/kg body weight per hour (2-4 hrs) never by intravenous bolus injection → lethal hypotension reduce quinine dose to 5-7 mg/kg if infusion last for more than 48 hrs or if pt develops renal failure monitor blood glucose levels and electrocardiographic features
Pre-referral drugs		
Suppository ** artesunate ** 50,100,400 mg	5 to 8 kg 1supp. 50 mg 9 to 19 kg 1supp.100 mg 20 to 29 kg 2supp 100 mg 30 to 39 kg 3supp 100 mg 40 to 59 kg 1 supp 400 mg 60 to 80 kg 2 supp 400 mg >80 kg 3 supp 400 mg	given once and followed as soon as possible by parenteral therapy
i.m. Quinine	10 mg/kg	Dilute 300 mg quinine (usually corresponding to 1 ml) into 5ml of sterile water for injection or saline (not dextrose) in the same syringe, that now contains 50mg quinine/ml
i.m. Artemether	3.2mg/kg	

§: please refer to drug package instruction before use
Parenteral antimalarials should be administered for a minimum of 24 h, once started. When parasitemia has decreased to less than 1% and patient can tolerate oral medication, treatment should be completed by giving a complete course of: (i) artemether plus lumefantrine, (ii) artesunate plus amodiaquine, (iii) dihydroartemisinin plus piperaquine, (iv) artesunate plus sulfadoxine-pyrimethamine, (v) artesunate plus clindamycin or doxycycline, (vi) quinine plus clindamycin or doxycycline

Figure 3: Treatment Regimen for Uncomplicated and Severe Malaria [Source: Castelli F. *et al.*, 2012]

Chloroquine continues to be the recommended treatment for *P. malariae*, *P. ovale* and *P. vivax* malaria once *P. falciparum* infection has been safely ruled out. It is advised to administer a total of 25 mg/kg (10 mg/kg at T0, followed by 5 mg/kg after 6, 24 and 48 hrs, or, alternatively, 10 mg/kg on the first and second days and then 5 mg/kg on the third day). Since *P. ovale* and *P. vivax* show a latent hepatic stage (hypnozoites), an effective cure that prevents relapses necessitates an adjuvant course of medication that kills hypnozoites. Only the 8-amino quinolines (buloquine, primaquine, and tafenoquine) exhibit notable efficacy against this parasite stage at the moment.

Regardless of the *Plasmodium* species causing the disease, the cornerstone of severe malaria treatment is an efficient, timely, parenteral anti-malarial treatment whose main objective is to prevent death and disability, with the prevention of recrudescence coming in second. Since 2006, the World Health Organization has recommended intravenous artesunate as the first line of treatment, whenever feasible, instead of intravenous quinine. When artesunate IV parenteral therapy is not readily available, intravenous quinine should be administered instead ^[13].

Drug Resistance

Resistance to antimalarial drugs such as 4-aminoquinolines (chloroquine, amodiaquine, piperazine), 8-aminoquinolines (primaquine), arylamino alcohols (quinine, mefloquine, lumefantrine), antifolates and artemisinin's have emerged and spread due to the genetic mutations adopted by the parasite, namely *pfcr*, *pfmdr1* and *K13*. There is an interplay between drug resistance and parasite fitness, and factors such as drug pressure, herd immunity, and ecological differences can influence the prevalence of drug-resistant parasites. Studies have found significant differences in the prevalence of drug resistance-associated mutations between parasites infecting mosquitoes and those infecting humans. This unexpected link between vector control and the prevalence of drug-resistant malaria parasites suggests a complex interplay between mosquito ecology, parasite genetics, and drug resistance ^[6].

Vaccines

RTS,S/AS01 vaccine (Mosquirix™), is the first malaria vaccine to reach Phase 3 clinical trials and pilot implementation. Developed through a collaboration between GlaxoSmithKline and the Walter Reed Army Institute of Research, the vaccine targets the pre-erythrocytic stage of *Plasmodium falciparum*. The vaccine's design is based on the circumsporozoite protein (CSP) of *P. falciparum*, combined with hepatitis B surface antigen and the AS01 adjuvant system. The Phase 3 trial of RTS,S/AS01, conducted from 2009-2014 in seven sub-Saharan African

countries, involved 15,459 participants, including infants and young children. The vaccine showed varying efficacy depending on the age group and dosing schedule. In children aged 5-17 months who received four doses, the vaccine demonstrated 36.3% efficacy against clinical malaria over a median follow-up of 48 months. The efficacy was lower in infants aged 6-12 weeks. The vaccine's safety profile was generally similar to other childhood vaccines, with a noted increased risk of febrile seizures. Despite its modest efficacy, the vaccine has significant public health potential, especially in high-transmission areas. The vaccine's development represents a major milestone in the fight against malaria, being the first to reach advanced clinical trials and implementation studies. There are ongoing efforts to improve the vaccine's efficacy, including alternative dosing schedules and adjuvants. The implementation challenges, including the need for a four-dose schedule and potential impact on other malaria interventions, underscore the complexity of introducing a new vaccine in resource-limited settings [14].

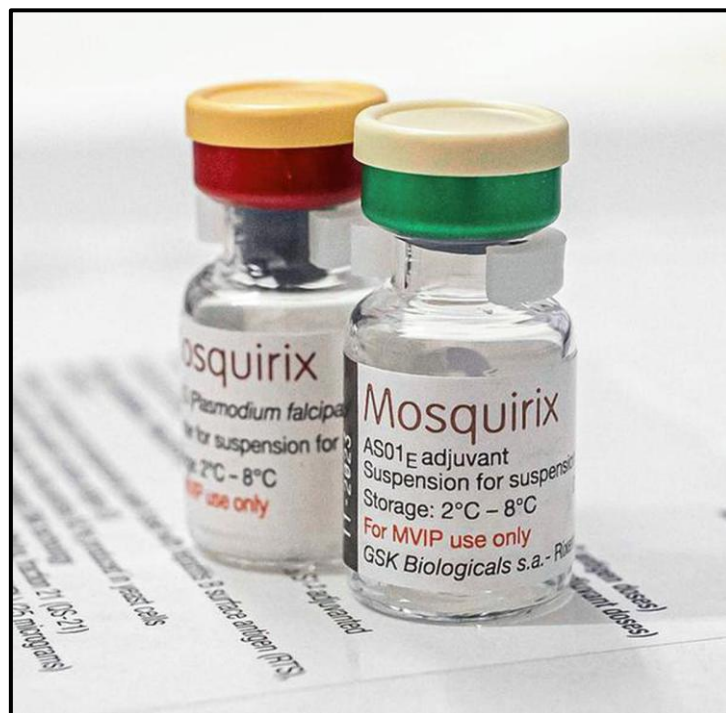


Figure 4: Mosquirix™ [The Hindu]

The RTS,S/AS01E booster vaccination was associated with increased levels of IL-2, IFN- γ , IL-17, IL-5 and IL-13. Notably, IL-2 responses to the Circumsporozoite Protein (CSP) remained elevated even after one year. There was also a moderate correlation between anti-CSP IgG and IL-2 one year after the booster. There is a moderate cell-mediated immunogenicity of the RTS,S/AS01E booster dose, which aligns with the partial recovery of vaccine efficacy observed in clinical trials. These findings contribute to our understanding of

the immune mechanisms underlying RTS,S/AS01E vaccine efficacy and may inform future strategies for improving malaria vaccines ^[15].

The Indian Council of Medical Research (ICMR) is developing an indigenous, multi-stage malaria vaccine candidate named AdFalcivax ^[16]. This vaccine is a significant advancement as it is a recombinant chimeric vaccine designed to target two critical phases of the *Plasmodium falciparum* parasite, which is responsible for the most lethal form of malaria ^[17]. This dual-stage approach aims to not only protect vaccinated individuals from infection but also to reduce community transmission by disrupting the parasite's life cycle in the mosquito vector ^[18].

- **Target:** AdFalcivax targets two stages of the parasite- the pre-erythrocytic stage (preventing infection in humans) and the sexual stage (preventing transmission via mosquitoes) ^[19].
- **Mechanism:** It utilizes a stable and functional recombinant chimera of antigenic components. This innovative design is hypothesized to offer broader protection and better long-term immunity compared to existing single-stage vaccines like RTS,S and R21 ^[18].
- **Production:** The vaccine is produced using a safe, food-grade bacterium, *Lactococcus lactis*.
- **Preclinical Findings:** Preclinical studies have shown promising results, indicating excellent efficacy, with some data suggesting over 90% protection in mice. The vaccine also demonstrated extended thermal stability, remaining functional for more than nine months at room temperature, which could be a major advantage for distribution in tropical regions ^[19].
- **Future Development:** The vaccine is currently in the early stages of development and has not yet been tested on humans. ICMR has issued an Expression of Interest (EoI) seeking collaboration with eligible organizations and manufacturers for technology transfer to undertake further development, manufacturing, and commercialization ^[17]. The projected timeline for the vaccine to be ready for clinical use is approximately 6-7 years ^[20].

2.1.8. Global Statistics:

An estimated 263 million cases of malaria were reported worldwide in 2023, with an incidence of 60.4 cases per 1000 at-risk individuals. This represents an increase in incidence from 58.6 cases per 1000 people at risk in 2022 and an increase of 11 million cases from the year before. With an expected 94% of all malaria cases globally in 2023 occurring in the WHO African Region, the region continues to bear the greatest burden of the illness. Since 2021, the incidence

in the WHO Eastern Mediterranean Region has increased by 57%, reaching 17.9 cases per 1000 people at risk in 2023. According to estimates, Nigeria accounted for 26% of the world's malaria cases in 2023, followed by the Democratic Republic of the Congo (13%), Uganda (5%), Ethiopia (4%), and Mozambique (4%).

With a projected 597000 deaths worldwide in 2023, the mortality rate was 13.7 per 100,000. In 2020, the number of deaths from malaria and the mortality rate fell steadily from 622,000 and 14.9 deaths per 100,000, respectively. 95% of estimated malaria deaths globally occur in the WHO African Region, which continues to bear the highest mortality burden. An estimated 2.2 billion cases and 12.7 million deaths from malaria were averted globally between 2000 and 2023, with the WHO African Region alone preventing 1.7 billion cases and 12 million deaths. Over 177 million illnesses and over 1 million deaths were prevented worldwide in 2023 alone.

With the exception of India and the Sudan, the 11 HBHI nations accounted for 66% of all malaria cases and 68% of all malaria-related deaths worldwide in 2023. Estimated malaria deaths in these 11 countries rose 2.3% from 399,000 to 408,000, and estimated malaria cases rose 13.8% from 152 million to 173 million between 2017-the year before the HBHI effort began-and 2023.

Significant strides were achieved in the eradication of malaria in 2023 and 2024. Due to Saudi Arabia and Timor-Leste retaining zero indigenous cases for three years in a row, the number of nations with malaria endemic status dropped from 85 in 2022 to 83. Additionally, 26 nations that had endemic malaria in 2000 went on to report zero indigenous cases for three years in a row by 2024. Tajikistan, Belize, Azerbaijan, and Cabo Verde were declared malaria-free in 2023. Additionally, Egypt became the third nation in the WHO Eastern Mediterranean Region to be malaria-free in 2024 ^[1].

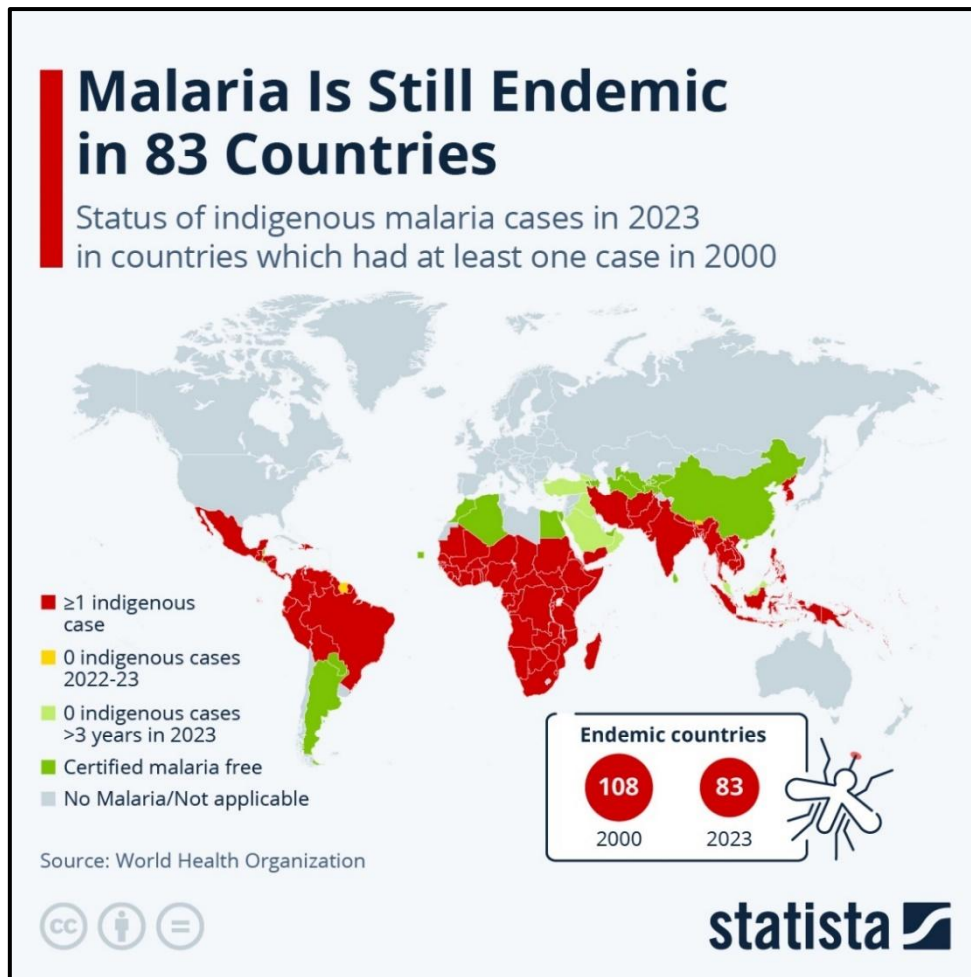


Figure 5: Global Statistics of Malaria [Source: Statista]

Among India's accomplishments is the notable decline in malaria infections and deaths from the disease from 2017-2023. India's departure from the WHO's High Burden to High Impact (HBHI) classification in 2024, which marked a significant change in its battle against malaria, serves as more evidence of this achievement. These accomplishments are a testament to the country's strong public health initiatives and its goal of eliminating malaria by 2030. Between 2015 and 2023, there was an almost 80% decrease in both malaria cases and deaths. Cases decreased from 11,69,261 in 2015 to 2,27,564 in 2023, while deaths decreased from 384 to just 83. The unrelenting attempts to fight the illness are reflected in this sharp deterioration. At the same time, the Annual Blood Examination Rate (ABER) increased significantly from 9.58 (2015) to 11.62 (2023) as a result of increased surveillance efforts. Early identification, prompt action, and more successful treatment have all been made possible by this enhanced surveillance ^[21].

2.1.9. Future of Antimalarials:

A multifaceted approach is required for effective antimalarial drug development for which it is important to understand the parasite's biology and metabolism to identify novel targets. Several promising drug targets have been found, including targeting parasite proteases, phosphatidylinositol 4-kinase (PfPI4K), various transporters (such as the *Plasmodium* sugar transporter and lactate transporter), P-Type Na⁺ + ATPase (PfATP4), V-Type H⁺ + -ATPase, Aquaporin-3, choline transport, dihydroorotate dehydrogenase (DHODH), isoprenoid biosynthesis, farnesyltransferase, and *P. falciparum* translational elongation factor 2 (pfEF2) [22].

The antimalarial properties of compounds isolated from *Dioscorea bulbifera* L. through *in vitro* studies and their potential interactions with *Plasmodium falciparum* lactate dehydrogenase (PfLDH) through molecular docking analysis was studied. It was found that the compounds quercetin, 2,4,3',5'-tetrahydroxybibenzyl, 3,5-dimethoxyquercetin, and quercetin-3-O- β -D-galactopyranoside, demonstrated antimalarial effect with quercetin showing the highest antimalarial activity and exhibiting remarkable binding affinity (-8.53 kcal/mol) to PfLDH [23].

Plasmepsins (PMs) are aspartic proteases in *Plasmodium* parasites that have been explored as potential drug targets for malaria treatment. This enzyme takes part in multiple processes of the parasite, such as haemoglobin degradation, protein export, and parasite egress from red blood cells. Non-haemoglobinase PMs (V, IX, X) as promising drug targets and should be incorporated in screening for drug targets to maximize chances of success. It is important to develop inhibitors that target multiple PMs simultaneously to slow down the evolution of drug resistance. There is a significant gap in research regarding PMs VI-VIII, which are expressed in mosquito-stage parasites which could be potentially targeted for malaria transmission-blocking strategies [24].

There is a need for drugs that can act on multiple stages of the parasite's life cycle, including both asexual blood stages and gametocytes, to achieve effective treatment and prevent transmission. Furthermore, the potential of combination therapies needs to be studied and it is important to develop drugs with novel mechanisms of action to combat resistance.

The availability of genome sequences has advanced our understanding of malaria parasites and opened new avenues for drug and vaccine development. The data has facilitated functional studies of malaria parasites, including transcriptional and proteomic analyses. These studies have revealed important insights into parasite biology, such as the role of translational control

in gene regulation during developmental transitions. Genome-wide analyses have contributed to our understanding of antigenic variation, a key mechanism for immune evasion in malaria parasites. Genetic diversity studies have informed drug resistance tracking and vaccine development strategies. The discovery of a conserved protein export motif (PEXEL/VTs) shared by malaria parasites and plant pathogens suggests a common mechanism for host cell manipulation across different eukaryotic pathogens. Genome-dependent methods have led to innovative vaccination strategies, such as genetically attenuated parasites that can induce protective immunity. Furthermore, the shift from single-enzyme screening approaches to cell-based methods in drug discovery, facilitated by genomic data, represents a significant advancement in malaria research. These insights demonstrate how genomic information has not only enhanced our understanding of parasite biology but also opened up new possibilities for therapeutic interventions against malaria [25].

2.1.10. *In vitro* study of malaria:

The development of continuous culture methods in 1976 dramatically expanded research possibilities in malaria. The protocol is designed for simplicity, minimal components, and reduced labour intensity, making it suitable for large-scale parasite preparations. Key aspects include the preparation of culture medium, washing of human blood, setting up cultures, changing medium, and making thin blood smears for microscopy, liquid nitrogen preservation and retrieval of parasites. Non-heat-treated plasma produces results as good as or better than serum, challenging the conventional use of serum in parasite cultivation [26].

Trager and Jensen invented two methods for the continuous cultivation of *Plasmodium falciparum* which are the most used for culturing the parasite worldwide. Both methods use human erythrocytes in a thin settled layer in RPMI 1640 medium with 10% human serum and a modified atmosphere. The first method, called the flow-vial method, provides a continuous slow flow of medium over settled cells, requiring no daily attention. It uses an atmosphere of 7% CO₂, 5% O₂, and 88% N₂. The second method uses plastic Petri dishes and requires daily manual medium changes. It employs a candle jar to create an atmosphere with 3% CO₂ and about 17% O₂ [27].

A groundbreaking method for the continuous *in vitro* propagation of *Plasmodium vivax* using human blood cells was developed. The Chesson strain of *P. vivax* was passaged from owl monkey erythrocytes to human reticulocytes in a modified McCoy's 5A medium supplemented with human serum. The key to the success of this method was the periodic addition of

reticulocytes during schizogony, providing merozoites with the necessary target cells for reinvasion. The cultures alternated between static candle jar and shaker conditions, with reticulocytes added during the shaker phase. This approach allowed for stable parasite densities and at least a doubling of parasite numbers every 48 hrs. The cultured parasites maintained typical *P. vivax* morphology and demonstrated infectivity when injected into an owl monkey. Prior to this breakthrough, the lack of a continuous culture system had significantly hindered research on *P. vivax* compared to *P. falciparum*. The ability to maintain *P. vivax* in culture opens up new opportunities for vaccine development, drug testing, and genome sequencing [28].

Researchers successfully established a long-term culture system for *Plasmodium berghei* using the Petri dish candle jar method, which was previously considered unsuitable for rodent malaria parasites. The researchers modified the RPMI 1640 medium by adding extra glucose and bactopeptone, which allowed mouse erythrocytes to survive for two weeks without damage. They tested different temperatures (22°C, 27°C and 37°C) and found that 27°C was optimal for parasite growth. Using this modified medium and temperature, they successfully maintained *P. berghei* cultures for 45 weeks, with a mean parasitaemia of 43.89% and a maximum parasitaemia of 50%. This achievement is significant as it allows for direct comparison between *in vivo* and *in vitro* experiments using the same host species [29].

While *in vitro* culturing of Pf has led to significant advancements in malaria research, the varying culturing conditions across different studies may influence experimental results and interpretations. There are several key factors in Pf culturing that can affect parasite biology, including the choice of laboratory strains or clinical isolates, human red blood cells (RBCs), culture media composition, serum or serum alternatives, haematocrit and parasitaemia levels, and incubation conditions. The use of lipid-rich alternatives like Albumax II® can cause significant impact as it can alter parasite protein expression and drug sensitivities. Incubation conditions, especially oxygen levels, are a critical factor that can induce transcriptional and translational changes in the parasite, potentially affecting its response to antimalarial drugs like artemisinin. There is a requirement for detailed reporting of culturing protocols and variations in culturing conditions should be acknowledged and discussed in multicentre research efforts. This emphasis on the subtle yet significant impacts of culturing conditions challenges researchers to reconsider their experimental designs and interpretations, potentially leading to more robust and translatable findings in malaria research [30].

2.1.11. Rodent Model of Malaria:

Both the mouse and human genomes are 85% identical. Mice have circulatory, reproductive, neurological, and other systems that are similar to those of humans. Because of their anatomical and physiological similarities to humans, laboratory rats and mice are useful animal models for biomedical research and comparative medicine studies. Rats, mice, and humans each have approximately 30,000 genes of which approximately 95% are shared by all three species. The use of rodents for study has economic benefits: mice and rats are relatively small and require minimal room or resources to maintain, have short gestation times but comparatively large numbers of offspring, and have a relatively rapid development to adulthood and shorter life spans. The *Plasmodium* rodent model is the most important tool in the primary phases of the investigation of anti-malarial drugs. The rodent *Plasmodium* species include *Plasmodium berghei*, *P. vinckei*, *P. yoelii* and *P. chabaudi*. In the mosquito, the cyclic transmission of *Plasmodium berghei* infection has benefitted the use of rodent malaria for chemotherapeutic studies. This model has been used to identify essential antimalarial activity with low cost and substantial reliability. The administration of new agents more efficiently and effectively, prediction of the likelihood of resistance development, and determining the effectiveness of new antimalarial agents came up against the existing resistance to established anti-drugs. Rodent models can be easily genetically manipulated by using various tools used for forward and reverse genetics and also the combination of efficient transfection.

Mouse models have been instrumental in validating observations from human studies and providing insights into the mechanisms of malaria resistance. For example, mouse studies have confirmed the protective effects of sickle cell trait and pyruvate kinase deficiency against malaria, which were initially observed in human populations. Additionally, mouse models have revealed novel mechanisms of malaria resistance, such as the role of heme oxygenase in protecting against cerebral malaria and the importance of host purine metabolism in parasite survival. There is a growing need of advanced genetic tools, such as CRISPR/Cas9 technology and the Collaborative Cross mouse lines, in improving the efficiency and relevance of mouse models for malaria research. These developments promise to enhance our understanding of host-parasite interactions and potentially lead to more effective strategies for malaria control and treatment ^[31].

Rodent malaria parasites (RMPs) in research are important due to their experimental tractability and genetic similarity to human-infecting *Plasmodium* species. These models have contributed to our understanding of the genetic basis of drug resistance and the development

of new antimalarial compounds. There is an emergence of liver-derived organoids as a promising *ex vivo* tool for studying *Plasmodium* liver stages, offering a bridge between *in vitro* systems and animal models. These advancements highlight the ongoing efforts to develop more accurate and ethically sound models for malaria research, potentially leading to better strategies for disease control and elimination [32].

2.1.12. Management Strategies:

A cluster-randomized trial of reactive, focal malaria interventions was conducted in Namibia's Zambezi region from January to December 2017. The trial evaluated the effectiveness of three interventions: chemoprevention using artemether-lumefantrine (rfMDA), indoor residual spraying with pirimiphos-methyl (RAVC), and a combination of both measuring both direct effects on intervention recipients within 500 m of index cases and spillover effects on non-recipients within 3 kms. There was limited evidence of direct effects, possibly due to small sample sizes; however, there was strong evidence of spillover effects, particularly for the combined intervention of chemoprevention and vector control. This combined approach reduced malaria incidence by 43% among non-recipients within 1 km of index cases and decreased infection prevalence by 79% among non-recipients within 3 kms. It was also revealed that spillover effects were stronger in areas with lower baseline malaria incidence, suggesting these focal interventions could be more effective in lower-transmission settings. There is a potential synergistic effect when combining chemoprevention and indoor residual spraying, which could be particularly effective in low-transmission settings approaching elimination or in outbreak responses after elimination has been achieved. These findings have important implications for malaria elimination strategies, highlighting the potential of targeted interventions to indirectly benefit non-recipients and providing valuable information for policy decisions in malaria control and elimination efforts [33].

A study on the use of Antimicrobial Peptides (AMPs) as potential effector molecules to target sporogonic stages in mosquitoes. The researchers identified seven AMPs that showed significant killing effects against malaria parasites: Melittin, TP10 monomer and dimer, Vida3 dimer, Anoplin, Mastoparan X and Duramycin which were mainly derived from bee and wasp venom. The most effective AMPs were Melittin and TP10 dimer, which reduced ookinete viability to zero within 30 mins *in vitro*. Importantly, most of these peptides did not negatively impact mosquito longevity or fecundity when ingested, with only TP10 dimer showing a significant reduction in mosquito survivorship in long-term studies [34].

A transgenic *Anopheles stephensi* mosquitoes expressing a Mutant Phospholipase A₂ (mPLA₂) gene was created to interfere with *Plasmodium falciparum* development. Two transgenic mosquito lines, AP(-) and AP(+), were generated using piggyBac-mediated transformation. Both lines showed significant impairment of rodent malaria (*P. berghei*) development, but only the AP(+) line effectively inhibited human malaria (*P. falciparum*) parasite development. Surprisingly, both transgenic lines demonstrated a fitness advantage over non-transgenic mosquitoes when maintained on infected blood, with the percentage of transgenic mosquitoes increasing over generations. This fitness advantage was attributed to increased fecundity in transgenic mosquitoes when fed on malaria-infected blood. The researchers also identified the genomic insertion sites of the transgene and found that the AP(+) line's insertion caused increased expression of an AP4-like transcription factor, which may contribute to observed fitness effects. A unique insight from this study is that transgenic mosquitoes expressing an anti-*Plasmodium* effector gene can have a fitness advantage when exposed to malaria parasites, contrary to the common assumption that genetic modifications generally reduce fitness. This finding has significant implications for future applications of transgenic mosquito technology in malaria control [35].

2.2. PHOSPHOLIPASE A₂

Phospholipase A₂ (PLA₂) enzymes play pivotal roles in various biological processes by catalysing the hydrolysis of phospholipids at the sn-2 position, thereby releasing free fatty acids, including arachidonic acid and lysophospholipids. These enzymes are integral components of cellular membranes and are involved in diverse physiological functions such as membrane remodelling, cell signalling, inflammation and lipid metabolism [36]. The versatility of PLA₂ enzymes extends beyond their enzymatic activity, as they also participate in intricate cellular pathways regulating immune responses, neuronal function and host defence mechanisms [37].

PLA₂ enzymes are classified into several subtypes based on their structure, localization and calcium dependency. Among these, the secretory PLA₂s (sPLA₂s) are prominent members, primarily secreted by various cell types including immune cells, in response to inflammatory stimuli. They are implicated in the initiation and propagation of inflammatory responses through the generation of lipid mediators such as prostaglandins and leukotrienes [4]. Intracellular PLA₂s (iPLA₂s) and cytosolic PLA₂s (cPLA₂s) are involved in cellular membrane

homeostasis and signal transduction, with dysregulation of their activity linked to pathological conditions such as cancer, neurodegenerative diseases and cardiovascular disorders ^[38].

Understanding the structural characteristics, biochemical properties and physiological roles of PLA₂ enzymes is crucial for deciphering their involvement in health and disease. Insights into the intricate regulatory mechanisms governing PLA₂ activity provide avenues for the development of therapeutic interventions targeting inflammatory diseases, cancer and neurodegeneration.

2.2.1. Sources:

Phospholipase A₂ (PLA₂) enzymes are widely distributed throughout nature and can be sourced from various organisms, tissues and cellular compartments. PLA₂ enzymes are sourced from a diverse range of organisms, including animals, microorganisms, plants and marine species. Understanding the sources and characteristics of PLA₂ enzymes provides insights into their biological functions and potential applications in various fields, including medicine, biotechnology and environmental science.

Animal Sources

Animal tissues represent one of the primary sources of PLA₂ enzymes. Pancreatic PLA₂, also known as Group IB PLA₂, is abundantly found in mammalian pancreases and plays a crucial role in digestive processes by hydrolysing dietary phospholipids ^[39]. Moreover, snake venoms are rich sources of PLA₂ enzymes, which contribute to the toxic effects of snakebites. These venom PLA₂s exhibit diverse structural and functional properties and are classified into different groups based on their sequence homology and biological activities.

Microbial Sources

Microorganisms, including bacteria, fungi and algae, produce a wide array of PLA₂ enzymes. Bacterial PLA₂s are often involved in membrane remodelling and lipid metabolism, contributing to bacterial pathogenesis and survival. Fungal PLA₂s play a role in growth, development and pathogenicity, with some fungal species secreting PLA₂ enzymes as virulence factors. Algal PLA₂s are of interest due to their potential biotechnological applications, including biodiesel production and pharmaceutical development ^[40].

Plant Sources

PLA₂ enzymes are also present in various plant species, where they participate in lipid metabolism, stress responses and defence mechanisms. Plant PLA₂s are classified into several

families based on their sequence homology and biochemical properties. They are involved in diverse physiological processes, including seed germination, membrane lipid turnover and responses to environmental stresses such as drought, cold and pathogen attacks. Furthermore, plant PLA₂s contribute to the generation of lipid-derived signalling molecules involved in plant growth, development and immunity ^[41].

Marine Sources

Marine organisms, including fish, molluscs and crustaceans, produce PLA₂ enzymes with unique structural and functional properties. Marine PLA₂s play a role in lipid digestion, membrane maintenance and defence against pathogens and predators. Moreover, marine PLA₂s exhibit potential biotechnological applications in areas such as food processing, pharmaceuticals and nutraceuticals. Studies have focused on characterizing PLA₂ enzymes from marine sources to uncover their biochemical properties and explore their industrial and therapeutic potentials ^{[42][43]}.

2.2.2. Evolution of PLA₂:

A comprehensive study using various computational methods to understand the evolutionary relationships and functional aspects of the PLA₂ gene family was done. The study utilized genomic data from 49 PLA₂ sequences across 10 different vertebrate species, including humans, mice, rats, pigs, dogs, chickens, cattle, frogs, orangutans and zebrafish. Multiple phylogenetic analysis methods were employed, including neighbour-joining (NJ), minimum evolution (ME), maximum parsimony (MP), and Bayesian inference. The phylogenetic trees showed that PLA₂G7 is one of the most evolutionarily distant members of the family, indicating a fast-evolving lineage with numerous structural changes. The study identified closer lineages between certain PLA₂ groups, such as PLA₂G16 and PLA₂G6, PLA₂G7 and PLA₂G4, and PLA₂G3 and PLA₂G12. One of the most intriguing findings of this study is the potential link between the evolutionary history of the PLA₂ gene family and its diverse physiological functions. PLA₂G5 might be the origin of the PLA₂ family, while PLA₂G7 has undergone significant evolutionary changes. These findings not only contribute to our understanding of the PLA₂ gene family's evolution but also offer potential targets for future research in areas such as cardiovascular diseases, inflammation, and lipid metabolism ^[44].

2.2.3. Characterization:

Phospholipase A₂ (PLA₂) enzymes, a diverse group of enzymes crucial for various physiological processes, play fundamental roles in cellular membrane remodelling, lipid metabolism, cell signalling and inflammation. These enzymes have been extensively characterized to elucidate their structural features, catalytic mechanisms, regulatory pathways and physiological roles. The biochemistry of PLA₂ enzymes is intricate and multifaceted, with significant implications for health and disease. Further research into the regulation and functional diversity of PLA₂ enzymes promises to uncover novel therapeutic targets and treatment strategies for a wide range of human diseases. Continued research efforts aimed at elucidating the complex interplay between PLA₂ enzymes and cellular signalling pathways hold promise for the development of novel therapeutic strategies targeting inflammatory diseases and other related disorders [45].

Classification

The PLA₂ superfamily comprises 15 groups categorized into four main types: secreted sPLA₂, cytosolic cPLA₂, calcium-independent iPLA₂, and platelet activating factor (PAF) acetyl hydrolase/oxidized lipid lipoprotein associated (Lp) PLA₂. Secreted PLA₂s, the first type discovered, are characterized by their low molecular weight, histidine in the catalytic site, and calcium-dependent activity. They play roles in various biological processes, including antimicrobial activity and potential involvement in inflammatory responses. Cytosolic PLA₂, particularly the GIVA PLA₂, is highlighted for its preference for arachidonic acid and its central role in eicosanoid generation and inflammatory processes. The calcium-independent iPLA₂, specifically the GVIA PLA₂, is discussed in terms of its multiple splice variants and its involvement in various cellular functions such as bone formation, apoptosis, and insulin secretion. Lastly, the PAF acetyl hydrolase/oxidized lipid LpPLA₂ is described, noting its ability to cleave oxidized lipids and its association with coronary heart disease.

sPLA₂s are secreted by various cell types, primarily in response to inflammatory stimuli, and are implicated in the initiation and propagation of inflammatory responses [37]. iPLA₂s and cPLA₂s, on the other hand, are involved in maintaining cellular membrane homeostasis and signal transduction processes [4].

Structure

PLA₂ enzymes exhibit structural diversity, reflecting their classification into distinct subtypes and their varied physiological functions. Studies have employed various techniques, including

X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy and cryo-electron microscopy, to elucidate the three-dimensional structures of PLA₂ enzymes [46]. These structural studies have revealed conserved catalytic domains containing essential residues for substrate binding and catalysis, as well as variable regions involved in substrate specificity and regulation [4]. The catalytic domain contains a highly conserved active site responsible for the hydrolysis of phospholipid substrates, while the lipid-binding domain facilitates substrate recognition and enzyme-substrate interactions [36]. The structural diversity among PLA₂ enzymes contributes to their substrate specificity and functional versatility.

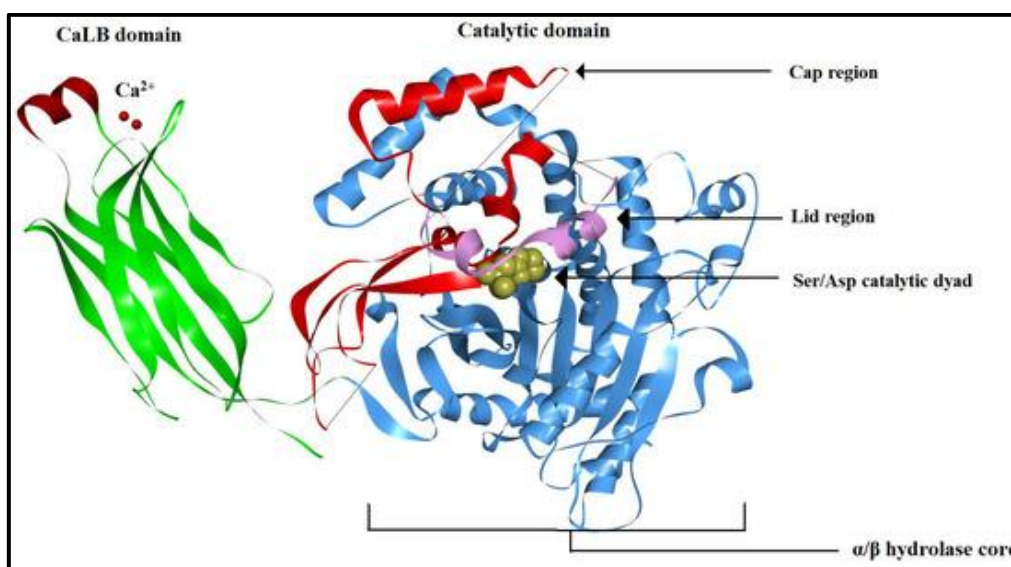


Figure 6: Structure of PLA₂ [Khan S. A. & Ilies M. A.]

Catalytic Mechanism

The catalytic mechanism of PLA₂ involves the hydrolysis of phospholipid substrates at the sn-2 position, resulting in the release of free fatty acids and lysophospholipids. Detailed mechanistic studies have highlighted the role of key catalytic residues, such as the conserved histidine, in facilitating nucleophilic attack on the phospholipid substrate and subsequent acyl-enzyme intermediate formation. Additionally, structural studies combined with kinetic analyses have provided insights into the stereochemistry of substrate binding and the transition state of the catalytic reaction [37]. The catalytic activity of PLA₂ enzymes is influenced by factors such as substrate composition, membrane fluidity and cellular localization.

Regulatory Pathways

PLA₂ activity is tightly regulated to maintain cellular homeostasis and prevent uncontrolled inflammation. Research has identified multiple regulatory pathways governing PLA₂ activity, including calcium binding, phosphorylation, lipid-protein interactions, and protein-protein

interactions. Regulation occurs at multiple levels, including post-translational modifications, calcium binding and interaction with regulatory proteins ^[4]. For instance, calcium ions serve as essential cofactors for the activation of certain PLA₂ isoforms, while phosphorylation-mediated activation regulates the activity of others. Furthermore, interaction with regulatory proteins, such as annexins and lipid-binding proteins, modulates PLA₂ localization and activity in response to cellular stimuli ^[36].

Physiological Roles

PLA₂ enzymes play diverse physiological roles, including membrane remodelling, lipid metabolism, cell signalling and inflammation. Research has demonstrated the involvement of PLA₂-derived lipid mediators, such as prostaglandins, leukotrienes and platelet-activating factor, in immune responses, inflammatory pathways and neuronal signalling. Dysregulation of PLA₂ activity is associated with numerous diseases, including inflammatory disorders, neurodegenerative diseases and cancer ^[38]. Therefore, understanding the complex interplay between PLA₂ enzymes and cellular signalling pathways holds significant therapeutic potential for the treatment of various diseases.

Role in immunity

PLA₂ enzymes are involved in various stages of the phagocytic process, from initial pathogen recognition to the degradation and presentation of antigens. For instance, cPLA₂ α plays a crucial role in eicosanoid signalling by generating arachidonic acid, which is further metabolized into inflammatory mediators. iPLA₂ β supports focal exocytosis during phagosome formation, while sPLA₂ forms (particularly IIA and V) have bactericidal properties and can directly kill pathogens. LPLA₂ and aiPLA₂ are involved in the processing of microbial lipids for antigen presentation and in repairing oxidative damage to phagosomal membranes. The most intriguing insight is the multifaceted nature of PLA₂ enzymes in immune function. While traditionally viewed as enzymes involved in lipid metabolism, they play critical roles in pathogen killing, membrane repair, and even antigen presentation. Studies have also raised interesting questions about the redundancy and specificity of different PLA₂ forms, as many phagocytes express multiple members of each PLA₂ group. This suggests that these enzymes may have non-overlapping functions or act at different stages of the phagocytic process, highlighting the complexity of lipid-mediated immune responses. Furthermore, the potential interplay between host and microbial PLA₂ enzymes during infection adds another layer of complexity to understanding the role of these enzymes in immune defence ^[47].

2.2.4. Assay Methods:

Phospholipase A₂ (PLA₂) enzymes are crucial regulators of various biological processes, making their detection and measurement essential for understanding their roles in health and disease. A diverse array of detection methods is available for studying PLA₂ enzymes, each offering unique advantages in terms of sensitivity, specificity and applicability. Integration of multiple detection approaches allows for comprehensive characterization of PLA₂ activity and its role in physiological and pathological processes.

Biochemical Assays

Biochemical assays represent one of the most widely used methods for detecting PLA₂ activity. These assays typically measure the hydrolysis of phospholipid substrates by PLA₂ enzymes, resulting in the release of free fatty acids and lysophospholipids. One commonly employed assay is the colorimetric assay using a synthetic chromogenic substrate, such as p-nitrophenylphosphorylcholine (p-NPPC), which yields a coloured product upon hydrolysis by PLA₂. Alternatively, radioactive substrates, such as [³H]-labelled phospholipids, can be utilized for sensitive detection and quantification of PLA₂ activity ^{[42][48][49]}.

Chromatography

A high-performance liquid chromatography (HPLC) method for assaying phospholipase A₂ (PLA₂) activity was studied and it involved extracting fatty acids released by the enzyme using heptane-isopropanol-H₂SO₄, with margaric acid as an internal standard, followed by precolumn derivatization with 9-anthryldiazomethane (ADAM). The ADAM derivatives of long-chain fatty acids are then accurately determined by reverse-phase HPLC with ultraviolet detection at 254 nm. The HPLC conditions were optimized to separate palmitic and oleic acid derivatives within 7 mins. Using 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG) and cholate as a substrate system was effective for sensitive detection of PLA₂ activities in rat tissue homogenates ^[39].

A modified isocratic reverse-phase high-performance liquid chromatography (RP-HPLC) method for phospholipase A₂ (PLA₂) activity assay involves using natural lecithin's and synthetic phospholipids as substrates, with release of fatty acids analysed after single-phase derivatization using p-bromophenacyl bromide at low temperature. This procedure allows for simultaneous determination of total and specific phospholipase activity. The researchers used PLA₂ isolated from the venom of *Vipera ammodytes meridionalis* (Bulgarian long-nosed viper) as a test enzyme. Both natural (soybean and egg yolk lecithin's) and synthetic (1,2-dipalmitoyl-

sn-glycero-3-phosphocholine) substrates were employed for quantitative determination of enzyme activity. The method demonstrates good analytical performance, with linearity in the studied concentration range (0-100 μ M palmitic acid), a limit of detection of 0.4 μ M, and a limit of quantification of 1.2 μ M. The reproducibility is excellent, with a relative standard deviation of less than 3%, and accuracy expressed as bias is less than 10% in the range of 10-100 μ M. One unique aspect of this method is the optimization of the derivatization procedure using p-bromophenacyl bromide (p-BPB) at lower temperatures (37°C) compared to previous methods (70-85°C). This modification is particularly beneficial for analysing unsaturated fatty acids, which are prone to oxidation and isomerization at higher temperatures. Additionally, the use of a liquid organic base (N,N-diisopropylethylamine) instead of a solid inorganic base eliminates the need for post-derivatization sample cleanup, streamlining the analysis process. These improvements make the method more suitable for routine biochemical PLA₂ activity analyses, especially when dealing with sensitive unsaturated fatty acids released by PLA₂ activity [50].

A study on the purification and characterization of phospholipase A₂ (PLA₂) enzyme from the venom of the Egyptian honey bee *Apis mellifera lamarckii*, isolated and purified PLA₂ using a two-step chromatographic method involving DEAE-cellulose and Sephacryl S-300 columns. The purification process resulted in an 8.9-fold increase in specific activity, yielding a final product with 6033 U/mg protein and 38% recovery. The purified PLA₂ was found to be a monomeric protein with a molecular weight of 16 kDa and an isoelectric point of 5.9 [51].

A HPLC-fluorimetric assay for measuring phospholipase A₂ (PLA₂) and platelet-activating factor acetylhydrolase (PAF-AH) activities in biological samples, aimed to overcome limitations of existing real-time fluorimetric assays, particularly for samples with high protein content, colour or turbidity. The method utilizes fluorescent NBD-PC derivatives as substrates and incorporates a reversed-phase HPLC step to separate the substrates from the reaction products. The study validated the method using standard porcine PLA₂ and human plasma PAF-AH as positive controls, and compared it with a radiometric assay and the real-time fluorimetric assay. The HPLC-fluorimetric method showed efficient separation of NBD-FAs from NBD-PCs, with good resolution and reproducibility. It demonstrated linearity for PLA₂ activities from 1 pmol to at least 250 nmol FA/h/mL sample, comparable to the radiometric assay. The HPLC-fluorimetric assay proved suitable for samples with high protein content where the real-time fluorimetric assay was insufficient. It was also evaluated under various conditions, including elevated temperatures, different pH values, and Ca²⁺ concentrations. The

method showed strong correlation with the reference radiometric method for both PLA₂ and PAF-AH determinations. Bland-Altman analysis further confirmed good agreement between the HPLC-fluorimetric and radiometric methods. The HPLC-fluorimetric assay overcomes limitations of existing methods, allowing for accurate measurement of PLA₂ and PAF-AH activities in complex biological samples. It offers versatility in experimental conditions, potentially enabling discrimination between different PLA₂ isoforms. The method's ability to function in the presence of fluorescent PLA₂ inhibitors expands its applicability. Moreover, compared to radiometric methods, this assay is more cost-effective and environmentally friendly, making it a valuable tool for researchers studying phospholipase activities in various biological contexts ^[52].

Mass Spectrometry

Mass spectrometry (MS) has emerged as a powerful tool for the detection and characterization of PLA₂ enzymes and their substrates. MS-based methods enable the direct measurement of PLA₂-mediated lipid hydrolysis products, including free fatty acids and lysophospholipids, providing quantitative information on PLA₂ activity. Moreover, MS allows for the identification of specific lipid species targeted by PLA₂ enzymes, offering insights into substrate selectivity and preferences ^{[53][39]}.

Fluorescence-Based Assays

Fluorescence-based assays offer sensitive and high-throughput methods for detecting PLA₂ activity. These assays utilize fluorescent probes, such as 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-PE) or 1,2-dioleoyl-sn-glycero-3-phosphocholine-N-(lissamine rhodamine B sulfonyl) (Rhodamine-PC), which exhibit changes in fluorescence intensity upon hydrolysis by PLA₂ enzymes. Fluorescence resonance energy transfer (FRET)-based assays, utilizing donor and acceptor fluorophores conjugated to lipid substrates, offer enhanced sensitivity and specificity for detecting PLA₂ activity in complex biological samples ^[54].

Immunological Assays

Immunological assays, such as enzyme-linked immunosorbent assays (ELISAs) and immunoblotting, enable the detection of PLA₂ enzymes based on their antigenic properties. Antibodies raised against specific PLA₂ isoforms or epitopes can be used to selectively capture and detect PLA₂ enzymes in biological samples. ELISAs offer quantitative measurements of

PLA₂ levels, while immunoblotting allows for the visualization and characterization of PLA₂ isoforms based on their molecular weights and post-translational modifications [48][55].

2.2.5. Therapeutic Advances:

Phospholipase A₂ (PLA₂) enzymes have garnered increasing attention for their diverse therapeutic potential in various disease contexts. PLA₂ enzymes offer diverse therapeutic opportunities across multiple disease areas, including inflammatory disorders, neurological diseases, cancer and cardiovascular diseases. Further exploration of the therapeutic potential of PLA₂ enzymes, as well as the development of innovative strategies for targeting PLA₂ activity, holds promise for advancing disease treatment and improving patient outcomes.

Inflammatory Disorders

One of the most well-established therapeutic applications of PLA₂ enzymes lies in the treatment of inflammatory disorders. PLA₂-derived lipid mediators, such as prostaglandins, leukotrienes and platelet-activating factor, play pivotal roles in the initiation and propagation of inflammatory responses. Targeting PLA₂ enzymes offers a promising approach to modulate the production of inflammatory mediators and mitigate inflammation-associated pathologies, including rheumatoid arthritis, inflammatory bowel disease and asthma [37].

Metabolic disorders

PLA₂G1B is primarily expressed in pancreatic acinar cells and facilitates postprandial lysophospholipid absorption, which suppresses hepatic fatty acid oxidation. This leads to increased VLDL synthesis, decreased glucose tolerance, and promotion of tissue lipid deposition, ultimately contributing to diet-induced metabolic disorders. While these enzymes play essential roles in normal physiology, such as digestion and host defence, they can also contribute to the development of inflammatory metabolic diseases when dysregulated. The potential of PLA₂G1B inhibition as a therapeutic strategy for metabolic disorders is particularly intriguing, as it addresses multiple aspects of metabolic syndrome simultaneously, including obesity, diabetes, and hyperlipidaemia. This multifaceted approach to treating metabolic diseases through a single target enzyme represents a promising avenue for future research and drug development [56].

Neurological Diseases

Emerging evidence suggests that PLA₂ enzymes are implicated in the pathogenesis of neurological diseases, including Alzheimer's disease, Parkinson's disease and multiple

sclerosis. PLA₂-mediated hydrolysis of membrane phospholipids leads to the release of arachidonic acid, which serves as a precursor for the synthesis of neuro-inflammatory mediators and reactive oxygen species. Inhibition of PLA₂ activity has been proposed as a therapeutic strategy to attenuate neuro-inflammation and neuronal damage in various neurological disorders [57].

Cancer Therapy

PLA₂ enzymes have emerged as potential targets for cancer therapy due to their roles in tumour progression, angiogenesis and metastasis. PLA₂-mediated hydrolysis of membrane phospholipids generates bioactive lipid metabolites that promote cancer cell proliferation, invasion and survival. Inhibition of PLA₂ activity or expression has been shown to suppress tumour growth and metastasis in preclinical cancer models, highlighting the therapeutic potential of targeting PLA₂ enzymes in cancer treatment [58].

On the other side of the spectrum, *Bothrops moojeni* PLA₂ (BmPLA₂) exhibited significant anticancer activity against human colorectal adenocarcinoma (Caco-2) and human rhabdomyosarcoma (RD) cell lines. The enzyme showed selectivity for cancer cells compared to healthy cells, with a selectivity index of 1.85 when compared to MRC-5 cells and 6.33 when compared to BV-2 cells. Importantly, BmPLA₂ displayed low haemolytic activity against mouse erythrocytes, suggesting a potentially safe profile for therapeutic use [59].

Cardiovascular Diseases

PLA₂ enzymes have been implicated in the pathogenesis of cardiovascular diseases, including atherosclerosis, myocardial infarction and stroke. PLA₂-derived lipid mediators modulate vascular inflammation, endothelial dysfunction and plaque instability, contributing to the development and progression of cardiovascular pathologies. Pharmacological inhibition or genetic deletion of PLA₂ isoforms has shown beneficial effects in preclinical models of cardiovascular diseases, suggesting the therapeutic utility of targeting PLA₂ enzymes to prevent or treat cardiovascular disorders [48]. For instance, the sPLA₂ inhibitor Varespladib and the Lp-PLA₂ inhibitor Darapladib have advanced to phase III clinical trials for cardiovascular diseases [60].

Antibacterial Activity

A study on the isolation and characterization of phospholipase A₂ (PLA₂) from the venom of *Montivipera bornmuelleri*, a Lebanese Viper demonstrated strong antibacterial activity,

particularly against the Gram-positive bacteria *Staphylococcus aureus*, followed by the Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* [61].

Murine intestinal phospholipase A₂ (iPLA₂) effectively killed both *Escherichia coli* and *Listeria monocytogenes*. The bactericidal activity of iPLA₂ against *L. monocytogenes* was enhanced by calcium, inhibited by EGTA, and abolished after reduction and alkylation, suggesting that enzymatic activity was crucial for its antimicrobial effects. A comparison of the bactericidal activity of iPLA₂ to that of defensins showed that iPLA₂ had similar potency on a molar basis. It was identified that iPLA₂ is identical to an 'enhancing factor' (EF) previously shown to promote fibroblast growth and increase EGF binding to receptor-positive cells. This suggests that Paneth cell-derived iPLA₂ may not only protect intestinal crypts from bacterial incursions but also foster vital processes of epithelial cell renewal within the crypts [62].

Purified ChPLA₂-IIA from chicken intestine mucosa was tested for its bactericidal activity against various gram-positive and gram-negative bacteria. It was found that ChPLA₂-IIA displays potent bactericidal activity against gram-positive bacteria but lacks effectiveness against gram-negative bacteria. ChPLA₂-IIA was particularly effective against *Bacillus cereus*, *Bacillus subtilis* and *Micrococcus luteus*, with inhibition diameters greater than 20 mm. The bactericidal activity of ChPLA₂-IIA was shown to be calcium-dependent, as it was inhibited by EGTA, a calcium chelator. One interesting insight is the synergistic effect observed between ChPLA₂-IIA and lysozyme. The researchers found that pre-treating bacteria with lysozyme increased the bactericidal efficacy of ChPLA₂-IIA. This suggests that lysozyme may disrupt the bacterial cell wall, allowing ChPLA₂-IIA better access to the cell membrane for phospholipid hydrolysis. Additionally, the study highlights the potential physiological role of ChPLA₂-IIA in defending the intestine against bacterial invasions, given its presence in intestinal secretions and its potent activity against gram-positive bacteria [63].

PLA₂ enzyme extracted from the marine snail *Hexaplex trunculus* was investigated for its potential as a new anti-infective agent by examining its effects on various bacterial strains, *Chlamydia trachomatis*, and mammalian cell lines. It was found that the marine snail digestive phospholipase A₂ (mSDPLA₂) exhibited significant antibacterial activity, particularly against Gram-positive bacteria and showed the highest efficacy against *Staphylococcus aureus* [64].

Antiviral Activity

Snake venom phospholipase A₂ (PLA₂) enzymes exhibit potent virucidal activity against SARS-CoV-2 at nanomolar concentrations. These enzymes can inhibit viral infection through multiple mechanisms, including destroying the viral envelope, interfering with the virus's ability to bind to host cell receptors, and inhibiting cell-cell fusion mediated by the SARS-CoV-2 spike glycoprotein. The antiviral activity of PLA₂s is primarily dependent on their phospholipolytic activity, as chemically inactivated versions of the enzymes displayed significantly reduced antiviral effects. Additionally, the researchers found that PLA₂s could inhibit the binding of both anti-ACE2 antibodies and the receptor-binding domain (RBD) of the SARS-CoV-2 spike protein to host cells expressing the ACE2 receptor. These enzymes are effective not only against SARS-CoV-2 but also against other enveloped viruses from different families. This suggests that PLA₂s could serve as a promising starting point for developing novel antiviral therapies with potential efficacy against a wide range of viral pathogens. Furthermore, the ability of PLA₂s to target the viral envelope, a structure common to many viruses, indicates a potential mechanism for overcoming viral mutations and reducing the likelihood of drug resistance [65].

Antimalarial Activity

The anti-plasmodial effect of the whole venom and the isolated fractions of *Bothrops asper* on *in vitro* cultures of *Plasmodium falciparum* showed that the whole venom, and purified fraction V and fraction VI were all active against the parasite. The study provided interesting insights into the potential of snake venom components as antimalarial agents. It was found that both catalytically-active PLA₂s and the PLA₂ homologue without enzymatic activity demonstrate anti-plasmodial effects, suggesting different mechanisms of action [12]. PLA₂s from *B. diporus* venom, particularly BdTX-II, show promising anti-plasmodial activity with low cytotoxicity. The PLA₂s did not exhibit haemolytic activity at the tested concentrations, suggesting their anti-plasmodial action is directly on the intra-erythrocytic forms of *P. falciparum* [66].

Researchers investigated the interactions between Bee venom PLA₂ (bvPLA₂), human triglyceride-rich lipoproteins, and infected erythrocytes to evaluate the potential involvement of host lipoproteins and secreted phospholipases A₂ (sPLA₂s) in malaria. The study found that bvPLA₂ preferentially associates with lipoproteins rather than with erythrocytes, even when the erythrocytes are infected with *Plasmodium falciparum*. The enzyme's anti-*Plasmodium* activity is dependent on the hydrolysis of lipoprotein phospholipids, which generates toxic lipid products, primarily polyunsaturated fatty acids (PUFAs). Among these, Arachidonic Acid

(AA) and Linoleic Acid (LA) were identified as the main actors in toxicity. The researchers also discovered that minimal oxidation of lipoproteins enhanced their toxicity and induced their interaction with both infected and normal erythrocytes [67][68].

An investigation in the role of Human Group II A sPLA₂ (hGIIA sPLA₂), which is increased in the plasma of malaria patients, in controlling *Plasmodium falciparum* growth demonstrated that hGIIA sPLA₂ exhibits inhibitory effects against *P. falciparum* in the presence of oxidized lipoproteins. Other sPLA₂s, including hGIIF, hGV, and hGX, also showed enhanced potency in the presence of oxidized lipoproteins. Importantly, malaria plasma containing oxidized lipoproteins was inhibitory to *P. falciparum* when supplemented with hGIIA sPLA₂. *In vivo* experiments using mice infected with *P. chabaudi* showed that injection of recombinant hGIIA reduced the peak of parasitaemia, but only when plasma peroxidation levels were increased during infection. This suggests that malaria-induced oxidation of lipoproteins converts them into preferential substrates for hGIIA sPLA₂, promoting its parasite-killing effect. This mechanism may contribute to host defence against *P. falciparum*, particularly in severe cases of malaria where high levels of hGIIA are observed. This finding is particularly interesting as it reveals a previously unknown mechanism of host defence against malaria [69].

Researchers created transgenic *Anopheles stephensi* mosquitoes that express the bee venom phospholipase A₂ (PLA₂) gene in their gut. This gene was placed under the control of a gut-specific and blood-inducible promoter from the *Anopheles gambiae* carboxypeptidase gene. The recombinant PLA₂ protein was detected in the midgut of transgenic mosquitoes, with peak expression occurring between 8 and 24 hrs after a blood meal. This timing coincides with the invasion of the midgut by *Plasmodium* ookinetes. Importantly, the expression of PLA₂ in transgenic mosquitoes significantly reduced *Plasmodium berghei* oocyst formation by an average of 87% and greatly impaired the mosquitoes' ability to transmit the parasite to naive mice [70].

Drug Development

PLA₂ enzymes themselves, as well as their inhibitors and modulators, represent promising candidates for drug development. Small-molecule inhibitors targeting PLA₂ enzymes have been developed and evaluated for their therapeutic efficacy in various disease models. Additionally, PLA₂ enzymes have been engineered for improved substrate specificity, stability and pharmacokinetic properties to enhance their therapeutic potential. Continued research efforts aimed at elucidating the biochemical properties and physiological functions of PLA₂

enzymes hold promise for the development of novel therapeutic agents targeting a wide range of diseases ^{[67][71][70][72][73]}.

PLA₂ enzyme, isolated from the venom of Egyptian honey bee, demonstrated significant anti-platelet aggregation and anti-coagulation activities. It was able to prevent the aggregation of platelet-rich plasma by reducing ADP-stimulated platelet aggregation by 60%. Additionally, the PLA₂ showed potent anti-coagulation effects, with just 6 µg of the enzyme completely preventing blood coagulation. These properties suggest that the Egyptian honey bee venom PLA₂ could be a promising candidate for developing novel anti-clot formation drugs in the future ^[51].

2.3. PHARMACOKINETIC STUDIES

The study of Pharmacokinetics (PK) examines how the body responds to chemicals that are provided over the course of exposure. While they are closely connected, pharmacodynamics-which looks more deeply at how the medicine affects the body-is very different from this. Absorption, Distribution, Metabolism and Excretion (ADME) are the four primary factors that are typically examined in this subject.

Given the diverse physiologies and lifestyles of their patients, practitioners can prescribe and administer medications that will yield the greatest benefit at the lowest risk by having a thorough understanding of these processes. They can also make appropriate modifications.

2.3.1. Absorption:

The process by which a medication enters the systemic circulation after being administered, such as by a pill or capsule, is called absorption. The rate and concentration at which a medicine may reach its intended site of action, such as plasma, are influenced by absorption. Oral, intravenous, intramuscular, intrathecal, subcutaneous, buccal, rectal, vaginal, ophthalmic, otic, inhaled, nebulized and transdermal are just a few of the numerous ways that drugs can be administered. Every administration technique has unique absorption properties, benefits and drawbacks.

Liberation, or the process by which the medication is liberated from its pharmaceutical dosage form, is another common component of the absorption process. This is particularly crucial when it comes to oral drugs. For example, an oral drug may cause mucosal injury or delay the onset of symptoms for hours after being administered in the throat or oesophagus. Before these

medications even reach the systemic circulation, the low pH in the stomach may start a chemical reaction with them.

2.3.2. Bioavailability:

Depending on the substance's characteristics and the method of administration, bioavailability is the percentage of the initially delivered medication that enters the systemic circulation. Medication absorption may be directly reflected in bioavailability. When medicine is administered intravenously, for instance, 100% of the drug enters circulation very immediately, resulting in a 100% bioavailability. In terms of bioavailability, intravenous injection is therefore the gold standard. This idea is particularly crucial for drugs that are taken orally.

Oral drugs must pass through the stomach's acidity after being ingested and be absorbed by the digestive system. For oral medications, the digestive enzymes start the metabolic process, reducing the quantity of drug that enters the bloodstream before it is absorbed. Following absorption by intestinal transporters, the drugs frequently experience "first-pass metabolism." The liver, intestinal wall, or digestive enzymes frequently process oral medications in substantial amounts, which reduces the amount of medicine that enters the bloodstream and, as a result, its bioavailability.

The Area Under the plasma concentration Curve (AUC) is used since other methods of administration (oral, transdermal and intramuscular) may cause some drug quantities to enter the bloodstream later than expected. The AUC measures the plasma concentration over a specified period of time and is a technique for determining the drug bioavailability of compounds with various dissemination properties. Bioavailability can be represented as a percentage of the intravenous administration's 100% bioavailability by computing the integral of that curve.

Volume of Distribution (Vd)

The Volume of Distribution (Vd) is a key metric used to describe how a drug spreads throughout the body. It is calculated by dividing the amount of drug in the body by the plasma drug concentration. The body consists of different theoretical fluid compartments like extracellular, intracellular, and plasma, and Vd aims to represent a uniform volume in a theoretical compartment. Vd varies based on the properties of the drug molecule. Larger, charged, or highly protein-bound molecules have low Vd and remain in the intravascular space, while smaller, hydrophilic molecules exhibit larger Vd by distributing into extracellular fluid.

Lipophilic molecules, such as chloroquine with a high Vd of 140 L/kg, can spread extensively throughout cells and adipose tissues.

Understanding Vd is crucial for determining dosing strategies. For instance, in cases like advanced infections, a loading dose of a drug like vancomycin may be necessary to rapidly achieve therapeutic concentrations. Loading doses are calculated based on Vd, desired plasma concentration, and bioavailability to ensure rapid and effective drug action.

Protein Binding

Proteins play a crucial role in drug efficacy and toxicity. Drugs in the body can exist as either protein-bound or free forms, with only the free drug being able to produce pharmacological effects, move across bodily compartments, or be eliminated. The clinical impact of a drug is more closely linked to its free concentration at receptor sites in plasma rather than its total concentration, highlighting the significance of protein binding. Reduction in plasma protein binding increases the available drug for receptor interaction, potentially intensifying the effect or the risk of toxicity.

Albumin and alpha-acid glycoprotein are critical proteins that bind to medications, influencing their distribution and activity. Factors like age, liver or kidney conditions, and nutritional health can affect protein levels, impacting drug binding and efficacy. In conditions like renal failure, reduced protein binding due to uraemia can lead to higher levels of unbound drug in circulation, resulting in increased drug effect and toxicity, such as respiratory depression.

2.3.3. Metabolism:

Metabolism involves the body processing the drug into different compounds through various methods-

Purpose of Metabolism

It is utilized to convert the drug into more water-soluble forms for renal clearance or to activate pro-drugs like codeine into active metabolites.

Sites of Metabolism

Metabolism can take place in different areas like the gastrointestinal tract, skin, plasma, kidneys or lungs, with a major focus on the liver where most metabolism occurs.

Liver Metabolism

In the liver, metabolism primarily happens through phase I (CYP450) and phase II (UGT) reactions. Phase I processes oxidize substances to form polar metabolites, enabling phase II conjugation reactions. These processes typically render the drug inactive, transform it into a more hydrophilic form, and facilitate its excretion through urine or bile.

2.3.4. Excretion:

Excretion involves eliminating drugs from the body, primarily managed by the kidneys but sometimes through the lungs, skin, or gastrointestinal tract for specific medications. The excretion process can vary in complexity.

Kidney Excretion

Drugs are often cleared through the kidneys via either passive filtration in the glomerulus or active secretion in the tubules. This process can be intricate due to the potential reabsorption of some compounds, affecting the elimination efficiency.

Clearance

Clearance, a crucial concept in the context of drug excretion, is defined as the ratio of a drug's elimination rate to its plasma concentration. It is impacted by factors such as the properties of the drug itself and the patient's organ function and blood flow, typically involving the kidneys. In scenarios where an organ can completely extract all drug from the blood, the clearance is constrained by the organ's blood flow.

Understanding clearance enables healthcare professionals to determine the appropriate dosage of medications tailored to individual patients. Maintenance dosing calculations rely on clearance values, considering the elimination of drug between doses. Maintenance doses are computed by multiplying clearance by the desired plasma concentration and then dividing by bioavailability.

2.3.5. Half-life (t):

The half-life (t) of a drug is the time required for the serum drug concentrations to reduce by 50%. It is calculated using the formula $t = (0.693 \times V_d) / \text{Clearance}$. The drug's half-life is directly related to the volume of distribution and inversely related to clearance. It is influenced by the volume of distribution and clearance of the drug. Changes in clearance parameters due to factors like disease or aging can alter the half-life of medications.

2.3.6. Drug Kinetics:

Drug kinetics, the graphical representation of metabolism and excretion, illustrates a medication's half-life and is categorized into zero-order and first-order kinetics.

Zero-order kinetics: Characterized by a constant rate of metabolism or elimination regardless of drug concentration, seen in alcohol and phenytoin elimination. Variable half-life decreases as serum concentrations reduce.

First-order kinetics: Depends on the proportion of drug plasma concentration, showing a constant 't' with decreasing plasma clearance over time, commonly used for most medications.

Drug kinetics models like zero-order and first-order are not exclusively applicable to all drugs. For instance, salicylates follow proportional elimination to serum concentrations at low levels, shifting to constant elimination at higher concentrations due to metabolic process saturation. These models aid in estimating steady states, where drug administration and clearance balance to maintain an unchanged plasma concentration over time. Ideal continuous infusion achieves steady state after four to five half-lives of the drug, affecting steady-state concentration alterations only by changes in dosing, total dose, or drug clearance.

Total elimination, measurable by half-lives, indicates that around 94 to 97% of a drug is eliminated after four to five half-lives for drugs with first-order elimination kinetics. For example, Morphine with a 't' of 120 mins implies negligible drug presence in the system after eight to ten hours post-administration.

These kinetics concepts are pivotal for understanding drug effectiveness, dosing adjustments, and predicting medication concentrations in clinical scenarios.

2.3.7. Pharmacokinetics in Medication Prescribing:

When healthcare providers prescribe medications, the primary aim is to achieve positive therapeutic results while minimizing negative reactions. Understanding pharmacokinetics plays a crucial role in developing effective treatment plans involving drugs.

Essence of Pharmacokinetics

Pharmacokinetics focuses on tracking the movement of drugs in the body and the body's interactions with the drug. By leveraging specific terms, theories and equations related to pharmacokinetics, practitioners can predict the drug's distribution and concentrations in various body regions.

Determining Drug Concentrations

Laboratory tests are used to ascertain the precise drug concentrations needed to achieve therapeutic effects and to avoid adverse reactions.

Safe Medication Dosing

Equations in pharmacokinetics aid clinicians in calculating safe dosages of medications over time and estimating how long a drug will stay in a patient's system. These estimates are statistically-driven and can vary based on factors such as the drug's form and the patient's physiological conditions.

Clinical Flexibility

Profound knowledge of pharmacokinetics is vital in medical practice, enabling healthcare providers to adapt treatment strategies, when necessary, based on the clinical scenario ^[74].

The reviewed literature indicates that Phospholipase A₂ enzymes have been extensively studied for their roles in lipid metabolism, membrane remodelling, and antimicrobial activity. Several studies have demonstrated that Phospholipase enzymes, particularly those derived from venom sources, can exhibit inhibitory effects on microbial and parasitic organisms.

However, most of these studies are limited to *in vitro* systems and often involve complex or non-standardized enzyme sources. There is limited information regarding the use of well-characterized, commercially available Phospholipase A₂ enzymes in controlled experimental settings for evaluating antimalarial activity.

Furthermore, the potential role of phospholipid hydrolysis in influencing malaria parasite survival within erythrocytes remains insufficiently explored, particularly in *in vivo* infection models.

Therefore, the present study seeks to address this gap by evaluating the effect of Bovine Phospholipase A₂ on malaria parasites using both *in vitro* and *in vivo* approaches, thereby providing preliminary insights into the interaction between phospholipid-modifying enzymes and parasite biology.

CHAPTER 3:

AIM AND

OBJECTIVES

3.1. AIM

The aim of the study is to evaluate the *in vitro* and *in vivo* activity of Phospholipase A₂ enzyme against *Plasmodium sp.*

3.2. OBJECTIVES

1. Determining the cytotoxic and haemolytic effect of the enzyme.
2. *In vitro* efficacy evaluation of Phospholipase A₂ enzyme against *Plasmodium sp.*
3. *In vivo* efficacy evaluation of Phospholipase A₂ enzyme against *Plasmodium sp.*

CHAPTER 4:

MATERIALS AND

METHODS

4.1. Enzyme:

Lyophilized Phospholipase A₂ enzyme from Bovine pancreas was obtained from Sigma Aldrich. It was solubilized in 0.9% saline and separated into aliquots. The aliquots were then stored in -80°C for further testing.

4.2. Cytotoxic Activity:

Peripheral Blood Mononuclear Cells (PBMC) were separated by adding Histopaque to Human blood (with anticoagulant), and centrifuged at 2000 rpm for 10 mins. PBMCs formed a layer between plasma and sedimented erythrocytes, with Histopaque forming layer and separating plasma from PBMCs. The plasma layer was discarded and the PBMC layer was carefully separated for the assay. The cells were then washed with PBS and 50 µL was transferred to a 96-well plate at a concentration of 3×10^5 cells/well. A volume 50 µL of different concentrations of the enzyme, i.e. 500 µg/mL, 250 µg/mL, 125 µg/mL, 62.5 µg/mL, 31.25 µg/mL, 15.63 µg/mL, 7.81 µg/mL, 3.91 µg/mL, 1.95 µg/mL and 0.98 µg/mL; were added to into the well plate with each concentration in triplicates. Cells were cultured with the enzyme for 24 hrs at 37°C at 5% CO₂. Then 40 µL of 0.5 mg/mL MTT was added and the plate was incubated for an additional 3 hrs under the same conditions. The reaction was halted by adding 130 µL of Dimethyl sulfoxide (DMSO) and the plate was then read in a microplate reader at 420 nm. The percentage viability was calculated for each concentration.

4.3. Indirect Haemolysis:

Indirect Haemolysis was evaluated on red blood cells in suspension. Human blood was collected and centrifuged at 3000 rpm for 10 mins and the plasma and buffy coat was discarded. An equal amount 0.9% saline was added to the RBC pellet and it was subjected to centrifugation again. This process was repeated 2 more times. The supernatant was discarded and 0.5 ml of RBC pellet was diluted with 9.5 mL of NS to prepare 5% RBC suspension. The suspension was stored at 4°C until the assay.

A volume of 200 µL of the sample at different concentrations was added in different the vials (each concentration in triplicates). PBS was used as a negative control and Distilled water as positive control (each in triplicates). A volume 50 µL of PBS was added to all tubes along with 200 µL of 1% RBC suspension. The tubes were incubated at 37°C for 30 mins and then centrifuged at 2000 rpm for 10 mins. A volume of 200 µL of the supernatant was added in a

labelled 96 well plate. The plate was then read in a micro-plate reader at 540 nm. The percentage haemolysis for each concentration with the following formula-

$$\% \text{ Haemolysis} = \frac{\text{Absorbance of sample} - \text{Absorbance of negative control}}{\text{Absorbance of positive control}} \times 100$$

4.4. Cultivation of *Plasmodium falciparum*:

Based on the procedure described by Trager and Jensen *et al.*, *P. falciparum* was grown at 37°C in human erythrocytes to a haematocrit of 2% and 3%-6% parasitaemia under an atmosphere of 3% CO₂, 6% O₂ and 91% N₂. Cultures were prepared in T-25 flasks with 2% haematocrit, diluted in RPMI 1640 medium culture [supplemented with 25mM HEPES, 21mM sodium bicarbonate, 11mM glucose, 40 µg/mL gentamicin and 0.5% (w/v) Albumax]. The cultures were maintained in a CO₂ incubator at 37°C. Culture media was changed daily and parasitaemia was monitored in blood smears, fixed with methanol, stained with Field's stain and visualized under an optical microscope at 100X. On reaching 2% parasitaemia, the cultures were passaged or cryopreserved.

Multiple attempts to revive existing cryopreserved cultures of *P. falciparum* cultures was done. However, each attempt failed as either the culture collapsed after initial growth or did not revive at all. After, considerable efforts, resources and time was put into culturing *P. falciparum*, it was decided, after due discussion and approval of the DRC panel, that we will go forward with the use of *P. berghei* to do the *In vitro* Antimalarial assay. A previous study done in the department itself showed that *P. berghei* can sustain for a short time interval in the same medium used for culturing *P. falciparum*, and hence the species was changed for the assay.

4.5. *In vitro* antimalarial assays against *Plasmodium berghei*:

A volume of 100 µL/well of parasite cultures (collected from infected mice) mixed with RPMI 1640 medium containing: 0.5% parasitaemia and 2% haematocrit was added into 96-well plates for the SYBR Green I test. SYBR Green dye binds selectively to double-stranded DNA. In malaria-infected erythrocytes, parasite replication leads to an increase in DNA content, resulting in higher fluorescence intensity. Therefore, fluorescence measurements were used as an indirect indicator of parasite growth.

Prior to adding the parasite suspension, 20 µL of the samples, previously diluted in PBS 1X, was added to the test plate in serial concentrations (each in triplicates). Chloroquine was used as a positive control, infected red cells was used as the negative control, and non-infected red

blood cells (blank) was used as the experimental control. The plates were incubated at 37°C for 48 hrs. After the incubation period, a fluorescence test was performed according to Vitorino *et al.* (2020) [66]. Afterwards, the supernatant was discarded and 100 µL of lysis buffer (20mM Tris, pH 7.5; 5mM EDTA; 0.008% w/v saponin; 0.08% and Triton X-100 v/v) with SYBR Green I (30 µL of SYBR in 15 mL of Lysis buffer) was added in dark and incubated further for 24 hrs. Fluorescence was measured using a spectrophotometer with an excitation of 485 nm and an emission of 590 nm. Percentage Inhibition was calculated for each concentration.

$$\% \text{ Inhibition} = \frac{\text{Fluorescence of negative control} - \text{Fluorescence of sample}}{\text{Fluorescence of negative control}} \times 100$$

4.6. Animals:

All animal-related work was performed after due approval from Institutional Animal Ethics Committee (IAEC) [HITRT/IAEC/05/2021]. Male/female (6-8 weeks) Swiss Albino mice were used in the experiments. Animals were housed at room temperature (25°C ± 2°C) and 12:12 light/dark cycle. Food and water were given ad libitum. Mice were exposed to Isoflurane for short-term anaesthesia. At the end of each experiment, anesthetized mice were humanely sacrificed by CO₂ asphyxia.

4.7. Parasite Maintenance *In vivo*:

Cryopreserved *P. berghei* cultures were revived and injected intraperitoneally into mice at a concentration of 3×10⁸ parasites/mL. The animals were observed daily for signs and symptoms and their weights were also recorded. The parasitaemia was counted by preparing thin blood smears of mice through tail prick method every alternate day. On reaching 5% parasitaemia, blood was collected through the retroorbital route and mice was bled till death. The blood was either cryopreserved for future maintenance or infection, or inoculated into another uninfected mice.

4.8. Pharmacokinetic studies:

For kinetic studies of the enzyme, the mice from *In vivo* Suppression study were used, i.e. group II and III were divided into sub groups of 3 mice each for 9 different time intervals viz 0, 6, 12, 24, 30, 36, 48, 54 and 60 hrs. The grouping of mice is given in Table 1. A volume of 0.1 mL of the enzyme at the concentration of 500 µg/mL was injected intraperitoneally in each mouse at 0, 24 and 48 hrs and the blood was drawn at above mentioned time intervals. Control

mice were injected with saline and their blood was drawn at the given time intervals. The blood samples were then subjected to centrifugation at 2000 rpm for 5 minutes and the plasma was separated under sterilized conditions. The plasma was then stored in -80°C Deep Freezer for SDS-PAGE and HPLC analysis.

Sr. No.	Time Interval (in hours)	Group I (3 mice)	Group II (3 mice)	Group III (3 mice)
	1st Dose:			
1.	0	✓		
2.	6		✓	
3.	12			✓
	2nd Dose:			
4.	24	✓		
5.	30		✓	
6.	36			✓
	3rd Dose:			
7.	48	✓		
8.	54		✓	
9.	60			✓

Table 1: Diagrammatic presentation of sparse sampling method for kinetic studies

Plasma samples were analyzed by SDS-PAGE to confirm the presence of the protein in circulation. SDS-PAGE using silver nitrate method was done using Pierce™ Silver Stain Kit. All the procedures and reagents used were from the kit.

There were 5 trials of HPLC done to detect PLA₂ in plasma samples. 4 trials were done with a few changes to yield results. The 5th trial was done after a preliminary test with SDS-PAGE, and followed the protocol of the 4th trial. Following are the methods used for each trial –

Trial 1:

- **Column:** Comosil Packed Column, 5PE-MS, 4.6 ID × 250 mm
- **Protocol:** Multi-step Gradient
- **Mobile phase:** A- 0.1% TFA in Water
B- Methanol
C- 0.1% TFA in Acetonitrile: Water (80:20)
D- 50:50 Acetonitrile: Water

Time (mins)	Flow (mL/min)	% A	% B	% C	% D
0.0	1.0	95.0	0.0	5.0	0.0
0.0	1.0	95.0	0.0	5.0	0.0
5.0	1.0	95.0	0.0	5.0	0.0
30.0	1.0	20.0	0.0	80.0	0.0
35.0	1.0	95.0	0.0	5.0	0.0

Trial 2:

- **Column:** Comosil Packed Columnn, 5PE-MS, 4.6 ID × 250 mm
- **Protocol:** Multi-step Gradient
- **Mobile phase:** A- 0.1% TFA in Water
B- Methanol
C- 0.1% TFA in Acetonitrile: Water (80:20)
D- 50:50 Acetonitrile: Water

Time (mins)	Flow (mL/min)	% A	% B	% C	% D
0.0	1.0	95.0	0.0	5.0	0.0
0.0	1.0	95.0	0.0	5.0	0.0
5.0	1.0	95.0	0.0	5.0	0.0
15.0	1.0	85.0	0.0	15.0	0.0
75.0	1.0	55.0	0.0	45.0	0.0
85.0	1.0	30.0	0.0	70.0	0.0
90.0	1.0	30.0	0.0	70.0	0.0
100.0	1.0	95.0	0.0	5.0	0.0

Trial 3:

- **Column:** Comosil Packed Columnn, 5PE-MS, 4.6 ID × 250 mm
- **Protocol:** Multi-step Gradient
- **Mobile phase:** A- 0.1% TFA in Water
B- Methanol
C- 0.1% TFA in Acetonitrile: Water (80:20)
D- 50:50 Acetonitrile: Water

Time (mins)	Flow (mL/min)	% A	% B	% C	% D
0.0	1.0	99.0	0.0	1.0	0.0
0.0	1.0	99.0	0.0	1.0	0.0
5.0	1.0	99.0	0.0	1.0	0.0
10.0	1.0	98.0	0.0	2.0	0.0
15.0	1.0	97.0	0.0	3.0	0.0
20.0	1.0	96.0	0.0	4.0	0.0
25.0	1.0	95.0	0.0	5.0	0.0
30.0	1.0	99.0	0.0	1.0	0.0

Trial 4:

- **Column:** Comosil Packed Column, 5PE-MS, 4.6 ID × 250 mm
- **Protocol:** Multi-step Gradient
- **Mobile phase:** A- 0.1% TFA in Water
B- Methanol
C- 0.1% TFA in Acetonitrile: Water (80:20)
D- 50:50 Acetonitrile: Water

Retention Time (min)	Flow (mL/min)	% B	% C	% D
0.0	1.0	0.0	5.0	0.0
0.0	1.0	0.0	5.0	0.0
5.0	1.0	0.0	5.0	0.0
15.0	1.0	0.0	15.0	0.0
75.0	1.0	0.0	45.0	0.0
85.0	1.0	0.0	70.0	0.0
90.0	1.0	0.0	70.0	0.0
100.0	1.0	0.0	5.0	0.0

4.9. *In vivo* Suppression Test:

Four-day parasite suppression test was performed to know the suppressive activity of the enzyme. This was done as per a method described by Valissery *et al.* (2020) [75]. Total 29 mice were used for the test. The mice were randomized based on body weight and divided into 3 groups, with Group I having 5 mice and Group II and III having 12 mice each (Table 2). The mice were injected intraperitoneally with parasites on Day 1 and the drug treatments were commenced on the same day. The treatment was continued for the next 4 days. Mice survival and signs and symptoms were observed daily for up to 28 days post-inoculation initiation. The mean survival time was calculated for each group. Field's-stained thin blood smears were prepared on the 4th Day, and the Percentage of Parasite Growth Suppression (PGS) was estimated using the following formula-

$$\text{Parasite Growth Suppression (\%)} = [(A-B)/A] \times 100$$

Where, A is the average parasitaemia of control (negative) group and B represents the parasitaemia of the experimental group.

Group no.	Group Name	Treatment	No. of animals
Group I	Treatment I	Chloroquine	5
Group II	Treatment II	Vehicle	12
Group III	Treatment III	Enzyme	12

Table 2: Group Distribution for *In vivo* Suppression Test

4.10. *In vivo* Antimalarial activity:

46 Swiss Albino mice were randomly divided into 6 groups, with Control group having 6 mice and other groups having 8 mice each (Table 3). Group 1 was Control group, where the animals were not infected and they did not receive any treatment. Group 2 was Sham group, where the animals received only the vehicle (0.9% saline). Group 3 to 5 were injected with selected enzyme concentrations, i.e. 500 µg/mL, 250 µg/mL and 125 µg/mL respectively. Group 6 was Standard Group, where the animals received 200 mg per kg per day of Chloroquine on the 4th and 5th day. The mice were injected intraperitoneally (I. P.) with 3×10^8 parasitized erythrocytes. Treatment was started 4 days post parasite inoculation. Treatment was continued for 5 days. The survival rate was monitored on a daily basis in all groups for 28 days post-treatment initiation. On day 5, Field's-stained thin blood smears were prepared from the tail of each animal to determine parasitaemia and percentage inhibition. The percentage of Parasite Growth Suppression (PGS) was estimated. The Mean Survival Time (MST) for each group was determined over a period of 28 days.

$$\% \text{ Parasitaemia} = \frac{\text{Number of Parasitized RBCs} \times 100}{\text{Total number of RBCs counted}}$$

$$\text{Mean Survival Time (MST)} = \frac{\text{Sum of survival time for all mice in a group}}{\text{Total number of mice in that group}}$$

Group no.	Group Name	Treatment	No. of animals
Group I	Normal Control	Without any treatment	6
Group II	Sham control	Vehicle	8
Group III	Treatment I	Concentration 1	8
Group IV	Treatment II	Concentration 2	8
Group V	Treatment III	Concentration 3	8
Group VI	Treatment IV	Chloroquine	8

Table 3: Group Distribution for *In vivo* Antimalarial Test

4.11. Statistical Analysis:

All experimental data are expressed as Mean $\pm$ Standard Deviation (SD) from replicate measurements. Due to the limited number of biological replicates, statistical analysis was restricted to descriptive statistics, and no inferential statistical tests were applied.

CHAPTER 5:

RESULTS

5.1. Cytotoxic Activity:

The percentage viability of Phospholipase A₂ against PBMCs was found to be higher than 80% in all concentrations, which is considered to be non-cytotoxic. The positive control, Chloroquine, showed a percentage viability of 57.58% which is considered moderately cytotoxic. Table 3 shows the Mean Absorbance and Percentage Viability of the enzyme.

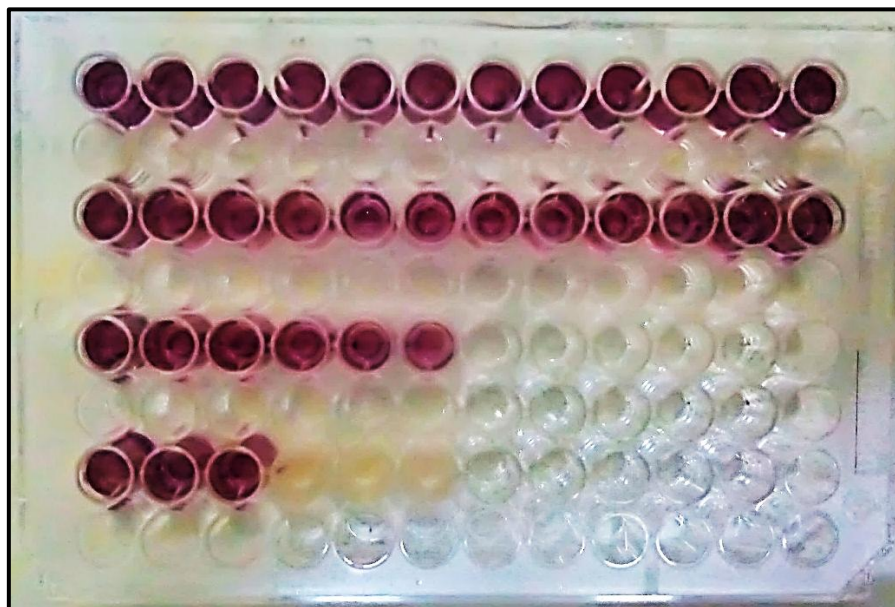


Figure 7: MTT Assay of PLA₂

CONCENTRATION ($\mu\text{g/mL}$)	MEAN	PERCENTAGE VIABILITY (%)
500	0.60	90.91 ± 0.01
250	0.59	89.39 ± 0.04
125	0.62	93.94 ± 0.05
62.5	0.62	93.94 ± 0.08
31.25	0.63	95.45 ± 0.04
15.63	0.60	90.91 ± 0.03
7.81	0.57	86.36 ± 0.03
3.9	0.52	78.79 ± 0.08
1.95	0.56	84.85 ± 0.05
0.97	0.56	84.85 ± 0.08
Negative Control	0.66	100 ± 0.10
Positive Control (Chloroquine)	0.38	57.58 ± 0.06

Table 4: Mean Absorbance and Percentage of Viability of enzyme

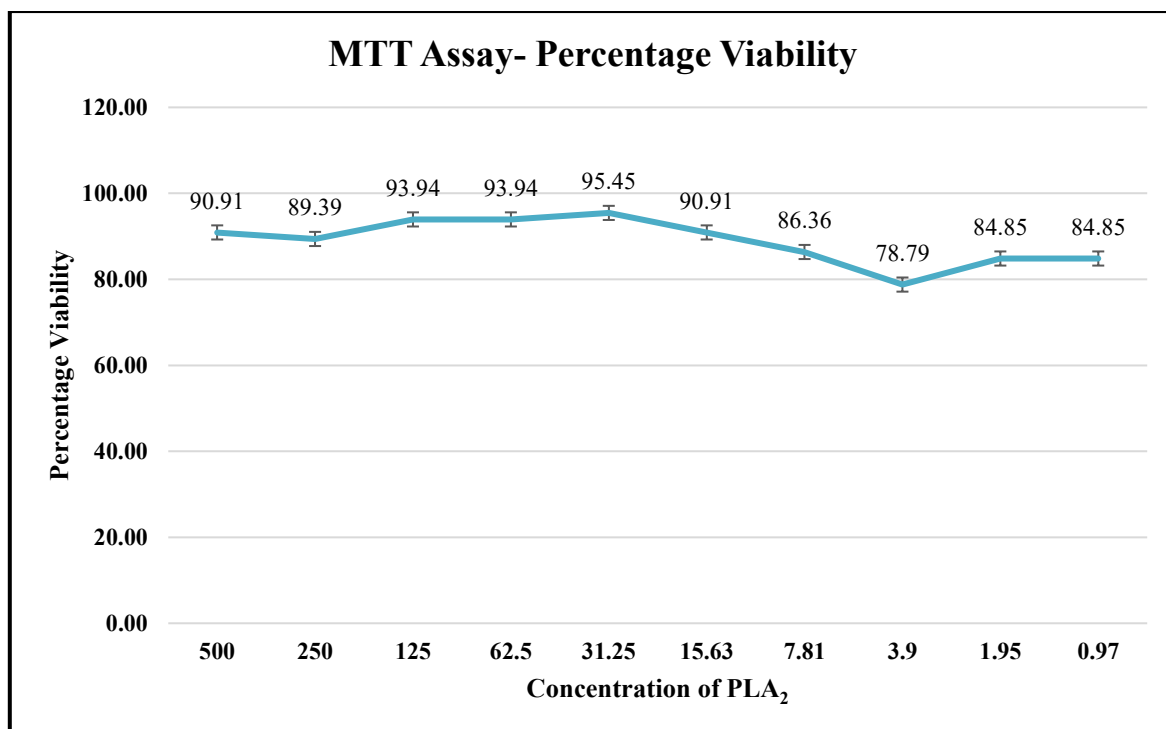


Figure 8: Graphical representation of Percentage Viability

5.1. Indirect Hemolysis:

It was found that the enzyme does not produce significant hemolysis and all concentrations have percentage hemolysis below 50%. Hence, the enzyme is not hemotoxic.

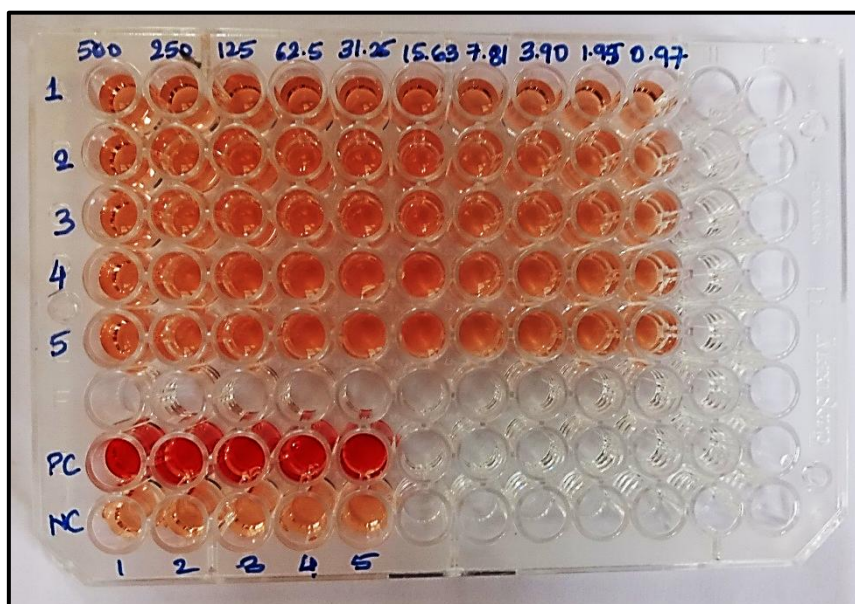


Figure 9: Indirect Haemolysis of PLA₂

CONCENTRATION ($\mu\text{g/mL}$)	MEAN	PERCENTAGE HEMOLYSIS
500	0.433	4.73 ± 0.001
250	0.414	3.80 ± 0.004
125	0.432	4.68 ± 0.001
62.5	0.417	3.95 ± 0.001
31.25	0.407	3.46 ± 0.002
15.63	0.415	3.85 ± 0.001
7.81	0.343	0.34 ± 0.003
3.9	0.337	0.05 ± 0.006
1.95	0.340	0.20 ± 0.001
0.97	0.343	0.34 ± 0.003

Table 5: Mean Absorbance and Percentage Haemolysis of PLA₂

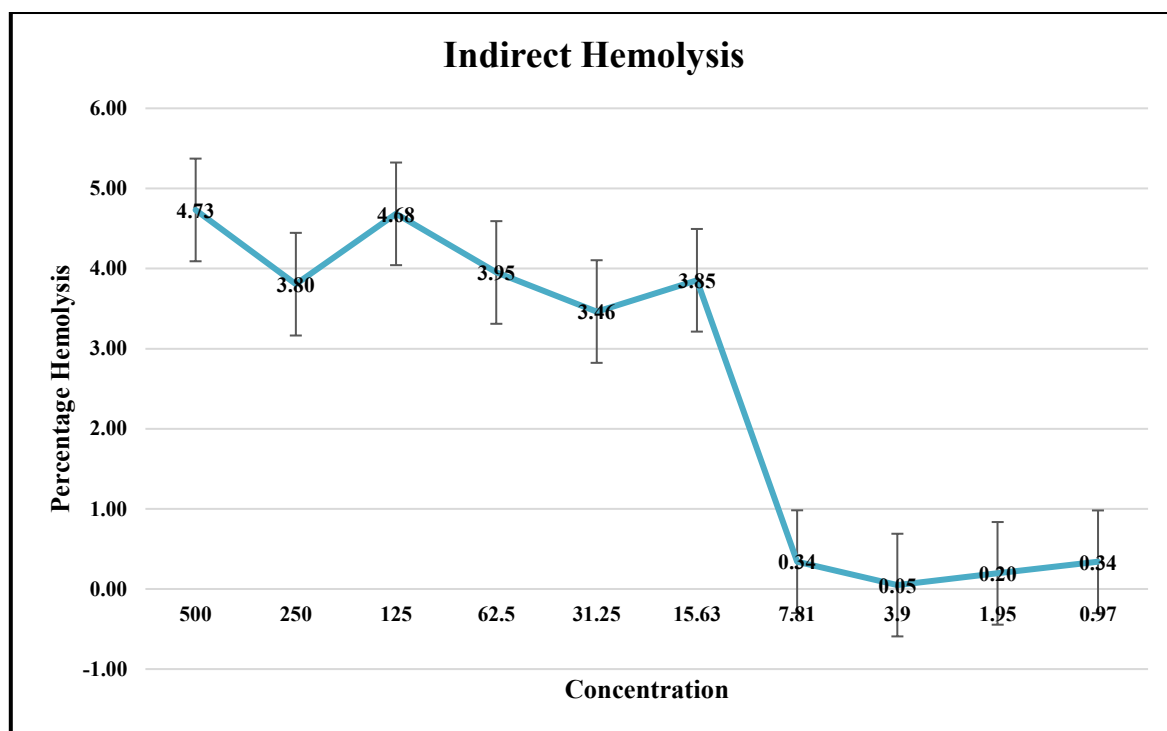


Figure 10: Graphical Representation of Indirect haemolysis

5.2. *In vitro* Antimalarial Activity:

It was observed that the concentrations 500 $\mu\text{g/mL}$, 1.95 $\mu\text{g/mL}$ and 0.98 $\mu\text{g/mL}$ demonstrate 100% inhibition. The observed negative inhibition values are likely due to experimental variability in fluorescence measurements rather than true biological proliferation. Since

erythrocytes are terminally differentiated cells lacking nuclei, they do not undergo proliferation. Variations in fluorescence signal may arise from assay noise, pipetting differences, or differences in parasite synchronization. Therefore, these values should be interpreted as experimental variability rather than increased parasite growth.

CONCENTRATION	MEAN	PERCENTAGE INHIBITION (%)
500	0	100 ± 0.0
250	5.33	-77.78 ± 5.51
125	0.33	88.89 ± 0.58
62.5	7	-133.33 ± 5.29
31.25	6	-100 ± 4.58
15.63	17.33	-477.78 ± 9.45
7.81	14	-366.67 ± 4.58
3.91	24.67	-722.22 ± 2.31
1.95	0	100 ± 0.0
0.98	0	100 ± 0.0
Control	3	
Chloroquine	0	100 ± 0.0

Table 6: Mean Absorbance and Percentage Inhibition of PLA₂

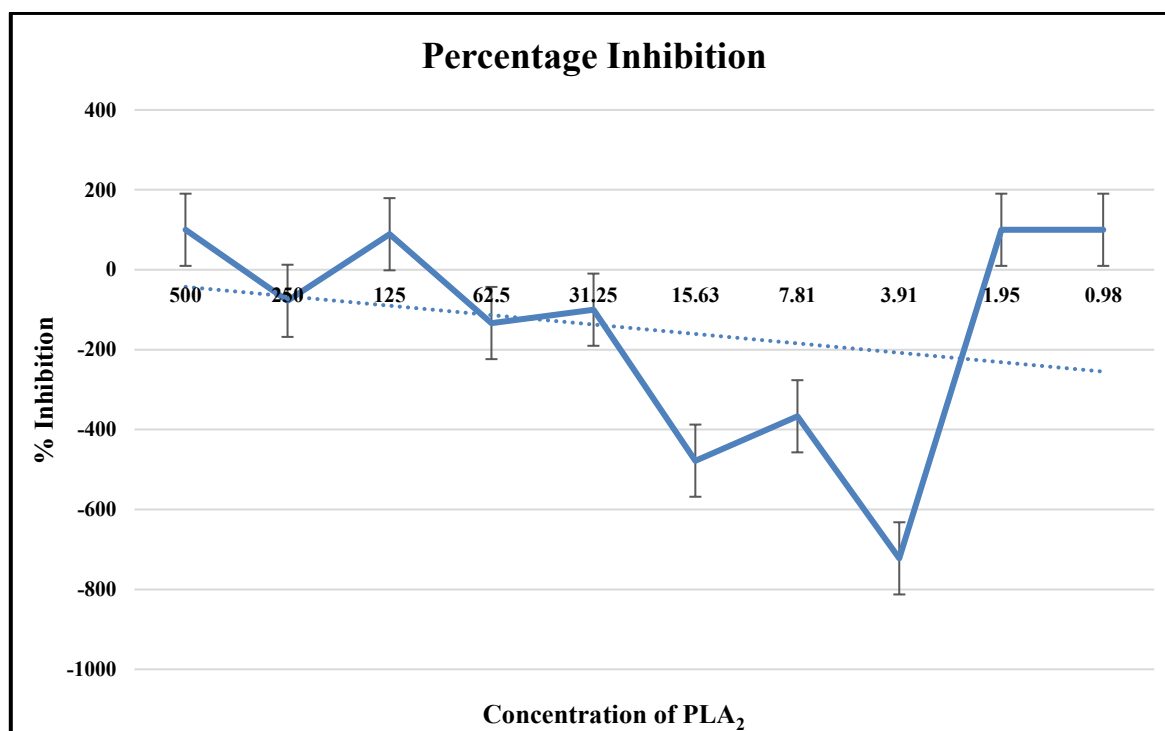


Figure 11: Graphical Representation of Percentage Inhibition

5.3. Pharmacokinetic Studies:

HPLC Analysis for detecting presence of PLA₂ was done. There were three different protocols used with repeatability, but none of them could detect the enzyme. A 4th trial yielded results but they were inconclusive. Hence, detection of PLA₂ using SDS PAGE with silver nitrate staining was done. The SDS PAGE yielded results for pure PLA₂, but on running plasma samples there was no distinction between bands. Based on this, another HPLC Analysis for detecting presence of PLA₂ was tried with the last protocol used. This time the trial yielded results, but for confirmation and clarity a MS detection is required, which is costly as well as would take more time for standardization.

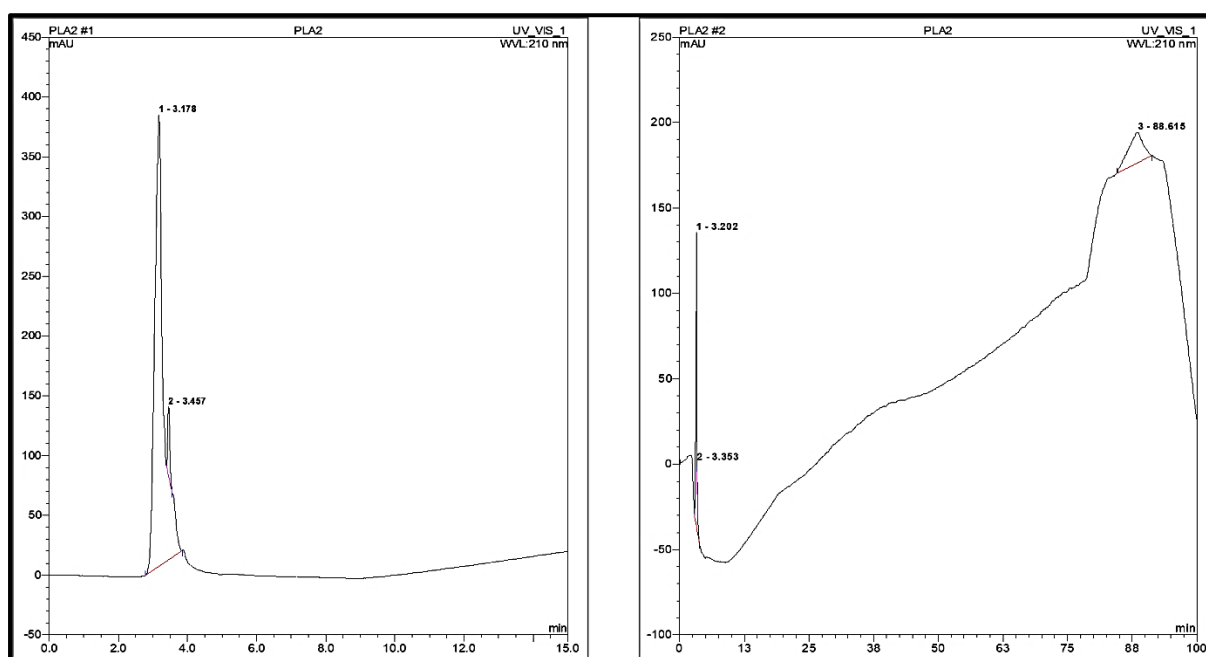


Figure 12: Results of Trial 1 (pure PLA₂)

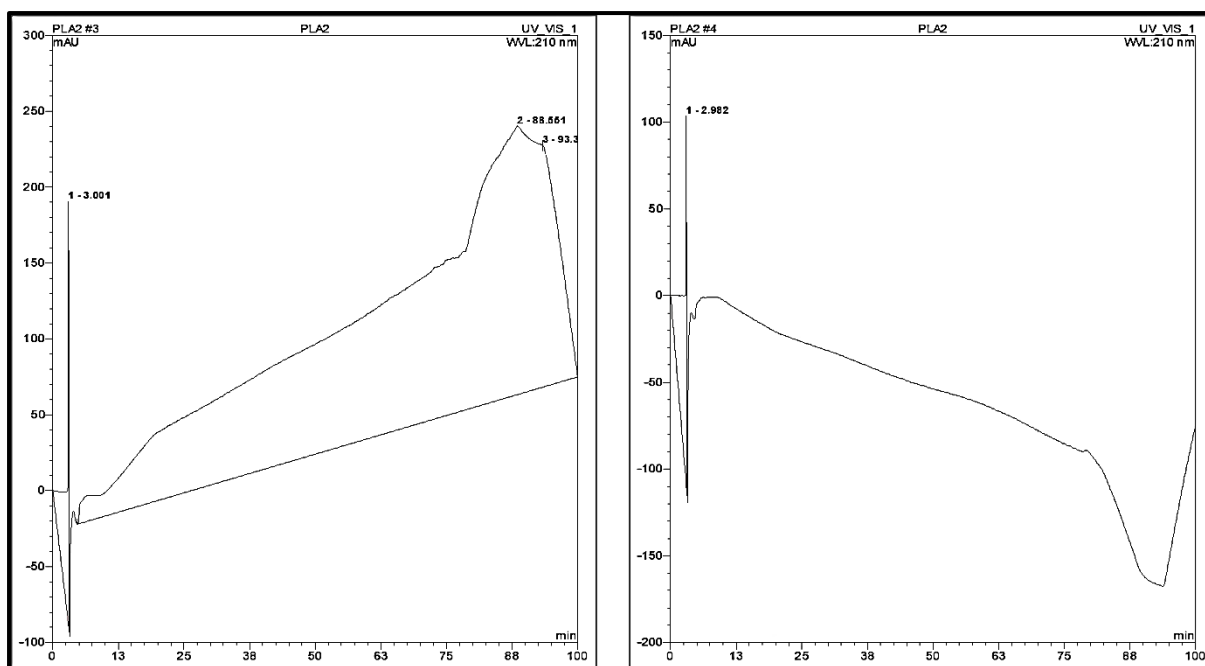


Figure 13: Results of Trial 2 (pure PLA₂)

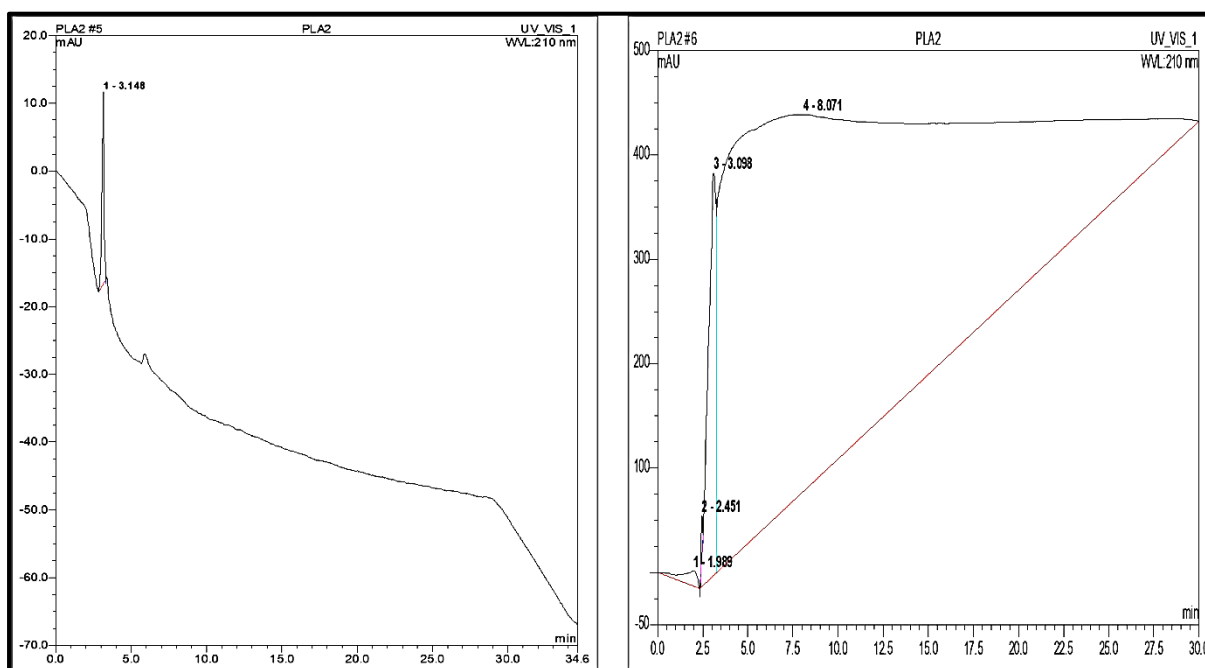


Figure 14: Results of Trial 3

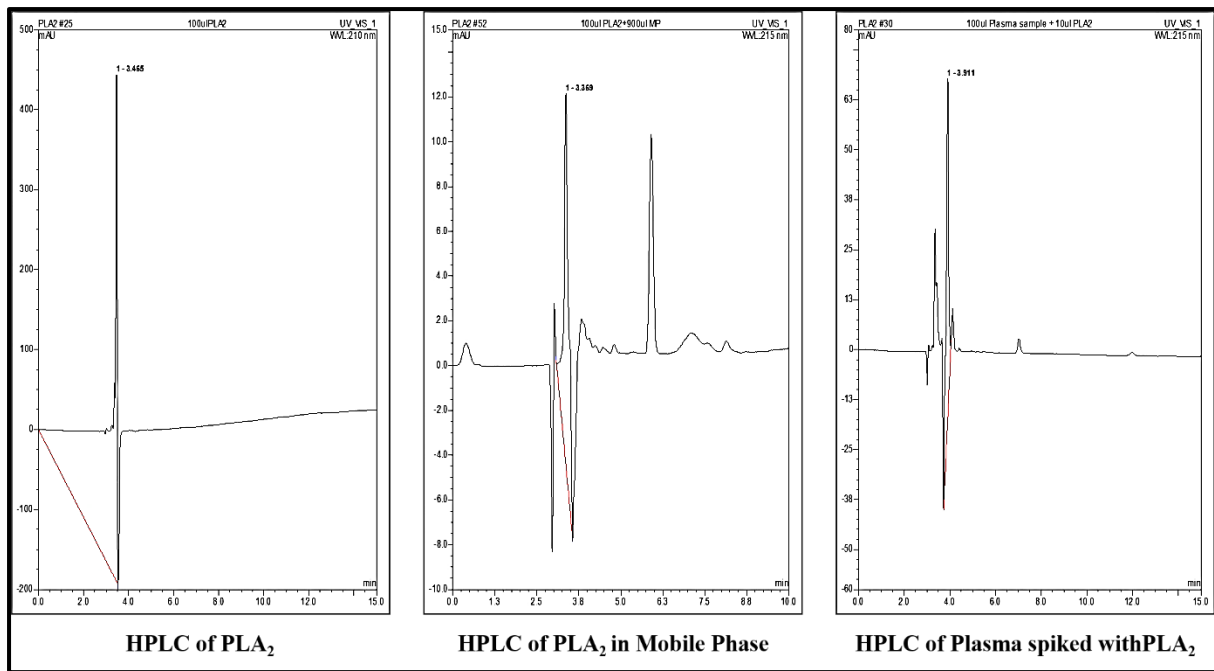


Figure 15: Results of Trial 4

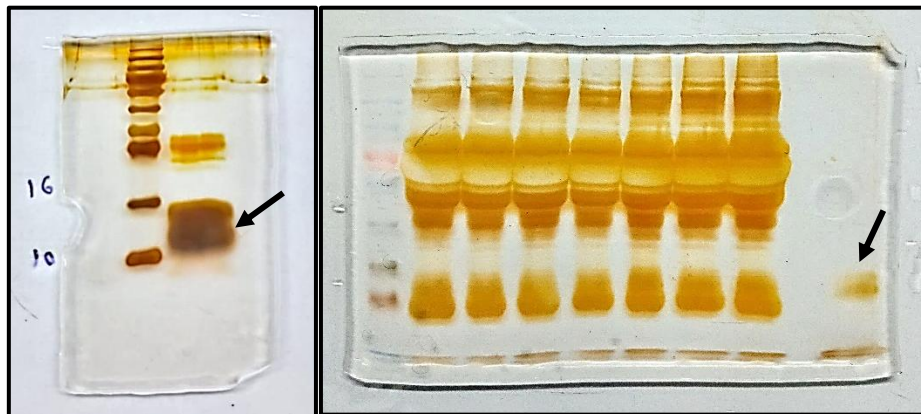


Figure 16: SDS PAGE of pure PLA₂ (left) and PLA₂ in plasma (right) [PLA₂ band has been marked]

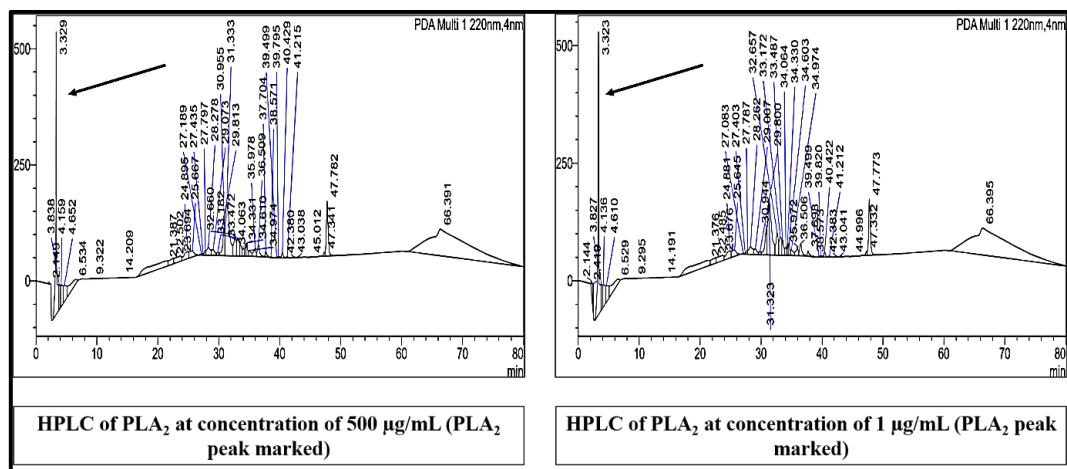


Figure 17: Results of Trial 5

5.4. *In vivo* Suppression Test:

It was found that PLA₂ showed considerable suppression at 0.11% followed by Chloroquine at 0.66%. It was in stark contrast to the vehicle group with 17.45% parasitaemia. The mean survival time indicates that PLA₂ does show considerable survival almost on par with Chloroquine. The signs and symptoms included shivering, piloerection, toe walking, slow gait, hypothermia, hunchback posture, weight loss and morbidity.

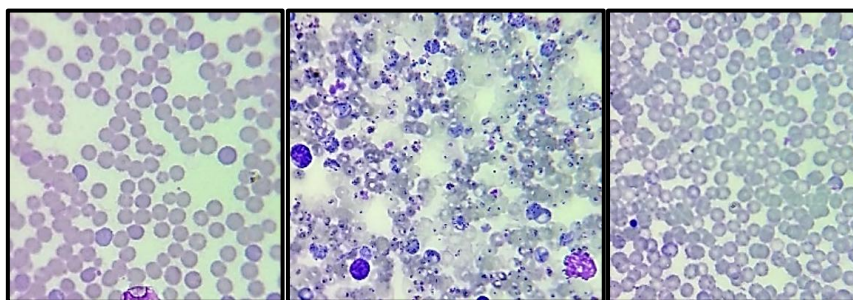


Figure 18: Slides of Chloroquine, Vehicle and PLA₂ respectively

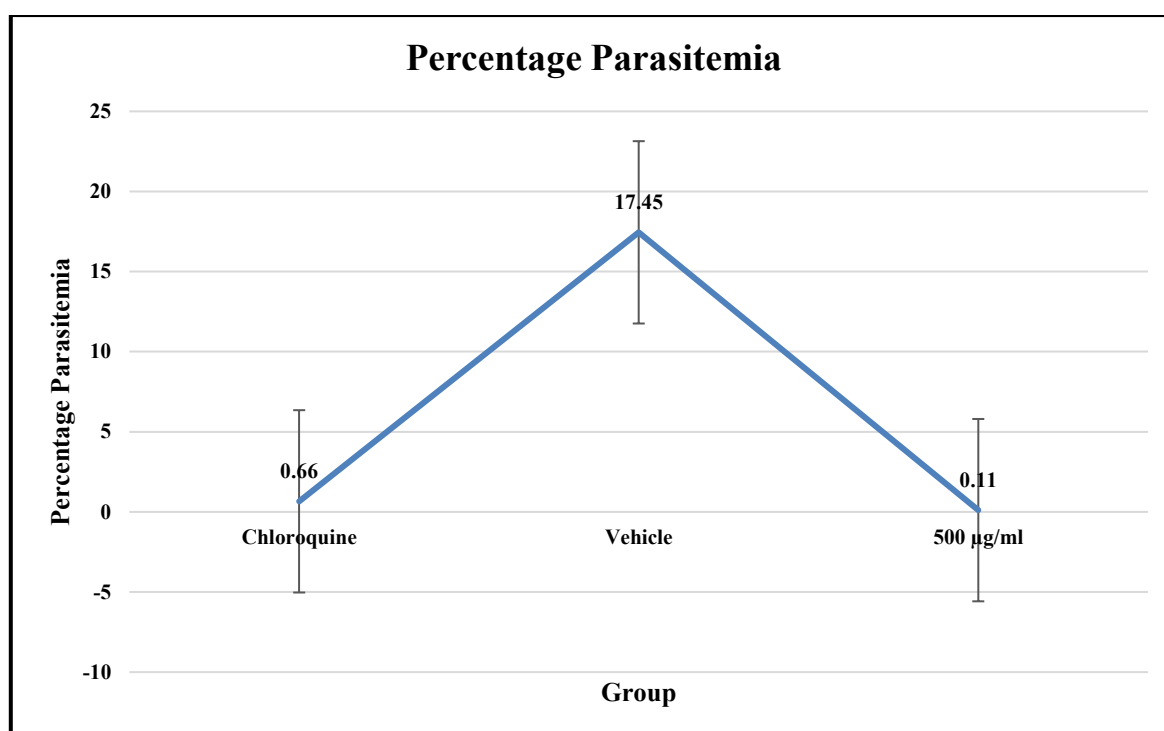


Figure 19: Graphical Representation of Percentage Parasitaemia

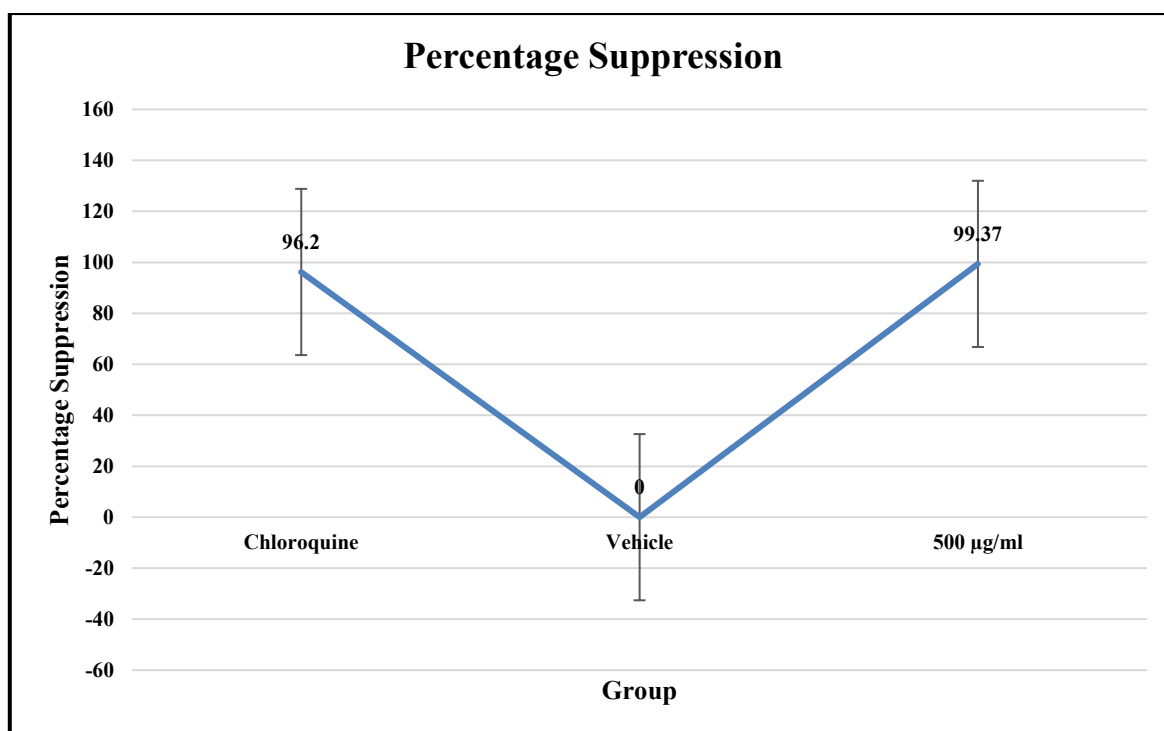


Figure 20: Graphical Representation of Percentage Suppression

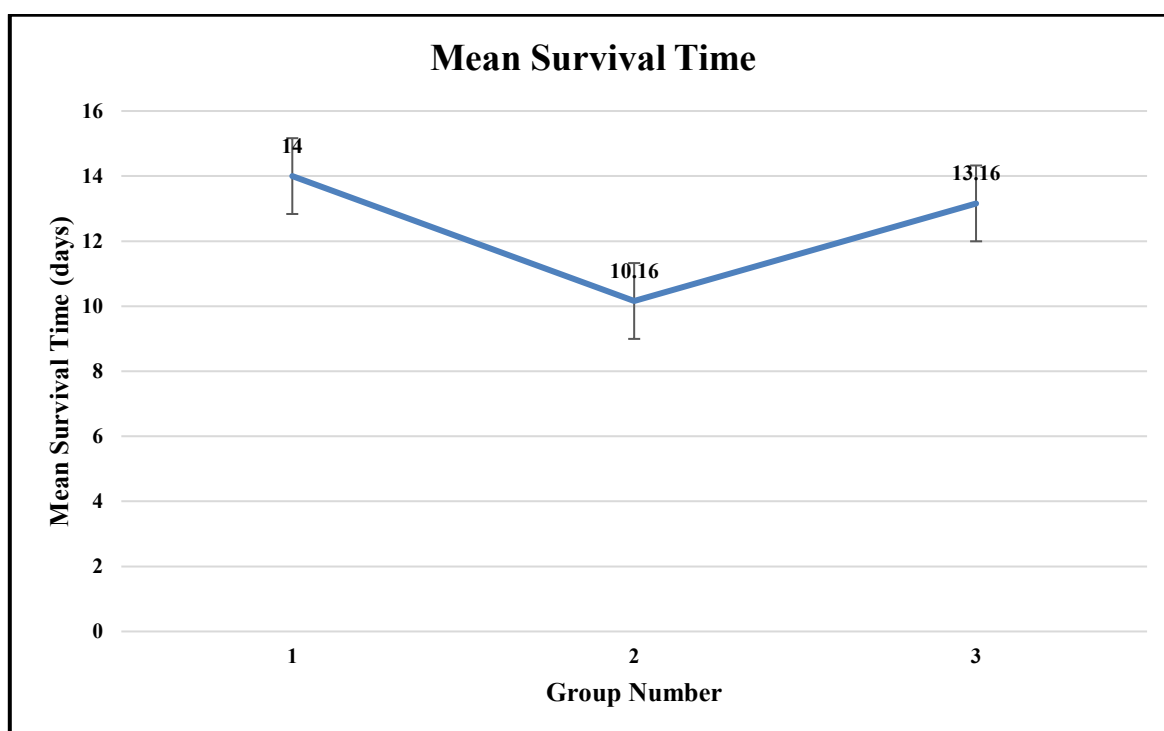


Figure 21: Graphical Representation of Mean Survival Time

5.5. *In vivo* Antimalarial Test:

It was observed that there was linearity in the activity of PLA₂, with 500 µg/mL showing maximum activity at 3.65%, followed by 250 µg/mL at 9.80% and 125 µg/mL at 34.87%

percentage parasitaemia. The activity of PLA₂ was near to but not equal to Chloroquine showing 0.81% percentage parasitaemia. The Mean Survival Time also showed the same linearity, but it was not better than Chloroquine. The signs and symptoms included shivering, piloerection, toe walking, slow gait, hypothermia, hunchback posture, weight loss and morbidity. After sometime the animals started showing signs of normality and their feed consumption also increased.

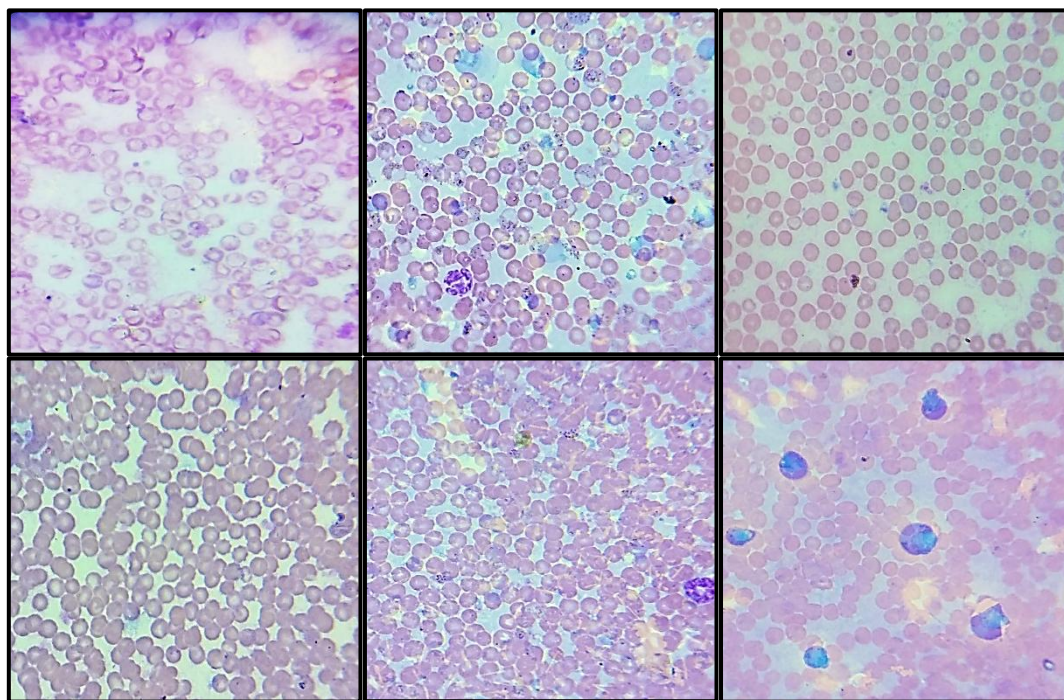


Figure 22: Slides of *In vivo* Antimalarial Test [In order from Top left corner- Control, Sham Control, 500 µg/mL, 250 µg/mL, 125 µg/mL and Chloroquine]

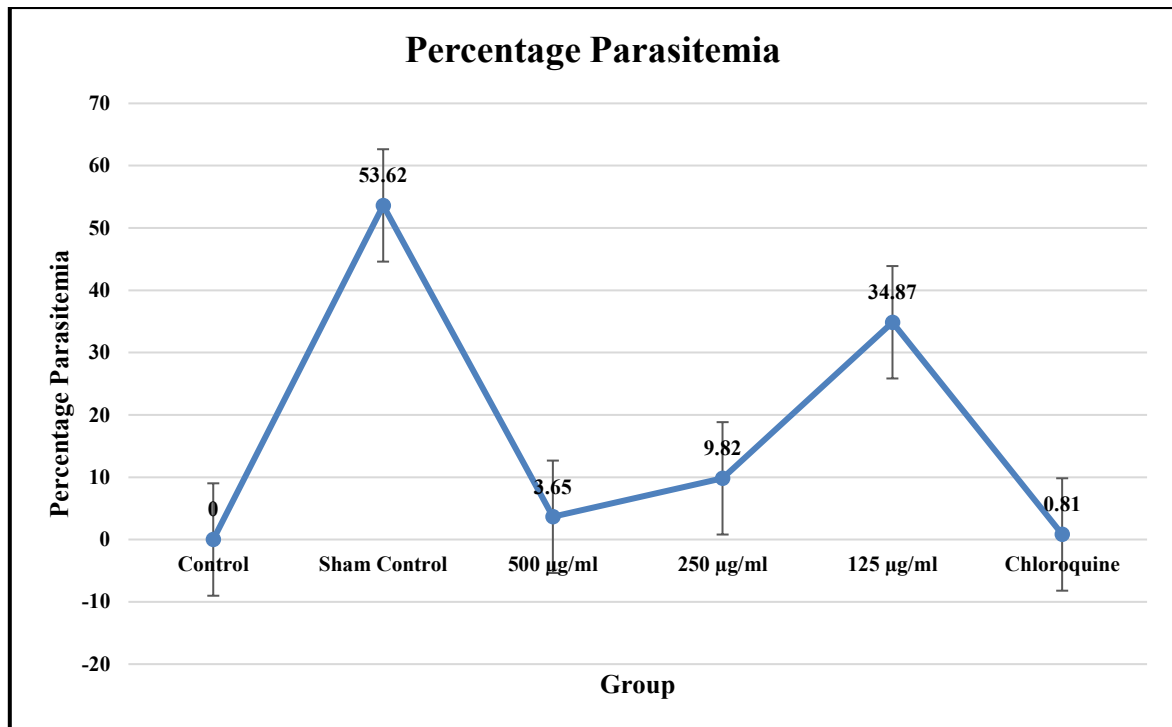


Figure 23: Graphical Representation of Percentage Parasitaemia

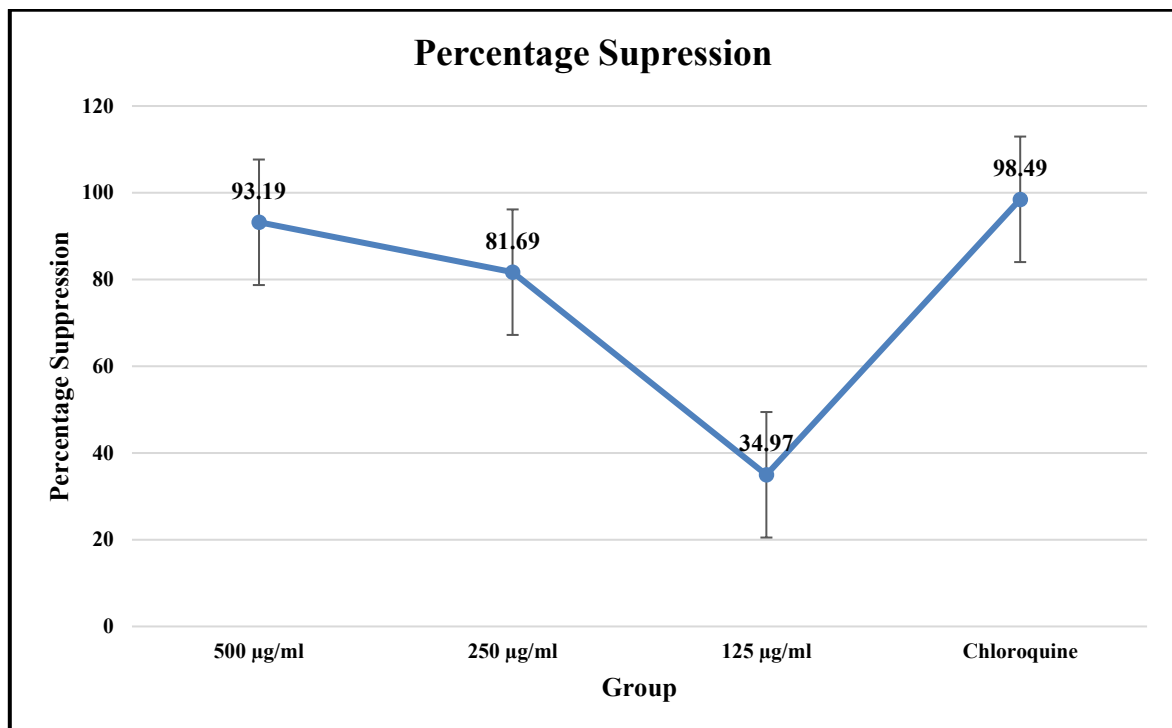


Figure 24: Graphical Representation of Percentage Suppression

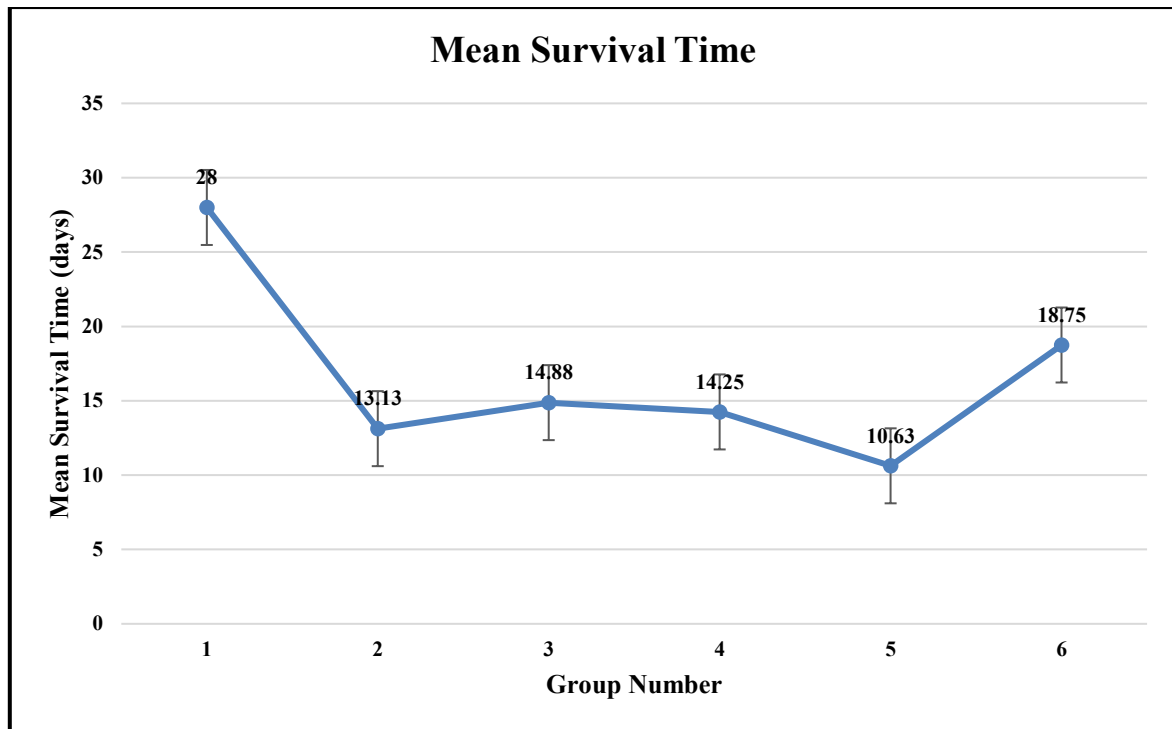


Figure 25: Graphical Representation of Mean Survival Time

CHAPTER 6:

DISCUSSION

Malaria is a life-threatening disease caused by *Plasmodium* parasites, transmitted to humans through the bites of infected female *Anopheles* mosquitoes. While it remains most common in tropical and subtropical regions, efforts to control the disease are increasingly challenged by the rise of drug-resistant strains, particularly of *Plasmodium falciparum*, the deadliest species. These resistant parasites reduce the effectiveness of key antimalarial medications, making treatment more difficult and increasing the risk of severe illness or death. Symptoms include fever, chills, headache, and fatigue, and the disease remains especially dangerous for young children and pregnant women. Preventive measures such as insecticide-treated bed nets, mosquito control, and the development of new treatments are essential to combat both the disease and its growing resistance to drugs.

The ≥ 80 % PBMC viability across all doses classifies the enzyme as non-cytotoxic under ISO-10993-5 criteria. Comparable safety has been reported for snake-venom PLA₂ homologues BdTX-II and BaPLA₂, which retained >85 % Vero-cell viability up to 100 $\mu\text{g/mL}$ while inhibiting *Plasmodium* growth [66][76].

Human erythrocytes tolerated the bovine enzyme with <5 % haemolysis at 500 $\mu\text{g/mL}$, consistent with the observation that catalytically active PLA₂s prefer unsaturated phospholipids and spare cholesterol-rich membranes [32]. Together these data confirm a wide safety window relative to classical antimalarials such as chloroquine, which is known to cause oxidative stress-related lysis at similar concentrations.

The variation observed in parasite inhibition across different concentrations of PLA₂ does not demonstrate a consistent dose-dependent trend. While fluctuations in response were noted, the data does not support a defined pattern such as a U-shaped dose-response relationship. Such variability may be attributed to experimental factors, differences in enzyme activity under assay conditions, or complex interactions between host cell membranes and parasite development. Therefore, no definitive mechanistic interpretation can be made from the current data, and further studies with improved experimental design and increased replicates would be required to investigate this observation in detail.

Failure to detect the enzyme in plasma is consistent with the rapid binding and clearance described for endogenous Group IIA PLA₂ (half-life 3-5 mins in rodents) [36]. The tight lipoprotein association – PLA₂ partitions into LDL/HDL has complicated HPLC recovery; LC–MS/MS with lipid-depletion pre-columns has proven superior [50]. The small 14-kDa enzyme

is filtered rapidly by the kidney; PEGylation or encapsulation in PLGA nanoparticles prolonged circulating levels four-fold in a recent bee-venom PLA₂ study [8].

Although PLA₂ demonstrated measurable parasite suppression at 99%, the level of inhibition and survival benefit was lower than that observed with chloroquine. Therefore, chloroquine remains significantly more effective under the conditions tested.

Although the top dose of 500 µg/mL (3.65% parasitaemia) did not match chloroquine (0.81%) in *in vivo* Antimalarial test, the dose-response was linear and survival times approached those of the standard drug. This echo results with *Bothrops asper* PLA₂ homologues, which lowered parasitaemia in *P. berghei*-infected mice but required higher mg/kg doses than chloroquine for radical cure [76].

The observed activity of PLA₂ may be associated with its ability to hydrolyze phospholipids in erythrocyte membranes. Malaria parasites depend on host cell membrane lipids for growth, membrane biogenesis, and intracellular survival. Enzymatic modification of these phospholipids may therefore alter the microenvironment required for parasite development. The study suggests that PLA₂ can elucidate effect for early antimalarial or prophylactic use but may need prolonged exposure or combination therapy for full clearance of late blood stages.

The study has several limitations that should be acknowledged. The number of biological replicates was limited, and statistical analysis was therefore restricted to descriptive representation. Variability observed in the in-vitro assays indicates the need for improved experimental standardization. Additionally, protein detection was performed using SDS-PAGE, which lacks the specificity of antibody-based methods such as Western blotting. These limitations suggest that the findings should be interpreted as preliminary observations requiring further validation.

CHAPTER 7:

CONCLUSION

This study demonstrates that Bovine Phospholipase A₂ exhibits measurable biological activity against *Plasmodium* parasites in both *in vitro* and *in vivo* experimental models. The enzyme was found to exert mild inhibitory effects against *Plasmodium berghei* across multiple concentrations and experimental models, with minimal cytotoxicity and haemolytic activity, confirming its favourable safety profile.

The magnitude of parasite inhibition and survival improvement observed was modest compared to the standard antimalarial drug chloroquine in prophylactic antimalarial efficacy. The results suggest that PLA₂ may influence parasite survival through interactions with host cell membrane phospholipids, but it should be considered as a preliminary biological observation rather than a direct therapeutic candidate. However, challenges in detecting the enzyme in plasma indicate pharmacokinetic limitations, likely due to rapid degradation or tissue sequestration, which warrant further optimisation through advanced drug delivery systems or formulation strategies.

These findings align well with existing literature on secretory PLA₂s derived from animal venoms, such as bee and snake venoms, which also demonstrate antimalarial effects through membrane-disruptive and immune-modulatory mechanisms.

In conclusion, this work substantiates the candidacy of bovine PLA₂ as a novel enzymatic antimalarial agent, highlighting its efficacy, safety, and mechanistic promise. Future studies should prioritise mechanistic elucidation, pharmacokinetic enhancement, and potential combination therapies to overcome its current limitations and advance its clinical translation.

CHAPTER 8:

FUTURE

PROSPECTS

Future studies should dissect the molecular interactions between PLA₂ and the *Plasmodium* cell membrane, with a focus on identifying whether lipid hydrolysis or immune modulation underpins its anti-plasmodial effects. The current challenge in detecting PLA₂ *in vivo* calls for advanced techniques such as LC-MS/MS. Furthermore, nano-formulations or enzyme encapsulation strategies may enhance PLA₂ stability and bioavailability. Longitudinal studies to assess the potential for resistance development by *Plasmodium* to PLA₂ would provide critical insights into its long-term viability as a therapeutic agent. Given the partial overlap in efficacy with chloroquine, PLA₂ could be tested in combination with standard antimalarials to evaluate synergistic effects, reduced resistance or enhanced therapeutic indices. Evaluating PLA₂'s effect on different *Plasmodium* life stages, including gametocytes and liver stages, would offer a more comprehensive understanding of its antimalarial spectrum. Detailed toxicological assessments and dose-escalation studies in higher-order animal models are necessary before considering human trials. Inspired by studies in transgenic mosquitoes expressing PLA₂ to block transmission, the gene encoding PLA₂ could be explored for vector control strategies, expanding its utility beyond therapeutic use.

CHAPTER 9:

BIBLIOGRAPHY

1. World malaria report 2024: addressing inequity in the global malaria response. Geneva: World Health Organization; 2024. Licence: CC BY-NC-SA 3.0 IGO.
2. White N. J., Pukrittayakamee S., Hien T. T., Faiz M. A., Mokuolu O. A. & Dondorp A. M. (2014). Malaria. *The Lancet*; 383 (9918): 723-735.
3. Dondorp A. M., Nosten F., Yi P., Das D., Ohyo A. P., Tarning J., Lwin K. M., Arie F., Hanpithakpong W., Lee S. J., Ringwald P., Silamut K., Imwong M., Chotivanich K., Lim P., Herdman T., An S. S., Yeung S., Singhasivanon P., Day N. P. J., Lindegardh N., Socheat D. & White N. J. (2009). Artemisinin Resistance in *Plasmodium falciparum* Malaria. *The New England Journal of Medicine*; 361: 455-467.
4. Six D. A. & Dennis E. A. (2000). The expanding superfamily of phospholipase A (2) enzymes: classification and characterization. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*; 1488 (1-2): 1-19.
5. Baron S. (1996). Medical Microbiology. University of Texas, Medical Branch, Galveston.
6. Cui L., Mharakurwa S., Ndiaye D., Rathod P. K. & Rosenthal P. J. (2015). Antimalarial Drug Resistance: Literature Review and Activities and Findings of the ICEMR Network. *Am. J. Trop. Med. Hyg.*; 93 (3 Suppl): 57-68.
7. Kini R. M. (2003). Excitement ahead: structure, function and mechanism of snake venom phospholipase A₂ enzymes. *Toxicon*; 42 (8): 827-840.
8. Samy R. P., Gopalakrishnakone P., Jois S. D. & Stiles B. G. (2013). Snake Venom Proteins and Peptides as Novel Antibiotics Against Microbial Infections. *Current Proteomics*; 10 (1): 10-28.
9. Vaughan A. M., Aly A. S. & Kappe S. H. (2008). Malaria parasite pre-erythrocytic stage infection: gliding and hiding. *Cell Host Microbe.*; 4 (3): 209-18.
10. Rénia L. & Goh Y. S. (2016). Malaria Parasites: The Great Escape. *Front. Immunol.*; 7: 463.
11. Beeson J. G., Drew D. R., Boyle M. J., Feng G., Fowkes F. J. & Richards J. S. (2016). Merozoite surface proteins in red blood cell invasion, immunity and vaccines against malaria. *FEMS Microbiol. Rev.*; 40 (3): 343-72.

12. Li Q., Lv K., Jiang N., Liu T., Hou N., Yu L., Yang Y., Feng A., Zhang Y., Su Z., Sang X., Feng Y., Chen R., Xu W., Cui L., Cao Y. & Chen Q. (2024). SOD3 suppresses early cellular immune responses to parasite infection. *Nature communications*; 15 (1): 4913.
13. Castelli F., Tomasoni L. R. & Matteelli A. (2012). Advances in the Treatment of Malaria. *Mediterr. J. Hematol. Infect. Dis.*; 4 (1): e2012064.
14. Laurens M. B. (2020). RTS,S/AS01 vaccine (Mosquirix™): an overview. *Hum. Vaccin. Immunother.*; 16 (3): 480-489.
15. Mitchell R. A., Macià D., Jairoce C., Mpina M., Naidu A., Chopo-Pizarro A., Vázquez-Santiago M., Campo J. J., Aide P., Aguilar R., Daubenberger C., Dobaño C. & Moncunill G. (2024). Effect of RTS,S/AS01_E vaccine booster dose on cellular immune responses in African infants and children. *NPJ vaccines*; 9 (1): 200.
16. *Indian Council of Medical Research*. (2025, August 17). Invitation for Expression of Interest (EoI) for Transfer of Technology of "A recombinant multi-stage malaria vaccine" (Vaccine Candidate) Ref:EoI No. ICMR/EoI/PM/05/Malaria vaccine (AdFalcivax)/2025. <https://www.icmr.gov.in/call-for-proposals>
17. *The Hindu*. (2025, July 19). ICMR seeks collaboration for malaria vaccine production through tech transfer. <https://www.thehindu.com/sci-tech/health/icmr-seeks-collaboration-for-malaria-vaccine-production-through-tech-transfer/article69831601.ece>
18. *The Indian Express*. (2025, July 24). How new ICMR vaccine gives new hope in fight against malaria. <https://indianexpress.com/article/explained/explained-health/how-new-icmr-vaccine-gives-new-hope-in-fight-against-malaria-10145512/>
19. *DD News*. (2025, July 20). ICMR developing indigenous two-stage malaria vaccine, AdFalcivax. <https://ddnews.gov.in/en/icmr-developing-indigenous-two-stage-malaria-vaccine-adfalcivax/>
20. *Hindustan Times*. (2025, August 4). ICMR's anti-malaria vaccine still 6-7 years away. <https://www.hindustantimes.com/india-news/icmr-anti-malaria-vaccine-still-6-7-years-away-101754248247192.html>

21. PIB Delhi (2024). Update on India's Progress on in Malaria Elimination. Ministry of Health and Family Welfare.
22. Belete T. M. (2020). Recent Progress in the Development of New Antimalarial Drugs with Novel Targets. *Drug Des. Devel. Ther.*; 14: 3875-3889.
23. Chaniad P., Mungthin M., Payaka A., Viriyavejakul P. & Punsawad C. (2021). Antimalarial properties and molecular docking analysis of compounds from *Dioscorea bulbifera L.* as new antimalarial agent candidates. *BMC Complement Med Ther*; 21: 144.
24. Cheuka P. M., Dziwornu G., Okombo J. & Chibale K. (2020). Plasmepsin Inhibitors in Antimalarial Drug Discovery: Medicinal Chemistry and Target Validation (2000 to Present). *J. Med. Chem.*; 63 (9): 4445-4467.
25. Winzeler E. A. (2008). Malaria research in the post-genomic era. *Nature*; 455 (7214): 751-756.
26. Read M. & Hyde J. E. (1993). Simple in vitro cultivation of the malaria parasite *Plasmodium falciparum* (erythrocytic stages) suitable for large-scale preparations. *Methods Mol. Biol.*; 21: 43-55.
27. Trager W. & Jensen J. B. Cultivation of erythrocytic stages. *Bull World Health Organ.* (1977); 55 (2-3): 363-5.
28. Golenda C. F., Li J. & Rosenberg R. (1997). Continuous in vitro propagation of the malaria parasite *Plasmodium vivax*. *Proc. Natl. Acad. Sci. U. S. A.*; 94 (13): 6786-91.
29. Ramaiya M. L., Kamath V. R. & Renapurkar D. M. (1987). Long-term in vitro cultivation of *Plasmodium berghei*. *Int. J. Parasitol.*; 17 (7): 1329-31.
30. Duffy S. & Avery V. M. (2018). Routine In Vitro Culture of *Plasmodium falciparum*: Experimental Consequences? *Trends Parasitol.*; 34 (7): 564-575.
31. Huang H. M., McMorran B. J., Foote S. J. & Burgio G. (2018). Host genetics in malaria: lessons from mouse studies. *Mamm. Genome.*; 29 (7-8): 507-522.
32. Simwela N. V. & Waters A. P. (2022). Current status of experimental models for the study of malaria. *Parasitology*; 149 (6): 1-22.

33. Benjamin-Chung J., Li H., Nguyen A. *et al.* (2024). Extension of efficacy range for targeted malaria-elimination interventions due to spillover effects. *Nat. Med.*; 30: 2813–2820.
34. Carter V., Underhill A., Baber I., Sylla L., Baby M., Larget-Thiery I., Zettor A., Bourgouin C., Langel U., Faye I., Otvos L., Wade J. D., Coulibaly M. B., Traore S. F., Tripet F., Eggleston P. & Hurd H. (2013). Killer bee molecules: antimicrobial peptides as effector molecules to target sporogonic stages of *Plasmodium*. *PLoS Pathog.*; 9 (11): e1003790.
35. Smith R. C., Kizito C., Rasgon J. L. & Jacobs-Lorena M. (2013). Transgenic mosquitoes expressing a phospholipase A₂ gene have a fitness advantage when fed *Plasmodium falciparum*-infected blood. *PLoS One*; 8 (10): e76097.
36. Dennis E. A. (1994). Diversity of group types, regulation, and function of phospholipase A₂. *Journal of Biological Chemistry*; 269 (18): 13057-13060.
37. Murakami M. & Kudo I. (2002). Phospholipase A₂. *The Journal of Biochemistry*; 131 (3): 285–292.
38. Schaloske R. H. & Dennis E. A. (2006). The phospholipase A₂ superfamily and its group numbering system. *Biochim. Biophys. Acta.*; 1761 (11): 1246-59.
39. Tojo H., Ono T. & Okamoto M. (1993). Reverse-phase high-performance liquid chromatographic assay of phospholipases: application of spectrophotometric detection to rat phospholipase A₂ isozymes. *J. Lipid Res.*; 34 (5): 837-844.
40. Yamamoto K., Takase K., Kojima H. & Urabe I. (2016). Cloning and expression of phospholipase A₂ from a marine bacterium, *Vibrio sp.* ABE-1, in *Escherichia coli*. *Journal of Biotechnology*; 223: 42-49.
41. Yang W., Devaiah S. P., Pan X., Isaac G., Welti R. & Wang X. (2017). AtPLAI is an acyl hydrolase involved in basal jasmonic acid production and Arabidopsis resistance to *Botrytis cinerea*. *Journal of Biological Chemistry*; 292 (47): 19687-19699.
42. Bacha A. B., Karray A., Bouchaala E., Gargouri Y. & Ali Y. B. (2011). Purification and biochemical characterization of pancreatic phospholipase A₂ from the common stingray *Dasyatis pastinaca*. *Lipids Health Dis.*; 10: 32.

43. Miles E. W., Rhee S., Davies D. R., Hochachka P. W. & Hand S. C. (2005). Structure of a lobster apo-vertebrate hemocyanin: apoprotein and carbohydrate at 5 Å resolution. *Journal of Biological Chemistry*; 280 (50): 41940-41946.
44. Huang Q., Wu Y., Qin C., He W. & Wei X. (2015). Phylogenetic and structural analysis of the phospholipase A₂ gene family in vertebrates. *Int. J. Mol. Med.*; 35 (3): 587-96.
45. Murakami M., Taketomi Y., Miki Y., Sato H., Yamamoto K. & Lambeau G. (2014). Emerging roles of secreted phospholipase A₂ enzymes: The 3rd edition. *Biochimie*; 1-9.
46. Fremont D. H., Anderson D. H., Wilson I. A., Dennis E. A. & Xuong N. H. (1993). Crystal structure of phospholipase A₂ from Indian cobra reveals a trimeric association. *Proc. Natl. Acad. Sci. U. S. A.*; 90 (1): 342-346.
47. Dabral D. & van den Bogaart G. (2021). The Roles of Phospholipase A₂ in Phagocytes. *Front. Cell Dev. Biol.*; 9: 673502.
48. Gomez-Cambronero J., Horwitz J. & Sha'afi R. I. (2003). Measurements of Phospholipases A₂, C, and D (PLA₂, PLC, and PLD). *Methods in Molecular Biology*; 218.
49. Sharp J. D. & Honig M. G. (1988). The structure and function of the soluble 14,000-dalton antigen of the nematode *Caenorhabditis elegans*. *Journal of Biological Chemistry*; 263 (19): 9106-9111.
50. Atanasov V., Petrova S. & Mitewa M. (2009). HPLC Assay of Phospholipase A₂ Activity Using Low-Temperature Derivatization of Fatty Acids. *Analytical Letters*; 42 (10): 1341-1351.
51. Darwish D. A., Masoud H. M. M., Abdel-Monsef M. M., Helmy M. S., Zidan H. A. & Ibrahim M. A. (2021). Phospholipase A₂ enzyme from the venom of Egyptian honey bee *Apis mellifera lamarckii* with anti-platelet aggregation and anti-coagulation activities. *J. Genet. Eng. Biotechnol.*; 19 (1): 10.
52. Karkabounas A., Kitsioulis E. I., Nakos G. & Lekka M. E. (2011). HPLC-fluorimetric assay of phospholipase A₂: Application to biological samples with high protein content and various reaction conditions. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.*; 879 (19): 1557-64.

53. Thompson C. R., Maher H. W. & Turk J. (2006). Characterization of the molecular specificity and kinetic properties of rat liver cytosolic phospholipase A₂-beta. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*; 1761 (5-6): 623-630.
54. Wijeyewickrema L. C., Mickleburgh I., Alkhatib H. & Schroit A. J. (2007). Phospholipid binding specificities of annexins. *Biochimica et Biophysica Acta (BBA)-Biomembranes*; 1768 (12): 2702-2711.
55. Sakurada S., Kato S. & Nishino H. (2004). Tryptophan 64 of phospholipase A₂ from porcine pancreas, a residue located on the surface of the protein, is critical for the binding of this enzyme to the substrate surface. *Biochemical and Biophysical Research Communications*; 313 (1): 98-104.
56. Hui D. Y. (2012). Phospholipase A (2) enzymes in metabolic and cardiovascular diseases. *Curr. Opin. Lipidol.*; 23 (3): 235-240.
57. Farooqui A. A., Ong W. Y., Horrocks L. A. & Farooqui T. (2006). Brain phospholipases A₂: a perspective on the history. *Journal of Lipid Research*; 47 (5): 1094-1113.
58. Nixon A. B., Pang H., Starr M. D., Friedman P. N., Bertagnolli M. M., Kindler H. L., Goldberg R. M., Venook A. P. & Hurwitz H. I. (2018). Prognostic and predictive blood-based biomarkers in patients with advanced pancreatic cancer: results from CALGB80303 (Alliance). *Clinical Cancer Research*; 24 (7): 1574-1584.
59. Frihling B. E. F., Boleti A. P. A., Oliveira C. F. R., Sanches S. C., Cardoso P. H. O., Verbiseck N., Macedo M. L. R., Rita P. H. S., Carvalho C. M. E. & Migliolo L. (2022). Purification, Characterization and Evaluation of the Antitumoral Activity of a Phospholipase A₂ from the Snake *Bothrops moojeni*. *Pharmaceuticals*; 15: 724.
60. Dennis E. A., Cao J., Hsu Y. H., Magrioti V. & Kokotos G. (2011). Phospholipase A₂ enzymes: physical structure, biological function, disease implication, chemical inhibition and therapeutic intervention. *Chem. Rev.*; 111 (10): 6130-85.
61. Accary C., Mantash A., Mallem Y., Fajloun Z. & Elkak A. (2015). Separation and Biological Activities of Phospholipase A₂ (Mb-PLA₂) from the Venom of *Montivipera bornmuelleri*, a Lebanese Viper. *Journal of Liquid Chromatography & Related Technologies*; 38 (8): 833-839.

62. Harwig S. S., Tan L., Qu X. D., Cho Y., Eisenhauer P. B. & Lehrer R. I. (1995). Bactericidal properties of murine intestinal phospholipase A₂. *J. Clin. Invest.*; 95 (2): 603-10.
63. Karray A., Ali Y. B., Gargouri Y. & Bezzine S. (2011). Antibacterial properties of chicken intestinal phospholipase A₂. *Lipids Health Dis.*; 10: 4.
64. Zarai Z., Gharsallah H., Hammami A., Mejdoub H., Bezzine S. & Gargouri Y. T. (2012). Antibacterial, anti-chlamydial and cytotoxic activities of a marine snail (*Hexaplex trunculus*) phospholipase A₂: an in vitro study. *Appl. Biochem. Biotechnol.*; 168 (4): 877-86.
65. Siniavin A. E., Streltsova M. A., Nikiforova M. A., Kudryavtsev D. S., Grinkina S. D., Gushchin V. A., Mozhaeva V. A., Starkov V. G., Osipov A. V., Lummis S. C. R., Tsetlin V. I. & Utkin Y. N. (2021). Snake venom phospholipase A₂s exhibit strong virucidal activity against SARS CoV-2 and inhibit the viral spike glycoprotein interaction with ACE2. *Cell Mol. Life Sci.*; 78 (23): 7777-7794.
66. Vitorino K. A., Alfonso J. J., Gómez A. F., Santos A. P. A., Antunes Y. R., Caldeira C. A. S., Gómez C. V., Teles C. B. G., Soares A. M. & Calderon L. A. (2020). Antimalarial activity of basic phospholipases A₂ isolated from Paraguayan *Bothrops diporus* venom against *Plasmodium falciparum*. *Toxicon*; 8: 100056.
67. Guillaume C., Calzada C., Lagarde M., Schrével J. & Deregnaucourt C. (2006). Interplay between lipoproteins and bee venom phospholipase A₂ in relation to their anti-*Plasmodium* toxicity. *J. Lipid Res.*; 47 (7): 1493-506.
68. Moraes L. A., Ampomah P. B., Lim L. H. & Chong A. S. (2020). Phospholipase A₂ receptors and their endogenous ligands: structure, function and pathophysiology. *Pharmacology & Therapeutics*; 220: 107719.
69. Dacheux M., Sinou V., Payré C., Jeammet L., Parzy D., Grellier P., Deregnaucourt C. & Lambeau G. (2019). Antimalarial Activity of Human Group IIA Secreted Phospholipase A₂ in Relation to Enzymatic Hydrolysis of Oxidized Lipoproteins. *Infect. Immun.*; 87 (11): e00556-19.

70. Moreira L. A., Ito J., Ghosh A., Devenport M., Zieler H., Abraham E. G., Crisanti A., Nolan T., Catteruccia F. & Jacobs-Lorena M. (2002). Bee venom phospholipase inhibits malaria parasite development in transgenic mosquitoes. *J. Biol. Chem.*; 277 (43): 40839-43.
71. Lambeau G. & Gelb M. H. (2008). Biochemistry and physiology of mammalian secreted phospholipases A₂. *Annual Review of Biochemistry*; 77: 495-520.
72. Simões-Silva R., Alfonso J. J., Gómez A. F., Sobrinho J. C., Kayano A. M., de Medeiros D. S. S., Teles C. B. G., Quintero A., Fuly A. L., Gómez C. V., Pereira S. S., da Silva S. L., Stábeli R. G. & Soares A. M. (2021). Synergism of *in vitro* plasmodicidal activity of phospholipase A₂ isoforms isolated from panamanian *Bothrops asper* venom. *Chemico-Biological Interactions*; 346: 109581.
73. Zieler H., Keister D. B., Dvorak J. A. and Ribeiro J. M. C. (2001). A snake venom phospholipase A₂ block malaria parasite development in the mosquito midgut by inhibiting ookinete association with the midgut surface. *The Journal of Experimental Biology*; 204: 4157-4167.
74. Grogan S. & Preuss C. V. (2025). Pharmacokinetics. StatPearls Publishing.
75. Valissery P., Thapa R., Singh J., Gaur D., Bhattacharya J., Singh A. P. & Dhar S. K. (2020). Potent *in vivo* antimalarial activity of water-soluble artemisinin nano-preparations. *RSC advances*; 10 (59): 36201-36211.
76. Castillo J. C., Vargas L. J., Segura C., Gutiérrez J. M. & Pérez J. C. (2012). In vitro anti-plasmodial activity of phospholipases A₂ and a phospholipase homologue isolated from the venom of the snake *Bothrops asper*. *Toxins (Basel)*; 4 (12): 1500-16.
77. Burke J. E. & Dennis E. A. (2009). Phospholipase A₂ structure/function, mechanism, and signalling. *J. Lipid Res.*; 50: 237-42.
78. Carter R. & Mendis K. N. (2002). Evolutionary and historical aspects of the burden of malaria. *Clinical microbiology reviews*; 15 (4): 564–594.
79. Hurt-Camejo E., Camejo G., Peilot H., Oörni K. & Kovanen P. (2001). Phospholipase A(2) in vascular disease. *Circulation research*; 89 (4): 298–304.

80. Michel M., Skourtanioti E., Pierini F., Guevara E. K., Mötsch A., Kocher A., Barquera R., Bianco R. A., Carlhoff S., Coppola Bove L., Freilich S., Giffin K., Hermes T., Hiß A., Knolle F., Nelson E. A., Neumann G. U., Papac L., Penske S., Rohrlach A. B., *et. al.* (2024). Ancient *Plasmodium* genomes shed light on the history of human malaria. *Nature*; 631 (8019): 125–133.
81. Milner D. A. Jr. (2018). Malaria Pathogenesis. *Cold Spring Harb. Perspect. Med.*; 8 (1): a025569.
82. Murakami M., Sato H., Miki Y., Yamamoto K. & Taketomi Y. (2015). A new era of secreted phospholipase A₂. *J. Lipid Res.*; 56 (7): 1248-61.
83. Murakami M., Nakatani Y., Atsumi G I., Inoue K. & Kudo I. (2017). Regulatory Functions of Phospholipase A₂. *Crit. Rev. Immunol.*; 37 (2-6): 127-195.
84. Nosten F., Richard-Lenoble D. & Danis M. (2022). A brief history of malaria. *Presse medicale (Paris, France: 1983)*; 51 (3): 104130.
85. Ramrakhiani L. & Chand S. (2011). Recent progress on phospholipases: different sources, assay methods, industrial potential and pathogenicity. *Appl. Biochem. Biotechnol.*; 164 (7): 991-1022.
86. Sanni L. A., Fonseca L. F. & Langhorne J. (2002). Mouse models for erythrocytic-stage malaria. *Methods Mol. Med.*; 72: 57-76.
87. Sato H., Taketomi Y. & Murakami M. (2016). Metabolic regulation by secreted phospholipase A₂. *Inflamm. Regen.*; 36: 7.
88. Schuster F. L. (2022). Cultivation of *Plasmodium spp.* *Clinical Microbiology Reviews*; 15 (3): 355-364.
89. Talapko J., Škrlec I., Alebić T., Jukić M. & Včev A. (2019). Malaria: The Past and the Present. *Microorganisms*; 7 (6): 179
90. Wang P., Li Y., Shao Q., Zhou W. & Wang K. (2015). Targeting human secretory phospholipase A₂ with designed peptide inhibitors for inflammatory therapy. *J. Drug Target.*; 23 (2): 40-6.



Assessment of the Anti-inflammatory and Analgesic Properties of *Tragia involucrata* Anti-inflammatory, Analgesic Activity of *Tragia involucrata*

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ABSTRACT

The diverse bioactive properties of *Tragia involucrata* are highlighted by numerous studies that reveal its anti-inflammatory, antioxidant, analgesic, and potential anti-diabetic effects. Leaves of *T. involucrata* were gathered, extracted with various solvents, and analyzed for phytochemical components through biochemical methods and high-performance thin-layer chromatography (HP-TLC). The extracts underwent evaluation for cytotoxicity, antioxidant capacity, and in vitro anti-inflammatory effects. An acute oral toxicity assessment was conducted to determine in vivo toxicity in rats, while carrageenan-induced paw edema was utilized to assess in vivo anti-inflammatory activity. The phytochemical constituents include tannins, flavonoids, saponins, terpenoids, and steroids. Notably, the aqueous extract exhibited significant membrane-stabilizing effects on human red blood cell membranes and demonstrated potent activity against stable free radicals. Results from acute toxicity studies and paw edema tests indicated that the extract was well tolerated at a dose of 2000 mg/kg body weight and effectively reduced edema. Additionally, the aqueous extract produced analgesic effects at an oral dosage of 300 mg/kg body weight. These results imply that AETI possesses promising analgesic, antioxidant, and anti-inflammatory properties, suggesting that *T. involucrata* may be a therapeutic option for managing pain and inflammation.

Keywords: *Tragia involucrata*, anti-inflammatory, analgesic, antioxidant activity, Ayurveda.

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DETERMINATION OF SPERMICIDAL ACTIVITY OF PLANT
EXTRACT ON GOAT SEMEN

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ABSTRACT

Fertility control is an issue of global & national public health concern. The traditional use of medicinal plants to treat different sorts of disease, including anti-fertility, is widespread throughout the world as many plant substances are known for their interference with male reproductive system. Approximately 50% of all pregnancies are unintended at conception; 50% of those occur in the 94% of sexually active couples who report using some method of contraception. The only male-specific contraceptive methods currently available are withdrawal, condoms and vasectomy. As concerns regarding side effects and inconvenience of the existing methods of contraception (both female and male) prevent their universal acceptance, the development of additional male methods of fertility control can provide tremendous social and public health benefits. Tests on goat

semen were done using *Mentha arvensis* & *Momordica charantia*. Mass activity and Initial Motility assays as well as live and dead percentages of number of sperms in the semen sample were determined. Hypo-osmotic swelling test was also carried out. It was observed that the plant extracts change the plasma membrane integrity of the sperms and lead to their

**EVALUATION OF ANTI-OSTEOARTHRITIC ACTIVITY OF
METHANOLIC EXTRACT OF CYNODON DACTYLON IN
MONOSODIUM IODOACETATE INDUCED RATS****Mrunal Ghag Sawant^{1*}, Anjana Kalpesh Yadav² and Sheetal Sudhakar Shetty³**

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ABSTRACT

Osteoarthritis is a common condition and is likely to become even more prevalent as the population ages. It involves focal areas of damage to the articular cartilage of synovial joints, with subsequent remodeling of the underlying bone and mild synovitis. Medicinal Plants are source of various potent and powerful drugs one such plant is *Cynodon dactylon* Linn. The plant contains flavonoids, glycosides, and alkaloids. Few studies on pharmacological activity of this plant such as toxicity studies, anti-diabetic, antiulcer, diuretic, antimicrobial, hepatoprotective, cardio protective, immunomodulatory, anti-inflammatory, rheumatoid arthritis can be found in scientific literature. Thus its activity as anti-inflammatory and rheumatoid arthritis could be further explored for its activity in Osteoarthritis. The present investigation was undertaken to demonstrate the activity of *C. dactylon*

in intra articularly induced Osteoarthritis using Monosodium Iodoacetate induced animal model.

KEYWORDS: Anti-inflammatory, Osteoarthritis, Monosodium Iodoacetate, Medicinal Plants, *Cynodon dactylon*, Methanolic Extract.



Certificate of Participation

This is to certify that

Sheetal Sudhakar Shetty

has participated at

12th International Conference of Laboratory Animal Scientists' Association (LASA), India

on

"Harmonization of Management of Animal Facilities and Improving Quality of Research Animal Production"

held on 29th- 30th November 2024 in CIDCO Convention Centre, Vashi, Navi Mumbai, India

Organized by: Tata Memorial Centre-ACTREC, Navi Mumbai, India & Laboratory Animal Scientists' Association (LASA), India.

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Dr. R. K. Shakthi Devan
Secretary, LASA

Dr. V. P. Singh

Dr. V. P. Singh
President, LASA

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Presented a **Poster/Oral Presentation** and won the **First/Second/Third Prize**

At the 12th International Conference of Laboratory Animal Scientists' Association (LASA), India

on the theme

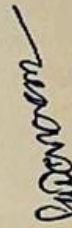
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30.11.2024

Dr. Arvind Ingle
Organizing Secretary



Dr. R. K. Shakti Devan
Secretary, LASA



Dr. V. P. Singh
President, LASA

CHAPTER 10:

SYNOPSIS

**SYNOPSIS OF THE THESIS TO BE SUBMITTED TO THE
UNIVERSITY OF MUMBAI FOR THE DEGREE OF MASTERS
BY RESEARCH IN THE SUBJECT OF APPLIED BIOLOGY**

Name of the Student : Ms. Sheetal Sudhakar Shetty

Title of the Thesis : *In vitro* and *In vivo* Activity of Phospholipase A₂ enzyme
against *Plasmodium* sp.

Degree & Subject : Masters by Research in Applied Biology

Registration No. / Date : 64/17-06-2022

**Name of the Research
Supervisor / Guide** : Dr. Mrunal Ghag Sawant

**Date of Superannuation
/ retirement** : 28/02/2039

**Name of the Research
Center** : Haffkine Institute for Training, Research and Testing

Signature of Student : 

**Signature of Research
Supervisor / Guide** :  **Head of The Department**
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INTRODUCTION

Malaria remains one of the most devastating infectious diseases globally, causing significant morbidity and mortality, particularly in tropical and subtropical regions. According to the World Health Organization (WHO), an estimated 263 million malaria cases and 597,000 deaths occurred in 2023, with *Plasmodium falciparum* being the most lethal species ^[80]. The growing resistance of *Plasmodium* parasites to conventional antimalarial drugs, such as chloroquine and artemisinin derivatives, presents a formidable challenge to global malaria control and eradication efforts ^{[77][21]}. Consequently, the search for novel therapeutic targets and agents is more critical than ever.

Phospholipase A₂ (PLA) enzymes, which hydrolyse the sn-2 acyl bond of phospholipids to release arachidonic acid and lysophospholipids, are widely distributed in various organisms, including mammals, plants, and venomous animals. These enzymes play pivotal roles in membrane remodelling, signal transduction, and inflammatory processes. Interestingly, PLA₂s derived from animal venoms-particularly snake venoms-have demonstrated potent antimicrobial, antiviral and antiparasitic properties ^{[37][59]}. Recent studies have shown that PLA₂s can disrupt parasite membranes, interfere with host-cell invasion, or modulate immune responses, making them promising candidates for antiparasitic drug development.

Although preliminary *in vitro* studies have indicated that PLA₂ exhibits activity against *Plasmodium* spp., comprehensive investigations into its efficacy, mechanisms of action, and potential toxicity in *in vivo* models remain limited. Exploring both *in vitro* and *in vivo* activities of PLA₂ against *Plasmodium* could provide valuable insights into its therapeutic potential and facilitate the development of novel antimalarial strategies.

This thesis aims to evaluate the *in vitro* and *in vivo* anti-plasmodial activity of PLA₂ enzymes, particularly focusing on their cytotoxicity, parasite growth inhibition and host immune response modulation. By integrating biochemical assays, cell culture models and murine malaria models, this study seeks to elucidate the therapeutic prospects and biological mechanisms of PLA₂ as an antimalarial agent.

REVIEW OF LITERATURE

MALARIA

Life Cycle of Parasite:

To understand malaria, it is important to understand the life cycle of the parasite. *Plasmodium* sp. goes through the life cycle in two organisms- the vector mosquito and the host. The life cycle is divided into two stages, namely sexual and asexual, with the asexual stage further divided into different developmental stages such as pre-erythrocytic stage, erythrocytic stage and schizogonic stage.

The life cycle of *Plasmodium* is complex and passes through different stages. The initiation of life cycles takes place through the bite of an infected female *Anopheles* mosquito, the sporozoites are passed through saliva into the bloodstream or lymphatic system. Upon deposition in the skin by a mosquito bite, sporozoites use gliding motility to reach blood vessels and enter the circulation. They then travel to the liver, where they cross the sinusoidal cell layer, often through Kupffer cells, before traversing through several hepatocytes. Finally, they invade a hepatocyte and form a parasitophorous vacuole, known as schizont, for further development ^[74]. When the mature schizonts rupture the merozoites are released into the liver sinusoid, thus ending the liver cycle. These released merozoites are the ones that invade the erythrocytes.

The blood phase of the life cycle is started when the merozoites are released into the bloodstream. Erythrocytes are immediately invaded by merozoites to enter the trophozoite stage. The characteristic ring form is formed inside a vacuole. As the parasite grows, the cytoplasm expands and the vacuole disappears in 12-24 hrs showing a characteristic parasite pigment in the cytoplasm. Further, as the nuclear division progresses the division produces two or more nuclei and the parasite enters the schizont stage. Until an appropriate number of merozoites are produced nuclear division continues. At the end of this phase, the schizogonic cycle is completed and erythrocytes are ruptured, releasing the merozoites into the bloodstream. The discharged merozoites enter into the blood circulation and invade other healthy erythrocytes and further continue their life cycle until there is any inhibition of the process by immune response or any drug. In the erythrocytes, the course of the schizogonic cycle may take another path and merozoites may differentiate into gametocytes- the sexual form.

The initiation of the sporogonic cycle occurs during the blood meal when the male gametocytes and female gametocytes are ingested by a suitable *Anopheles* species. As soon as the gametocytes reach the insect's midgut, the female gametocytes shed red blood cells and freely rain as macrogamete in the extracellular space. The male gametocytes divide into sperm-like flagellated microgametes, leave the erythrocytes and move to fertilize the macrogametes in the midgut. The active penetration of ookinete into the peritrophic membrane and epithelium of the midgut initiates the development of non-motile oocytes in the basal lamina of the outer gut wall where ookinetes settle, after 24-72 hrs of the blood meal. The products of mature oocytes are the sporozoites that are 10-15 μm in length, actively motile, narrow and curved shaped. Through small perforation without disturbing the cell wall, sporozoites actively pass and leave the cyst, at least till most of the sporozoites exit the membrane and further enter into the hemocelomic space of the insect. The sporozoites reach the salivary gland after migration and penetrate the basal membrane, pass intracellularly through a secretory cell and settle in the salivary gland. When the mosquito takes its next blood meal, the contents of saliva and sporozoites present in the saliva are injected into the host to start another asexual replicative cycle.

Epidemiology:

The primary manner that malaria is spread is through the bite of an infected *Anopheles* mosquito. Additionally, it can spread congenitally and by the inoculation of contaminated blood. The majority of Anophelines' breeding grounds are in rural settings, and they feed at night. Therefore, in rural regions, the hours between nightfall and dawn are the most perilous for malaria. Urban locations in many malaria-endemic areas pose little or no danger. However, in other regions of the world, particularly Africa, urban transmission is widespread. Malaria endemic transmission can occur seasonally or year-round. Malaria parasites are constantly present in the blood of 90 to 100 percent of children under five in various parts of Africa. Children are the main victims of malaria in endemic places because naturally acquired immunity grows with increased exposure. On the other hand, malaria affects people of all ages during epidemics because naturally acquired immunity declines in epidemic areas ^[4].

Symptoms:

Fever is malaria's most prevalent symptom. Myalgias, headaches, nausea, vomiting and chills are some of the other typical symptoms. Sometimes there is coughing, diarrhoea and stomach ache. As the sickness worsens, some individuals may experience the characteristic malaria paroxysm, which alternates between periods of illness and times when they don't have any

symptoms. There are three stages in a malaria paroxysm. Shivering and a cold feeling are the hallmarks of the initial cold stage, which lasts for 15 to 60 mins. The heated stage, which lasts for 2 to 6 hrs, is followed by flushed, dry skin, fever (which can reach 41°C), headache, nausea and vomiting. Lastly, there is the sweating stage, which lasts for two to four hours and is characterized by a quick drop in fever and perspiration.

Delays or missed diagnoses of malaria can lead to complex malaria, which can be lethal, particularly when *P. falciparum* is present. Severe anaemia and cerebral malaria are the most common deadly side effects of malaria. Any aberration in mental status in a person with malaria is referred to as cerebral malaria, which has a 15-50% case fatality rate. Other complications can include: severe hypoglycaemia; lactic acidosis; prolonged hyperthermia; shock; pulmonary, cardiac, hepatic, or renal dysfunction; seizures; spontaneous bleeding; high-output diarrhoea or vomiting; or hyperparasitemia (more than 3 to 5 % of the erythrocytes parasitized) [4].

Diagnosis:

In most cases, direct detection of malaria parasites in thick and thin blood smears stained with Giemsa is necessary for a definitive diagnosis of malaria. As more blood is analysed, thick blood smears are far more sensitive than thin blood smears, but they are also more difficult to interpret. To identify the type of parasite causing the infection, thin blood smears are utilized, where parasites are visible inside erythrocytes. The existence of diagnostic forms might differ significantly depending on the stage of parasite, particularly in the initial stages of illness.

Additional diagnostic approaches include polymerase chain reaction/DNA and RNA probe methods, as well as assays that identify malaria antibodies and antigens. Rarely are these methods utilized for individual patient diagnosis; instead, they are mostly employed in epidemiologic research and vaccination trials [4].

Treatment:

WHO now recommends the use of five typical ACT combinations as the initial treatment of uncomplicated *P. falciparum* malaria in endemic areas. The suggested combinations, with the exception of artesunate-sulfadoxine-pyrimethamine, are currently offered as fixed-dose therapies, which are better due to increased adherence and convenience of usage. Atovaquone-proguanil is also considered as one of the first-line treatments for travellers returning to non-endemic areas and for regions with confirmed artemisinin resistance, provided that it has not been used for prophylaxis. When ACT and atovaquone-proguanil are unavailable or

contraindicated, oral quinine plus clindamycin or doxycycline may be used as a second line of treatment. If ACTs are not available, chloroquine treatment for *P. falciparum* uncomplicated malaria is only permitted for patients returning from the few regions where *P. falciparum* is still susceptible to this medication (Haiti, Dominican Republic, Middle East, and Central America - north of the Panama Canal). Chloroquine continues to be the recommended treatment for *P. malariae*, *P. ovale* and *P. vivax* malaria once *P. falciparum* infection has been safely ruled out. *P. vivax* show a latent hepatic stage (hypnozoites), an effective cure that prevents relapses necessitates an adjuvant course of medication that kills hypnozoites. Only the 8-amino quinolines (buloquine, primaquine, and tafenoquine) exhibit notable efficacy against this parasite stage at the moment.

Global Statistics:

An estimated 263 million cases of malaria were reported worldwide in 2023, with an incidence of 60.4 cases per 1000 at-risk individuals. This represents an increase in incidence from 58.6 cases per 1000 people at risk in 2022 and an increase of 11 million cases from the year before. With an expected 94% of all malaria cases globally in 2023 occurring in the WHO African Region, the region continues to bear the greatest burden of the illness. Since 2021, the incidence in the WHO Eastern Mediterranean Region has increased by 57%, reaching 17.9 cases per 1000 people at risk in 2023. According to estimates, Nigeria accounted for 26% of the world's malaria cases in 2023, followed by the Democratic Republic of the Congo (13%), Uganda (5%), Ethiopia (4%), and Mozambique (4%).

Among India's accomplishments is the notable decline in malaria infections and deaths from the disease from 2017 to 2023. India's departure from the WHO's High Burden to High Impact (HBHI) classification in 2024, which marked a significant change in its battle against malaria, serves as more evidence of this achievement. These accomplishments are a testament to the country's strong public health initiatives and its goal of eliminating malaria by 2030. Between 2015 and 2023, there was an almost 80% decrease in both malaria cases and deaths. Cases decreased from 11,69,261 in 2015 to 2,27,564 in 2023, while deaths decreased from 384 to just 83. Early identification, prompt action, and more successful treatment have all been made possible by this enhanced surveillance ^[53].

PHOSPHOLIPASE A₂

Phospholipase A₂ (PLA₂) enzymes play pivotal roles in various biological processes by catalysing the hydrolysis of phospholipids at the sn-2 position, thereby releasing free fatty

acids, including arachidonic acid and lysophospholipids. These enzymes are integral components of cellular membranes and are involved in diverse physiological functions such as membrane remodelling, cell signalling, inflammation and lipid metabolism ^[19]. The versatility of PLA₂ enzymes extends beyond their enzymatic activity, as they also participate in intricate cellular pathways regulating immune responses, neuronal function and host defence mechanisms ^[47]. Phospholipase A₂ (PLA₂) enzymes are widely distributed throughout nature and can be sourced from various organisms, tissues and cellular compartments. PLA₂ enzymes are sourced from a diverse range of organisms, including animals, microorganisms, plants and marine species. Understanding the sources and characteristics of PLA₂ enzymes provides insights into their biological functions and potential applications in various fields, including medicine, biotechnology and environmental science.

Classification

The PLA₂ superfamily comprises 15 groups categorized into four main types: secreted sPLA₂, cytosolic cPLA₂, calcium-independent iPLA₂, and platelet activating factor (PAF) acetyl hydrolase/oxidized lipid lipoprotein associated (Lp) PLA₂. Secreted PLA₂s, the first type discovered, are characterized by their low molecular weight, histidine in the catalytic site, and calcium-dependent activity. They play roles in various biological processes, including antimicrobial activity and potential involvement in inflammatory responses. Cytosolic PLA₂, particularly the GIVA PLA₂, is highlighted for its preference for arachidonic acid and its central role in eicosanoid generation and inflammatory processes. The calcium-independent iPLA₂, specifically the GVIA PLA₂, is discussed in terms of its multiple splice variants and its involvement in various cellular functions such as bone formation, apoptosis, and insulin secretion. Lastly, the PAF acetyl hydrolase/oxidized lipid LpPLA₂ is described, noting its ability to cleave oxidized lipids and its association with coronary heart disease.

Antimalarial Activity

The anti-plasmodial effect of the whole venom and the isolated fractions of *Bothrops asper* on *in vitro* cultures of *Plasmodium falciparum* showed that the whole venom, and purified fraction V and fraction VI were all active against the parasite. The study provided interesting insights into the potential of snake venom components as antimalarial agents. It was found that both catalytically-active PLA₂s and the PLA₂ homologue without enzymatic activity demonstrate anti-plasmodial effects, suggesting different mechanisms of action ^[12]. PLA₂s from *B. diporus* venom, particularly BdTX-II, show promising anti-plasmodial activity with low cytotoxicity.

The PLA₂s did not exhibit haemolytic activity at the tested concentrations, suggesting their anti-plasmodial action is directly on the intra-erythrocytic forms of *P. falciparum* [75].

Researchers investigated the interactions between Bee venom PLA₂ (bvPLA₂), human triglyceride-rich lipoproteins, and infected erythrocytes to evaluate the potential involvement of host lipoproteins and secreted phospholipases A₂ (sPLA₂s) in malaria. The study found that bvPLA₂ preferentially associates with lipoproteins rather than with erythrocytes, even when the erythrocytes are infected with *Plasmodium falciparum*. The enzyme's anti-*Plasmodium* activity is dependent on the hydrolysis of lipoprotein phospholipids, which generates toxic lipid products, primarily polyunsaturated fatty acids (PUFAs). Among these, arachidonic acid (AA) and linoleic acid (LA) were identified as the main actors in toxicity. The researchers also discovered that minimal oxidation of lipoproteins enhanced their toxicity and induced their interaction with both infected and normal erythrocytes [29].

An investigation in the role of Human Group II A sPLA₂ (hGIIA sPLA₂), which is increased in the plasma of malaria patients, in controlling *Plasmodium falciparum* growth demonstrated that hGIIA sPLA₂ exhibits inhibitory effects against *P. falciparum* in the presence of oxidized lipoproteins. Other sPLA₂s, including hGIIF, hGV, and hGX, also showed enhanced potency in the presence of oxidized lipoproteins. Importantly, malaria plasma containing oxidized lipoproteins was inhibitory to *P. falciparum* when supplemented with hGIIA sPLA₂. *In vivo* experiments using mice infected with *P. chabaudi* showed that injection of recombinant hGIIA reduced the peak of parasitaemia, but only when plasma peroxidation levels were increased during infection. This suggests that malaria-induced oxidation of lipoproteins converts them into preferential substrates for hGIIA sPLA₂, promoting its parasite-killing effect. This mechanism may contribute to host defence against *P. falciparum*, particularly in severe cases of malaria where high levels of hGIIA are observed. This finding is particularly interesting as it reveals a previously unknown mechanism of host defence against malaria [17].

Researchers created transgenic *Anopheles stephensi* mosquitoes that express the bee venom phospholipase A₂ (PLA₂) gene in their gut. This gene was placed under the control of a gut-specific and blood-inducible promoter from the *Anopheles gambiae* carboxypeptidase gene. The recombinant PLA₂ protein was detected in the midgut of transgenic mosquitoes, with peak expression occurring between 8 and 24 hrs after a blood meal. This timing coincides with the invasion of the midgut by *Plasmodium* ookinetes. Importantly, the expression of PLA₂ in transgenic mosquitoes significantly reduced *Plasmodium berghei* oocyst formation by an

average of 87% and greatly impaired the mosquitoes' ability to transmit the parasite to naive mice ^[46].

PHARMACOKINETIC STUDIES

The study of pharmacokinetics (PK) examines how the body responds to chemicals that are provided over the course of exposure. While they are closely connected, pharmacodynamics- which looks more deeply at how the medicine affects the body-is very different from this. Absorption, distribution, metabolism and excretion (ADME) are the four primary factors that are typically examined in this subject.

Given the diverse physiologies and lifestyles of their patients, practitioners can prescribe and administer medications that will yield the greatest benefit at the lowest risk by having a thorough understanding of these processes. They can also make appropriate modifications.

AIM

The aim of the study is to evaluate the *in vitro* and *in vivo* activity of Phospholipase A₂ enzyme against *Plasmodium sp.*

OBJECTIVES

4. Determining the cytotoxic and haemolytic effect of the enzyme.
5. *In vitro* efficacy evaluation of Phospholipase A₂ enzyme against *Plasmodium sp.*
6. *In vivo* efficacy evaluation of Phospholipase A₂ enzyme against *Plasmodium sp.*

MATERIALS AND METHODS

Enzyme:

Lyophilized Phospholipase A₂ enzyme from Bovine pancreas was obtained from Sigma Aldrich. It was solubilized in 0.9% saline and separated into aliquots. The aliquots were then stored in -80°C for further testing.

Cytotoxic Activity:

Peripheral Blood Mononuclear Cells (PBMC) were separated from Human blood, and centrifuged at 2000 rpm for 10 mins. The plasma layer was discarded and the PBMC layer was carefully separated for the assay. The cells were then washed with PBS and 50 µL was

transferred to a 96-well plate at a concentration of 3×10^5 cells/well. A volume 50 μL of different concentrations of the enzyme, i.e. 500 $\mu\text{g/mL}$, 250 $\mu\text{g/mL}$, 125 $\mu\text{g/mL}$, 62.5 $\mu\text{g/mL}$, 31.25 $\mu\text{g/mL}$, 15.63 $\mu\text{g/mL}$, 7.81 $\mu\text{g/mL}$, 3.91 $\mu\text{g/mL}$, 1.95 $\mu\text{g/mL}$ and 0.98 $\mu\text{g/mL}$; were added into the well plate with each concentration in triplicates, and it was incubated for 24 hrs at 37°C at 5% CO_2 . Then 40 μL of 0.5 mg/mL MTT was added and the plate was incubated for an additional 3 hrs under the same conditions. The reaction was halted by adding 130 μL of Dimethyl sulfoxide (DMSO) and the plate was then read in a microplate reader at 420 nm. The percentage viability was calculated for each concentration.

Indirect Haemolysis:

Indirect Haemolysis was evaluated on red blood cells in suspension. A volume of 0.5 ml of processed and washed RBC pellet was diluted with 9.5 mL of NS to prepare 5% RBC suspension. A volume of 200 μL of the sample at different concentrations was added in different the vials (each concentration in triplicates). PBS was used as a negative control and Distilled water as positive control (each in triplicates). A volume 50 μL of PBS was added to all tubes along with 200 μL of 1% RBC suspension. The tubes were incubated at 37°C for 30 mins and then centrifuged at 2000 rpm for 10 mins. A volume of 200 μL of the supernatant was added in a labelled 96 well plate. The plate was then read in a micro-plate reader at 540 nm. The percentage haemolysis for each concentration with the following formula-

$$\% \text{ Haemolysis} = \frac{\text{Absorbance of sample} - \text{Absorbance of negative control}}{\text{Absorbance of positive control}} \times 100$$

Cultivation of *Plasmodium falciparum*:

Based on the procedure described by Trager and Jensen *et al.*, *P. falciparum* was grown at 37°C in human erythrocytes to a haematocrit of 2% and 3%-6% parasitaemia under an atmosphere of 3% CO_2 , 6% O_2 and 91% N_2 . Cultures were prepared in T-25 flasks with 2% haematocrit, diluted in RPMI 1640 medium culture [supplemented with 25mM HEPES, 21mM sodium bicarbonate, 11mM glucose, 40 $\mu\text{g/mL}$ gentamicin and 0.5% (w/v) Albumax]. The cultures were maintained in a CO_2 incubator at 37°C . Culture media was changed daily and parasitaemia was monitored in blood smears, fixed with methanol, stained with Field's stain and visualized under an optical microscope at 100X. On reaching 2% parasitaemia, the cultures were passaged or cryopreserved.

***In vitro* antimalarial assays against *Plasmodium berghei*:**

A volume of 100 µL/well of parasite cultures mixed with RPMI 1640 medium containing: 0.5% parasitaemia and 2% haematocrit was added into 96-well plates for the SYBR Green I test. Prior to adding the parasite suspension, 20 µL of the samples, previously diluted in PBS 1X, was added to the test plate in serial concentrations (each in triplicates). Chloroquine was used as a positive control, infected red cells was used as the negative control, and non-infected red blood cells (blank) was used as the experimental control. The plates were incubated at 37°C for 48 hrs. Afterwards, the supernatant was discarded and 100 µL of lysis buffer (20mM Tris, pH 7.5; 5mM EDTA; 0.008% w/v saponin; 0.08% and Triton X-100 v/v) with SYBR Green I (30 µL of SYBR in 15 mL of Lysis buffer) was added in dark and incubated further for 24 hrs. Fluorescence was measured using a spectrophotometer with an excitation of 485 nm and an emission of 590 nm. Percentage Inhibition was calculated for each concentration.

Animals:

All animal-related work was performed after due approval from Institutional Animal Ethics Committee (IAEC) [HITRT/IAEC/05/2021]. Male/female (6-8 weeks) Swiss Albino mice were used in the experiments. Animals were housed at room temperature (25°C ± 2°C) and 12:12 light/dark cycle. Food and water were given ad libitum. Mice were exposed to Isoflurane for short-term anaesthesia. At the end of each experiment, anesthetized mice were humanely sacrificed by CO₂ asphyxia.

Pharmacokinetic studies:

For kinetic studies of the enzyme, the mice from *In vivo* Suppression study were used, i.e. group II and III were divided into sub groups of 3 mice each for 9 different time intervals viz 0, 6, 12, 24, 30, 36, 48, 54 and 60 hrs. The grouping of mice is given in Table 1. A volume of 0.1 mL of the enzyme at the concentration of 500 µg/mL was injected intraperitoneally in each mouse at 0, 24 and 48 hrs and the blood was drawn at above mentioned time intervals. Control mice were injected with saline and their blood was drawn at the given time intervals. The plasma was separated and stored for further testing.

Sr. No.	Time Interval (in hours)	Group I (3 mice)	Group II (3 mice)	Group III (3 mice)
	<u>1st Dose:</u>			
1.	0	✓		
2.	6		✓	
3.	12			✓
	<u>2nd Dose:</u>			
4.	24	✓		
5.	30		✓	
6.	36			✓
	<u>3rd Dose:</u>			
7.	48	✓		
8.	54		✓	
9.	60			✓

Table 1: Diagrammatic presentation of sparse sampling method for kinetic studies

***In vivo* Suppression Test:**

Four-day parasite suppression test was performed to know the schizonticidal activity of the enzyme. Total 29 mice were used for the test. The mice were randomized based on body weight and divided into 3 groups, with Group I having 5 mice and Group II and III having 12 mice each (Table 2). The mice were injected with parasites on Day 1 and the drug treatments were commenced after 4 days. The treatment was continued for the next 4 days. Mice survival and signs and symptoms were observed daily for up to 28 days post-treatment initiation. The mean survival time was calculated for each group. Field's-stained thin blood smears were prepared on the 4th Day, and the Percentage of Parasite Growth Suppression (PGS) was estimated using the following formula-

$$\text{Parasite Growth Suppression (\%)} = [(A-B)/A] \times 100$$

Where, A is the average parasitaemia of control (negative) group and B represents the parasitaemia of the experimental group.

Group no.	Group Name	Treatment	No. of animals
Group I	Treatment I	Chloroquine	5
Group II	Treatment II	Vehicle	12
Group III	Treatment III	Enzyme	12

Table 2: Group Distribution for *In vivo* Suppression Test

***In vivo* Antimalarial activity:**

46 Swiss Albino mice were randomly divided into 6 groups, with Control group having 6 mice and other groups having 8 mice each (Table 3). Group 1 was Control group, where the animals were not infected and they did not receive any treatment. Group 2 was Sham group, where the animals received only the vehicle (0.9% saline). Group 3 to 5 were injected with selected enzyme concentrations, i.e. 500 µg/mL, 250 µg/mL and 125 µg/mL respectively. Group 6 was Standard Group, where the animals received 200 mg per kg per day of Chloroquine on the 4th and 5th day. The mice were injected intraperitoneally (I. P.) with 3×10^8 parasitized erythrocytes. Treatment was started 4 days post parasite inoculation. Treatments continued for 5 days. The survival rate was monitored on a daily basis in all groups for 28 days post-inoculation. On day 5, Field's-stained thin blood smears were prepared from the tail of each animal to determine parasitaemia and percentage inhibition. The percentage of Parasite Growth Suppression (PGS) was estimated. The Mean Survival Time (MST) for each group was determined over a period of 28 days.

$$\text{Mean Survival Time (MST)} = \frac{\text{Sum of survival time for all mice in a group}}{\text{Total number of mice in that group}}$$

Group no.	Group Name	Treatment	No. of animals
Group I	Normal Control	Without any treatment	6
Group II	Sham control	Vehicle	8
Group III	Treatment I	Concentration 1	8
Group IV	Treatment II	Concentration 2	8
Group V	Treatment III	Concentration 3	8
Group VI	Treatment IV	Chloroquine	8

Table 3: Group Distribution for *In vivo* Antimalarial Test

Statistical Analysis:

The results are presented as mean $\pm$ S.E.M of three replicates, and experimental differences between means were determined by Analysis of Variance (ANOVA) followed by Dunnett's test for intragroup comparisons. Significance was set up at $p < 0.05$.

RESULTS

Cytotoxic Activity:

The percentage viability of Phospholipase A₂ against PBMCs was found to be higher than 80% in all concentrations, which is considered to be non-cytotoxic. The positive control,

Chloroquine, showed a percentage viability of 57.58% which is considered moderately cytotoxic. Table 3 shows the Mean Absorbance and Percentage Viability of the enzyme.

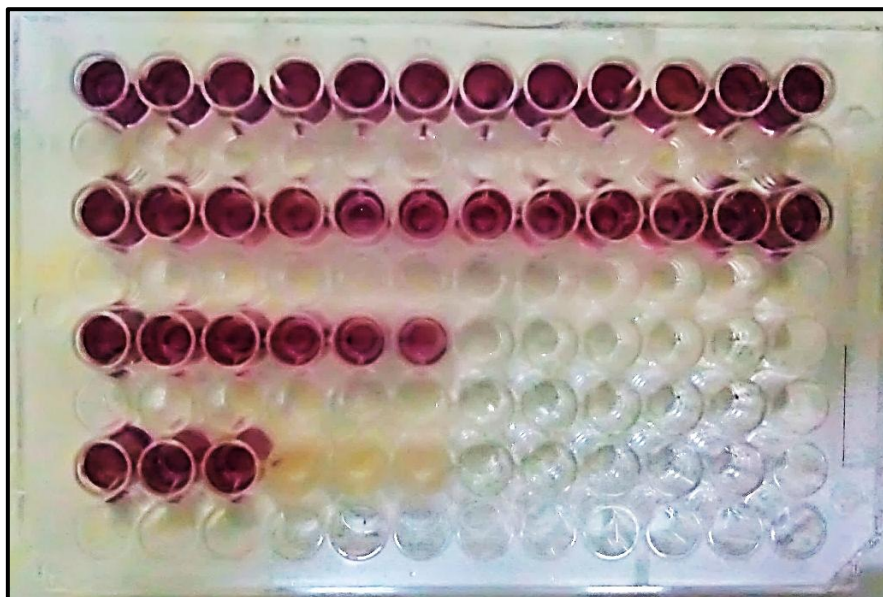


Figure 5: MTT Assay of PLA₂

CONCENTRATION ($\mu\text{g/mL}$)	MEAN	PERCENTAGE VIABILITY (%)
500	0.60	90.91
250	0.59	89.39
125	0.62	93.94
62.5	0.62	93.94
31.25	0.63	95.45
15.63	0.60	90.91
7.81	0.57	86.36
3.9	0.52	78.79
1.95	0.56	84.85
0.97	0.56	84.85
Negative Control	0.66	100
Positive Control (Chloroquine)	0.38	57.58

Table 4: Mean Absorbance and Percentage of Viability of enzyme

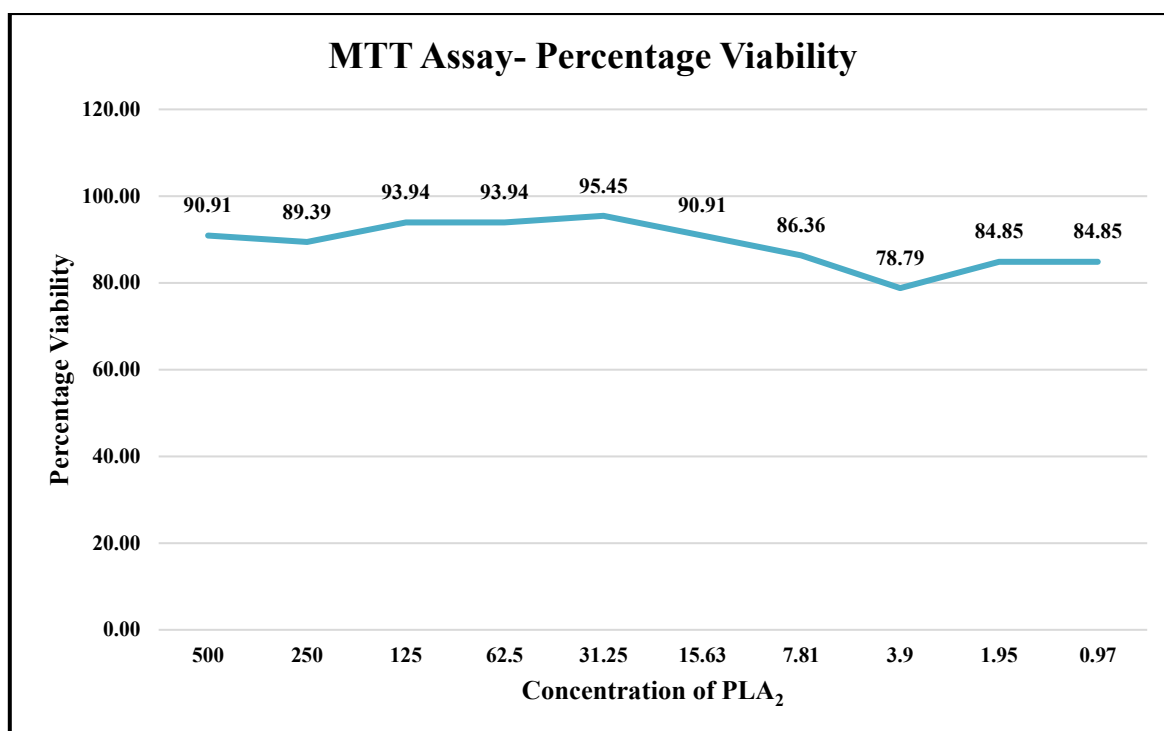


Figure 6: Graphical representation of Percentage Viability

Indirect Hemolysis:

It was found that the enzyme does not produce significant hemolysis and all concentrations have percentage hemolysis below 50%. Hence, the enzyme is not hemotoxic.

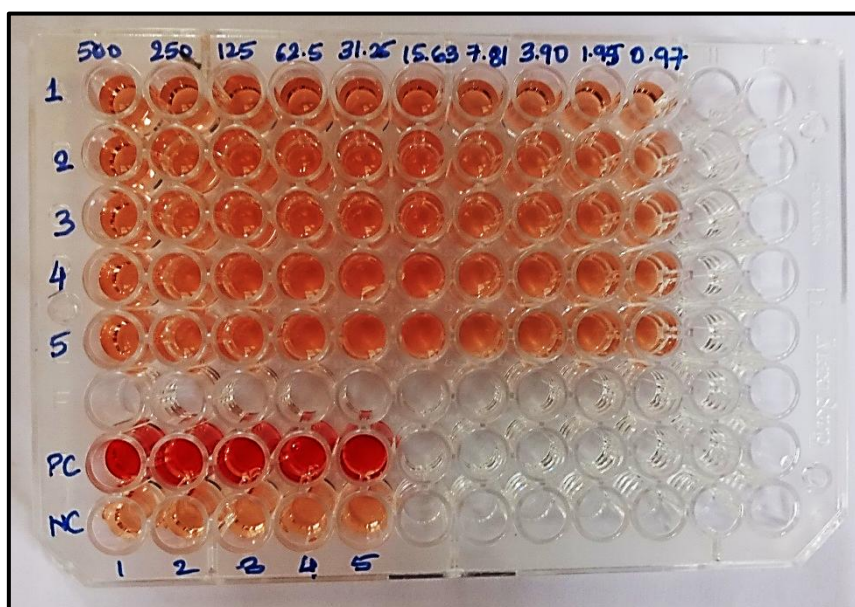


Figure 7: Indirect Haemolysis of PLA₂

CONCENTRATION ($\mu\text{g/mL}$)	MEAN	PERCENTAGE HEMOLYSIS
500	0.433	4.73
250	0.414	3.80
125	0.432	4.68
62.5	0.417	3.95
31.25	0.407	3.46
15.63	0.415	3.85
7.81	0.343	0.34
3.9	0.337	0.05
1.95	0.340	0.20
0.97	0.343	0.34

Table 5: Mean Absorbance and Percentage Haemolysis of PLA₂

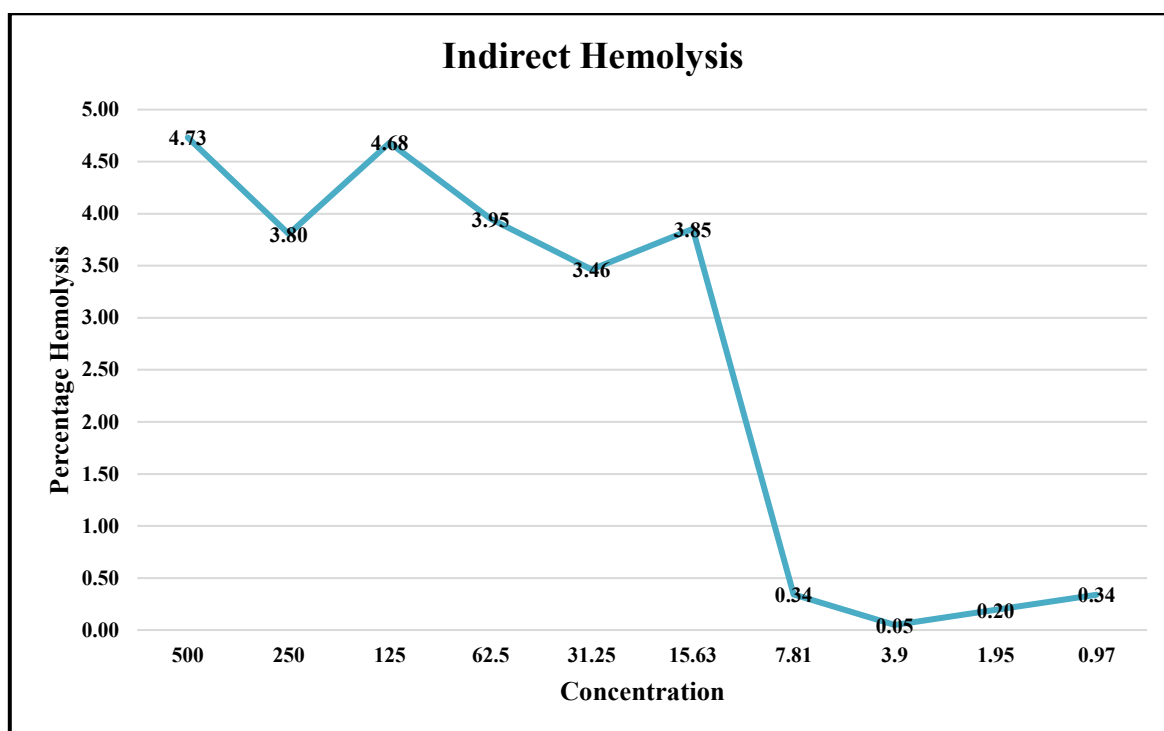


Figure 8: Graphical Representation of Indirect haemolysis

***In vitro* Antimalarial Activity:**

It was observed that the concentrations 500 $\mu\text{g/mL}$, 250 $\mu\text{g/mL}$, 1.95 $\mu\text{g/mL}$ and 0.98 $\mu\text{g/mL}$ demonstrate 100% inhibition. While the other consecutive concentration displayed negative values which could indicate possible cell proliferation.

CONCENTRATION	MEAN	PERCENTAGE INHIBITION (%)
500	0	100
250	5.33	-77.78
125	0.33	88.89
62.5	7	-133.33
31.25	6	-100
15.63	17.33	-477.78
7.81	14	-366.67
3.91	24.67	-722.22
1.95	0	100
0.98	0	100
Control	3	
Chloroquine	0	100

Table 6: Mean Absorbance and Percentage Inhibition of PLA₂

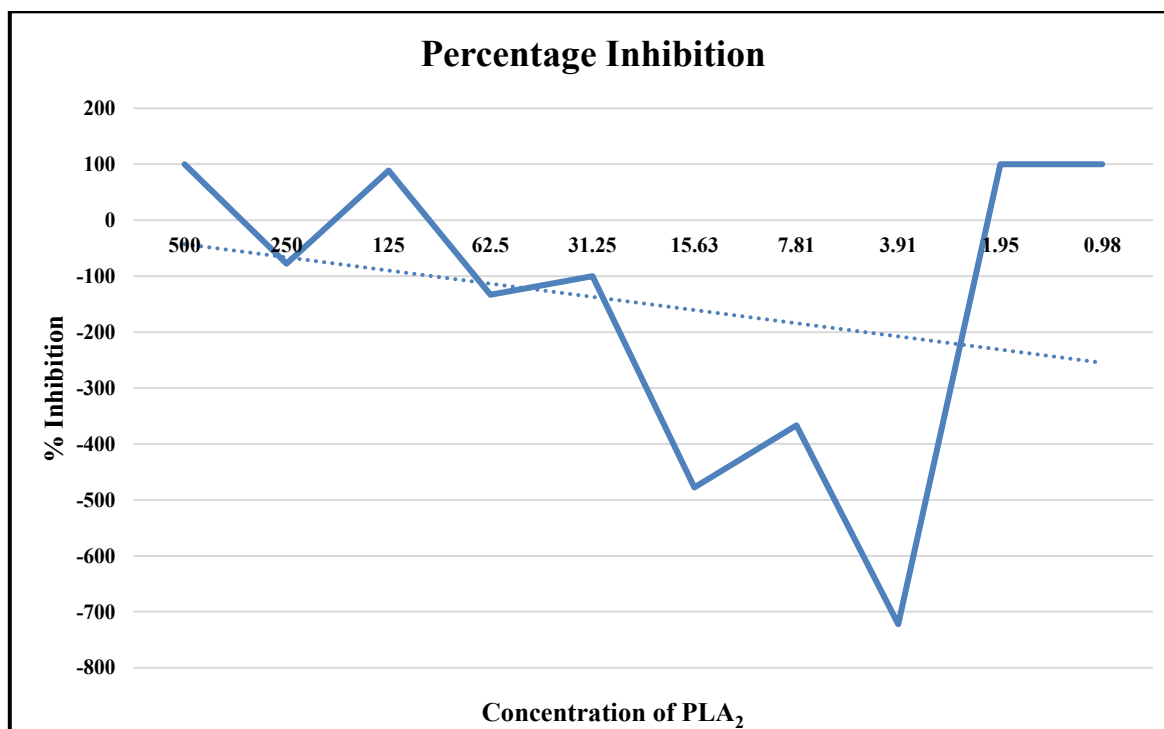


Figure 9: Graphical Representation of Percentage Inhibition

Pharmacokinetic Studies:

HPLC Analysis for detecting presence of PLA₂ was done. There were three different protocols used with repeatability, but none of them could detect the enzyme. Another attempt yielded

results but they were inconclusive. Hence, detection of PLA₂ using SDS PAGE with silver nitrate staining was done. The SDS PAGE yielded results for pure PLA₂, but on running plasma samples there was no distinction between bands. Based on this, another HPLC Analysis for detecting presence of PLA₂ was tried. This time the trials yielded results, but for confirmation and clarity a MS detection is required, which is costly as well as would take more time for standardization.

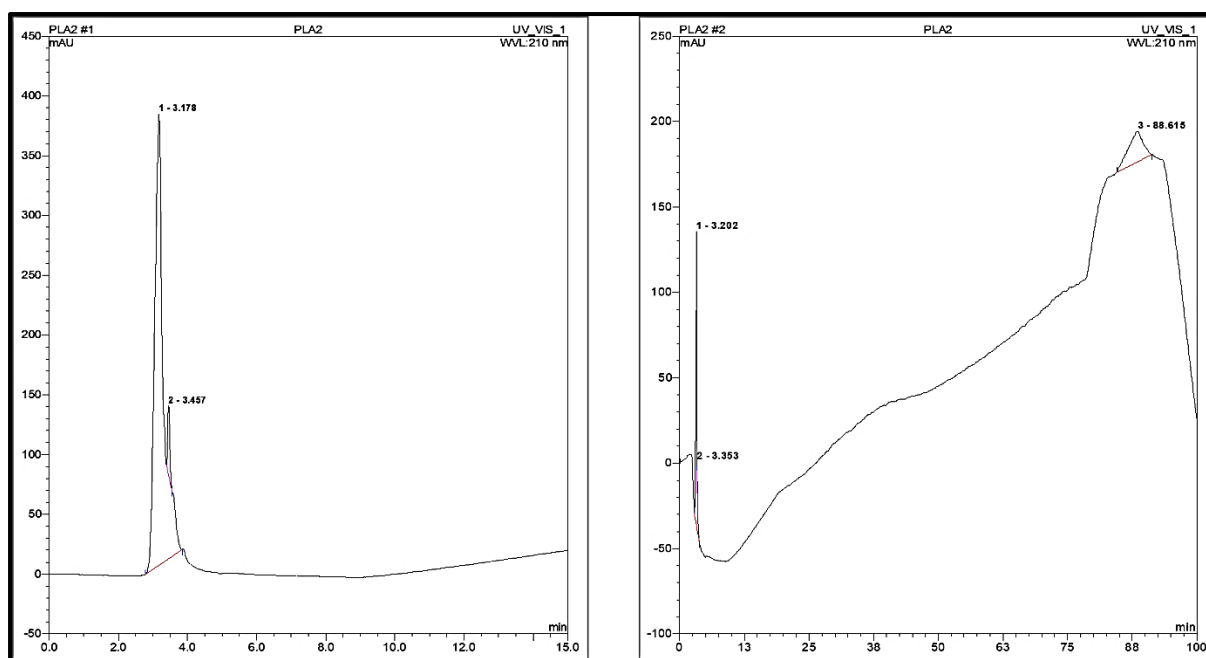


Figure 10: Results of Trial 1 (pure PLA₂)

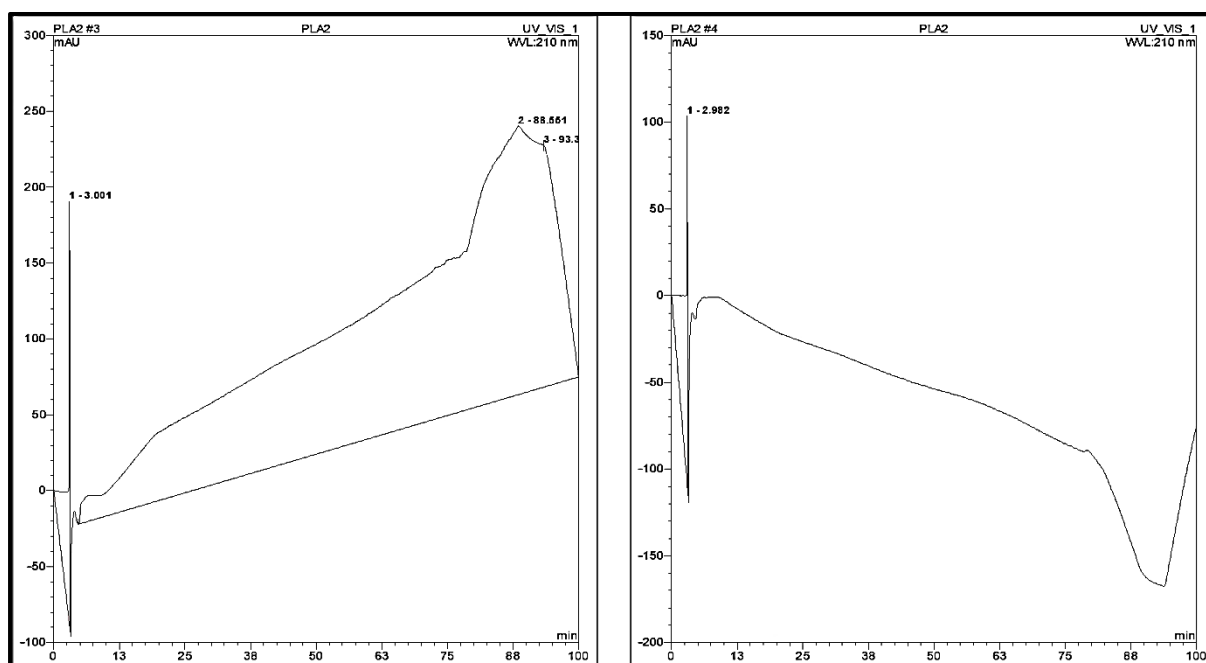


Figure 11: Results of Trial 2 (pure PLA₂)

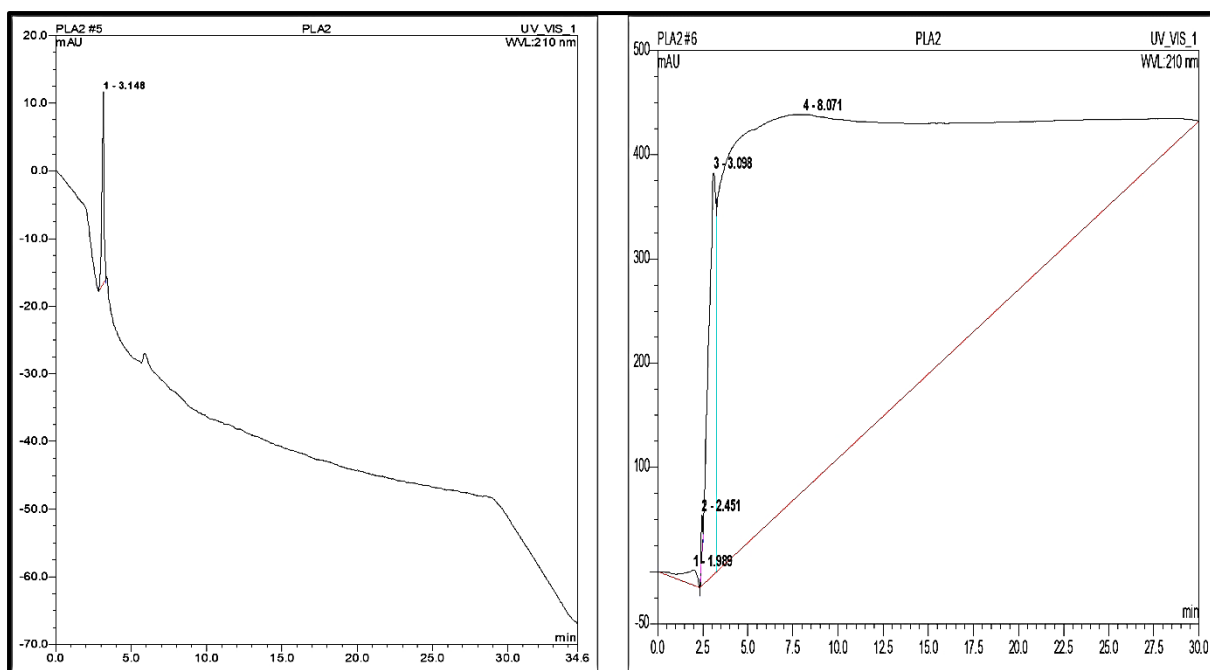


Figure 12: Results of Trial 3

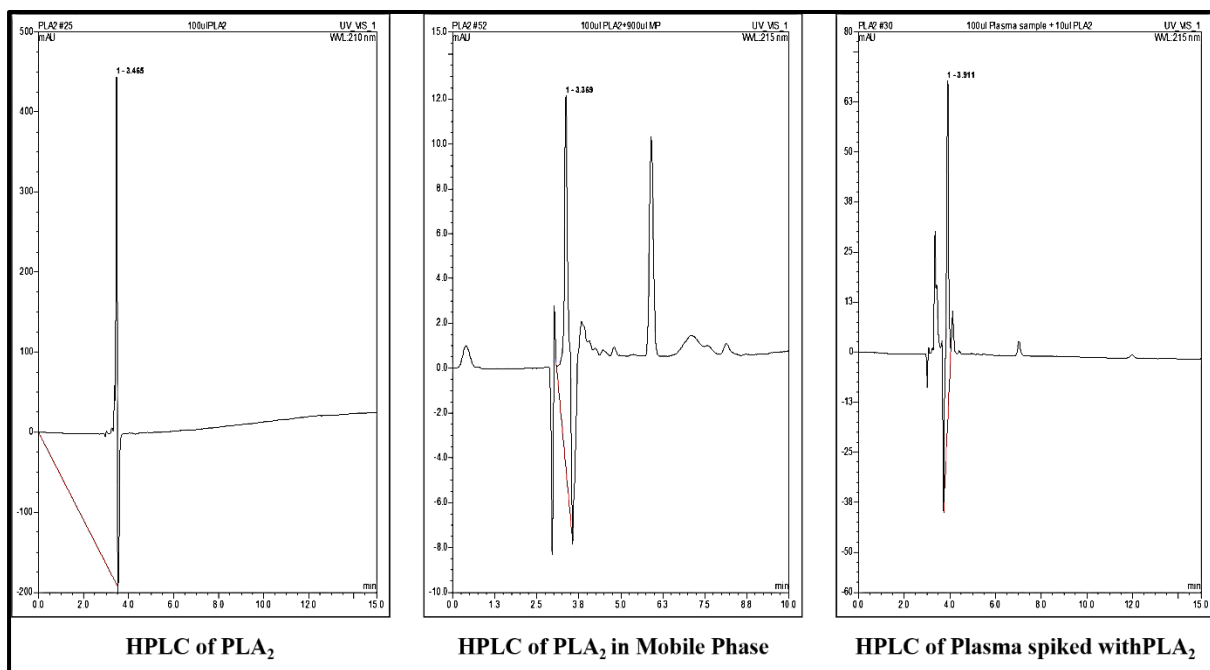


Figure 13: Results of Trial 4

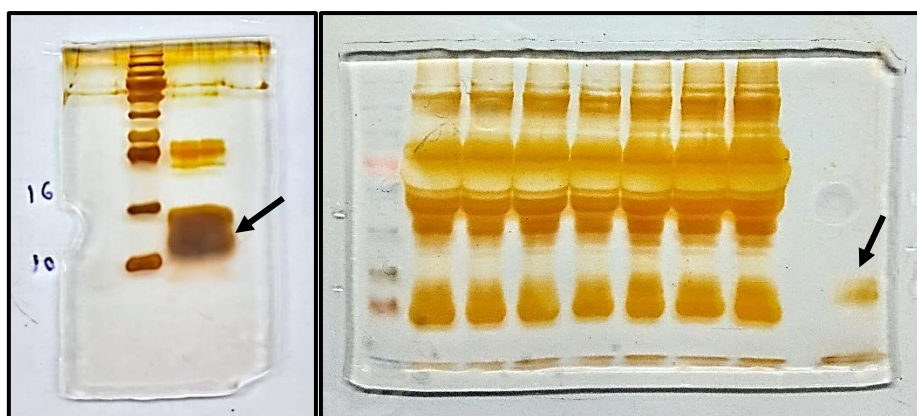


Figure 14: SDS PAGE of pure PLA₂ (left) and PLA₂ in plasma (right) [PLA₂ band has been marked]

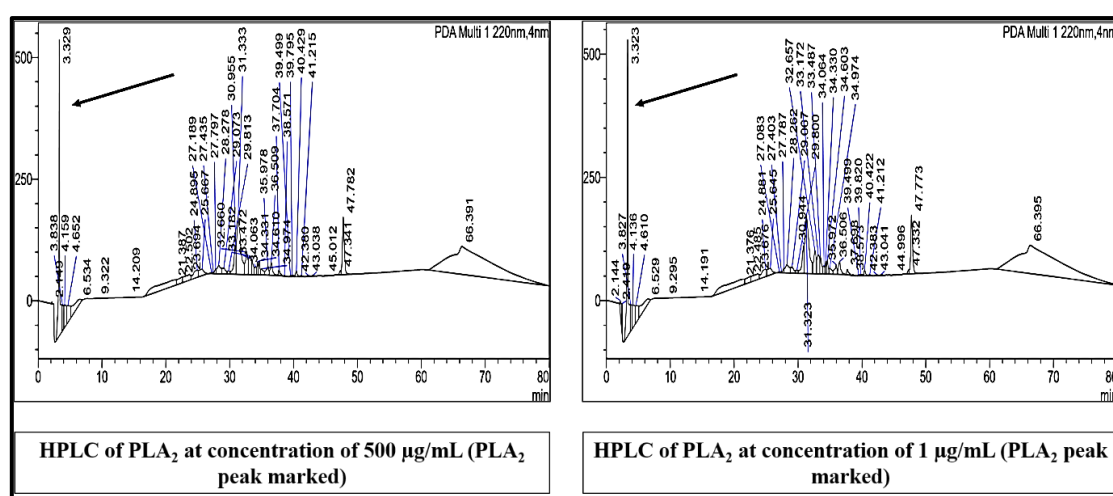


Figure 15: Results of Trial 5

In vivo Suppression Test:

It was found that PLA₂ showed considerable suppression at 0.11% followed by Chloroquine at 0.66%. It was in stark contrast to the vehicle group with 17.45% parasitaemia. The mean survival time indicates that PLA₂ does show considerable survival almost on par with Chloroquine. The signs and symptoms included shivering, piloerection, toe walking, slow gait, hypothermia, hunchback posture, weight loss and morbidity.

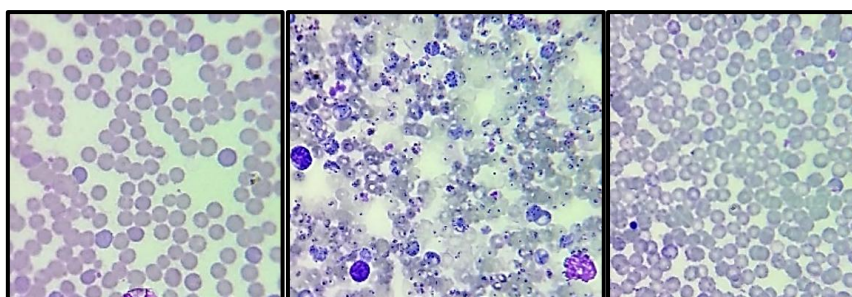


Figure 16: Slides of Chloroquine, Vehicle and PLA₂ respectively

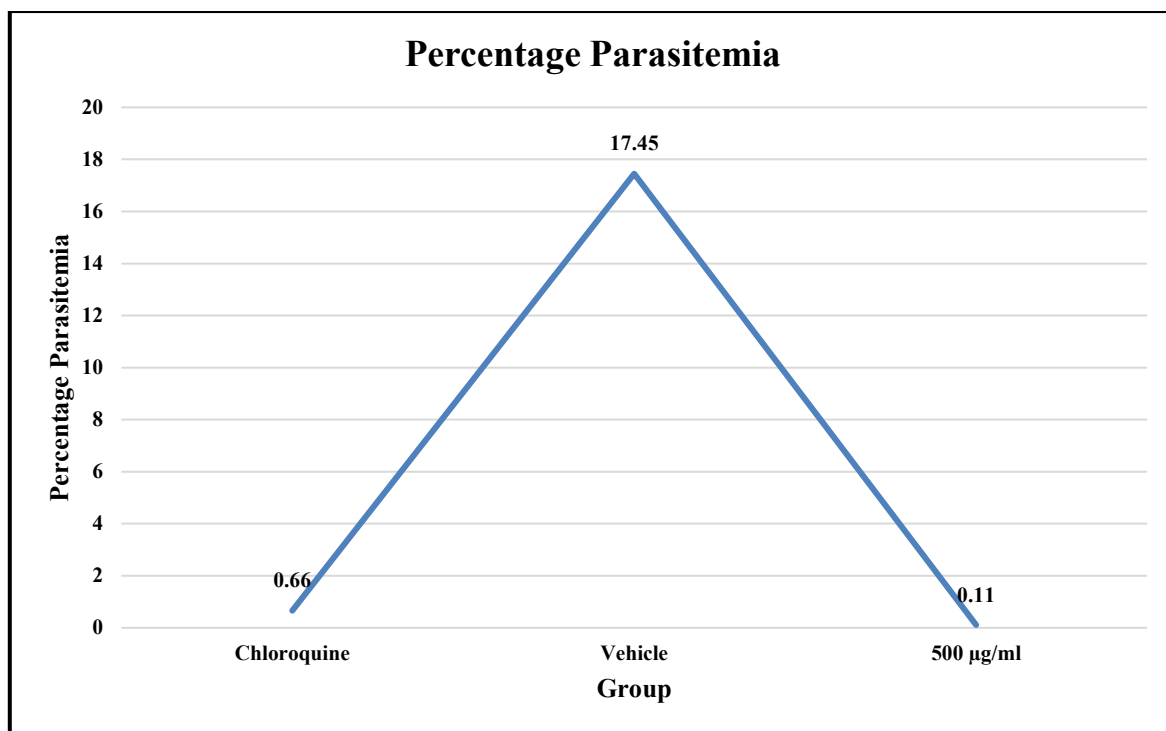


Figure 17: Graphical Representation of Percentage Parasitaemia

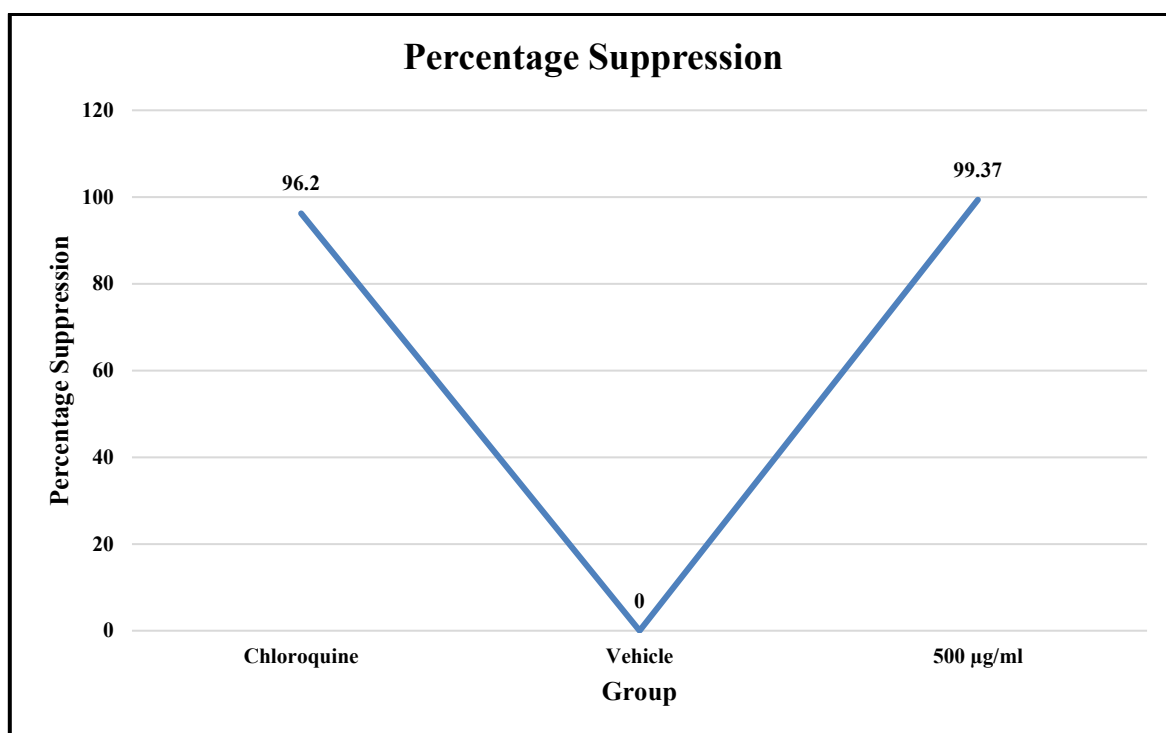


Figure 18: Graphical Representation of Percentage Suppression

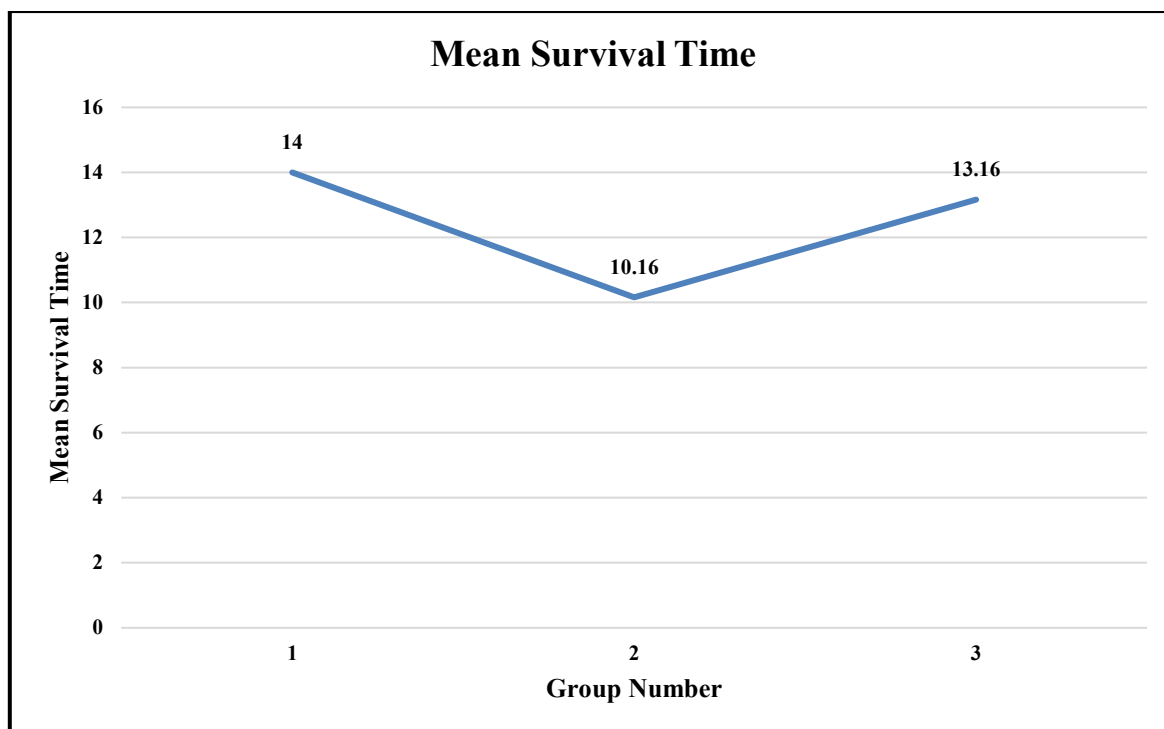


Figure 19: Graphical Representation of Mean Survival Time

***In vivo* Antimalarial Test:**

It was observed that there was linearity in the activity of PLA₂, with 500 µg/mL showing maximum activity at 3.65%, followed by 250 µg/mL at 9.80% and 125 µg/mL at 34.87% percentage parasitaemia. The activity of PLA₂ was near to but not equal to Chloroquine showing 0.81% percentage parasitaemia. The Mean Survival Time also showed the same linearity, but it was not better than Chloroquine. The signs and symptoms included shivering, piloerection, toe walking, slow gait, hypothermia, hunchback posture, weight loss and morbidity. After sometime the animals started showing signs of normality and their feed consumption also increased.

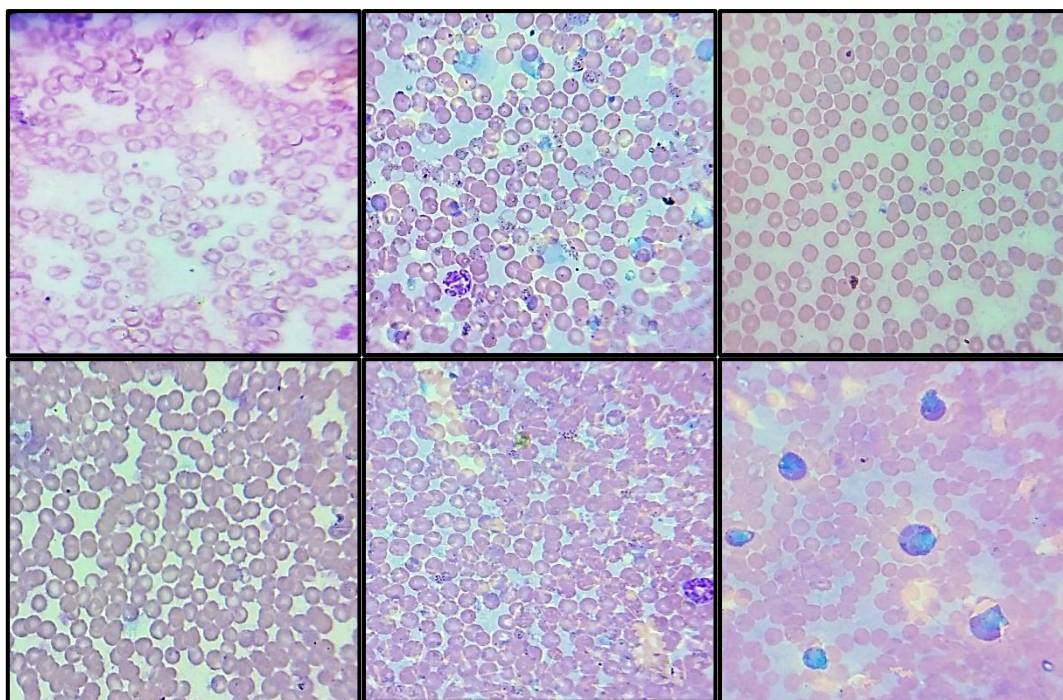


Figure 20: Slides of *In vivo* Antimalarial Test [In order from Top left corner- Control, Sham Control, 500 µg/mL, 250 µg/mL, 125 µg/mL and Chloroquine]

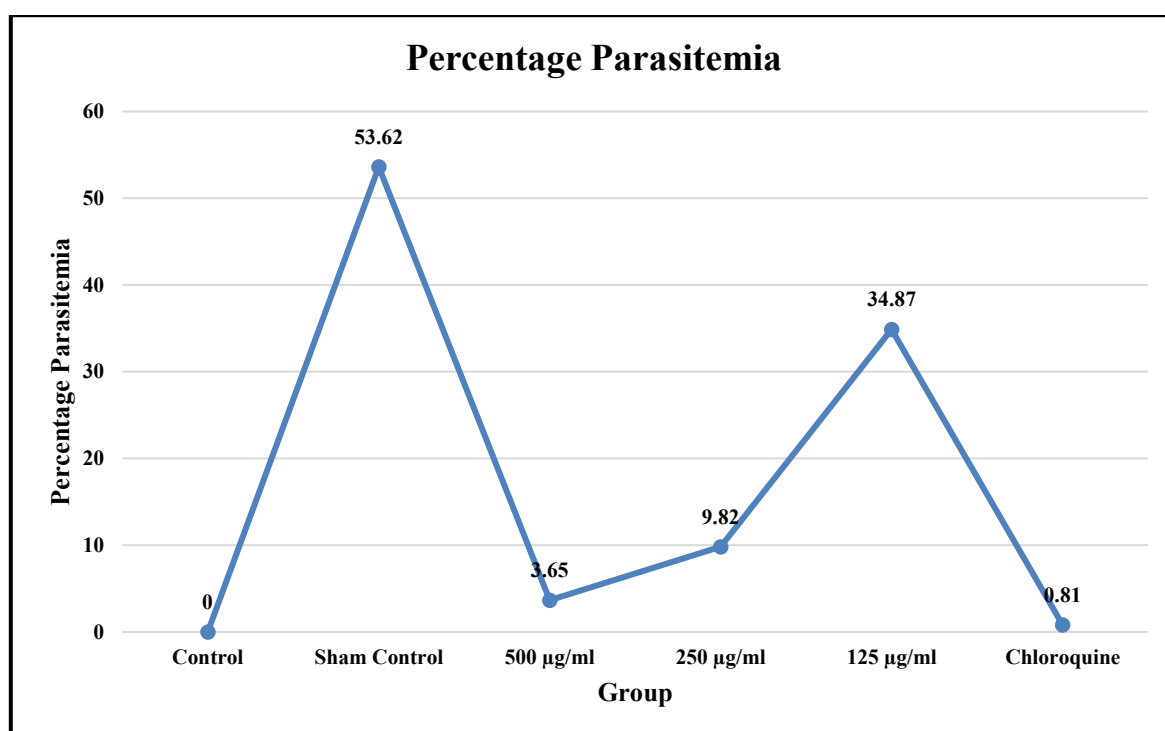


Figure 21: Graphical Representation of Percentage Parasitaemia

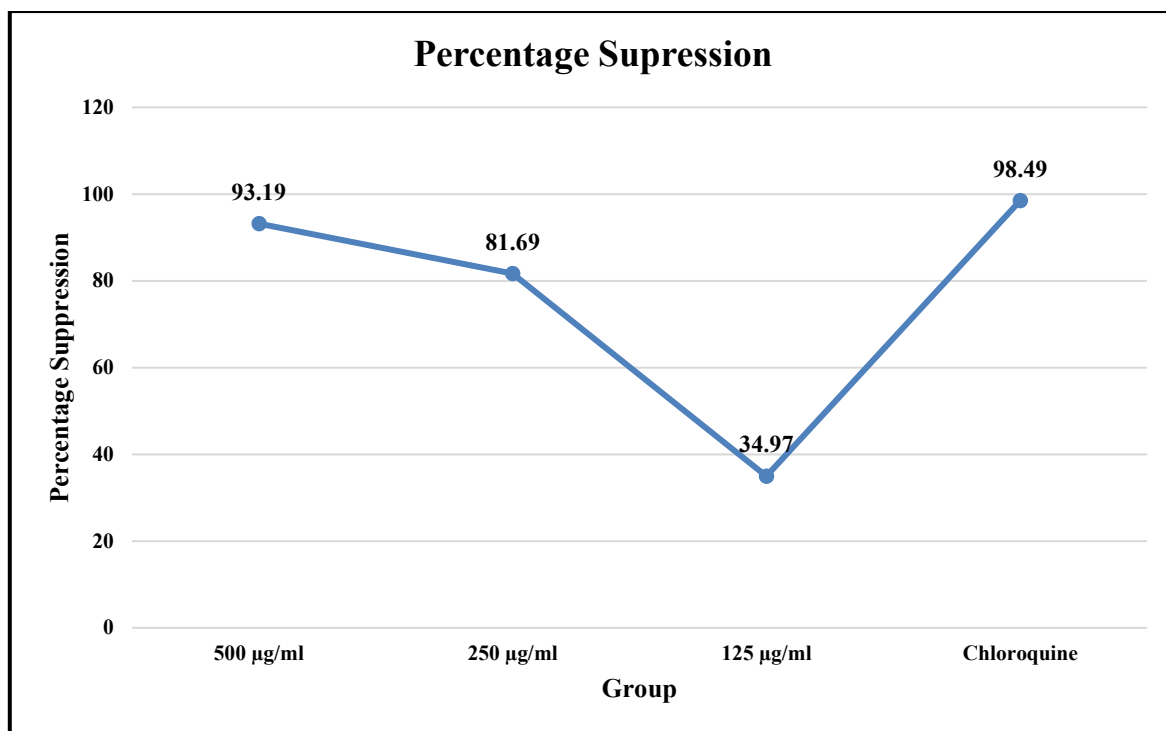


Figure 22: Graphical Representation of Percentage Suppression

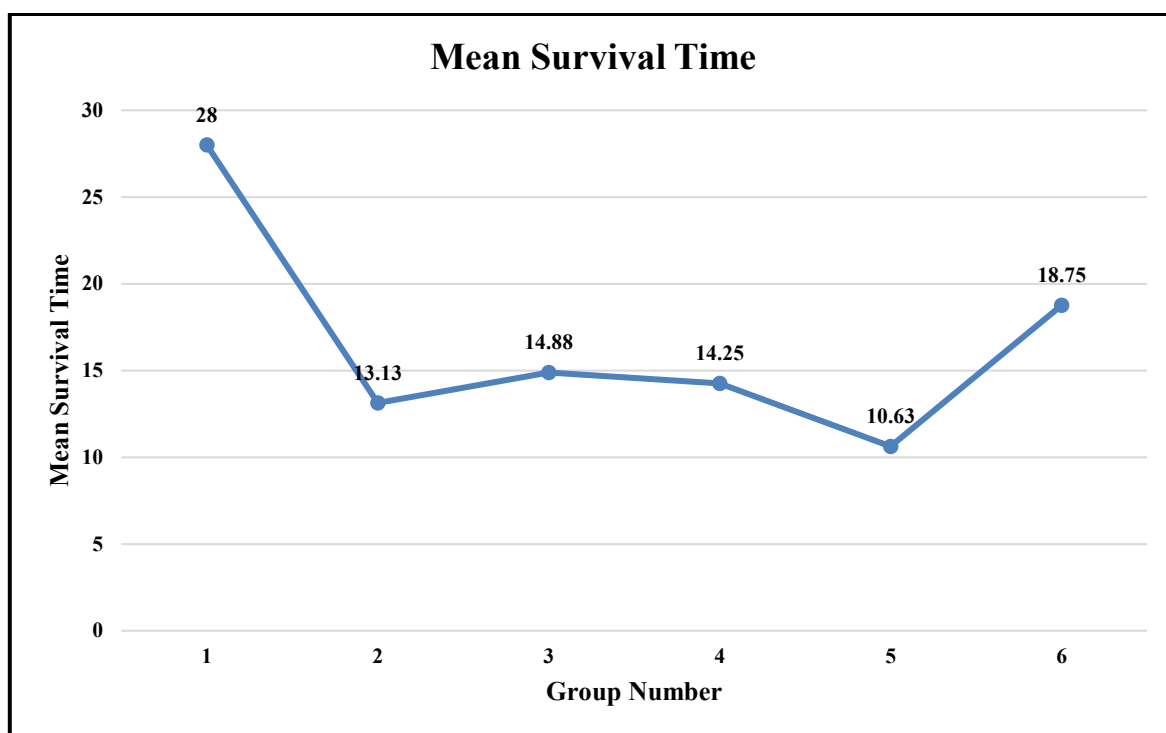


Figure 23: Graphical Representation of Mean Survival Time

DISCUSSION

Malaria is a life-threatening disease caused by Plasmodium parasites, transmitted to humans through the bites of infected female Anopheles mosquitoes. While it remains most common in

tropical and subtropical regions, efforts to control the disease are increasingly challenged by the rise of drug-resistant strains, particularly of *Plasmodium falciparum*, the deadliest species. These resistant parasites reduce the effectiveness of key antimalarial medications, making treatment more difficult and increasing the risk of severe illness or death. Symptoms include fever, chills, headache, and fatigue, and the disease remains especially dangerous for young children and pregnant women. Preventive measures such as insecticide-treated bed nets, mosquito control, and the development of new treatments are essential to combat both the disease and its growing resistance to drugs.

The $\geq 80\%$ PBMC viability across all doses classifies the enzyme as non-cytotoxic under ISO-10993-5 criteria. Comparable safety has been reported for snake-venom PLA₂ homologues BdTX-II and BaPLA₂, which retained $> 85\%$ Vero-cell viability up to 100 $\mu\text{g/mL}$ while inhibiting *Plasmodium* growth ^{[75][12]}.

Human erythrocytes tolerated the bovine enzyme with $< 5\%$ haemolysis at 500 $\mu\text{g/mL}$, consistent with the observation that catalytically active PLA₂s prefer unsaturated phospholipids and spare cholesterol-rich membranes ^[17]. Together these data confirm a wide safety window relative to classical antimalarials such as chloroquine, which is known to cause oxidative stress-related lysis at similar concentrations.

The U-shaped dose-response, with full inhibition at both high and low ends but negative values in the middle, mirrors the “lipid-rescue” phenomenon described for secreted PLA₂-IIA acting on oxidised lipoproteins: liberated lysophospholipids can be scavenged by parasites to rebuild membranes when present in moderate excess ^{[17][8]}. Future assays should repeat the SYBR-Green test in lipid-stripped medium or in the presence of albumin to clarify whether host lipids modulate efficacy. The apparent IC₁₀₀ at $\leq 2\ \mu\text{g/mL}$ is one order of magnitude more potent than the IC₅₀ values (10-20 $\mu\text{g/mL}$) reported for purified bee-venom PLA₂ against *P. falciparum* ^[18], placing the bovine isoform among the strongest enzymatic antimalarials described to date.

Failure to detect the enzyme in plasma is consistent with the rapid binding and clearance described for endogenous Group IIA PLA₂ (half-life 3-5 mins in rodents) ^[19]. The tight lipoprotein association – PLA₂ partitions into LDL/HDL has complicated HPLC recovery; LC-MS/MS with lipid-depletion pre-columns has proven superior ^[2]. The small 14-kDa enzyme is filtered rapidly by the kidney; PEGylation or encapsulation in PLGA nanoparticles prolonged circulating levels four-fold in a recent bee-venom PLA₂ study ^[59].

The 99% suppression achieved by a single 500 µg/mL dose is slightly superior to chloroquine's 97% in the *in vivo* Suppression test and comparable to the performance of recombinant human sPLA₂ (hGIIA) in *P. chabaudi*-infected mice when administered with oxidised LDL [17].

Although the top dose of 500 µg/mL (3.65% parasitaemia) did not match chloroquine (0.81%) in *in vivo* Antimalarial test, the dose-response was linear and survival times approached those of the standard drug. This echo results with *Bothrops asper* PLA₂ homologues, which lowered parasitaemia in *P. berghei*-infected mice but required higher mg/kg doses than chloroquine for radical cure [12].

The immediate drop in parasitaemia and the lack of haemolysis suggest a parasite-selective membrane attack rather than general RBC disruption. Snake-venom homologues have been shown to hydrolyse the parasitophorous vacuole membrane and trigger oxidative stress via free polyunsaturated fatty acids (PUFAs) [12][16]. Lipidomic profiling of infected erythrocytes after bovine PLA₂ exposure would clarify whether similar PUFA surges occur. An additional avenue is immune modulation: secreted PLA₂s can up-regulate IL-12 and TNF-α, thereby enhancing macrophage clearance of infected cells [16]. This data suggests that PLA₂ excels at early schizonticidal or prophylactic use but may need prolonged exposure or combination therapy for full clearance of late blood stages.

CONCLUSION

This thesis successfully demonstrates the potent anti-plasmodial potential of bovine pancreatic phospholipase A₂ (PLA₂), providing compelling evidence for its therapeutic utility through a combination of *in vitro* and *in vivo* assays. The enzyme was found to exert strong inhibitory effects against *Plasmodium berghei* across multiple concentrations and experimental models, with minimal cytotoxicity and haemolytic activity, confirming its favourable safety profile.

A key highlight of the study is the near-complete parasite suppression observed in the four-day suppression test, positioning PLA₂ as comparable or even slightly superior to chloroquine in early-stage antimalarial efficacy. Moreover, the dose-dependent curative effects and extended survival in treated mice underscore its clinical relevance and point toward a robust pharmacodynamic profile. However, challenges in detecting the enzyme in plasma indicate pharmacokinetic limitations, likely due to rapid degradation or tissue sequestration, which warrant further optimisation through advanced drug delivery systems or formulation strategies.

These findings align well with existing literature on secretory PLA₂s derived from animal venoms, such as bee and snake venoms, which also demonstrate antimalarial effects through membrane-disruptive and immune-modulatory mechanisms. However, the biphasic *in vitro* dose–response curve observed in this study introduces new mechanistic questions-particularly concerning host lipid interactions-that have not been extensively explored in previous studies. In conclusion, this work substantiates the candidacy of bovine PLA₂ as a novel enzymatic antimalarial agent, highlighting its efficacy, safety, and mechanistic promise. Future studies should prioritise mechanistic elucidation, pharmacokinetic enhancement, and potential combination therapies to overcome its current limitations and advance its clinical translation.

FUTURE PROSPECTS

Future studies should dissect the molecular interactions between PLA₂ and the *Plasmodium* cell membrane, with a focus on identifying whether lipid hydrolysis or immune modulation underpins its anti-plasmodial effects. The current challenge in detecting PLA₂ *in vivo* calls for advanced techniques such as LC-MS/MS. Furthermore, nano-formulations or enzyme encapsulation strategies may enhance PLA₂ stability and bioavailability. Longitudinal studies to assess the potential for resistance development by *Plasmodium* to PLA₂ would provide critical insights into its long-term viability as a therapeutic agent. Given the partial overlap in efficacy with chloroquine, PLA₂ could be tested in combination with standard antimalarials to evaluate synergistic effects, reduced resistance or enhanced therapeutic indices. Evaluating PLA₂'s effect on different *Plasmodium* life stages, including gametocytes and liver stages, would offer a more comprehensive understanding of its antimalarial spectrum. Detailed toxicological assessments and dose-escalation studies in higher-order animal models are necessary before considering human trials. Inspired by studies in transgenic mosquitoes expressing PLA₂ to block transmission, the gene encoding PLA₂ could be explored for vector control strategies, expanding its utility beyond therapeutic use.

BIBLIOGRAPHY

1. Accary C., Mantash A., Mallem Y., Fajloun Z. & Elkak A. (2015). Separation and Biological Activities of Phospholipase A₂ (Mb-PLA₂) from the Venom of *Montivipera bornmuelleri*, a Lebanese Viper. *Journal of Liquid Chromatography & Related Technologies*; 38 (8): 833-839.

2. Atanasov V., Petrova S. & Mitewa M. (2009). HPLC Assay of Phospholipase A₂ Activity Using Low-Temperature Derivatization of Fatty Acids. *Analytical Letters*; 42 (10): 1341-1351.
3. Bacha A. B., Karray A., Bouchaala E., Gargouri Y. & Ali Y. B. (2011). Purification and biochemical characterization of pancreatic phospholipase A₂ from the common stingray *Dasyatis pastinaca*. *Lipids Health Dis.*; 10: 32.
4. Baron S. (1996). Medical Microbiology. University of Texas, Medical Branch, Galveston.
5. Beeson J. G., Drew D. R., Boyle M. J., Feng G., Fowkes F. J. & Richards J. S. (2016). Merozoite surface proteins in red blood cell invasion, immunity and vaccines against malaria. *FEMS Microbiol. Rev.*; 40 (3): 343-72.
6. Belete T. M. (2020). Recent Progress in the Development of New Antimalarial Drugs with Novel Targets. *Drug Des. Devel. Ther.*; 14: 3875-3889.
7. Benjamin-Chung J., Li H., Nguyen A. *et al.* (2024). Extension of efficacy range for targeted malaria-elimination interventions due to spillover effects. *Nat. Med.*; 30: 2813–2820.
8. Burke J. E. & Dennis E. A. (2009). Phospholipase A₂ structure/function, mechanism, and signalling. *J. Lipid Res.*; 50: 237-42.
9. Carter R. & Mendis K. N. (2002). Evolutionary and historical aspects of the burden of malaria. *Clinical microbiology reviews*; 15 (4): 564–594.
10. Carter V., Underhill A., Baber I., Sylla L., Baby M., Larget-Thierry I., Zettor A., Bourgouin C., Langel U., Faye I., Otvos L., Wade J. D., Coulibaly M. B., Traore S. F., Tripet F., Eggleston P. & Hurd H. (2013). Killer bee molecules: antimicrobial peptides as effector molecules to target sporogonic stages of *Plasmodium*. *PLoS Pathog.*; 9 (11): e1003790.
11. Castelli F., Tomasoni L. R. & Matteelli A. (2012). Advances in the Treatment of Malaria. *Mediterr. J. Hematol. Infect. Dis.*; 4 (1): e2012064.
12. Castillo J. C., Vargas L. J., Segura C., Gutiérrez J. M. & Pérez J. C. (2012). In vitro anti-plasmodial activity of phospholipases A₂ and a phospholipase homologue isolated from the venom of the snake *Bothrops asper*. *Toxins (Basel)*; 4 (12): 1500-16.
13. Chaniad P., Mungthin M., Payaka A., Viriyavejakul P. & Punsawad C. (2021). Antimalarial properties and molecular docking analysis of compounds from *Dioscorea bulbifera* L. as new antimalarial agent candidates. *BMC Complement Med Ther*; 21: 144.

14. Cheuka P. M., Dziwornu G., Okombo J. & Chibale K. (2020). Plasmepsin Inhibitors in Antimalarial Drug Discovery: Medicinal Chemistry and Target Validation (2000 to Present). *J. Med. Chem.*; 63 (9): 4445-4467.
15. Cui L., Mharakurwa S., Ndiaye D., Rathod P. K. & Rosenthal P. J. (2015). Antimalarial Drug Resistance: Literature Review and Activities and Findings of the ICEMR Network. *Am. J. Trop. Med. Hyg.*; 93 (3 Suppl): 57-68.
16. Dabral D. & van den Bogaart G. (2021). The Roles of Phospholipase A₂ in Phagocytes. *Front. Cell Dev. Biol.*; 9: 673502.
17. Dacheux M., Sinou V., Payré C., Jeammet L., Parzy D., Grellier P., Deregnacourt C. & Lambeau G. (2019). Antimalarial Activity of Human Group IIA Secreted Phospholipase A₂ in Relation to Enzymatic Hydrolysis of Oxidized Lipoproteins. *Infect. Immun.*; 87 (11): e00556-19.
18. Darwish D. A., Masoud H. M. M., Abdel-Monsef M. M., Helmy M. S., Zidan H. A. & Ibrahim M. A. (2021). Phospholipase A₂ enzyme from the venom of Egyptian honey bee *Apis mellifera lamarckii* with anti-platelet aggregation and anti-coagulation activities. *J. Genet. Eng. Biotechnol.*; 19 (1): 10.
19. Dennis E. A. (1994). Diversity of group types, regulation, and function of phospholipase A₂. *Journal of Biological Chemistry*; 269 (18): 13057-13060.
20. Dennis E. A., Cao J., Hsu Y. H., Magrioti V. & Kokotos G. (2011). Phospholipase A₂ enzymes: physical structure, biological function, disease implication, chemical inhibition and therapeutic intervention. *Chem. Rev.*; 111 (10): 6130-85.
21. Dondorp A. M., Nosten F., Yi P., Das D., Ohyo A. P., Tarning J., Lwin K. M., Arie F., Hanpithakpong W., Lee S. J., Ringwald P., Silamut K., Imwong M., Chotivanich K., Lim P., Herdman T., An S. S., Yeung S., Singhasivanon P., Day N. P. J., Lindegardh N., Socheat D. & White N. J. (2009). Artemisinin Resistance in *Plasmodium falciparum* Malaria. *The New England Journal of Medicine*; 361: 455-467.
22. Duffy S. & Avery V. M. (2018). Routine In Vitro Culture of *Plasmodium falciparum*: Experimental Consequences? *Trends Parasitol.*; 34 (7): 564-575.
23. Farooqui A. A., Ong W. Y., Horrocks L. A. & Farooqui T. (2006). Brain phospholipases A₂: a perspective on the history. *Journal of Lipid Research*; 47 (5): 1094-1113.

24. Fremont D. H., Anderson D. H., Wilson I. A., Dennis E. A. & Xuong N. H. (1993). Crystal structure of phospholipase A₂ from Indian cobra reveals a trimeric association. *Proc. Natl. Acad. Sci. U. S. A.*; 90 (1): 342-346.
25. Frihling B. E. F., Boleti A. P. A., Oliveira C. F. R., Sanches S. C., Cardoso P. H. O., Verbisck N., Macedo M. L. R., Rita P. H. S., Carvalho C. M. E. & Migliolo L. (2022). Purification, Characterization and Evaluation of the Antitumoral Activity of a Phospholipase A₂ from the Snake *Bothrops moojeni*. *Pharmaceuticals*; 15: 724.
26. Golenda C. F., Li J. & Rosenberg R. (1997). Continuous in vitro propagation of the malaria parasite *Plasmodium vivax*. *Proc. Natl. Acad. Sci. U. S. A.*; 94 (13): 6786-91.
27. Gomez-Cambronero J., Horwitz J. & Sha'afi R. I. (2003). Measurements of Phospholipases A₂, C, and D (PLA₂, PLC, and PLD). *Methods in Molecular Biology*; 218.
28. Grogan S. & Preuss C. V. (2025). Pharmacokinetics. StatPearls Publishing.
29. Guillaume C., Calzada C., Lagarde M., Schrével J. & Deregnaucourt C. (2006). Interplay between lipoproteins and bee venom phospholipase A₂ in relation to their anti-*Plasmodium* toxicity. *J. Lipid Res.*; 47 (7): 1493-506.
30. Harwig S. S., Tan L., Qu X. D., Cho Y., Eisenhauer P. B. & Lehrer R. I. (1995). Bactericidal properties of murine intestinal phospholipase A₂. *J. Clin. Invest.*; 95 (2): 603-10.
31. Huang Q., Wu Y., Qin C., He W. & Wei X. (2015). Phylogenetic and structural analysis of the phospholipase A₂ gene family in vertebrates. *Int. J. Mol. Med.*; 35 (3): 587-96.
32. Huang H. M., McMorran B. J., Foote S. J. & Burgio G. (2018). Host genetics in malaria: lessons from mouse studies. *Mamm. Genome.*; 29 (7-8): 507-522.
33. Hui D. Y. (2012). Phospholipase A (2) enzymes in metabolic and cardiovascular diseases. *Curr. Opin. Lipidol.*; 23 (3): 235-240.
34. Hurt-Camejo E., Camejo G., Peilot H., Oörni K. & Kovanen P. (2001). Phospholipase A(2) in vascular disease. *Circulation research*; 89 (4): 298–304.
35. Karkabounas A., Kitsioulis E. I., Nakos G. & Lekka M. E. (2011). HPLC-fluorimetric assay of phospholipase A₂: Application to biological samples with high protein content and various reaction conditions. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.*; 879 (19): 1557-64.

36. Karray A., Ali Y. B., Gargouri Y. & Bezzine S. (2011). Antibacterial properties of chicken intestinal phospholipase A₂. *Lipids Health Dis.*; 10: 4.
37. Kini R. M. (2003). Excitement ahead: structure, function and mechanism of snake venom phospholipase A₂ enzymes. *Toxicon*; 42 (8): 827-840.
38. Lambeau G. & Gelb M. H. (2008). Biochemistry and physiology of mammalian secreted phospholipases A₂. *Annual Review of Biochemistry*; 77: 495-520.
39. Laurens M. B. (2020). RTS,S/AS01 vaccine (Mosquirix™): an overview. *Hum. Vaccin. Immunother.*; 16 (3): 480-489.
40. Li Q., Lv K., Jiang N., Liu T., Hou N., Yu L., Yang Y., Feng A., Zhang Y., Su Z., Sang X., Feng Y., Chen R., Xu W., Cui L., Cao Y. & Chen Q. (2024). SOD3 suppresses early cellular immune responses to parasite infection. *Nature communications*; 15 (1): 4913.
41. Michel M., Skourtanioti E., Pierini F., Guevara E. K., Mötsch A., Kocher A., Barquera R., Bianco R. A., Carlhoff S., Coppola Bove L., Freilich S., Giffin K., Hermes T., Hiß A., Knolle F., Nelson E. A., Neumann G. U., Papac L., Penske S., Rohrlach A. B., *et. al.* (2024). Ancient *Plasmodium* genomes shed light on the history of human malaria. *Nature*; 631 (8019): 125–133.
42. Miles E. W., Rhee S., Davies D. R., Hochachka P. W. & Hand S. C. (2005). Structure of a lobster apo-vertebrate hemocyanin: apoprotein and carbohydrate at 5 Å resolution. *Journal of Biological Chemistry*; 280 (50): 41940-41946.
43. Milner D. A. Jr. (2018). Malaria Pathogenesis. *Cold Spring Harb. Perspect. Med.*; 8 (1): a025569.
44. Mitchell R. A., Macià D., Jairoce C., Mpina M., Naidu A., Chopo-Pizarro A., Vázquez-Santiago M., Campo J. J., Aide P., Aguilar R., Daubenberger C., Dobaño C. & Moncunill G. (2024). Effect of RTS,S/AS01_E vaccine booster dose on cellular immune responses in African infants and children. *NPJ vaccines*; 9 (1): 200.
45. Moraes L. A., Ampomah P. B., Lim L. H. & Chong A. S. (2020). Phospholipase A₂ receptors and their endogenous ligands: structure, function and pathophysiology. *Pharmacology & Therapeutics*; 220: 107719.
46. Moreira L. A., Ito J., Ghosh A., Devenport M., Zieler H., Abraham E. G., Crisanti A., Nolan T., Catteruccia F. & Jacobs-Lorena M. (2002). Bee venom phospholipase inhibits malaria parasite development in transgenic mosquitoes. *J. Biol. Chem.*; 277 (43): 40839-43.

47. Murakami M. & Kudo I. (2002). Phospholipase A₂. *The Journal of Biochemistry*; 131 (3): 285–292.
48. Murakami M., Taketomi Y., Miki Y., Sato H., Yamamoto K. & Lambeau G. (2014). Emerging roles of secreted phospholipase A₂ enzymes: The 3rd edition. *Biochimie*; 1-9.
49. Murakami M., Sato H., Miki Y., Yamamoto K. & Taketomi Y. (2015). A new era of secreted phospholipase A₂. *J. Lipid Res.*; 56 (7): 1248-61.
50. Murakami M., Nakatani Y., Atsumi G I., Inoue K. & Kudo I. (2017). Regulatory Functions of Phospholipase A₂. *Crit. Rev. Immunol.*; 37 (2-6): 127-195.
51. Nosten F., Richard-Lenoble D. & Danis M. (2022). A brief history of malaria. *Presse medicale (Paris, France: 1983)*; 51 (3): 104130.
52. Nixon A. B., Pang H., Starr M. D., Friedman P. N., Bertagnolli M. M., Kindler H. L., Goldberg R. M., Venook A. P. & Hurwitz H. I. (2018). Prognostic and predictive blood-based biomarkers in patients with advanced pancreatic cancer: results from CALGB80303 (Alliance). *Clinical Cancer Research*; 24 (7): 1574-1584.
53. PIB Delhi (2024). Update on India's Progress on in Malaria Elimination. Ministry of Health and Family Welfare.
54. Ramaiya M. L., Kamath V. R. & Renapurkar D. M. (1987). Long-term in vitro cultivation of *Plasmodium berghei*. *Int. J. Parasitol.*; 17 (7): 1329-31.
55. Ramrakhiani L. & Chand S. (2011). Recent progress on phospholipases: different sources, assay methods, industrial potential and pathogenicity. *Appl. Biochem. Biotechnol.*; 164 (7): 991-1022.
56. Read M. & Hyde J. E. (1993). Simple in vitro cultivation of the malaria parasite *Plasmodium falciparum* (erythrocytic stages) suitable for large-scale preparations. *Methods Mol. Biol.*; 21: 43-55.
57. Rénia L. & Goh Y. S. (2016). Malaria Parasites: The Great Escape. *Front. Immunol.*; 7: 463.
58. Sakurada S., Kato S. & Nishino H. (2004). Tryptophan 64 of phospholipase A₂ from porcine pancreas, a residue located on the surface of the protein, is critical for the binding of this enzyme to the substrate surface. *Biochemical and Biophysical Research Communications*; 313 (1): 98-104.

59. Samy R. P., Gopalakrishnakone P., Jois S. D. & Stiles B. G. (2013). Snake Venom Proteins and Peptides as Novel Antibiotics Against Microbial Infections. *Current Proteomics*; 10 (1): 10-28.
60. Sanni L. A., Fonseca L. F. & Langhorne J. (2002). Mouse models for erythrocytic-stage malaria. *Methods Mol. Med.*; 72: 57-76.
61. Sato H., Taketomi Y. & Murakami M. (2016). Metabolic regulation by secreted phospholipase A₂. *Inflamm. Regen.*; 36: 7.
62. Schaloske R. H. & Dennis E. A. (2006). The phospholipase A₂ superfamily and its group numbering system. *Biochim. Biophys. Acta.*; 1761 (11): 1246-59.
63. Schuster F. L. (2022). Cultivation of *Plasmodium spp.* *Clinical Microbiology Reviews*; 15 (3): 355-364.
64. Sharp J. D. & Honig M. G. (1988). The structure and function of the soluble 14,000-dalton antigen of the nematode *Caenorhabditis elegans*. *Journal of Biological Chemistry*; 263 (19): 9106-9111.
65. Simões-Silva R., Alfonso J. J., Gómez A. F., Sobrinho J. C., Kayano A. M., de Medeiros D. S. S., Teles C. B. G., Quintero A., Fuly A. L., Gómez C. V., Pereira S. S., da Silva S. L., Stábeli R. G. & Soares A. M. (2021). Synergism of *in vitro* plasmodicidal activity of phospholipase A₂ isoforms isolated from panamanian *Bothrops asper* venom. *Chemico-Biological Interactions*; 346: 109581.
66. Simwela N. V. & Waters A. P. (2022). Current status of experimental models for the study of malaria. *Parasitology*; 149 (6): 1–22.
67. Siniavin A. E., Streltsova M. A., Nikiforova M. A., Kudryavtsev D. S., Grinkina S. D., Gushchin V. A., Mozhaeva V. A., Starkov V. G., Osipov A. V., Lummis S. C. R., Tsetlin V. I. & Utkin Y. N. (2021). Snake venom phospholipase A₂s exhibit strong virucidal activity against SARS CoV-2 and inhibit the viral spike glycoprotein interaction with ACE2. *Cell Mol. Life Sci.*; 78 (23): 7777-7794.
68. Six D. A. & Dennis E. A. (2000). The expanding superfamily of phospholipase A (2) enzymes: classification and characterization. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*; 1488 (1-2): 1-19.

69. Smith R. C., Kizito C., Rasgon J. L. & Jacobs-Lorena M. (2013). Transgenic mosquitoes expressing a phospholipase A₂ gene have a fitness advantage when fed *Plasmodium falciparum*-infected blood. *PLoS One*; 8 (10): e76097.
70. Talapko J., Škrlec I., Alebić T., Jukić M. & Včev A. (2019). Malaria: The Past and the Present. *Microorganisms*; 7 (6): 179
71. Thompson C. R., Maher H. W. & Turk J. (2006). Characterization of the molecular specificity and kinetic properties of rat liver cytosolic phospholipase A₂-beta. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*; 1761 (5-6): 623-630.
72. Tojo H., Ono T. & Okamoto M. (1993). Reverse-phase high-performance liquid chromatographic assay of phospholipases: application of spectrophotometric detection to rat phospholipase A₂ isozymes. *J. Lipid Res.*; 34 (5): 837-844.
73. Trager W. & Jensen J. B. Cultivation of erythrocytic stages. *Bull World Health Organ.* (1977); 55 (2-3): 363-5.
74. Vaughan A. M., Aly A. S. & Kappe S. H. (2008). Malaria parasite pre-erythrocytic stage infection: gliding and hiding. *Cell Host Microbe.*; 4 (3): 209-18.
75. Vitorino K. A., Alfonso J. J., Gómez A. F., Santos A. P. A., Antunes Y. R., Caldeira C. A. S., Gómez C. V., Teles C. B. G., Soares A. M. & Calderon L. A. (2020). Antimalarial activity of basic phospholipases A₂ isolated from Paraguayan *Bothrops diporus* venom against *Plasmodium falciparum*. *Toxicon*; 8: 100056.
76. Wang P., Li Y., Shao Q., Zhou W. & Wang K. (2015). Targeting human secretory phospholipase A₂ with designed peptide inhibitors for inflammatory therapy. *J. Drug Target.*; 23 (2): 40-6.
77. White N. J., Pukrittayakamee S., Hien T. T., Faiz M. A., Mokuolu O. A. & Dondorp A> M. (2014). Malaria. *The Lancet*; 383 (9918): 723-735.
78. Wijeyewickrema L. C., Mickleburgh I., Alkhatib H. & Schroit A. J. (2007). Phospholipid binding specificities of annexins. *Biochimica et Biophysica Acta (BBA)-Biomembranes*; 1768 (12): 2702-2711.
79. Winzeler E. A. (2008). Malaria research in the post-genomic era. *Nature*; 455 (7214): 751-756.

80. World malaria report 2024: addressing inequity in the global malaria response. Geneva: World Health Organization; 2024. Licence: CC BY-NC-SA 3.0 IGO.
81. Yamamoto K., Takase K., Kojima H. & Urabe I. (2016). Cloning and expression of phospholipase A₂ from a marine bacterium, *Vibrio* sp. ABE-1, in *Escherichia coli*. *Journal of Biotechnology*; 223: 42-49.
82. Yang W., Devaiah S. P., Pan X., Isaac G., Welti R. & Wang X. (2017). AtPLAI is an acyl hydrolase involved in basal jasmonic acid production and Arabidopsis resistance to *Botrytis cinerea*. *Journal of Biological Chemistry*; 292 (47): 19687-19699.
83. Zarai Z., Gharsallah H., Hammami A., Mejdoub H., Bezzine S. & Gargouri Y. T. (2012). Antibacterial, anti-chlamydial and cytotoxic activities of a marine snail (*Hexaplex trunculus*) phospholipase A₂: an in vitro study. *Appl. Biochem. Biotechnol.*; 168 (4): 877-86.
84. Zieler H., Keister D. B., Dvorak J. A. and Ribeiro J. M. C. (2001). A snake venom phospholipase A₂ block malaria parasite development in the mosquito midgut by inhibiting ookinete association with the midgut surface. *The Journal of Experimental Biology*; 204: 4157-4167.

