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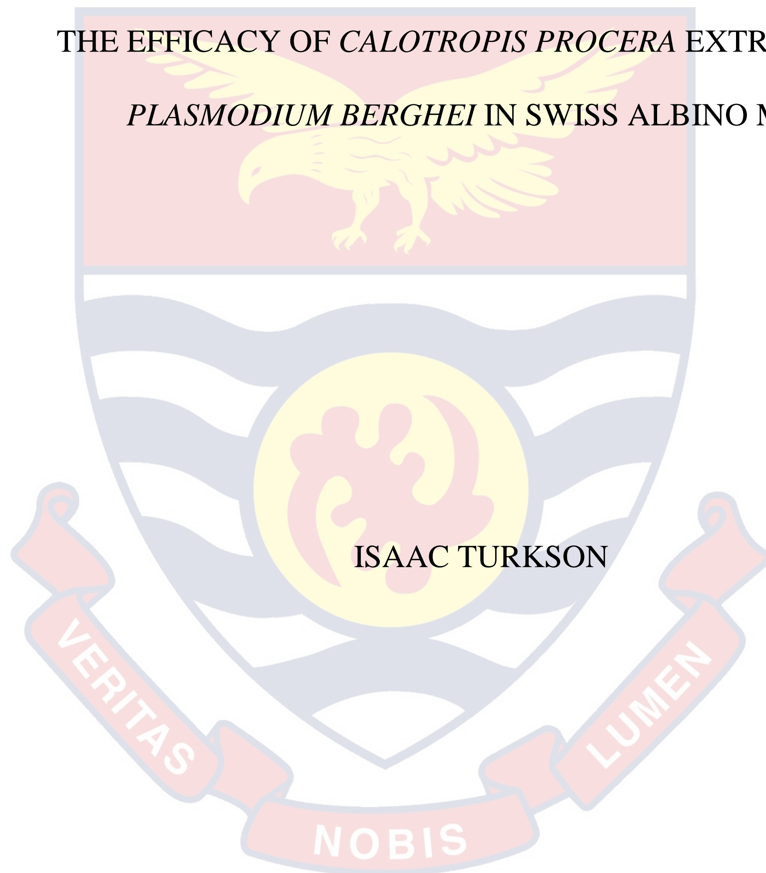

THE EFFICACY OF *CALOTROPIS PROCERA* EXTRACTS ON *PLASMODIUM BERGHEI* IN SWISS ALBINO MICE

ISAAC TURKSON

2021

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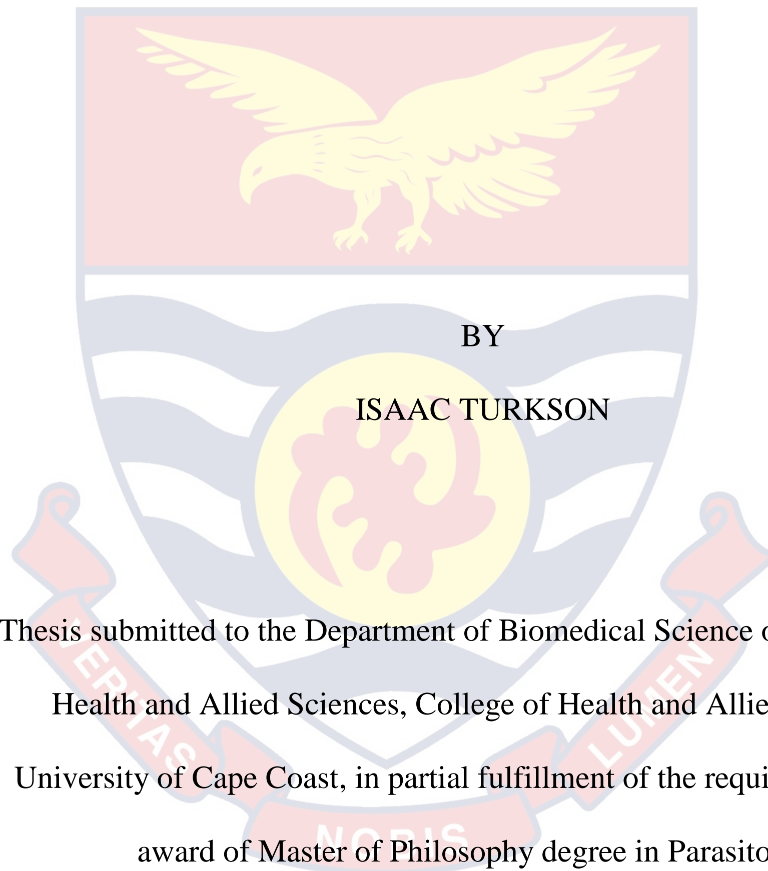

BY

ISAAC TURKSON

Thesis submitted to the Department of Biomedical Science of the School of Health and Allied Sciences, College of Health and Allied Sciences, University of Cape Coast, in partial fulfillment of the requirement for the award of Master of Philosophy degree in Parasitology

AUGUST 2021

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DECLARATION

Candidate's Declaration

I hereby declare that this thesis is the result of my own original research and that no part of it has been presented for another degree in this University or elsewhere.

Candidate's Signature ………………………………. Date ………………...

Name: Isaac Turkson

Supervisors' Declaration

We hereby declare that the preparation and presentation of the thesis were supervised in accordance with the guidelines on supervision of thesis laid down by the University of Cape Coast.

Supervisor's Signature ………………………… Date ………………….

Name: Prof. Elvis Ofori Ameyaw

Co-Supervisor's Signature ………………………… Date……………..

Name: Prof. Desmond Omane Acheampong

VERITAS LUMEN NOBIS

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ABSTRACT

Malaria is one of the most prevalent human infections worldwide and treatment has been com-promised by drug resistance to the parasite. *Calotropis procera*, is known for its pharmacological importance for many years. This study sought to determine efficacy of *Calotropis procera* extracts on *Plasmodium berghei* in swiss mice. *In vitro* antimalarial activity was carried out. *In vivo* parasite growth inhibitory effect of crude extract was assessed in mice inoculated with *Plasmodium berghei* (ANKA strain). The *in vivo* antiplasmodial activity of the test extract was performed against early infection (4-day suppressive test), curative effect against established infection and prophylactic effect against residual infection. Acute toxicity was carried out according to OECD guidelines. Anti-inflammatory and anti-pyretic activities were carried out on the extract. The *in vitro* parasitaemia suppression was IC_{50}= 83.91± 0.375μg/ml. *In vivo* parasite growth inhibition effect of crude extract of *C. procera* was evaluated at 200, 400 and 600 mg/kg dose levels. It suppressed parasitaemia by $P< 0.0001$ at 600 mg/kg dose level in the 4-day test. In curative and prophylactic potential tests, it suppressed parasitaemia by $P< 0.001$ and $P< 0.0015$ at 600 mg/kg dose level, respectively. *In vivo* toxicity tests revealed mild toxicity at high dose. All parasitaemia suppressions were statistically significant at $P< 0.05$ as compared to the vehicle-treated group. The antipyretic and anti-inflammatory activities showed $P< 0.0002$ and $P< 0.0144$ at dose 300 mg/kg. The crude extract also prolonged survival time in a dose-dependent manner. The investigation results suggest that the leave extract of *C. procera* possesses antimalarial activity. It is therefore necessary for the government to create funds for research institutions to carry out a series of work on other plants to elucidate its potency in treating the malaria disease.

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KEYWORDS

In vitro antiplasmodial activity

In vivo antiplasmodial activity

Calotropis procera

Antipyretic activity

Anti-inflammatory activity

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DEDICATION

This work is dedicated to all who will get the chance to read this thesis.

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LIST OF ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
AIM	African Initiative for Malaria
AL	Arthemeter-Lumefantrin
ANOVA	Analysis of variance
CDC	Centre for Disease Control
CPD	Citrate-Phosphate-Dextrose
CPM	Complete Parasite Media
COX	Cyclooxygenase
DHPS	Deoxyhypusine Synthase
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
GIT	Gastrointestinal tract
GPI	Glycosylphosphatidylinositol
HIV	Human Immuno-deficiency Virus
IC_{50}	Inhibitory concentration
Kg	Kilogram
mL	Millilitre
µL	Microlitre
µg	Microgram
ng	Nanogram
NMIMR	Noguchi Memorial Institute for Medical Research
NSAIDs	Nonsteroidal anti-inflammatory drugs
OECD	Organisation for Economic Cooperation and Development
PCV	Packed cell volume

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PfCRT	*Plasmodium falciparum* resistance transporter
PGE	Prostaglandin E
pLDH	*Plasmodium* lactate dehydrogenase
RBCs	Red Blood Cells
TRAP	Thrombospondin-related Anonymous Protein
WHO	World Health Organisation

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CHAPTER ONE

INTRODUCTION

This chapter describes into the background of the study. It further chronologically provides the purpose of the study, objectives, hypothesis, justification, delimitation, limitation, organisation of the study and the chapter summary.

Background to the study

The exploration of traditional medicine as a therapeutic option for the treatment of malaria is not a new practice as the current active ingredient against *Plasmodium*, artemesinin was discovered via this means (Miller, Baruch, Marsh, & Doumbo, 2002). One of such plants with a potential antimalarial activity is *Calotropis procera*, commonly referred to as 'apple of Sodom'. Previously, its antibacterial, antifungal, and anti-inflammatory effects in both humans and animal models have been investigated (Basu, Chaudhuri, 1991). Nonetheless, its therapeutic potential in the treatment of malaria remains largely unexplored. In Ghana, it is popularly known as 'mpasatu asa' in Twi and 'Gbeke biiawuo' in Ga. Formerly, *Calotropis procera* was classified under family Asclepiadaceae (the milkweed family). However, it is now, considered to belong to subfamily Apocynaceae (Acevedo-Rodriguez, Strong, 2012).

Calotropis procera is a well-known plant and has been used traditionally to treat diarrhoea, sinus fistula, and skin diseases (Alikhan & Khanum, 2005). The leaf is also used to treat jaundice (Alikhan et al 2005). The water, ethanol, acetone and some other organic solvent extracts of this plant possess insecticidal (Moursy, 1997), larvicidal (Markouk, Bekkouche,

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Larhsini, Bousaid, Lazrek & Jana, 2000), anti-bacterial and anti-parasitic (Larhsini, Oumoulid, Lazrek, Wataleb, Bousaid, Markouk & Jana, 1999) properties. The milky white latex obtained from the plant exhibits potent anti-inflammatory activity in various animal models that is comparable to standard anti-inflammatory drugs (Sangraula, Dewan & Kumar, 2002).

Compounds such as calotropon, calotropin, uscharine, calotoxin, calctin, uscharidin and calotropagenin are some of the important compounds obtained from the leaves and latex of *Calotropis procera* plant. The phytochemical studies on the aerial parts of the plant also showed the presence of alkaloids, anthocyanins, proteolytic enzymes, cardiac glycosides, tannins, flavonoids, sterols and triterpenes (Mossa, Tariq, Mohin, Ageel, Al-Yahya & Al-Said, 1991). Formerly, cardenolides, phytosterols, and triterpenoids saponins have been isolated from the roots of *Calotropis procera* (Khan & Malik, 1989). This medicinal plant has been reported to contain some essential elements such as Aluminium (Al), Arsenic (As), Copper (Cu), Calcium (Ca), Chromium (Cr), Cadmium (Cd), Iron (Fe), Potassium (K), Manganese (Mn), Sodium (Na), Lead (Pb), and Zinc (Zn) in varying amounts, with approximately 27-32 percent total protein (Mossa, Tariq, Mohin, Ageel, Al-Yahya, & Al-Said, 1991).

Malaria is one of the most prevalent human infections worldwide. Over forty percent (40%) of the world's population lives in malaria-endemic areas (World Health Organisation, 2015). An estimate of 300 to 500 million malaria cases and 1.5 to 2.7 million malaria-associated deaths occur each year (WHO, 2015). Ninety percent (90%) of malaria-associated deaths occur in sub-Saharan Africa, majority involving children less than 5 years of age

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(WHO, 2016). Malaria disproportionately affects the poor, in whom higher morbidity and mortality can be largely attributed. Because of a lack of access to effective treatment, sixty percent (60%) of malaria deaths worldwide occur in the poorest twenty percent (20%) of the population (The World Bank, 2015). In addition, children and pregnant women (particularly primigravidae) and immunocompromised people are at a higher risk of severe disease. This situation is undermining the achievements of the four millennium sustainable goals, that is, eradication of extreme poverty, reduction of child mortality rates, improvement of maternal health, and combating of HIV/AIDS and other diseases (Mishra, Dash, Swain & Dey, 2009).

There is growing international agreement on how best to use preventive and treatment methods that are available (WHO, 2008). The most effective preventive measures include the use of mosquito bed nets treated with long-lasting insecticides, to avoid the mosquito bites and spraying the inside walls of houses with similar insecticides to kill *P. falciparum* carrying mosquitoes (Acevedo-Rodriguez, Strong, 2012). The most effective treatment for malaria is the artemisinin-based combination therapy consisting of artemisinin backbone combined with other anti-malarial active agents (WHO, 2015). Preventive treatment of pregnant women with anti-malarial drugs can also reduce the harmful effects of malaria both on the mother and on the unborn child (WHO, 2015).

Several international organisations have set up ambitious objectives for large-scale malaria control. The target set by the World Health Organisation (WHO) in 2005, is to offer malaria prevention and treatment services by the year 2010, to at least 80% of the people who need them. By doing so, it aims

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to reduce at least by half the proportion of people who become ill or die from malaria by the year 2010 and at least by three quarters by the year 2015 compared to the year 2005.

Statement of the problem

It has been suggested that the tendency at which the malaria parasites develop resistance to purified anti-malarial drugs is far higher compared to unpurified whole plant extract with anti-malaria activity (Krettli, Adebayo & Krettli, 2009). The parasite's resistance against chloroquine was reported back in Thailand in 1957 and this consequently spread all over the world (Kshirasagar, 2006). Due to the reports of resistance, most malaria endemic countries have stopped using chloroquine as first line of treatment for malaria. Presently, artemisinin and its derivatives are used as first line treatment (Mutabingwa, 2005). Unfortunately, artemisinin-resistant strains have been also reported from Thai-Cambodia region in 2009, highlighting the need for new anti-malarial drug (Maude, Woodrow & White, 2010). There has also been a full resistance to quinine, the only other drug sufficiently fast-acting to deal with severe malaria (Tyagi, Gleeson, Arnold, Tahar, Prieur, Decosterd, Perignon, Olliaro & Druilhe, 2018). The discovery and development of new anti-malaria drug candidates from plants is still on-going since the parasite has persistently been gaining resistance against orthodox anti-malarial drugs.

Calotropis procera has long been recognized for its pharmacological significance. This coarse shrub has anticancer, ascaricidal, schizonticidal, anti-microbial, antihelmintic, insecticidal, anti-inflammatory, anti-diarrhoeal, and larvicidal properties, among many others (Palwa, & Chatterjee, 1988; Nenaah & Ahmed, 2011; Pattnaik, Kar, Chhatoi, Shahbazi, Ghosh, & Kuanar, 2017).

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Nonetheless, the antimalarial properties of *Calotropis procera* remain largely unexplored. The previous reports on the medicinal properties of *Calotropis procera* pointed to the need for further investigation on its potential as an antimalarial agent

Study Aim

The aim of the study was to determine the efficacy of *Calotropis procera* extracts on *Plasmodium berghei* in Swiss albino mice.

Specific Objectives

The specific objectives of this study were;

to evaluate the *in vitro* antiplasmodial activity of the crude ethanol leaf extract of *Calotropis procera* against chloroquine-sensitive *Plasmodium falciparum* (3D strain).

to evaluate the *in vivo* antiplasmodial activity of the crude ethanol leaf extract of *Calotropis procera* against *Plasmodium berghei* (ANKA strain).

to investigate the toxic effect of crude ethanol leaf extract of *Calotropis procera* on the brain, kidney, liver and spleen of Swiss albino mice.

Hypothesis

The study hypothesised that *Calotropis procera* possesses anti-malarial activities against *Plasmodium berghei*.

Significance of study

Malaria is one of the most severe public health problems worldwide. It is a leading cause of death and disease in many developing countries, where young children and pregnant women are the groups most affected. According to WHO report in 2017, nearly half the world's population lives in areas at risk of malaria transmission. In 2016, malaria caused an estimated 216 million

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clinical episodes, and 445,000 deaths. An estimated 90% of deaths in 2016 were in the African Region (WHO, 2017).

Malaria places huge costs on people and on governments alike (WHO, 2017). Costs for individuals and their families include the cost of malaria prevention medications at home, expenses for transportation to and care at hospitals and clinics, missed working days, school absence, expenses for preventive measures and funeral expenses in the event of deaths.

Costs to governments include maintenance, supply and staffing of health facilities, and purchase of drugs and supplies. It also includes public health interventions against malaria such as insecticide spraying or distribution of insecticide-treated bed nets, lost days of work with resulting loss of income and lost opportunities for joint economic ventures and tourism (WHO, 2017). Direct costs (for example, illness, treatment, premature death) have been estimated to be at least US$ 12 billion per year according to WHO report 2017. The cost in terms of lost economic growth is many times higher.

Malaria occurs mostly in poor tropical and subtropical areas of the world. Africa is the most affected due to the abundance of *Anopheles gambiae* which is responsible for high transmissions (De Mendonça, Goncalves, & Barral-Netto, 2012). Malaria has been a major source of concern in Sub-Saharan Africa, with an increasing mortality rate (WHO, 2014). In this region, efforts to eradicate malaria have clearly been unsuccessful and current efforts to control the disease focus on reducing attributable morbidity and mortality (WHO, 2014). The Roll Back Malaria program, — a global partnership established by the World Health Organization, the World Bank, the United Nations Development Programme and UNICEF — has been expanded to

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include national governments, non-governmental organisations, private sector groups and researchers to effectively combat malaria. The four main preventive and control components of the program include: improving access to effective treatment, preventing malaria during pregnancy, reducing mosquito–human contact by widespread use of insecticide-treated bed nets, and ensuring timely and appropriate action during malaria epidemics (WHO, 2008).

Pivotal among these preventive and control strategies is the use of antimalarial drugs either as a prophylactic agent or curative agent. However, the alarming rate at which malaria parasites develop resistance to most of the available and affordable anti-malarial drugs is a major concern that urgently requires the development of newer and more effective alternatives (Veiga, Ferreira Malmberg, Jörnhagen Bjorkman, Nosten, & Gil 2012).

Delimitation of the Study

The total number of mice under each treatment group was five and this was adequately powered to ascertain reliable results. Further, mice that were susceptible to *Plasmodium berghei* infection were used in this present study reducing the possibility of low infectivity or immune resistant of the mice against *Plasmodium berghei*. In addition to the direct investigation of antiplasmodial effect of *Calotropis procera*, the study further explored the effectiveness of the extract against different pathophysiological conditions induced by *Plasmodium berghei* infections including pyrexia.

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Limitation

Though the protocol followed was appropriate for the study due to time constraints other methods were not carried out to find out if they could also work effectively on the parasite.

Organisation of the Study

The study examines the efficacy and mechanism of action of *Calotropis procera* extract on *Plasmodium berghei* in mice. The study is organised into five chapters. The first chapter is an introduction to the study. It involves background to the study, statement of the problem, purpose of the study, hypothesis and the justification.

Chapter two focuses on the review of related literature. It comprises both the theoretical framework and empirical evidence of the study. It reviews literature on subtopics such as introduction and distribution of malaria, epidemiology of malaria in Ghana, determinants of malaria infections, and life cycle of malaria parasite, pathophysiology of malaria, antimalarial agents, and *Calotropis procera* plant.

Chapter three is about the materials and methods employed in the study. This includes the research design, population, sample and sampling procedure. It also looks at the design and administration of the instrument used for the study. The chapter also describes the procedure adopted in collecting data and how the data was analysed.

Chapter four provided the results for the study and further discussed the results in relation to existing relevant literature. Chapter five provided summary and conclusion the study. It also made some recommendations and future perspectives that can be considered.

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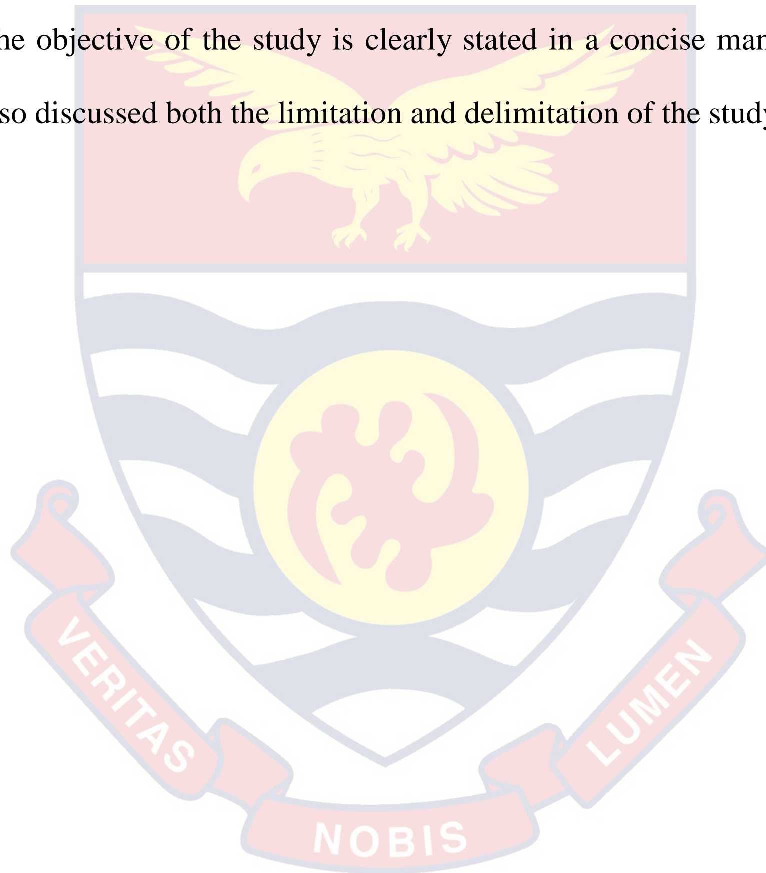
Chapter Summary

The chapter touched on the background to the study. The chapter further looked at the need for this research work since the *Plasmodium* parasite is building resistance to most of the refined drugs. A review of the literature revealed that there has been little research on this plant's ability to attack the *Plasmodium* parasite. It is for this reason that the study is aimed at the efficacy of *Calotropis procera* extracts on *Plasmodium berghei* in mice. The objective of the study is clearly stated in a concise manner. The chapter also discussed both the limitation and delimitation of the study.



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CHAPTER TWO

REVIEW OF RELATED LITERATURE

In this chapter, the study reviewed the occurrence of malaria, its prevalence and further considered some drugs to combat the menace. The chapter explored available literature on *Calotropis procera.* In a coda, the chapter presents the traditional use of *Calotropis procera,* therapeutic prospects and toxicity against organs of animals. It further discusses literatures on malaria: epidemiology, *Plasmodium* forms, determinants of malaria, and antiplasmodium compounds.

Calotropis procera Plant

Commonly referred to as 'sodom apple' or milkweed, *Calotropis procera* is a seed-producing shrub found mostly in the tropical regions especially West Africa (Alrheam, 2015). However, it can also be found in the Asia, Middle East including India, Pakistan, Afghanistan, Iran, Saudi Arabia, Jordan and Australia (Galal, Farahat, El-Midany, & Hassan, 2016; Lottermoser, 2011). The vernacular names given to *Calotropis procera* vary from one geographical location to the other. It is called crown flower (English), madar (Hindi), babadi or bamanbi-bauwami (senegal), arbre à soie (French), bawane (The Gambia), korunka (Mali), ganganpi or furo fogo (Ivory Coast), mapatu-asa (Ghana, Akan), wolaporhu (Ghana, Dagbani), unablapong (Ghana, Kokomba) (Burkill, 1985). It belongs to the family *Asclepiadaceae*.

Structurally, *Calotropis procera* is a shrub with height ranging from 2.5-6 m, possessing milking latex throughout. The stem is round and simple (rarely branched), pale green, thickly covered with rubbing hoary pubescence

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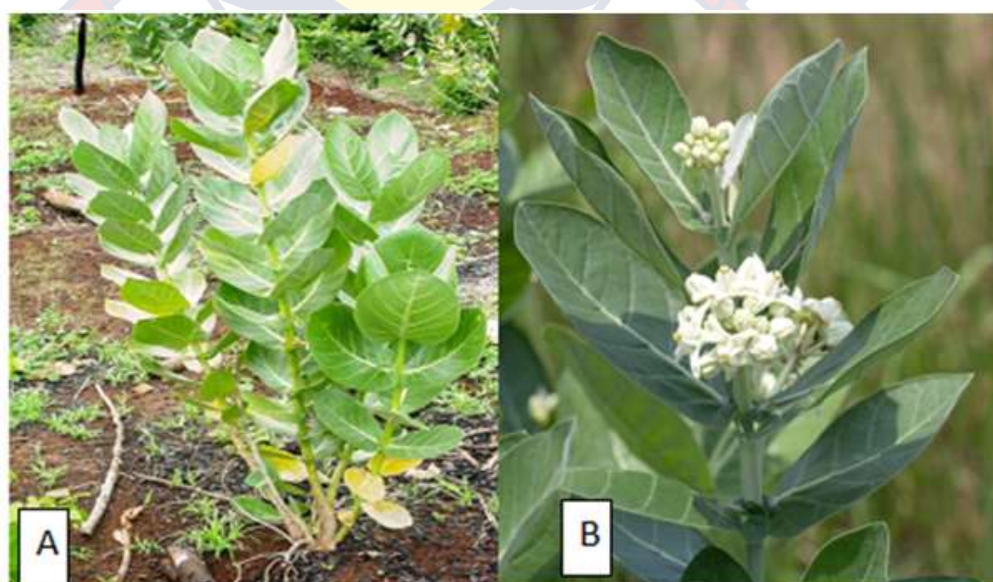
(Kumar & Roy, 2007). Leaves are decuss, obovate, 10-20 cm long and 4-10 cm thick, coriaceous and auriculate. Inflorescence of a thick, multiflowered, umbellate cyme arising from the nodes and axillary or terminal appearance. Corolla is slightly campanulate, with 5 sepals 4-5mm long; ovate, acute, rather concave, dull purple segments bordered on the upper side by white, silvery on the underside. Subglobose, ellipsoid or ovoid buds, recurved follicles, 7.5-10.0cm. Light-brown seeds, roughly ovate, flattened 3.2cm silky hairs. A white milky sap is exuded from any wound on the plant (Murti, Yogi, & Pathak, 2010). It is xerophytic perennial plant capable of growing in wide range of soil types and climatic conditions (Galal *et al*., 2016). The plant is considered antropogene and usually grows in villages or deserted villages. The young foliage is generally considered poisonous and therefore they are usually cut to wilt, making it safe to be grazed upon by goats and other herbivorous animals (Kerharo & Adam, 1964). The structure of *Calotropis procera* is shown in figure 1.



(A) = Aerial of *C. procera*, (B) = Flowering *Calotropis procera*

Figure 1: Structure of *Calotropis procera* growing from the soil
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Phytochemicals of *Calotropis procera*

Plants are rich sources of bioactive chemicals of medicinal importance. Many medicinal phytochemicals are isolated directly from plant. A typical example is the extraction of artemisinin from *Artemisia annua*. Similarly, *Calotropis procera* is deemed to possess phytochemicals of medicinal value. Studies indicate that, the phytochemical constituents differ from one part of the plant to the other although some constituents are common to all parts (Nigg, & Seigler, 2013; Tiwari, Brunton, & Brennan, 2013). The investigation carried out by Morsy, Al Sherif and Abdel-Rassol (2016), on a *Calotropis procera* in Egypt revealed the presence of sterols, triterpenes, cardiac glycosides and saponins in all parts of the plants: leave, flower, fruit, stem, and roots. However, they found the presence of glycosides and flavonoids only in the leaves and fruits whereas tannins were present in leaves, fruits and flowers. The presence of alkaloids: calotropin, catotoxin, calcinin and gigiantin are considered poisonous (Devasari, 1965). It has been shown that the presence of these phytochemicals vary according to the solvent used for the extraction (Javanmardi, Stushnoff, Locke, & Vivanco, 2003). Evidently, the presence of phenols, tannins, flavonoids, steroids, triterpens, saponins and alkaloids differed between latex extract of hexane, dichloromethane, ethyl acetate, butanol and water. Nonetheless, the influence of geographical location on phytochemical constituent of *Calotropis procera* remains largely unknown. Chemical studies of fresh and dry leaves have shown the presence of calotropenyl acetate, procesterol, and proceragenin, an antibacterial cardinoline (Yogi, Gupta, & Mishra, 2016). A chemometric analysis of root ethanolic extract revealed the presence of 10 chemotypesnamely: 9-

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octadecenoic acid (Z)-methylester, Alpha-D-glucopyranoside, methyl 2,3,4-Tris-O-(trimethylsilyl), hexadecanoate, hexadecanoic acid, ethyl ester, 9,12-octadecadienoic acid (Z,Z) and L-glutamic acid (8.10%) which were found to be major constituents whereas 2-hydroxyhexadecyl butanoate, ethyl (9Z,12Z) - 9,12-octadecadienoate, butane, 2,2-dimethyl and 1-dodecene were the other few constituents (Pattnaik, Kar, Chhatoi, Shahbazi, Ghosh, & Kuanar, 2017). Flavonoids are important group of polyphenols widely distributed among the plant. They are made of more than one benzene ring in its structure. There are numerous reports that support their use as antioxidants. Flavonoids play a variety of biological activities in plants, animals and bacteria. In plants, flavonoids have long been known to be synthesised in particular sites and are responsible for the colour and aroma of flowers, and in fruits to attract pollinators and consequently fruit dispersion to help in seed and spore germination, and the growth and development of seedlings (Iwashina, 2013). Flavonoids have been ascribed positive effects on human and animal health and the current interest is for disease therapy and chemoprevention. Kumar and Pandey (2013) reviewed the protective roles of flavonoids against human diseases as well as their functions in plants

Saponins possess ‘soap like’ behaviour in water, i.e. they produce foam. On hydrolysis, an aglycone is produced, which is called sapogenin (Hostettmann, & Marston, 1995). Saponins are extremely poisonous, as they cause heamolysis of blood and are known to cause cattle poisoning. They possess a bitter and acrid taste, besides causing irritation to mucous membranes (Kar, 2003). Saponins are also important therapeutically as they are shown to have hypolipidemic and anticancer activity, and also necessary

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for activity of cardiac glycosides (Netala, Ghosh, Bobbu, Anitha, & Tartte, 2014).

Tannins are phenolic compounds of high molecular weight. They are soluble in water and alcohol and have a characteristic feature to tan, i.e. to convert things into leather (Kar, 2003). They are acidic, used as antiseptic and also as healing agents in a number of diseases. They are used in the treatment of diseases like leucorrhoea, rhinnorhoea and diarrhea (Kar, 2003).

Terpenoids exhibit various important pharmacological activities such as anti-inflammatory, anticancer, anti-malarial, inhibition of cholesterol synthesis, anti-viral and antibacterial activities (Perry, Houghton, Theobald, Jenner, & Perry, 2000).

Steroids have been observed to promote nitrogen retention in osteoporosis and in animals with wasting illness (Maurya, Mohan, Sharma, & Srivastava, 2008).

Medicinal importance of *Calotropis procera*

Calotropis procera like many other shrubs has been traditionally explored for their medicinal properties. Its medicinal importance spans from the treatment of infectious diseases to non-infectious diseases.

Local administration of *Calotropis procera* induces severe inflammatory process characterised by oedema, increased infiltration of proinflammatory-associated immune cells and increased vascular permeability (Sehgal & Kumar, 2005). The presence of traces of histamine produced in local mast cells mediates the inflammatory process, hence the use of anti-histaminergic drug has the potential to reduce *Calotropis procera*-induced inflammation (Silva *et al.*, 2010). On the contrary, methanolic and aqueous

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extract has proven to possess anti-inflammatory properties capable of inhibiting inflammation induced by different inflammogens including histamine, serotonin, compound 48/80, bradykinin and prostaglandin E_2 (Arya & Kumar, 2005). In fact, it is shown that latex from *Calotropis procera* is more potent in inhibiting the infiltration of pro-inflammatory cells than phenylbutazone but comparable to chlorpheniramine in carrageenan-induced subcutaneous oedema. This phenomenon is deemed to be effected via the inhibition of local histamine and bradykinin, and partly prostaglandin (Soneera Arya & Vijay L Kumar, 2005; Silva et al., 2010). The anti-inflammatory property of *Calotropis procera* has been demonstrated as it reduced oedema induced by Freund's complete adjuvant in the paw of rats (Kumar & Roy, 2007).

In addition, Kumaar and Roy (2007) showed that, the plant is capable of reducing the tissue levels of TNF-α and nitric oxide remarkably. The ability of *Calotropis procera* to maintain the levels of antioxidants have been described in alloxan-induced diabetes rats (Roy, Sehgal, Padhy, & Kumar, 2005). The antioxidant property of *Calotropis procera* has been identified to be key in the reduction of beta-amyloid deposited in the brain hence posited to be useful in the treatment of Alzeimer’s disease.

Antimicrobial activity of *Calotropis procera* has been shown against both Gram-positive and Gram negative bacteria including *Escherichia coli*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Bacillus spp*., *Pseudomonas aeruginosa*, *Salmonella enteritidis*, and fungi including *Candida albicans*, *Candida tropicalis*, *Penicillium chrysogenum*, and *Saccharomyces cerevisiae* (Nenaah & Ahmed, 2011; Nenaah, 2013).

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Exploration for the bioactive antimicrobial phytochemical revealed that four flavonoid glycosides: 3-O-rutinosides of quercetin, kaempferol, and isorhamnetin, 5-hydroxy-3,7-dimethoxyflavone-4'-O-β-glucopyranoside were the most active antimicrobial compounds (Nenaah, 2013).

Toxic effect of *Calotropis procera* on organs

Charu and Trivedi (1997) have posited that the chopped leaves and latex of *Calotropis procera* show tremendous promise of nematicides *in vitro* and *in vivo* whereas a poultice of the plant leaves heal rheumatism when applied to joints. *C. procera* has also been found to contain secondary metabolites which are of no apparent importance to the plants own life but rather have prominent therapeutic as well as toxic effect on an animal's system (Mgbojikwe, 2004). The gross and histopathological examination on consumption of *C. procera* has revealed lesions. Exposure to the latex accidentally has been revealed to cause inflammation of both the skin and eyes (Shivkar & Kumar, 2003; Al-Mezaine, Al-Rajhi, Al-Assiri, Wagoner, 2005). Much is reported about the pharmacological and poisonous properties of the plant (Nsekuye, 1994; Basu, Sen, Pal, Mascolo, Capasso, Chaudhuri, & Nagchaudhuri, 1997). When *C. procera* is consumed fresh, it results in poisoning to the livestock as well as humans (Lewis & Elvin-Lewis, 1977). It was observed by Dada, Lamidi, Eghianruwa and Adepoju (2002), that animals that consumed fresh *C. procera* had fast and abnormal heartbeat, bradycardia and gasped for breathe which are indicative of some damage inflicted on the heart, and can be attributed to cardiac glycosides. Engorgement of blood vessels of the ears shows that there was circulatory disturbance (Jato, Jacob, Zainab, Okewole, Atiku, Shamaki, Umar, & Chudy, 2016). The lesions

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observed in the lungs, liver, brain and kidney grossly confirmed the clinical signs observed. Mahmoud, Adam and Tartour (1979), reported that animals that consumed *C. procera* were observed to have had catarrhal enteritis. Milky latex has strong bacteriolytic enzymes, toxic glycoside calactin, calotropin D1, calotropin D2, calotropin F11, and a nontoxic powerful proteolytic enzyme, and it has local anesthetic activity, according to studies (Samar, Arup, Ayan & Prashant, 2009). The latex's irritant and pro-inflammatory properties are well known (Alencar, Oliveira, Mesquita, Lima, Vale, Etchells, 2006).

Badshah, Farmanullah, Saljoqi and Shakur (2004), asserted that *C. procera* contain most alkaloids which are nitrogenous heterocyclic compounds have strong effects on the nervous system of animals and may result in death. De Lima, De Freitas, Amorim, Camara, Batista and Soto-Blanco (2011), also reported the plant as hepatotoxic and cardiotoxic. The renal toxicity and the hepatic toxicity of the plant is well documented by other researchers (Basak, Bhaumik, Mohanta & Singhal, 2009; Lin & Will, 2012).

Malhi and Tridedi (1972) described *Calotropis procera* as an abortifacient and an anti-fertility agent. Studies on the effects of the plant extract on the ultrastructure of the kidney as well as histology of the skin and reproductive organs of Wistar rats are reported (Al-Robai, Abo-khatwa, & Davish, 1993, Akinloye, Abatan, Alaka, Oke, 2001a).

It was revealed through phytochemical studies that *C. procera* contains a mixture of cardenolides, including proceragenin and 2”-oxovoruscharin (Akhtar, Malik, Ali, & Kazmi, 1992; Hanna, Shalaby, Morsy, Simon, Toth, Malik, & Duddeck, 2002; Van Quaquebeke et al., 2005). Cardenolides are

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cardiac-active compounds that inhibit the cellular membrane Na^+/K^+ ATPase, resulting in an electrolytic disturbance that affects the electrical conductivity of the heart (Joubert, 1989; Aslani, Movassachi, Mohri, Abbasian, Zarehpou, 2004; Poindexter, Feng, Dasgupta, Bick, 2007).

Studies which were conducted by Palwa and Chatterjee (1988), as well as Singhal and Kumar (2009), showed that animals that were treated with aqueous extracts of *C. procera* had lesions in the liver and kidneys after they had the animals sacrificed and these organs were harvested. Studies that was carried out by Buraimoh (2011), revealed that aqueous leaves extract of *C. procera* is detrimental to the integrity of the liver tissues as evident in the necrotic nature of liver based on the histological observation.

A study conducted by Akinloye et al. (2002), brought to light that the plant does not only have toxic effect on organs but also on the testes. In that, the extract of the plant has destructive effect on the germ cells which are actively dividing. It was reported that testosterone maintained the viability of spermatozoa (Bhargava, 1989).

Introduction to malaria

Malaria is a mosquito-borne disease which is caused by five protozoa and these are pathogenic to humans: *Plasmodium falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi* in humans. *Plasmodium vivax* is the most progressive of the human malarias, reaching historical latitudinal extremes of 64° north and 32° south (Snow, 2015). Four of these including *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale* are human malaria species that are spread from one person to another through the bite of female mosquitoes that belong to the genus Anopheles.

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There are about 400 different species of Anopheles mosquitoes, but only 30 of these are vectors of major importance in studies (Sinka, Bangs, Manguin, Rubio-Palis, Chareonviriyaphap, Coetzee, Mbogo, Hemingway, Patil, & Temperley, 2012). Recent studies have shown that malaria is caused by *P. knowlesi* in humans (Fornace, Abidin, & Alexander, 2016). This species was originally known to cause malaria in monkeys in certain forest areas in the South-East Asia. It was believed that *P. knowlesi* malaria could not spread from person to person, but rather occurs in people when an Anopheles mosquito infected by a monkey bites and infect humans (zoonotic transmission) (Bronner, Divis, Färnert, & Singh, 2009). They were known to typically infect pig-tailed and long-tailed macaques (Singh et al., 2004). Nonetheless, this perception changed with the emergence of molecular techniques to distinguish between species (Singh et al., 2004). In 2004, the first case of *P. knowlesi* in humans was reported in Sarawak, Malaysia Borneo (Singh et al., 2004). Since then there have been increasing reported cases of *P. knowlesi* mainly in Asia and as a result, *P. knowlesi* is now recognised as the fifth *Plasmodium* species causing malaria (Singh & Daneshvar, 2013).

P. falciparum and *P. vivax* malaria pose the greatest public health challenge (WHO, 2019). *P. falciparum* is most prevalent on the African continent, and it is recognised as the causative agent for malignant malaria worldwide (Akhwale, Lum, Kaneko, Eto, Obonyo, Björkman, & Kobayakawa, 2004; Westenberger, McClean, Chattopadhyay, Dharia, Carlton, Barnwell, Collins, Hoffman, Zhou, Vinetz, & Winzeler, 2010). *P. vivax* has a wider geographical distribution than *P. falciparum* because it can develop in the Anopheles mosquito vector at lower temperatures, and can survive at higher

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altitudes and in cooler climates (Dayananda, Achur, & Gowda, 2018). It also has a dormant liver stage (known as a hypnozoite) that can activate months after an initial infection, causing a relapse of symptoms. The dormant stage enables *P. vivax* to survive for long periods when Anopheles mosquitoes are not present (e.g. during winter months) (Dayananda, et al., 2018). Although *P. vivax* can be found in Africa, the risk of infection with this species is quite low due to the absence of the Duffy gene in many African populations, which produces a protein required for *P. vivax* to invade red blood cells (WHO, 2015). In many areas outside Africa, infections due to *P. vivax* are more common than those due to *P. falciparum*, and cause substantial morbidity (WHO, 2015).

Distribution of malaria

The species *Plasmodium falciparum*, *P. malariae*, and *P. ovale*, are all present in tropical Africa except *P. vivax*, which are found in both tropical and temperate regions of the world. It is believed *P. vivax* had been introduced from Europe and Africa to the New World during the seventeenth century (Dugacki, 2005). The *P. vivax* and *P. ovale* distributions rarely overlap. Figure 2, shows the global malaria distribution of *P. falciparum* and *P.vivax* of which the continent most affected is Africa. In the African region where the majority of malaria cases and deaths occur, 74% of the population live in endemic areas and 19% in epidemic prone areas (Centre for Disease Control and Prevention, 2011). Just 7% of the population of the country live in low-risk or malaria-free areas as shown in the figure 2 below.

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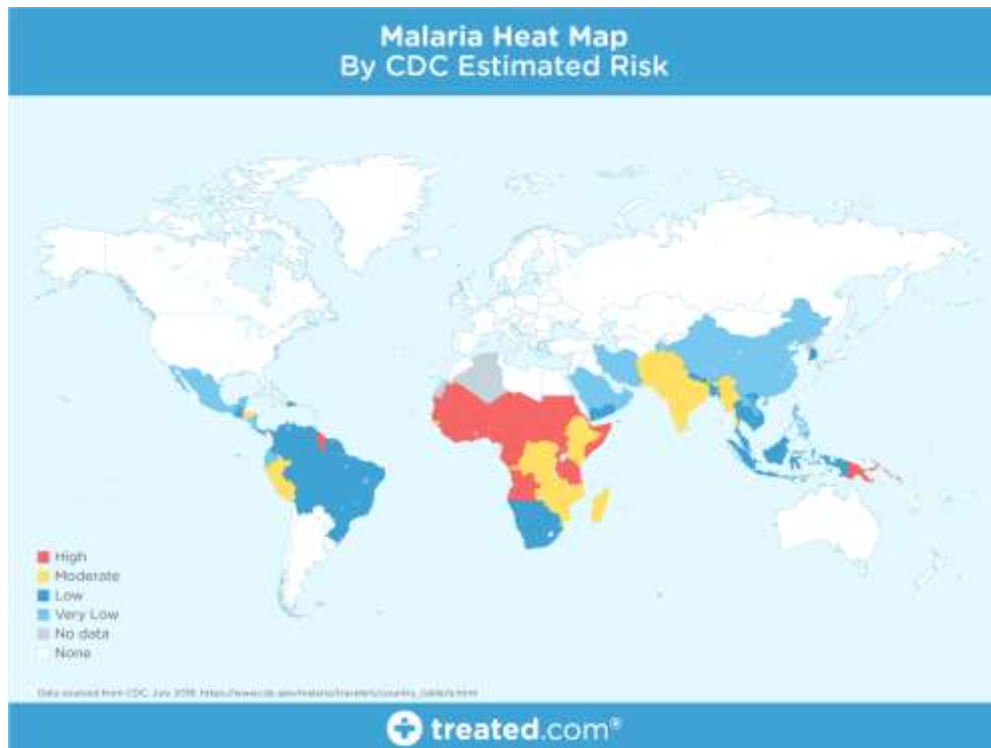

Figure 2: World Distribution of Malaria
Source: Centre for Disease Control and Prevention, 2011, U.S.A

To understand the dynamics of malaria infections, it is first important to take a comprehensive look at the malaria from global perspective. The global threat of malaria has existed for a very long time. Its persistence from the time of its first description as found in ancient Chinese medical records of 2700 BC (White, Pukrittayakamee, Hien, Faiz, Mokuolu, & Dondorp, 2014) till today attests to the resilience of the etiological agent, pathogenicity and the efficiency of its transmission. The robust resistance mechanisms exhibited by *Plasmodium* parasites have helped the parasite to survive a number of eradicating programs. Persistence of *Plasmodium* parasites has resulted in its spread to over 90 countries worldwide (WHO, 2016b).

As shown in figure 3 below, there is heterogeneity of malaria infection across difference regions and countries (WHO, 2018b). Strikingly, the burden of malaria infection is skewed to certain regions. For instance, in the recent

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malaria report by WHO released in December 2019, the Sub-Saharan region disproportionately represented a 93% of global malaria cases and 94% malaria death (WHO, 2020). The countries most affected by malaria included Nigeria, Democratic Republic of Congo, Uganda, Cote d'lvoire, Mozambique and Niger accounting for about halve of malaria cases globally (WHO, 2020). Previous annual documentation released in 2018 showed Nigeria, Democratic Republic of Congo, Mozambique, India and Uganda as the top five most malaria-endemic countries (figure 4). This therefore requires the institution of a robust, preventive and eradicating strategy in the sub-Saharan region.

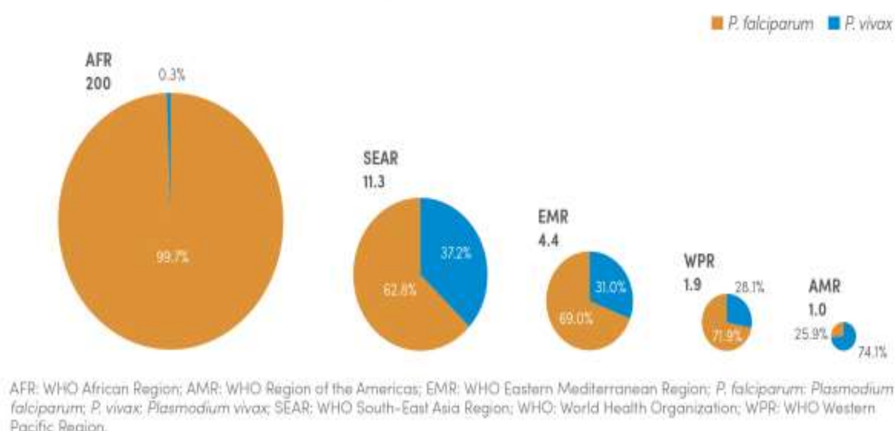

Figure 3: Global estimate of malaria cases by WHO, 2017

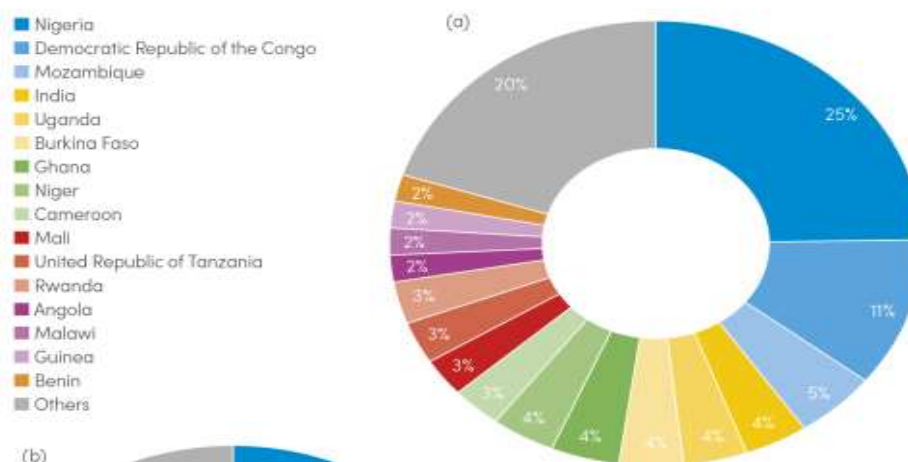

Figure 4: Estimated country share of total malaria cases
Source: WHO, 2017

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For the past decade or two, malaria incidence and mortality appears to be on an intermittent decline especially in the most endemic region of the world, including Sub-Saharan Africa (Meyrowitsch et al., 2011). Available data suggest that global malaria incidence reduced by 18% from 2010-2015 (WHO, 2016b). Thus, from the year 2010, malaria cases reduced from 262 million to 214 million cases in 2015 (Diagana, 2015). Similar observation was made in a 2020 report by the World Health Organisation (WHO, 2020). In their report, malaria case fell from 231 million cases in 2017 to 228 million in 2018. A corresponding malaria death of 405,000 was recorded for the year 2018 as opposed to 416,000 deaths in 2017. However, emerging studies have posited possible consequences that may accompany the observed decline in malaria transmission. One mathematical modelling study suggested that the decline in malaria transmission may result in a paradigm shift of global malaria epidemiology, a shift in population at risk (Bouyou-Akotet, Offouga, Mawili-Mboumba, Essola, Madoungou, & Kombila, 2014). Another study also predicted a shift in global burden from younger to older children (Carneiro, Roca-Feltrer, Griffin, Smith, Tanner, Schellenberg and Schellenberg, 2010). Although the global decline of malaria transmission is desirable, there is the need for stakeholders to anticipate and prepare adequately for the predictions made by these studies.

Over the last decade, there have been increased efforts towards the fight against malaria. Investments have been made to widen the coverage of malaria control programs worldwide. The World Health Organisation instituted the African Initiative for Malaria Control program (AIM) in African countries including Harare (Zimbabwe) and Burkina Faso aimed at increasing

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the awareness of the existing control strategies and promoting their implementation (Snow, Trape, & Marsh, 2001). More recently, the "Global Technical Strategy for Malaria 2016–2030" program which places emphasis on the need for universal malaria elimination was introduced to further strengthen the global course of fighting malaria. As a result, most endemic countries are undergoing reduction in malaria transmission and its associated morbidities and mortalities (WHO, 2016a). For instance, in malaria endemic countries where malaria control strategies have been rolled-out, studies have shown a remarkable reduction of malaria in pregnant women, (Mulamba & Mash, 2010), and the morbidities and mortality of malaria among vulnerable children (Hemingway, Shretta, Wells, Bell, Djimdé, Achee, & Qi, 2016). Evidently, Clark and his colleagues (2017), showed a positive association between the knowledge of malaria intervention strategies and cognitive function among school children.

The deployment of varied preventing and treatment strategies against malaria only point to the complexity and plasticity of the etiological agent (WHO, 2014). In fact, the control and preventing strategies is not only informed by the biology of the etiological agent but also the biology of its vector (Gachelin, Garner, Ferroni, Troehler, Chalmers, 2017). Malaria preventing and treatment tools include but not limited to long-lasting insecticide nets, indoor residual spraying, rapid diagnostic testing, artemisinin-based combination therapy and vaccine.

Epidemiology of malaria in Ghana

Ghana which is part of the Sub-Saharan region is malaria-endemic country. This means that there are approximately 30 million inhabitants who

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are all at risk of contracting malaria (National Malaria Control Program, 2013). Ghana together with Nigeria reported the highest increase in malaria cases from 2017-2018, although previous evidence indicates a global decline of malaria infection in the last decade (Fançony, et al., 2012; WHO, 2019). Nonetheless, Ghana alone recorded just a slight decrease in total reported malaria cases from 2016-2017. However, there appeared to be a steady increase in malaria cases from 2002-2016 (as shown in figure 5). In 2017, the total malaria cases reported in the health facilities, community level and private sector in Ghana stood at 4,348 694; 1,235 491; 1,391,725 respectively. Overall estimated cases and death stood at 7,800,000 (5,000,000–11,900,000) and 10,900 (10,100-11,800) respectively (WHO, 2017).

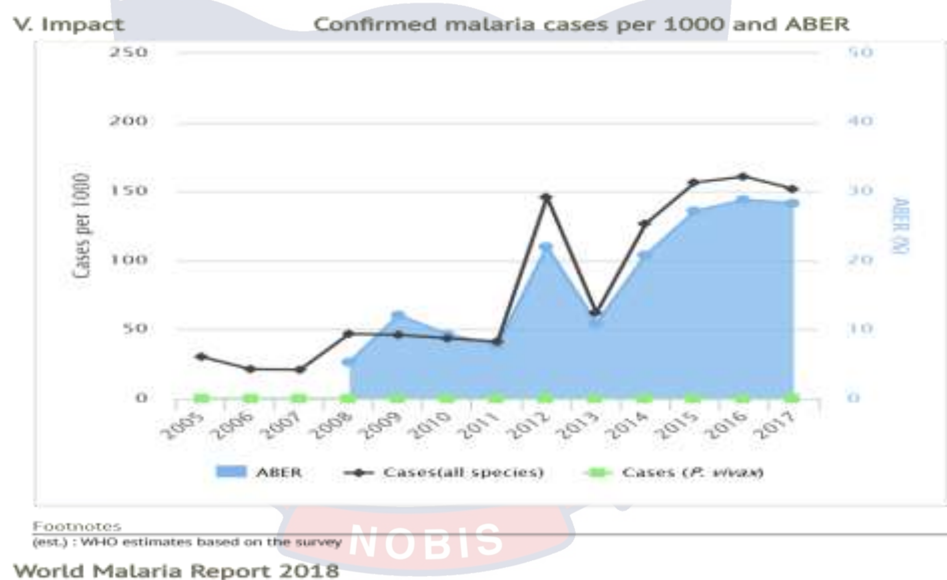

Figure 5: Confirmed cases from 2005-2017, Ghana
Source: WHO, 2018

Although the number malaria admission in Ghana seemed to steadily increase from 2005-2017, there appeared to be corresponding decrease in malaria mortality in the same period (figure 6)

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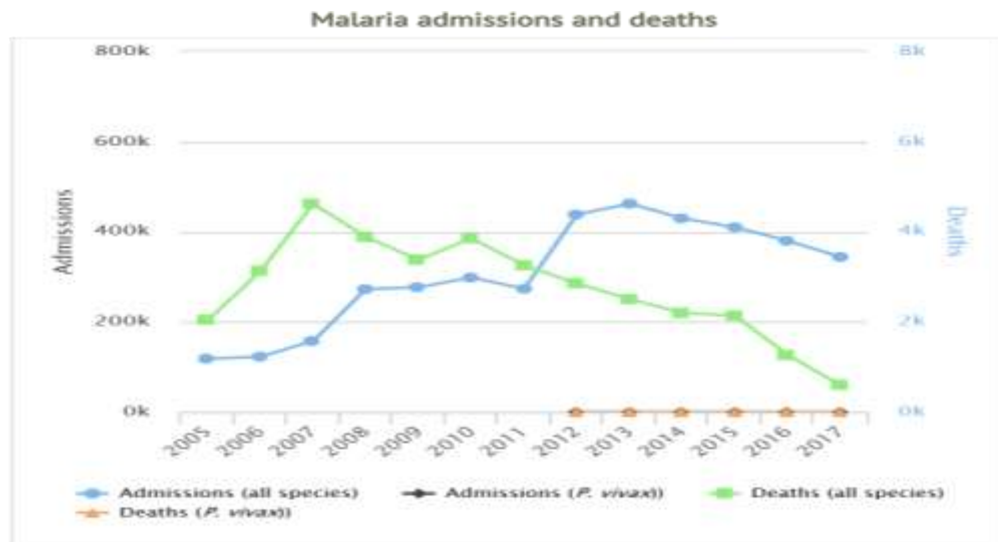

Figure 6: Malaria admissions and death in Ghana, 2005-2017
Source: WHO, 2018

Ghana adopts World Health Organization intervention strategies including the use of insecticide, chemoprophylaxis, improvement of drainage system, education, use of insecticide treated net, larval control and intermittent preventive treatment in pregnant women with sulfadoxine-pyrimethamine. Evidently, the implementation of the intermittent preventive treatment of malaria in pregnant women have been shown to have significantly reduced the development of placental malaria (Hommerich, Von Oertzen, Bedu-Addo, Holmberg, Acquah, Eggelte, & Mockenhaupt, 2007). Local and international collaborations have been key to the observed decline in malaria transmission in Ghana. For instance, in the past years, the Roll Back Malaria initiative by WHO in partnership with Ghana's national malaria control programs has worked together to facilitate the eradication of malaria since the African malaria summit in Abuja. As a result, most people in Ghana appear to be aware of the available malaria preventing strategies. In fact, it has been

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reported that, school children in Ghana have good understanding of the control strategies against malaria (Diema Konlan, Amu, Konlan, & Japiong, 2019).

Epidemiological profile of malaria reveals high transmission rates particularly in the western parts of Ghana (Figure 7). The eastern borders of Ghana appear to have relatively low transmission rates.

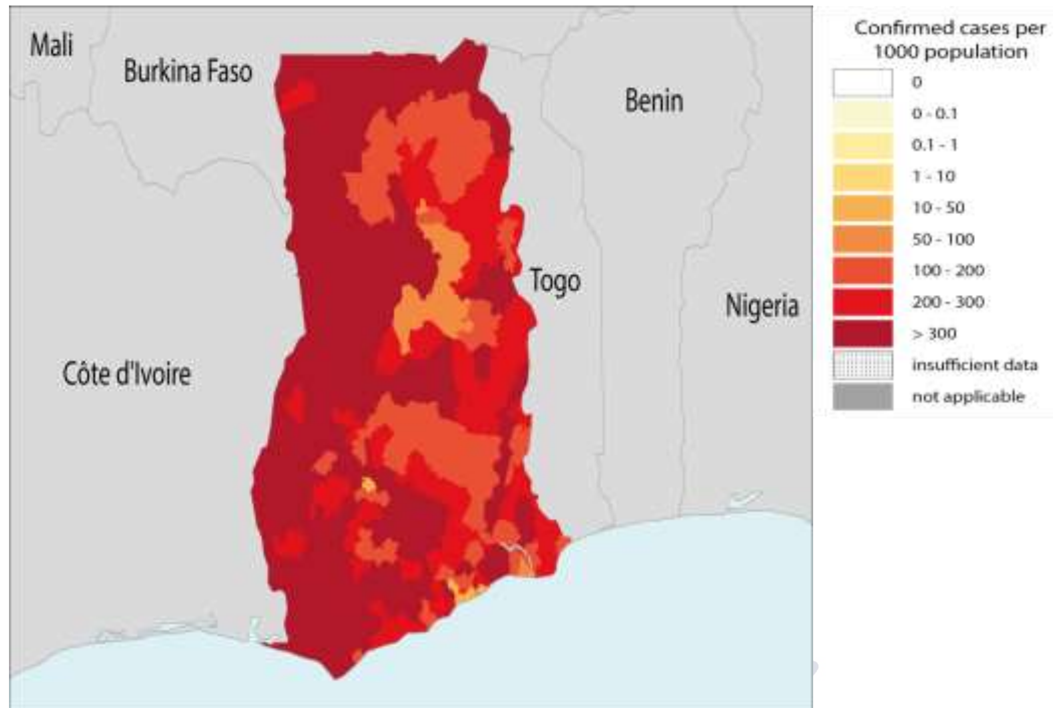

Figure 7: Epidemiological profile of Malaria Transmission in Ghana, 2017
Source: WHO, 2018

So far, of the five *Plasmodium* species known to infect humans, *Plasmodium falciparum, Plasmodium malariae and Plasmodium vivax* have been reported in Ghana. Of the three species reported in Ghana, *Plasmodium falciparum* appear to be the dominant species representing 90%-98% of all infections whereas *Plasmodium malariae* and *Plasmodium vivax* represent 3%-9% and 1% respectively (Owusu, Brown, Grobusch, & Mens, 2017). According to the country's malaria profile in 2017, *Plasmodium falciparum* was responsible for 100% of malaria cases in Ghana (WHO, 2017). This

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species is primarily transmitted by *Anopheles gambiae, Anopheles funestus, and Anopheles arabiensis* (WHO, 2017).

Determinants of malaria infection

The determinant and distribution of malaria is dependent on the epidemiological setting (WHO, 2017). The burden of malaria is relative higher in developing countries. For instance, children living in hyperendemic area are somewhat prone to 4-6 weeks repeated infections of *P. falciparum* (Rono, Färnert, Murungi, Ojal, Kamuyu, Guleid, & Olotu, 2015). The determinant of malaria can be placed under three main levels: individual level, household and community level and environmental and institutional level (Lawpoolsri, Sattabongkot, Sirichaisinthop, Cui, Kiattibutr, Rachaphaew, & Kaewkungwal, 2019). Individual level determinants include demographics, genetic makeup, immunological status and also the pathophysiological mechanism of the *Plasmodium* host. The second level of malaria determinants, household community level, includes social factors, economic factors, occupational factors and healthcare seeking behaviour. The third level, environmental and institutional level includes climate, geographical location, political factors and legal conflicts resulting in migration and displacement (Tusting, et al., 2013).

Individual level determinants and malaria infections

Gender is considered one of the individual level determinants of malaria infections. However, evidence from literature indicates paradoxical reports of malaria infections associated with gender (WHO, 2019). Studies conducted in Malaysia , Indonesia, Ethiopia and Rwanda showed higher levels of malaria infection in males than to females (Agegnehu, Shimeka, Berihun, & Tamir, 2018; Hasyim, Dale, Groneberg, Kuch, & Müller, 2019; Jusoh & Shah,

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2007; Kateera, Ingabire, Hakizimana, Rulisa, Karinda, Grobusch, & Mens, 2015). Other reports, on the other hand, claim that females are at a higher risk of malaria infection than males. (Kateera et al., 2015). For instance, a study carried out in Kisumu county, Kenya, revealed a 50% increase risk of malaria infection in females than to males (Jenkins,Omollo, Ongecha, Sifuna, Othieno, Ongeri, & Ogutu, 2015). A study conducted by Diiro and colleagues (2016) in a rural community showed that females are less knowledgeable about malaria transmission than males. As such females are less likely to adopt malaria preventive or control strategies making them more prone to malaria infection (Diiro et al., 2016). Perhaps this finding could be different in the urban areas as both males and females have equal chance of acquiring knowledge (WHO, 2007). A different study has explained this using the difference in sickle cell status between males and females. Veiga and his colleagues (2012) hypothesized that the lower level of Hb S sickle cell status in females than in males could explain the higher parasitaemia in females.

In malaria hyperendemic areas, studies have established that malaria infections decline with increasing age, indicating that acquired immunity is achieved with increasing age (Doolan, et al., 2009). Agegnehu et al. (2018), showed that malaria is widespread among people aged above age 15 than those below 15 years. Similar findings have been documented by Yukich, Taylor, Eisele, Reithinger, Nauhassenay, Berhane, and Keating, (2013). Contrary to these findings, majority of findings indicate that people below the age of 5 are the most vulnerable group to malaria infection (Mugwagwa, et al, 2015; Mutsigiri-Murewanhema, et al., 2017; Roberts et al., 2016; Tanner et al., 2008; WHO, 2018a).

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Malaria has been associated with genes selective pressure and other genetic disorders including thalassemia, glucose-6-phosphate dehydrogenase (G6PD) deficiency and genetic anemia in human host (Mackinnon, Mwangi, Snow, Marsh, & Williams, 2005). In addition, hereditary disorders such as spherocytosis, elliptocytosis, ovalocytosis, pyropoikilocytosis and acanthosis are also deemed as factors associated with resistance against malaria infection (De Mendonça, Goncalves, & Barral-Netto, 2012). Furthermore, the absence of the Duffy antigen correlates with protection against malaria infection caused by *P. vivax* (Maestre,Muskus, Duque, Agudelo, Liu, Takagi, & Hoffman, 2010). Details on the mechanism of resistance employed by these genetic disorders can be seen in the review article by Maestra et al. (2010).

Immune response to *Plasmodium* involves both the innate and adaptive immune system. The innate immunity is first activated followed by the adaptive immune system which includes T-cell and B-cell responses. Immune response to *Plasmodium* parasite culminates into a complex interplay of immune cells and their secretions including cytokines and chemokines (Zheng, Tan, & Xu, 2014). A number of studies have provided evidence suggesting that repeated malaria infection is associated with modified immune response in human host (Bediako, et al., 2019). Initial exposure to plasmodium species induces the development of partial protection or non-sterile immunity (Doolan, et al., 2009). Continuous exposure results in the gradual development of stronger immunity against the *Plasmodium* parasite. Also, there are evidences showing differential pathophysiology of malaria between immunocompetent and immunocompromised patients (Zheng, 2014). HIV infection has been described to increase the risk of severe malaria attacks

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thereby increasing malaria transmission (Alemu, et al., 2013; Laufer, et al., 2006). In addition, a selected case of severe malaria infection has been reported in patients receiving immunosuppressive treatment such as corticosteroids (Andriyani, 2018).

Household and community level determinants and malaria infection

At the household level, factors including the choice of building, use of long-lasting insecticide net, indoor residual spraying have been found to influence the transmission level (WHO, 2014). A study in Equatorial Guinea revealed a significant association between the use of insecticide-treated net and indoor residual spraying and the risk of malaria infection (Guerra, de Sousa, Ndong-Mabale, Berzosa, & Arez, 2018). In the study, reduced use of long-lasting insecticide net and indoor residual spraying correlated with high risk of malaria infection. Improvement on the quality of building like screening of windows and nets, improving roofing and housing design are important to the preventing of malaria infection. This is essential especially in areas where the inhabitants are exposed to *Plasmodium* vector, female Anopheles mosquito (WHO, 2017).

Malaria has long been referred to as a disease of poverty. This suggests that economic factors are key determinants of the transmission of malaria. It is purported that more than 50% of malaria-associated mortalities occurs among the populace from the poorest part of the world (Gwatkin & Guillot, 2000). Socio-economic factors do not only serve as distribution determinants of malaria but may also provide alternative ways to tackle malaria (WHO, 2017). This is because economic hardships including high cost of malaria treatment hinder patients from seeking proper treatment. In addition, poor nutritional

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status as a result of poverty is known to be associated with greater malaria morbidity and mortality in malaria endemic areas (Friedman, Kwena, Mirel, Kariuki, Terlouw, Phillips-Howard, & Ter Kuile, 2005; Mockenhaupt, Ehrhardt, Burkhardt, Bosomtwe, Laryea, Anemana, & Gellert, 2004). Nonetheless there are other paradoxical reports from other studies suggesting no association between malaria and under-nutrition (Snow, Byass, Shenton, & Greenwood, 1991). Improving the socio-economic status will increase better access to healthcare, long lasting insecticide treated nets, housing quality and food security thereby reducing the risk of malaria (Tusting, Willey, Lucas, Thompson, Kafy, Smith, & Lindsay, 2013).

In addition, the healthcare seeking behaviour of an individual is deemed an important factor to malaria infection and its treatment at the household and community level (WHO, 2014). For instance, the findings of a recent study conducted in Indonesia suggested that participants who were unaware of available health facilities were 4.2 more times likely to have malaria infection than participants who were aware of the availability of health facilities (Hasyim et al., 2019). Similar documentations have been made by Chinweuba, Agbapuonwu, Onyiapat, Israel, Ilo, and Arinze, (2017); Gerald, (2015).

Environmental and Institutional level determinant and malaria infection

One of the key determinants of malaria transmission is environmental factors. Unlike the other levels of determinant, most of the environmental determinants including climatological and hydrological factors are difficult to control. There has been a longstanding association of malaria transmission and factors such as humidity, rainfall and temperature (Craig, Kleinschmidt,

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Nawn, Sueur, & Sharp, 2004). Evidence from existing literature has established that, high temperatures and heavy rainfall are key determinant of high malaria burden especially in hyperendemic areas (Chitunhu & Musenge, 2015) although mathematical models do not always agree (Lunde, Bayoh, & Lindtjørn, 2013). A mathematical model study in China concluded that, high temperature fluctuations in cooler regions increases malaria incidence whereas high temperature fluctuations in warmer regions decreases malaria incidence (Zhao, Chen, Feng, Li, & Zhou, 2014). Empirical evidence from laboratory studies indicates that fluctuations in temperature can affect both the mosquito and parasite (Lyons, Coetzee, & Chown, 2013; Paaijmans, Blanford, Bell, Blanford, Read, & Thomas, 2010).

In addition, high mean temperatures rather than temperature variations have also been reported to increase malaria transmission (Craig, Snow, & le Sueur, 1999; Rogers & Randolph, 2000). Available evidence suggests that each 1°C increase in temperature correlates with a 0.9% increase in malaria cases. Also, a 1% increase in humidity was shown to correspond to approximately 4.0% increase in malaria cases (Li, Yang & Wang, 2013). Similar to temperature, increasing humidity is also deemed to affect both the mosquito and *Plasmodium* parasite (Kotepui & Kotepui, 2018).

Life cycle of the malaria parasite

The malaria parasite has a complex, multistage life cycle occurring within two living organisms, the invertebrate and the vertebrate hosts. The survival and development of the parasite within the invertebrate and vertebrate hosts, in intracellular and extracellular environments, is made possible by a toolkit of more than 5,000 genes and their specialised proteins that help the

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parasite to invade and grow within multiple cell types and to evade host immune responses (Greenwood, Fidock, Kyle, Kappe, Alonso, Collins, & Duffy, 2008). The parasite passes through several stages of development such as the sporozoites, merozoites, trophozoites, and gametocytes (sexual stages) and all these stages have their own unique shapes, structures and protein complements. The surface proteins and metabolic pathways keep changing during these different stages that help the parasite to evade the immune clearance, while also creating problems for the development of drugs and vaccines (Florens, Washburn, & Raine, 2002).

Mosquitoes are the definitive hosts for the malaria parasites, wherein the sexual phase of the parasite's life cycle occurs. The sexual phase is called sporogony and results in the development of innumerable infecting forms of the parasite within the mosquito that induce disease in the human host following their injection with the mosquito bite (Adams, Brown, & Turner, 2002).

When the female Anopheles draws a blood meal from an individual infected with malaria, the male and female gametocytes of the parasite find their way into the gut of the mosquito. The molecular and cellular changes in the gametocytes help the parasite to quickly adjust to the insect host from the warm-blooded human host and then to initiate the sporogonic cycle (Adams, et al., 2002). The male and female gametes fuse in the mosquito gut to form zygotes, which subsequently develop into actively moving ookinetes that burrow into the mosquito midgut wall to develop into oocysts. Growth and division of each oocyst produces thousands of active haploid forms called sporozoites. After the sporogonic phase of 8–15 days, the oocyst bursts and

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releases sporozoites into the body cavity of the mosquito, from where they travel to and invade the mosquito salivary glands. When the mosquito thus loaded with sporozoites takes another blood meal, the sporozoites get injected from its salivary glands into the human bloodstream, causing malaria infection in the human host (Florens, et al., 2002). It has been found that the infected mosquito and the parasite mutually benefit each other and thereby promote transmission of the infection. The *Plasmodium*-infected mosquitoes have a better survival and show an increased rate of blood-feeding, particularly from an infected host (Ferguson, & Read, 2004).

Man is the intermediate host for the parasite, wherein the asexual phase of the life cycle occurs. The sporozoites inoculated by the infested mosquito initiate this phase of the cycle from the liver, and the latter part continues within the red blood cells, which results in the various clinical manifestations of the disease (Centre for Disease Control and Prevention, 2011).

With the mosquito bite, tens to a few hundred invasive sporozoites are introduced into the skin. Following the intradermal deposition, some sporozoites are destroyed by the local macrophages, some enter the lymphatics, and some others find a blood vessel (Vaughan, Aly, & Kappe, 2008). The sporozoites that enter a lymphatic vessel reach the draining lymph node wherein some of the sporozoites partially develop into exoerythrocytic stages (Vaughan, et al, 2008) and may also prime the T cells to mount a protective immune response (Good & Doolan, 2007).

The sporozoites that find a blood vessel reach the liver within a few hours. It has recently been shown that the sporozoites travel by a continuous sequence of stick-and-slip motility, using the thrombospondin-related

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anonymous protein (TRAP) family and an actin–myosin motor (Baum, Richard, & Heale, 2006). The sporozoites then pass through the liver sinusoids, and migrate into a few hepatocytes, and then multiply and grow within parasitophorous vacuoles. Each sporozoite develops into a schizont containing 10,000–30,000 merozoites or more in case of *P. falciparum* (Kebaier, Voza, & Vanderberg, 2009). The growth and development of the parasite in the liver cells is facilitated by a favourable environment created by the circumsporozoite protein of the parasite (Singh, Buscaglia, & Wang, 2007). The entire pre-eryhrocytic phase lasts about 5–16 days depending on the parasite species, on an average 5-6 days for *P. falciparum*, 8 days for *P. vivax*, 9 days for *P. ovale*, 13 days for *P. malariae* and 8-9 days for *P. knowlesi* (Collins & Jeffery, 2007).

The merozoites that develop within the hepatocyte are contained inside the host cell-derived vesicles called merosomes that exit the liver intact, thereby protecting the merozoites from phagocytosis by Kupffer cells. These merozoites are eventually released into the blood stream in the lung capillaries and initiate the blood stage also known as erythrocytic stage of infection thereon (Vaughan et al., 2008).

In *P. vivax* and *P. ovale* malaria, some of the sporozoites may remain dormant for months within the liver termed as hypnozoites (Wells, Burrows, & Baird, 2010). These forms develop into schizonts after some latent period, usually of a few weeks to months. It has been suggested that these late developing hypnozoites are genotypically different from the sporozoites that cause acute infection soon after the inoculation by a mosquito bite (Cogswell,

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1992) and in many patients cause relapses of the clinical infection after weeks to months.

Red blood cells are the 'centre stage' for the asexual development of the malaria parasite. Within the red cells, repeated cycles of parasitic development occur with precise periodicity, and at the end of each cycle, hundreds of fresh daughter parasites are released that invade more number of red cells (Cogswell, 1992). The merozoites released from the liver recognise, attach, and enter the red blood cells (RBCs) by multiple receptor-ligand interactions in as little as 60 seconds. This quick disappearance from the circulation into the red cells minimises the exposure of the antigens on the surface of the parasite, thereby protecting these parasite forms from the host immune response (Cowman, & Crabb, 2006). The invasion of the merozoites into the red cells is facilitated by molecular interactions between distinct ligands on the merozoite and host receptors on the erythrocyte membrane (Wells et al., 2010). *P. vivax* invades only Duffy blood group-positive red cells, using the Duffy-binding protein and the reticulocyte homology protein, found mostly on the reticulocytes (Wells et al., 2010). The more virulent *P. falciparum* uses several different receptor families and alternate invasion pathways that are highly redundant. Varieties of Duffy binding-like (DBL) homologous proteins and the reticulocyte binding-like homologous proteins of *P. falciparum* recognize different RBC receptors other than the Duffy blood group or the reticulocyte receptors (Vaughan, et al., 2008). Such redundancy is helped by the fact that *P. falciparum* has four Duffy binding-like erythrocyte-binding protein genes, in comparison to only one gene in the DBL-EBP family

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as in the case of *P. vivax*, allowing *P. falciparum* to invade any red cell (Mayera, Cofiea, Jiangb, Hartlc, Tracya, Kabatd, Mendozaa, & Millera, 2009).

The process of attachment, invasion, and establishment of the merozoite into the red cell is made possible by the specialised apical secretory organelles of the merozoite, called the micronemes, rhoptries, and dense granules (Mayera, et al., 2009). The initial interaction between the parasite and the red cell stimulates a rapid "wave" of deformation across the red cell membrane, leading to the formation of a stable parasite–host cell junction (Cowman et al., 2006). Following this, the parasite pushes its way through the erythrocyte bilayer with the help of the actin–myosin motor, proteins of the thrombospondin-related anonymous protein family (TRAP) and aldolase, and creates a parasitophorous vacuole to seal itself from the host-cell cytoplasm, thus creating a hospitable environment for its development within the red cell. At this stage, the parasite appears as an intracellular "ring" (Cowman et al, 2006).

Within the red cells, the parasite numbers expand rapidly with a sustained cycling of the parasite population. Even though the red cells provide some immunological advantage to the growing parasite, the lack of standard biosynthetic pathways and intracellular organelles in the red cells tend to create obstacles for the fast-growing intracellular parasites (Zheng et al., 2014). These impediments are overcomed by the growing ring stages by several mechanisms: restriction of the nutrient to the abundant haemoglobin, by dramatic expansion of the surface area through the formation of a tubovesicular network, and by export of a range of remodeling and virulence factors into the red cell (Silvie, Mota, Matuschewski, & Prudêncio, 2008).

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Haemoglobin from the red cell, the principal nutrient for the growing parasite, is ingested into a food vacuole and degraded. The amino acids thus made available are used for protein biosynthesis and the remaining toxic heme is detoxified by heme polymerase and sequestrated as haemozoin (malaria pigment). The parasite depends on anaerobic glycolysis for energy, utilising enzymes such as pLDH, *Plasmodium* aldolase etc. As the parasite grows and multiplies within the red cell, the membrane permeability and cytosolic composition of the host cell is modified. These new permeation pathways induced by the parasite in the host cell membrane help not only in the uptake of solutes from the extracellular medium but also in the disposal of metabolic wastes, and in the origin and maintenance of electrochemical ion gradients (Wells et al., 2010). At the same time, the premature haemolysis of the highly permeabilised infected red cell is prevented by the excessive ingestion, digestion, and detoxification of the host cell haemoglobin. It is then discharged out of the infected RBCs through the new permeation pathways, thereby preserving the osmotic stability of the infected red cells (Lew, Tiffert, & Ginsburg, 2003).

The erythrocytic cycle occurs every 24 hours in case of *P. knowlesi*, 48 h in cases of *P. falciparum, P. vivax* and *P. ovale* and 72 h in case of *P. malariae*. During each cycle, each merozoite grows and divides within the vacuole into 8–32 (average 10) fresh merozoites, through the stages of ring, trophozoite, and schizont (Lew et al., 2003). At the end of the cycle, the infected red cells rupture, releasing the new merozoites that in turn infect more RBCs. With sunbridled growth, the parasite numbers can rise rapidly to levels as high as 10^{13} per host (Greenwood et al., 2008).

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A small proportion of asexual parasites do not undergo schizogony but differentiate into the sexual stage gametocytes. These male or female gametocytes are extracellular and nonpathogenic and help in transmission of the infection to others through the female Anopheline mosquitoes, wherein they continue the sexual phase of the parasite's life cycle (Greenwood et al., 2008). Gametocytes of *P. vivax* develop soon after the release of merozoites from the liver, whereas in case of *P. falciparum*, the gametocytes develop much later with peak densities of the sexual stages typically occurring one week after peak asexual stage densities (Pukrittayakamee, Imwong, Singhasivanon, Stepniewska, Day, & White, 2008).

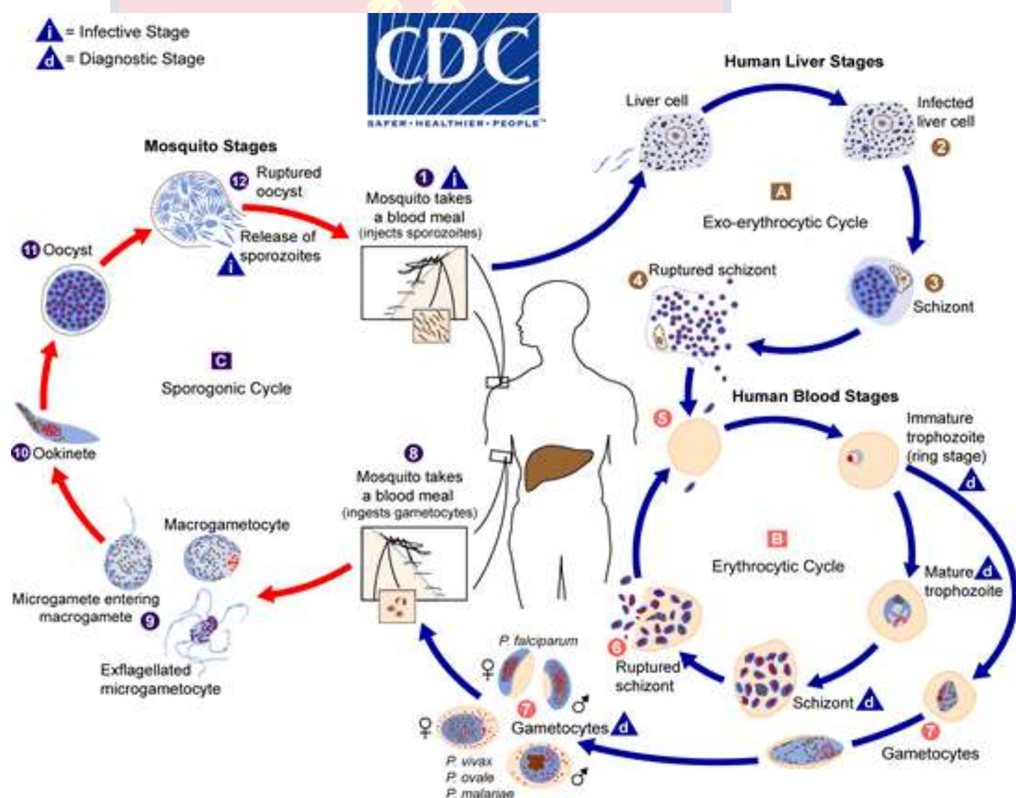

Figure 8: Schema of the Life Cycle of Malaria Parasite
Source: Global Health, Division of Parasitic Diseases and Malaria December, 2017

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Pathophysiology of malaria

Malaria is mostly associated with periodic fever, chills, shivering, headache, nausea, vomiting, and many other clinical conditions. Nonetheless, clinical complications that include severe anaemia, respiratory distress, cerebral malaria and other organ dysfunction are also common in *P. falciparum* (WHO, 2014; von Seidlein, Olaosebikan, Hendriksen, Lee, Adedoyin, Agbenyega, 2012). *P. vivax* infections are relatively benign and associated with mild clinical symptoms. Parasites do not sequester in the deep capillaries of organs but recent studies have suggested a possible sequestration in organs as evidenced by the *P. vivax* infection-associated severe illnesses and deaths (Kochar, Das, Kochar, Saxena, Sirohi, & Garg, 2009).

When the schizogony is completed within the red blood cells, each cycle lasting 24-72 hours depending on the species of the infecting parasite, newly developed merozoites are released by the lysis of infected erythrocytes and along with them, numerous known and unknown substances, such as red cell membrane products, haemozoin pigment and other toxic factors such as glycosylphosphatidylinositol (GPI) are also released into the blood. These products, particularly the GPI, activate macrophages and endothelial cells to secrete cytokines and inflammatory mediators such as tumor necrosis factor, interferon-γ, interleukin-1, IL-6, IL-8, macrophage colony-stimulating factor, and lymphotoxin, as well as superoxide and nitric oxide (NO) (Mackintosh, Beeson, & Marsh, 2004)). Many studies have implicated the GPI tail, common to several merozoite surface proteins such as MSP-1, MSP-2, and MSP-4, as a key parasite toxin (Chakravorty, Hughes, & Craig, 2008). The systemic manifestations of malaria such as headache, fever and rigors, nausea and

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vomiting, diarrhoea, anorexia, tiredness, aching joints and muscles, thrombocytopenia, immunosuppression, coagulopathy, and central nervous system manifestations have been largely attributed to the various cytokines released in response to these parasite and red cell membrane products (Clark, Budd, Alleva, Cowden, 2006). Also, the plasmodial DNA is presented by haemozoin (produced during the parasite development within the red cell) to interact intracellularly with the Toll-like receptor-9, leading to the release of proinflammatory cytokines that in turn induce COX-2-upregulating prostaglandins leading to the induction of fever (Parroche et al, 2007). Haemozoin has also been linked to the induction of apoptosis in developing erythroid cells in the bone marrow, which causes anaemia.

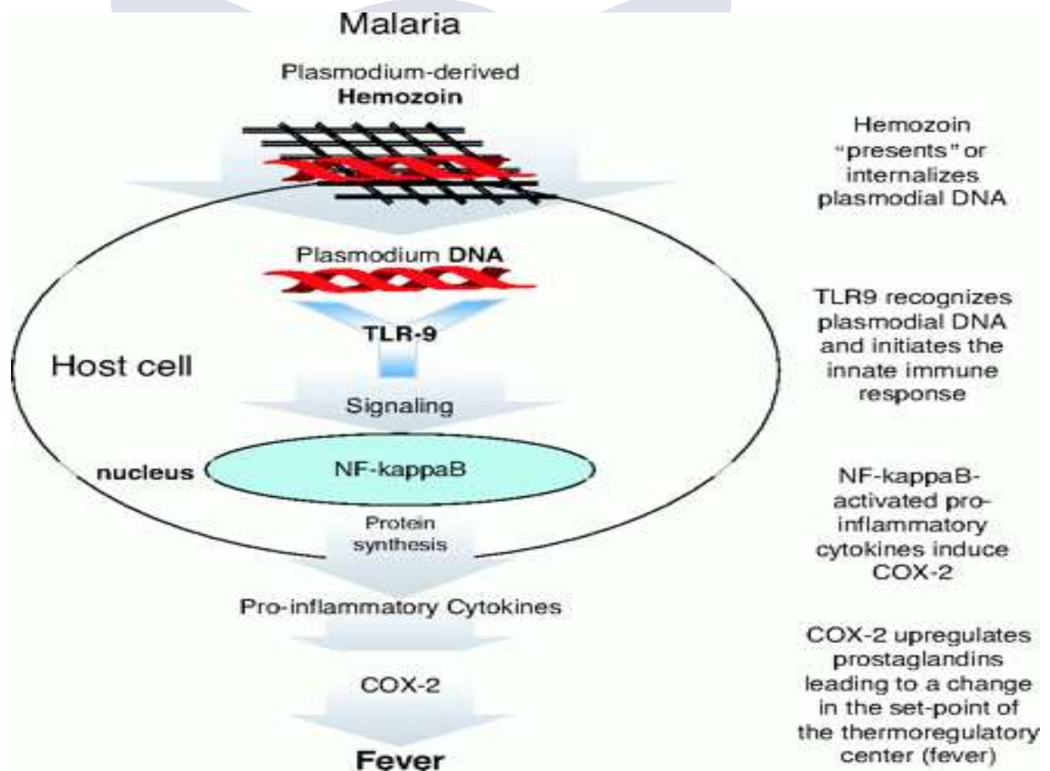

Figure 9: Induction of fever by malaria parasites
Source: Global Health, Division of Parasitic Diseases and Malaria December, 2017

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Mosquito Vector

Malaria is transmitted to humans by female mosquitoes of the genus *Anopheles*. Female mosquitoes take blood meals for egg production, and these blood meals are the link between the human and the mosquito hosts in the parasite life cycle. The successful development of the malaria parasite in the mosquito (from the "gametocyte" stage to the "sporozoite" stage) depends on several factors. The most important is ambient temperature and humidity (higher temperatures accelerate the parasite growth in the mosquito) and whether the *Anopheles* survives long enough to allow the parasite to complete its cycle in the mosquito host ("sporogonic" or "extrinsic" cycle, duration 9 to 18 days). In contrast to the human host, the mosquito host does not suffer noticeably from the presence of the parasites (Centre for Disease Control and Prevention, 2011).

Mosquitoes belong to the Phylum Arthropoda, Class Insecta and Order Diptera. The mosquito's lifespan typically lies between 3-4 weeks. During their developmental period they go through full stages of metamorphosis, i.e. from the embryo, larval, and pupal stages to a mature adult. Anopheline mosquitoes are the only carriers of malaria in humans (Arrow, Panosian, & Gelband, 2004.).

The Anopheles vectors

There are about 465 separate Anopheles mosquito species worldwide, many of which are sibling species which can only be described using genetic techniques (Sinka, 2013). Of these, nearly 70 are vectors of malaria but about 41 are of great importance (Sinka, 2013). The ability to spread *Plasmodium* parasites depends on whether the mosquito will live long enough to complete

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its developmental period, the tendency of the mosquito to feed on humans, and whether or not the mosquito is physiologically suitable for the parasite (Centre for Disease Control and Prevention, 2011). Most Anopheles mosquitoes are thought to be able to support at least one of the *Plasmodium* species to normal development (Sinka, 2013). They eat at night though few feed at daytime as well.

Anopheles mosquitoes are reclining with the body sloping upward. They exploit different breeding environments; prefer breeding in and around deteriorating infrastructures, such as broken water pipes, open tins / cans, poorly maintained drains, culverts, markets, gardens, urban agricultural sites, pools at construction sites, lorry tyre tracks, on unpaved roads, low-lying flood-prone areas, hydrants, catch pits, among others (Mattah, Futagbi, Amekudzi, Mattah, de Souza, Kartey-Attipoe, Bimi & Wilson, 2017).

Plasmodium species responsible for malaria

Plasmodium falciparum

This species causes *falciparum* malaria (formerly called malignant tertian malaria), and usually kills through cerebral malaria or renal failure (Wiser, 2004). Fever usually associated with this malaria occurs every 48 hours, but this periodicity is often masked because the stages are asynchronous. This periodicity is termed tertian, because there is fever on the first day, no fever on the second and then return of fever on the following day (Geleta & Ketema, 2016). *Plasmodium falciparum* needs an average ambient temperature of at least 20ºC hence common in the hotter and humid regions of the world (Blanford, Crane, Mann, Paaijmans, Schreiber & Thomas, 2013). It is found in tropical and subtropical Africa and parts of Central America and

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South America, Bangladesh, Pakistan, Afghanistan, Nepal, Sri Lanka, East Asia, Indonesia, Philippines, Haiti, Solomon Islands, Papua New Guinea and many islands in Melanesia (Cheesbrough, 2000). It is also found in parts of India, the Mediterranean and countries of North Africa (Cheesbrough, 2000; Wiser, 2004; Osei Tutu, 2009). Available record indicates that about 1–3 million mortalities per year, mainly in children and pregnant women, are due to severe malaria caused by *P. falciparum* (Geleta & Ketema, 2016).

Plasmodium vivax

This species causes *vivax* malaria (formerly called benign tertian malaria) which barely kills. This species is not found in tropical Africa mainly because black Africans lack the red cell surface Duffy antigen that *P. vivax* requires for cell invasion (Chessbrough, 2000; Miller, Baruch, Marsh, & Doumbo, 2002; Wiser, 2004). It can reside in places with an average temperature of only 16ºC. *Plasmodium vivax* is predominant in the Amazonian region of Brazil and in Central America primarily Nicaragua, Honduras and Mexico (Howes, Battle, Mendis, Smith, Cilbulskis, Kevin Baird & Hay, 2016). It is also found in Madagascar, Ethiopia, tropical and subtropical Africa, Central and South East Asia, China, small pockets of India, Myanmar, Indonesia, and the Solomon Islands, Papua New Guinea (Gething, Elyzar, Moyers, Smith, Battle, Guerra, Patil, Tatem, Howes, Myers, George, Horby, Heiman, Weithem, Hay, 2012).

P. ovale and *P. vivax* are implicated in causing relapse malaria because they can remain in a dormant hypnozoite stage for very long periods (years) within the liver. The adaptive value of this ability is that the parasite can persist in certain areas that experience long winters with no opportunities for transmission (Gething et al, 2012)

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Plasmodium ovale

This species cause rare *ovale* malaria (formerly called tertian malaria) with a long incubation period and relapses at three-month intervals (White, 2004). *P. ovale* malaria is endemic in tropical Western Africa. It is relatively unusual outside Africa and comprises less than 1% of isolates where found. It is also seen in the Philippines, Indonesia, and Papua New Guinea, however, relatively rare in these areas (Okafor & Finnigan, 2019). Just like the *P.vivax*, this species is also recurrent with a dormant liver stage.

Plasmodium malariae

This species produces quartan malaria with fever that returns every 72 hours. It is remarkable, in that it can persist in the blood of a host for many years at very low densities but does not have a dormant stage in the liver (White, 2017). Although it is well known that malaria episodes due to *P. malariae* can occur even after 30–50 years following a previous malaria infection, the mechanism responsible for its persistence and late recurrence still remains a medical mystery (Grande, Antinori, Menori, Menegon, Severini, 2019). *P. malariae* is widespread in the sub-Saharan Africa and the southeast of the Pacific region where its prevalence has surpassed 30% however, cases are rare in South America, Central America, Asia, and the Middle East having prevalence not above 2% (Camargo-Ayala et al, 2016).

Antimalarial agents

Antimalarial agents are used as prophylaxis and treatment of *falciparum* malaria and non-*falciparum* malaria. Since the isolation of quinine the first potent chemical for the treatment of malariain the year 1820, many other potent chemical compounds have been isolated or synthesised in the

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laboratory (Edwin, Korsik, & Todd, 2019). As in most cases, the discovery of new therapeutic agents provided good alternatives for the treatment of malaria until the parasite developed resistance to these compounds. The currently available antimalarial agents can be grouped into: 8-aminoquinoline, 4-aminoquinoline aryl-amino alcohols, antifolates, artemisinins, antibiotics and inhibitors of the cytochrome bc1 (Cowell & Winzeler, 2019). The chemical structure of the currently used antimalarials, artesunate, arthemeter, dihydroartemessin, amodiaquinn, meflouin, piperaquine, lumefantrine, atovaquone, proquanil, pymethamine, sulfadoxine, pyronaridine, tefinoquine is shown in figure 10**.** The development of resistance against antimalarial agents has resulted in cessation of the use of some chemical compounds or restriction of the compounds to particular situations (Nosten & White, 2007). Artimisinin combination therapy constitutes the treatment of choice recommended by the World Health Organisation. However, ongoing research has sought to produce hybrid compounds by combining antimalarial drugs with other compounds to increase their antiplasmodial efficacies and potencies (WHO, 2019). For instance, scientists are exploring the efficacies of quinolines combined with other compounds including trixolanes, ferrocene, and artemisine derivatives (Nqoro, Tobeka, & Aderibigbe, 2017).

***Artemisinin* compounds and its derivatives**

First isolated by Tu Youyou in 1971 from *Artemisia annua,* artemisinine represents the most widely used antimalarial compound (Su & Miller, 2015). Artemisinine and its derivatives including artesunate, artemether, arteether and dihydroartemisinine possess profound anti-parasitic effect against both late stage and early stage *Plasmodium* parasites forms in

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humans (Tse, Korsik, & Todd, 2019). The derivatives of artemisine are prodrugs which are converted into active form, dihydroartemisinine. They are usually combined with other long-lasting antimalarial compounds such as amodioquine, lumefantrine, mefloquine etc, to delay the onset of resistance (Tse et al., 2019).

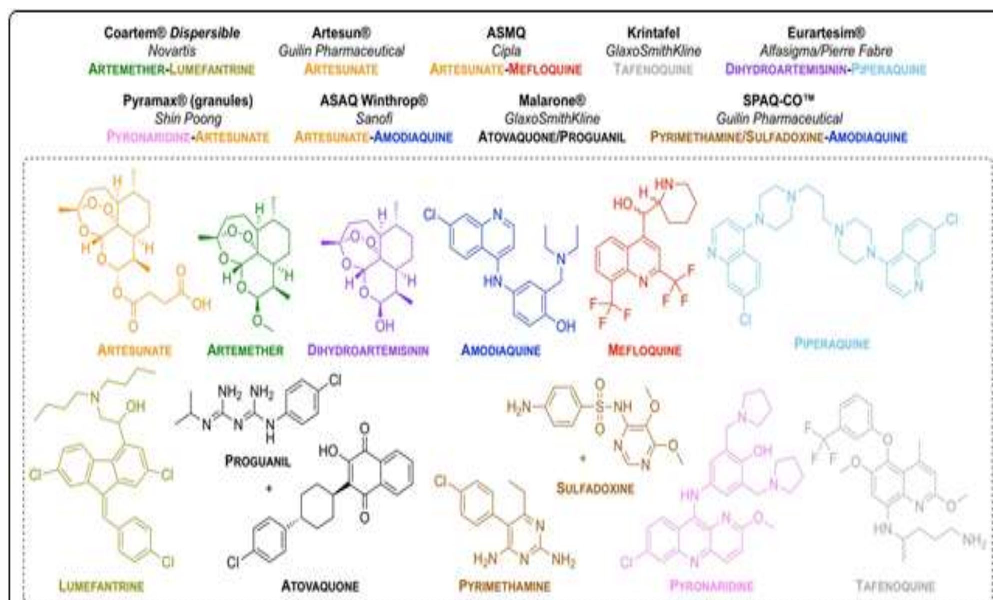

Figure10: New drug combinations that have been approved for use.
Source: Brand name of the drug (in bold), partnered company (in italics). (Tse et al., 2019).

The underlining mechanism of action of artemisinin compounds still remains obscure. There are multiple proposed pathways through which artemisinin works. The leading accepted theory proposed that artemisinin compound is activated by heme which leads to the production free oxygen radicals which inhibits proteins vital for the survival of the parasite (O'Neill, Barton & Ward, 2010). Other studies posit alternate pathways via the activation of the upregulation of unfolded protein response (Arnou, Montigny, Morth, Nissen, Jaxel, Moller, & Maire, 2011; Nagasundaram, Chakraborty, Karthick, Balaji, Siva, Lu, & Zhu, 2016).

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Antifolate compounds

Sulfadoxine and other antifolate compound inhibit enzymes involved in the folate pathway. Inhibition of the folate pathway results in decreased pyrimidine synthesis which in turn slows the synthesis of DNA, and amino acids including serine and methionine (Fernandez-Villa, Aguilar & Rojo, 2019). There are two types of antifolate compounds. Type I antifolate compounds (sulfonamides and sulfones) hampers the synthesis of dihydropterote from hydromethlydihydroperote by competing for active site of DHPS (an important enzyme in *Plasmodium* parasite) (Triglia, Menting, Wilson, Cowman, 1997). On the other hand, type 2 hydrofolates (pyrimethamines and quinazolines) inhibits dihydrofolate reductase (a bifunctional enzyme in plasmodium) resulting in the death of the *Plasmodium* parasite (Bilsland, Vliet, Williams, Feltham, Carrasco, Fotoran, Cubillos, Wunderlich, Grotli, Hollfelder, Jackson, King & Oliver, 2018).

8-aminoquinoline compounds

8-aminoquinolones were the first antiplasmodial compound to be isolated (Grewal, 1981). Later primaquine was developed (Grewal, 1981). Primaquine possesses antiplasmodial effect against intrahepatic form (hypnozoites) of *Plasmodium ovale* and *Plasmodium vivax*(Schaefer, Peters, & Miller, 2014). It is postulated that, primaquine act by inferring with plamodial mitochondrial activity by inhibiting electron transport chain and pyrimidine synthesis (Kuhlmann & Fleckenstein, 2017). Specifically, it is known to inhibit the function of ubiquinone and coenzyme Q in the mitochondria (Maddison, Page, & Church, 2008). When administered, primaquine undergoes metabolism and is converted to an unstable active form 5,6-

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quinones which is later stabilised at the second position of the quinoline rings (Anand & Sharma, 1997).

4-aminoquinoline compounds

The 4-aminoquinoline compounds emerged during the World War 2 where synthetic quinacrine was produced to treat malaria (Tse, Korsik, & Todd, 2019). The chemical structure of 4-aminoquinoline reveals 4-subtituted quinoine skeleton which is believed to be responsible for its antiplasmodial effect (Anand & Sharma, 1997). Compounds of 4-aminoquinoline include chloroquine, quinine, amodiquine, piperaquine and mefloquine. Chloroquine is known to inhibit the polymerization of ferroprotophorins generating oxygen radicals that causes oxidative stress and consequently membrane damage (Ryan, 2018). However, chloroquine is only effective against the erythrocytic forms of plasmodium hence ineffective against intrahepatic forms (Ryan, 2018). Chloroquine act on mature ring forms and trophozoites. The mechanism of action of the other 4-aminoquinoline compounds has not been well studied but it's likely to be the same as chloroquine.

Antimarial drug resistance to antimalarial drugs

For many years, the advent of antimalarial drug underpins many of the successes in treating mild and severe malaria. However, the emergence of antimalarial drug resistance possesses serious health threat to the successful treatment, control and elimination of malaria (Birth, Kao, & Hunte, 2014). Continued development of drug resistance would inevitably render most of the widely used antimalarial drugs ineffective and may leave highly vulnerable group like pregnant women and children under five to fight for their lives when infected (White, 2004). This may take us back to the era where malaria

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was deemed as one of the deadliest disease worldwide-high rate of morbidity and mortality. According to the Centre for Disease Control and Prevention (2018), antimalarial drug resistance has been confirmed in two of five known *Plasmodium* species known to infect man: *Plasmodium falciparum* and *Plasmodium vivax.* Drug resistance by *Plasmodium ovale* and *Plasmodium malariae* is yet to be described (Ryan, 2018). The emergence of antimalarial resistance results from spontaneous mutation and duplication of less susceptible genes which is selected in the absence of antimalarial drug pressure (White, 2004). Clones of these resistance strains are then produced which later disseminate. Although drug resistance could emerge from both the sexual and asexual *Plasmodium* forms, it is as though resistance strains are more likely to emerge from asexual forms in patients with poor adherence to drug regimen (Pongtavornpinyo,Imwong, Singhasivanon, Stepniewska, Day, & White, 2009).

Antimarial drug resistance in *Plasmodium falciparum* and *Plasmodium vivax*

The first report of chloroquine-resistance *Plasmodium falciparum* was documented in the Southeast Asia, Oceania, and South America in the late 1950's and early 1960's (Centers for Disease Control and Prevention, 2018). Subsequent reports suggested the spreading of resistance for these low transmission areas to high transmission areas including sub-Saharan Africa (White, 2004). Similar resistance pattern and distribution for sulphadoxine-pyrimethamine was observed resulting in high mortality rate among vulnerable children in sub-Saharan Africa (Korenromp, Williams, Gouws, Dye, & Snow, 2003). The relatively low events of emerging resistance in high

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transmission region to low transmission may be explained by the high immunity in high transmission areas resulting in resistant parasite clearance (Lopera-Mesa,Doumbia, Chiang, Zeituni, Konate, Doumbouya, & Diakite, 2013; Menard & Dondorp, 2017). Choroloquine resistance in *Plasmodium falciparum* and *Plasmodium vivax* is associated with point mutation in the gene encoding *Plasmodium falciparum* resistance transporter (PfCRT) protein (Chinappi, Via, Marcatili, & Tramontano, 2010). After erythrocyte invasion by *Plasmodium* it forms a lysosomal compartment called digestive vacuoles. Mutations including K77T, K76N, and K76I reduce the amount of chloroquine that could be absorbed in the digestive vacuole resulting in low chloroquine concentration (Chinappi et al., 2010). This enhances the survival of intraeeythrocyte forms of the parasite.

Artemisinin is the most effective antimalarial drug against the ring-stage parasite (Day & Dondorp, 2007). Therefore, resistance to artemisinin drugs is shown by reduced sensitivity or susceptibility of the ring-stage to artemisinin. Studies suggest that resistance to artemesinine drugs is due to mutation in the K13 gene, e.g., R539T and C580Y, which encodes for the properller region of the *Plasmodium falciparum* Kelch protein (Breglio, Rahman, Sá, Hott, Roberts, & Wellems, 2018; Menard & Dondorp, 2017). The K13 gene plays an important role in protein-protein interaction including increasing the rate of polyubiquination leading to degradation in proteasomes (White, 2014). The intraerythrocyte cell cycle of parasite carrying K13 resistant gene is altered- longer ring-stage and shorter trophozoite stage relative to the wild type (Coppée, Jeffares, Miteva, Sabbagh, & Clain, 2019).

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Resistance to chloroquine led to increase reliance on the antifolates as alternative treatment option (Menard et al., 2017). In particular, sulfadoxine/pyrimethamine is relied upon for the treatment especially in children and pregnant women. Resistance to antifolates correlated with mutations in their target enzymes, dihydrofolate reductase, dihydrofolate–thymidylate synthase and dihydropteroate synthase encoded in the pyrophosphokinase-dihydropteroate synthase gene of *Plasmodium* (Mkulama et al., 2008; Juma et al., 2019). The point mutation that confers resistance to sulfadoxine pyrimethamine is discussed by Mkulama and his colleagues (2008).

Chapter summary

Drawing from existing literature, this chapter reviewed the occurrence of malaria, its prevalence and further considered some drugs that are used to combat the menace. This chapter further discussed into details epidemiology, *Plasmodium* forms, determinants of malaria, antiplasmodium compounds, and *Calotropis procera.*

Literature revealed that there was an increase in malaria cases from 2017-2018 in Ghana, though it slightly decreased in 2016–2017. But there was a steady increase from 2002–2016. This could be due to antimalarial drug resistance confirmed in two of five known plasmodium species known to infect man: *Plasmodium falciparum* and *Plasmodium vivax.* Drug resistance by *Plasmodium ovale* and *Plasmodium malariae* is yet to be described. Additionally, literature has further revealed that the *Calotropis procera* plant has anti-inflammatory, anti-microbial, lavicidal, anticancer, antipyretic properties.

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CHAPTER THREE

MATERIALS AND METHODS

This chapter describes into detail the methodological approach and research methods used in the study. Emphasis is placed on ethical issues as well as the laboratory work. The chapter fully describes the various steps in each method used in the study.

The figure 11 (flow chart) below shows the sequence of the procedures carried out in the study.

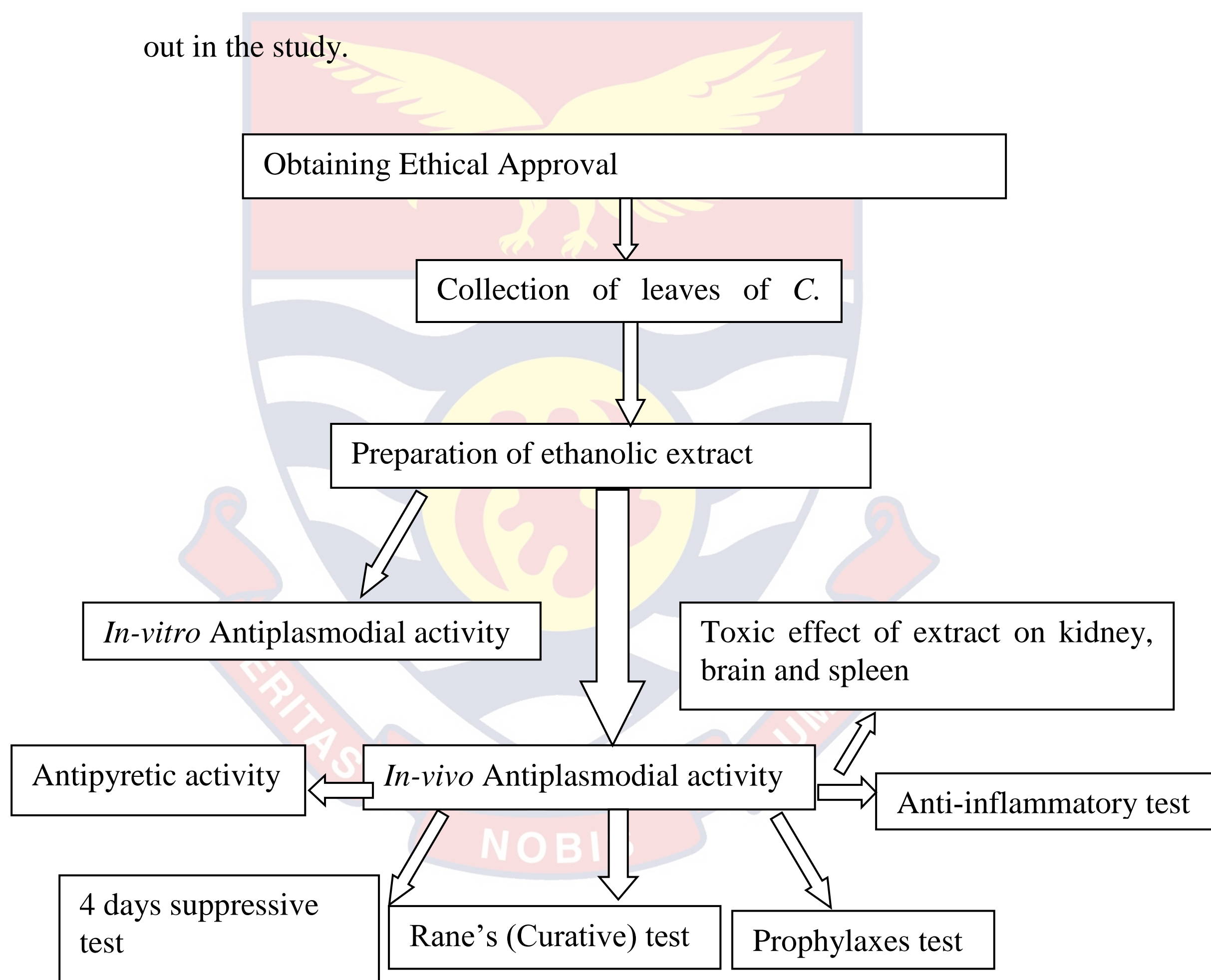

Figure11: Flow Chart summarizing the Methodology used in this study

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Ethical Approval

Ethical clearance was sought and obtained from the Departmental Animal Research Review Board of College of Health and Allied Sciences of the University of Cape Coast.

Collection of leaves of *Calotropis procera*

The fresh leaves of *Calotropis procera* were collected from Elimina in the Komenda Edina Eguafo Abirim District in the Central Region of Ghana during the month of November 2018. The fresh leaves were wrapped with plastic sheet during transportation. The collected leaves were identified as *Calotropis procera* by Mr. Michael Fynn, a botanist, who works in the Herbarium, School of Biological Sciences, University of Cape Coast. The specimen was given a voucher number (ATC number 3050) and deposited in the Herbarium, School of Biological Science, University of Cape Coast for future reference.

Plant Extraction

After collection, the fresh leaves were thoroughly washed with distilled water to remove dirt. They were then air-dried under shade for thirty days and pulverised into powder using the leave mill (Glen Creston, Canada). The powder (290g) was cold macerated with 2L of 70% methanol in a round bottom flask for three consecutive days at room temperature. The extraction process was facilitated using a mechanical shaker. The rotary evaporator was used to desiccate the resulting mixture leaving a pure extract that was subsequently dried in a laboratory oven. The extract (10% w/w) was transferred into bottled containers and kept in a refrigerator until use.

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In vitro antimalarial activity of *C. procera* crude ethanol extract against *P. falciparum*

The efficacy of *C. procera* extract was tested on 3D7 chloroquine-sensitive strain of *P. falciparum* in a complete parasite media (CPM) to quantitatively measure how much of *C. procera* will be required to impede *P. falciparum* by 50%.

Blood collection and red blood cells (RBC) preparation

Blood was collected aseptically from an O Rh^+ positive donor and transferred into vacutainer tubes (BD Vacutainer, USA) containing an anticoagulant 'Citrate-Phosphate-Dextrose' (CPD), the blood sample was mixed very well and stored at 2-8℃ for up to 48h before use. Fifteen milliliters of the whole blood was transferred into a sterile conical tube and centrifuged for 10 min. at 2000rpm. The plasma together with the buffy coat was removed and the RPMI medium added to each tube containing the packed cells to make the volume up to14mL and was mixed well. The mixture was then centrifuged for 10 mins at 200rpm, the supernatant discarded and the process of washing was repeated for 3 times. An equal volume of RPMI medium was added packed cells and stored at 2-8℃ until used. It is very important to note that the washed RBCs could only be stable at this storage condition for up to 2 weeks, hence all the procedures were carried out in a biological safety cabinet (BSC), with all the necessary precautionary measures followed.

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Culturing and maintenance of 3D7 chloroquine-sensitive strain of *P. falciparum*

Chloroquine sensitive strain of *P. falciparum* 3D7 was obtained from the Noguchi Memorial Institute for Medical Research (NMIMR, Legon) and maintained in continuous culture as described earlier by Trager and Jensen (1997). The parasites were cultivated in O Rh^+ RBC using complete parasite medium which composed of RPMI 1640, reinforced with HEPES, $NaHCO_3$, L-glutamine, gentamycin, glucose and Albumax II. In the experiment, the following incubation conditions were followed: 5.5% CO_2, 2% O_2, and 92.5% N_2 of a special gas mixture. The parasites cultivated in O Rh^+ were sustained in the incubator with daily media change till parasitaemia of more than 4% was obtained. Treating the culture with 5% sorbitol resulted in the synchronization of ring-stage *P. falciparum*. The parasitaemia had been reduced to1% after the parasites were sub-cultured after 48 h of synchronization.

Determination of parasitaemia in the *in vitro* assessment

Parasitaemia was checked daily to monitor culture conditions as well as at the point when the culture medium was being changed. The process involves making thin blood smears on cleaned grease-free microscope slides. After smears are air-dried, they were fixed in absolute methanol by dips for 5 seconds and were allowed to air dry. The methanol-fixed smears were then stained with 10% Giemsa stain in phosphate buffer for 15 minutes, excess stains washed and air-dried. The stained smears were examined for parasitized RBC and non-infected RBC at the monolayer of the film prepared using the 100x oil immersion objective of a light microscope. With the aid of a

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laboratory cell counter, the total numbers of RBCs was counted per field as well as the number of infected RBCs and were recorded in the worksheet. Other fields were visited and counting of both infected RBCs and uninfected RBCs continued until 500 RBCs have been counted. The percentage of parasitaemia was calculated by dividing the number of infected RBCs by the total number of RBCs and multiplied by 100. However, cultures with above 4% parasitaemia were sub-cultured at least twice daily to prevent culture from 'crashing'.

Preparation of plant crude extract

The crude extract of *C. procera* was weighed (10mg) and transferred into 1.5 mL-capacity Eppendorf tube and 1 ml of 100% DMSO added and vortexed to obtain a homogeneous solution to achieve a concentration of 10 mg/mL (stock). The extract (stock) was diluted further with CPM to obtain a concentration of 1000 µg/mL (extract). The diluted extract solution was filtered into new sterile tubes. Artesunate was used as the standard drug and was diluted with CPM to achieve a concentration of 1000 ng/mL

***In vitro* parasite growth inhibition assay**

The crude extract of *C. procera* was tested for anti-plasmodial activity using the SYBR Green I fluorescence assay as described by Smilktein et al., 2004). The stock concentrations of 10 mg/mL (100% DMSO) of *C. procera* extract were diluted with CPM to achieve a working concentration of 1000 µg/mL (0.1% DMSO). Serial dilutions of the extract (100, 50, 25, 12.5, 6.25 and 3.125 µg/mL) were prepared for the treatment and Artesunate (reference drug) was also serially diluted (50 to 1.56 ng/mL). The test wells (96-well tissue culture plate) were initially seeded with 90 µL of ring-stage

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(synchronized) pRBCs (1% parasitaemia), CPM and packed RBCs at 2% haematocrit. An aliquot of 10 µL of the extract was dispensed into each well in triplicate. But the wells containing RBCs (2% haematocrit), the parasitized RBCs (pRBCs) and CPM served as negative and blank controls respectively. The plate was then incubated for 48-72 h. An aliquot of 100 µLof 4x buffered SYBR Green I (0.20 µL of 10,000X SYBR Green I/mL of 1x phosphate buffer saline) was added further incubated for 30 min at 37°C. The presence and amount of pRBCs were detected using the Guava EasyCyte HT FACS machine (Millipore, USA) and parasitaemia recorded in percentage. The 50% inhibitory concentration (IC_{50}) was extrapolated from non-linear regression curves of percentage inhibition versus log-concentration curves from Graph Pad Prism 7.0 using algorithms obtained from flow cytometers (FACS) data.

***In vivo* Antimalarial Screening**

Experimental Animal and Parasite

Two hundred healthy Swiss albino mice (18–34 g), aged 4-6 weeks, were purchased from the animal house near Korle-bu Teaching Hospital in Accra. Mice were maintained in the laboratory under standard condition (temperature of 22 ± 3^{0}C, relative humidity of 40-50% and 12 h light/12 h dark cycle) with a commercial food and water *ad libitum*. Mice were acclimatized for a week before the study. All procedures and techniques used in this study were in accordance with the eighth edition of the book guide for care and use of laboratory animals (National Research Council of the National Academies, 2011)

Plasmodium berghei (ANKA) parasites obtained from the Noguchi Memorial Institute for Medical Research (NMIMR, Legon) were maintained

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by serial passage of blood from infected mice to the non-infected ones, on weekly basis.

Grouping of Animals

The animals that were used numbered one hundred and ninety-five. For each of the experiment, twenty-five animals were used. The twenty-five animals were divided into five groups of five each. Each group was made up of two males and three females. Groups I and II served as positive and negative controls respectively whereas groups III, IV and V were treated with the crude extract at different concentrations.

Parasite inoculation

Albino mice previously infected with *P. berghei* having different levels of parasitaemia (30 - 37%) were used as donors. Donor mice were placed in closed chamber and euthanized by inhalation of anesthesia gas (Nitrous Oxide, N_2O) and infected blood collected by cardiac puncture. The blood was then diluted in normal saline (0.9%) based on parasitaemia level and the red blood cell (RBC) count of normal mice so that the final suspension would contain about 1×10^7 parasitized red blood cells (pRBCs) in every 0.2 mL suspension. Each mouse used in the study was infected intraperitoneally with 0.2 mL infected blood containing about 1×10^7 *P. berghei* parasitized RBCs.

The antimalarial activity of crude extract was evaluated by intraperitoneally infecting a total of twenty-five mice with 0.2 mL infected blood containing about 1×10^7 *P. berghei* parasitized RBCs. The mice were randomly divided into five groups each consisting of five mice. Groups III, IV and V were treated with the crude extract at 200 mg/kg, 400 mg/kg, and 600 mg/kg, respectively. The remaining two groups I and II served as positive and

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negative controls and received Arthemeter-Lumifantrin (Bliss GVS Pharma, India) 25 mg/kg and 8 mL/kg of vehicle, respectively. Each dose was reconstituted by distilled water and administered orally.

The 4-Day Suppressive Test

Twenty five mice were randomly divided into five groups each consisting five mice as described earlier. Evaluation of schizonticidal activity of the leaves of *C. procera* crude extract on early infection was carried out by intraperitoneally infecting the mice with 0.2 mL infected blood containing about 1×10^7 *P. berghei* parasitized RBCs and were randomly divided into five groups each consisting five mice. Treatment began after 2 hours post-infection (D0), and continued daily at 24 h time intervals for four days (D1-D3). On the 5th day (D4), blood was collected from the tail of each mouse using clean, non-greasy slides and then thin films were made accordingly to determine parasitaemia.

Percentage parasitaemia suppression of the extract was compared with respect to the controls. Percentage parasitaemia suppression was calculated using the following formula as described by Knight and Peters (1980):

Average % of Parasitaemia Suppression

$$= \frac{\text{Parasitaemia in negative control} - \text{Parasitaemia in treatment}}{\text{Parasitaemia in negative control}}$$

Packed cell volume (PCV) and haemoglobin levels were determined as a measure of the effectiveness of the extract in preventing the haemolysis resulting from the increased parasitaemia due to malaria infection. After 4 days of treatment with extract of *Calotropis procera*, blood was taken from the tail of the mice via decapitation. The blood was collected into sterile test

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tube and subsequently analysed for PCV and haemoglobin level using automated Mission Hb Haemoglobin Testing System (Acon, USA).

Rane's (Curative) Test

Twenty-five mice were randomly grouped into five as earlier described. The mice were intraperitoneally inoculated with standard inoculums of 1×10^7 *P. berghei* PRBCs on the first day (Day 0). Seventy-two hours later, infected animals were administered with 200 mg/kg, 400 mg/kg and 600 mg/kg of *C. procera* extract per body weight. The treatment was continued once daily until the 7th day (D3-D6). The weight of the animals after parasitaemia check was also determined using the weighing balance (Adam equip, UK). Subsequently, Geimsa stained thin blood film was prepared from the tail of each mouse at the end of the 7th day to monitor parasitaemia level. The percentage parasitaemia was obtained by counting the number of infected RBC out of the total RBC in random microscopic fields (Olympus, Japan). Two stained slides (CAT, China) for each mouse were examined. Five different fields on each slide were counted to determine the percentage parasitaemia using the following formula as described by Ryley and Peters (1970):

$$\% \text{ parasitaemia} = \frac{\text{Number of PRBC}}{\text{Total number of RBC}} \times 100$$

Prophylactic Test

The prophylactic activity of the extract was tested using the residual infection procedure (Peters, 1965) with slight modifications. In brief, twenty adult male and female mice were weighed, randomized into five groups with four mice in each group as described in section 3.5.2, and treated for four consecutive days. On the 5th day (D4), all mice were infected with 0.2 mL

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blood containing about 1×10^7 *P. berghei* parasitized RBCs. Seventy-two hour post-infection parasitaemia and temperature levels were determined. Subsequently, the mice were closely observed for 28 days for their survival to calculate mean survival time.

The percentage parasitaemia was obtained by counting the number of infected RBCs out of the total RBCs in random microscopic fields. Two stained slides for each mouse were examined. Five different fields on each slide were counted to determine the percentage parasitaemia using the following formula:

$$\%\ \text{parasitaemia} = \frac{\text{Number of PRBC}}{\text{Total number of RBC}} \times 100$$

Antipyretic Activity of the ethanol leaf-extract of *Calotropis procera* against yeast-induced pyrexia

To investigate the anti-pyretic activity of the ethanol leaf (when it was done) extracts of *C. procera* in yeast-induced pyrexia, twenty-five Swiss albino mice (18–34 g), were divided into five groups of five animals each as previously described. Initial rectal temperature was measured by introducing a 3cm digital thermometer (Greenlife Medics, Canada) coated with glycerine as lubricant into the rectum of the mice to obtain the baseline. Before 18 h treatment with the extracts, 10% Baker's yeast solution in 0.9% normal saline was injected intraperitoneally to induce pyrexia and mice fasted overnight in maintained conditions with free access to drinking water.

Subsequently, the mice groups were orally administered with *C. procera* (30 mg/kg, 100 mg/kg, 300 mg/kg), paracetamol reference standard (150 mg/kg) and 10 mL/kg of 0.9% normal saline (Oxoid, England) for the

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control group, respectively. Body temperature was measured via the rectum hourly from 0 to 5 hours of treatment.

Anti-inflammatory effects of the ethanol leaf-extract of *C. procera* against Carrageenan-induced paw oedema in mice

To investigate the anti-inflammatory effects of ethanol leaf extract of *C. procera* against carrageenan-induced paw oedema, twenty-five Swiss albino mice were kept on fasting for a night with free access to water. The albino mice were divided into five equal groups as earlier described. All the mice with freshly prepared 0.1 mL of carrageenan in 0.9% normal saline into the sub plantar surface of the right hind paw of each animal to induce paw swelling. After 1 h of paw swelling induction, the animals were treated with the first group receiving 10 mL/kg of 0.9% normal saline; the second group received Diclofenac (100 mg/kg) as the reference drug. Group III, IV and V received 30 mg/kg, 100 mg/kg and 300 mg/kg respectively of the ethanol extract of *C. procera*. The inflammation was quantified by measuring the volume displaced by the paw using a venier calliper (Oneonta, USA) at time 1-5 h.

Assessing the Acute toxicity of *C. procera* on the brain, kidney, liver and spleen

The acute toxicity study was conducted under Organisation for Economic Cooperation and Development 423 guidelines (OECD, 2008). The mice were randomized into 5 groups and each group contained 5 animals as earlier described. After crude extract administration, the animals were observed continuously for the first 4 h to detect eventual behavioural changes. Then, they were observed periodically for 72 h for any mortality,

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changes in physical appearance, injury, pain and signs of illness. At the end, mice were sacrificed by cerebral dislocation and organs such as liver, spleen, kidney and brain were harvested and observed for possible histhological change as described in the eight edition of the book Bancroft's theory and practice of histological techniques (Suvarna, et al., 2019).

Data Analysis

GraphPad Prism version 7.0 (GraphPad, Software Inc., USA) was used for the analysis of the data. Statistical analysis was performed on data using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test for *in vitro* antiplasmodial test, Rane's test, 4-day suppressive test and prophylactic test. Data on rectal temperature, body weight, carragenaan, pyrexia, haemoglobin level and PCV were performed using two-way analysis of variance (ANOVA) followed by Tukey–Kramer's multiple comparison test. Data on the survival curve was carried out using chi square (log-rank test). Results were deemed statistically significant if $P < 0.05$ at 95% confidence intervals.

Chapter Summary

The leaves of the *C. procera* were collected in November, 2018 from Elmina in the Central Region of Ghana. The leaves were macerated and crude ethanol extract was prepared from it. Parasites were obtained from the Noguchi Memorial Institute for Medical Research (Legon) and animals were infected with them.

In vitro antiplasmodial activity was carried out using *P. falciparum*. *In vivo* antiplasmodial activity that was carried out considered the 4-day suppressive test, rane's (curative) test, prophylactic test, antipyretic activity

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and carrageenan-induced paw oedema in mice. Histopathology was also carried out on the brain, kidney, liver and spleen to assess the toxic effect of the extract on these organs.

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CHAPTER FOUR

RESULTS AND DISCUSSION

This chapter presents the results of the analyses and discussion of the findings of the study. The results presented in this chapter are presented in tables. Categorical variables are presented in count (percentage) whereas continuous variables are shown in mean±standard deviation. Graphs, Charts and pictures from the laboratory work were also used to describe the results.

Results

The crude ethanolic extract of leaves showed an antimalarial activity of IC_{50}= 83.91± 0.375µg/ml against *P. falciparum* strain 3D7. A = the IC_{50} of Arthemeter/Lumifantrin (positive control); B = the IC_{50} of *C. procera*; C = repeat of the IC_{50} of *C. procera*

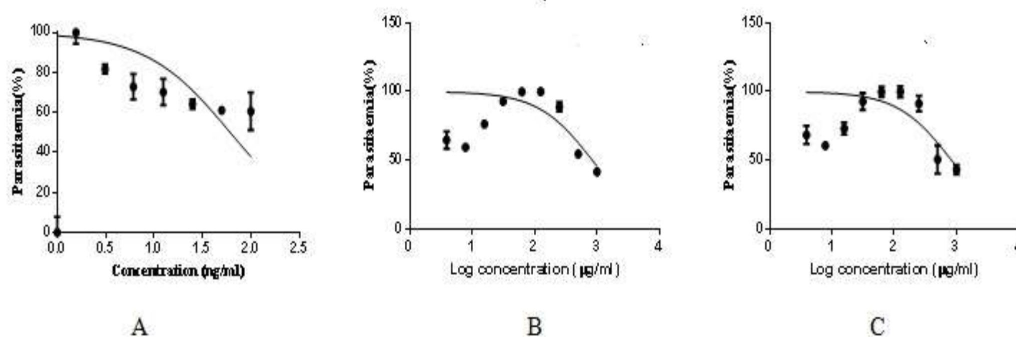

Figure 12. *Calotropis procera* demonstrated activity against *P. falciparum* strain 3D7

Figure 12 shows the IC_{50} values of the tested plant extracts against *P. falciparum*. The *invitro* antiplasmodial activity of biological active substances for the crude extract (B) was repeated to get (C). The IC_{50} value of the arthemeter/lumefantrin was found to be 61.41 µg/mL. The IC_{50}value of the crude extract was 84.28 µg/mL. The IC_{50} value for the crude extract was 83.53

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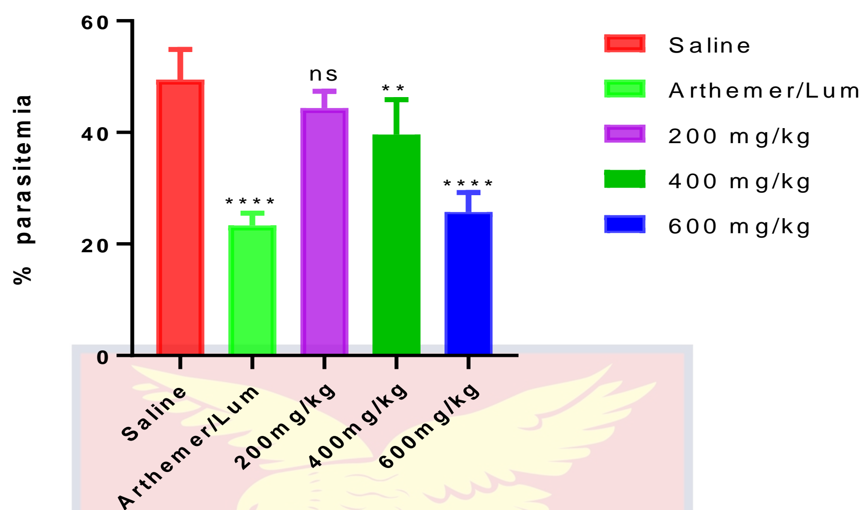
μg/mL when it was repeated against chloroquine-sensitive *P. falciparum* strain.



Data are expressed as mean±SEM (n=5); a, normal saline; b, art/lum; c, 200 mg/kg; d, 400 mg/kg; e, 600 mg/kg: [1]p< 0.0048 [ns], [2]p< 0.0066 **, [3]p< 0.0001 ****

Figure 13*: Calotropis procera* showed a dose dependent suppressing activity against *Plasmodium berghei*

As shown in figure 13, the 4-day suppressive study revealed that the crude extract exhibited a significant reduction in parasitaemia (P< 0.0001). The 600 mg/kg dose of *C. procera* produced a higher significance of reduction in parasitaemia (P< 0.0001). At dose 400 mg/kg, the level of significantly was high (P< 0.0066). There was no significance difference (P> 0.05) in chemosupression between 200 mg/kg. The standard drug markedly (P< 0.0001) reduced parasite count to undetectable level.

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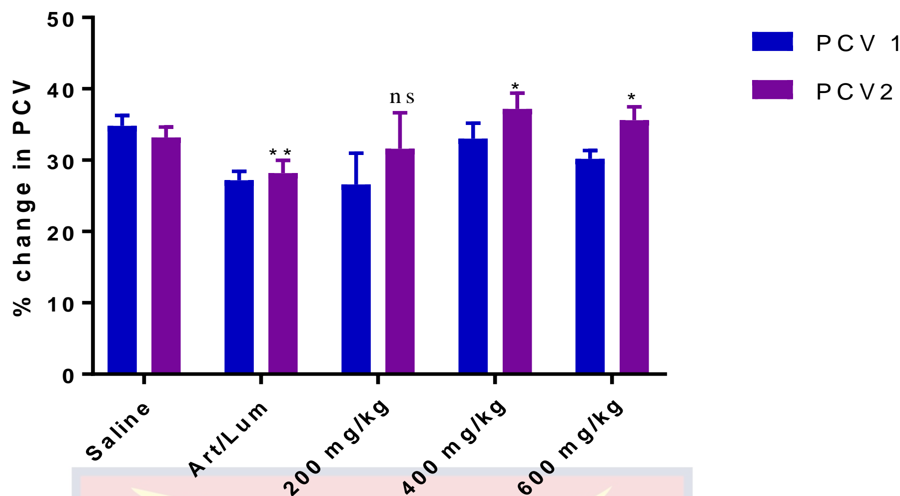

Comparison of packed cell volume in malaria-infected mice treated with crude extract in 4-day suppressive test. Data are expressed as mean±SEM (n=5); a, negative control; b, art/lum; c, 200 mg/kg; d, 400 mg/kg; e, 600 mg/kg; [1]p<0.0117 *; [2]p<0.0112 **; PCV1 = Packed Cell Volume before treatment; PCV 2 = Packed Cell Volume after treatment

Figure 14: Packed cell volume during 4-day suppressive test before and after administration of ethanol leaf extract of *Calotropis procera* against *Plasmodium berghei* infected mice

Figure 14 revealed that after infection, the PCV 1 levels for arthemer/lumefantrin, 200 mg/kg, 400 mg/kg and 600 mg/kg were all reduced with the exception saline. After treatment, PCV 2 of all doses (200 mg/kg, 400 mg/kg and 600 mg/kg) increased but that of the saline decreased. A high level of significance occurred in the positive control (arthemeter/lumefantrin) (P< 0.0112). The doses 400 mg/kg and 600 mg/kg were significant (P< 0.0117)

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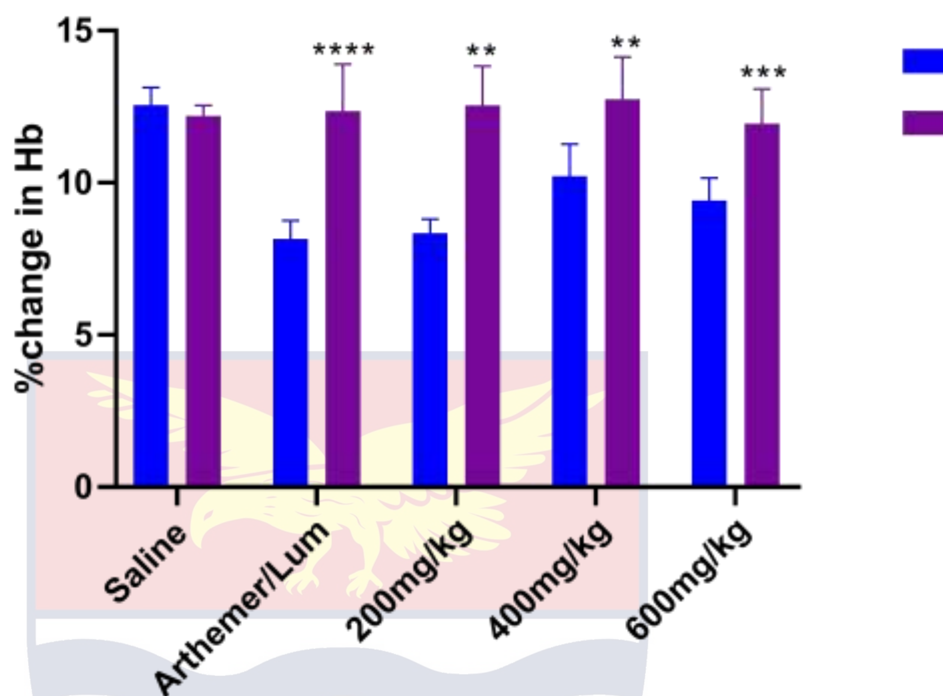

Data are expressed as mean±SEM (n=5); a, normal saline; b, art/lum; c, 200 mg/kg; d, 400 mg/kg; e, 600 mg/kg; [1]p< 0.0054**, [2]p< 0.001***, [3]p< 0.0001 ****, Hb 1= Haemoglobin before treatment; Hb 2 = Haemoglobin after treatment

Figure 15: Haemoglobin during 4-day suppressive test before and after administration of ethanol leaf extract against *Plasmodium berghei* infected mice

As shown in figure 15, the haemoglobin (Hb 1) levels decreased in all groups following the establishment of infection. After treatment the haemoglobin (Hb 2) levels of all the doses increased whereas that of saline decreased. The haemoglobin levels for the treatment groups were significantly higher than the saline group 200 mg/kg(P< 0.0054); 400 mg/kg(P< 0.0054); and at 600 mg/kg the (P< 0.001), with the p-value of A/L being (P<0.0001).

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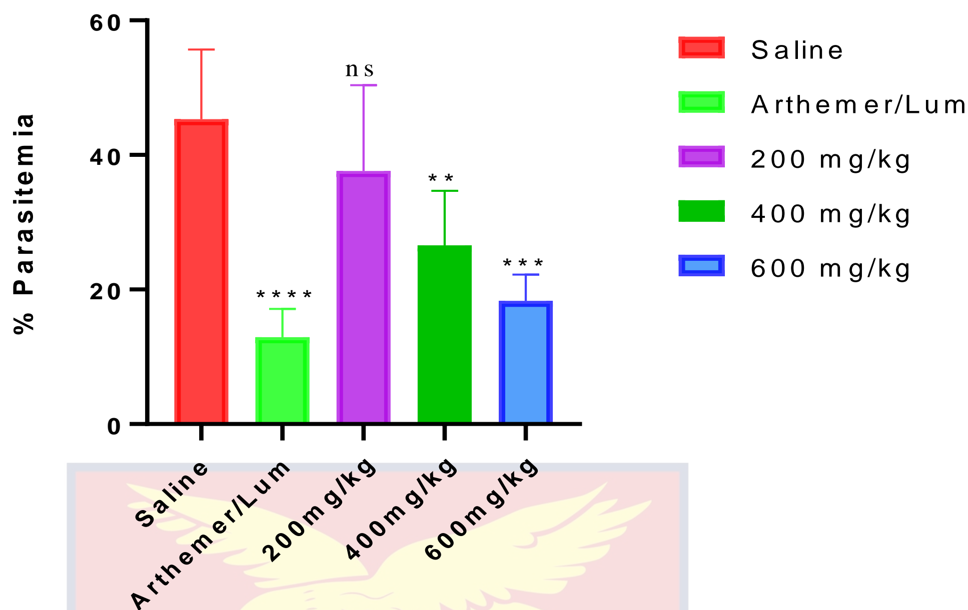

Data are expressed as mean±SEM (n=5); a, normal saline b, art/lum; c, 200 mg/kg; d, 400 mg/kg; e, 600 mg/kg: [1]p<0.4425 ns, [2]p< 0.0087 **, [3]p< 0.001 ***, [4]p< 0.0001 ****

Figure 16: *Calotropis procera* shows curative antimalarial activity against *Plasmodium berghei*

Figure 16 revealed that the of dose 400 mg/kg significantly (P< 0.0087) reduced parasitaemia when compared with the normal saline. At dose 600 mg/kg, the level of significance (P<0.001) was relatively high. The arthemeter/lumefatrine was highly significant (P<0.0001). At 200 mg/kg the extract did not exhibit any significance (P<0.4425).

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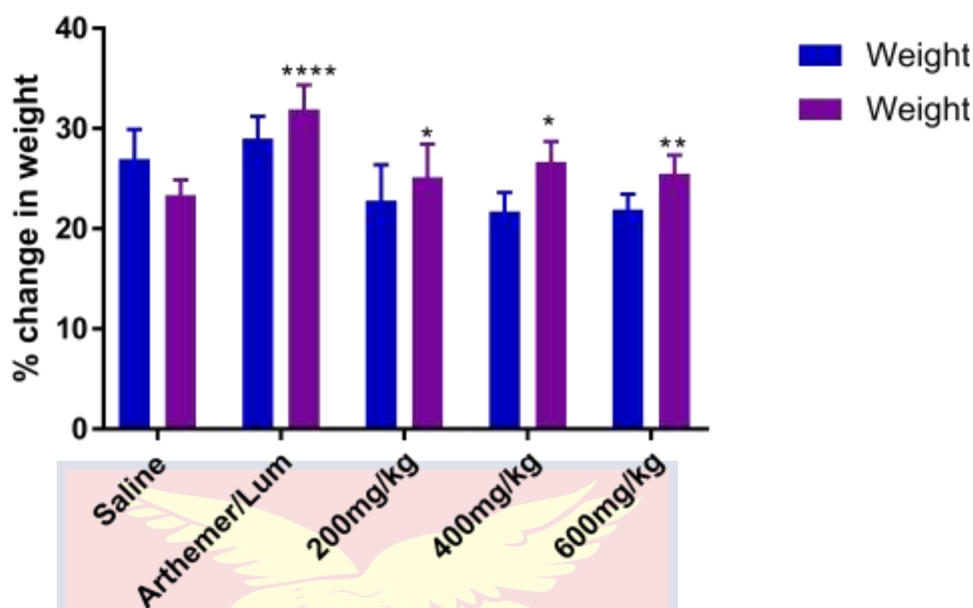

Data are expressed as mean±SEM (n=5); a, normal saline (control); b, art/lum; c, 200 mg/kg; d, 400 mg/kg; e, 600 mg/kg; [1]p< 0.0119 *, [2]p< 0.0013 **, [3]p< 0.0001 ****, Weight 1 = Weight before treatment; Weight 2 = Weight after treatment.

Figure 17*:* Body weight during Rane's test before and after administration of ethanol leaf extract of *C. procera* against *P. berghei* infected mice

Figure 17 revealed that, the weight (Weight 1) following the establishment of infection reduced in all the groups. After treatment, the weight (Weight 2) of all the doses increased whereas that of saline decreased. The weight in all the treatment groups were significantly higher than the saline group. 200 mg/kg (P< 0.0119); 400 mg/kg (P< 0.0119); 600 mg/kg (P< 0.0013) with that of the arthemeter/lumefantrine (P< 0.0001).

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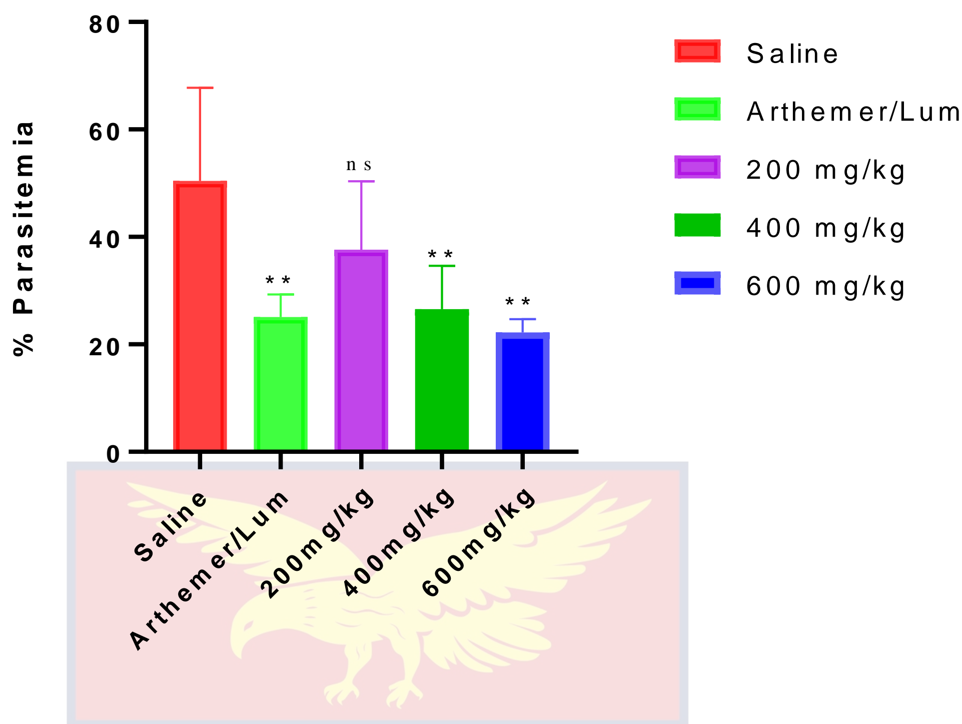

Data are expressed as mean±SEM (n=5); a, normal saline (control) b, art/lum; c, 200 mg/kg; d, 400 mg/kg; d, 600 mg/kg: [1]p< 0.2032 ns, [2]p< 0.0015 **

Figure 18: *Calotropis procera* shows prophylactic antimalarial activity against *Plasmodium berghei*

Figure 18 revealed that 200 mg/kg showed no significance (P< 0.2032) . At 400 mg/kg, the extract significantly (P< 0.0015) increase thereby reducing parasitaemia. At dose 600 mg/kg significantly high (P< 0.0015). The arthemeter/lumefantrine was significantly (P< 0.0015) high. This means that the crude extract of 400 mg/kg and 600 mg/kg significantly suppressed the parasite. Although complete eradication was not achieved, maximum suppression of parasitemia was noted by the positive control (arthemeter/lumefantrine).

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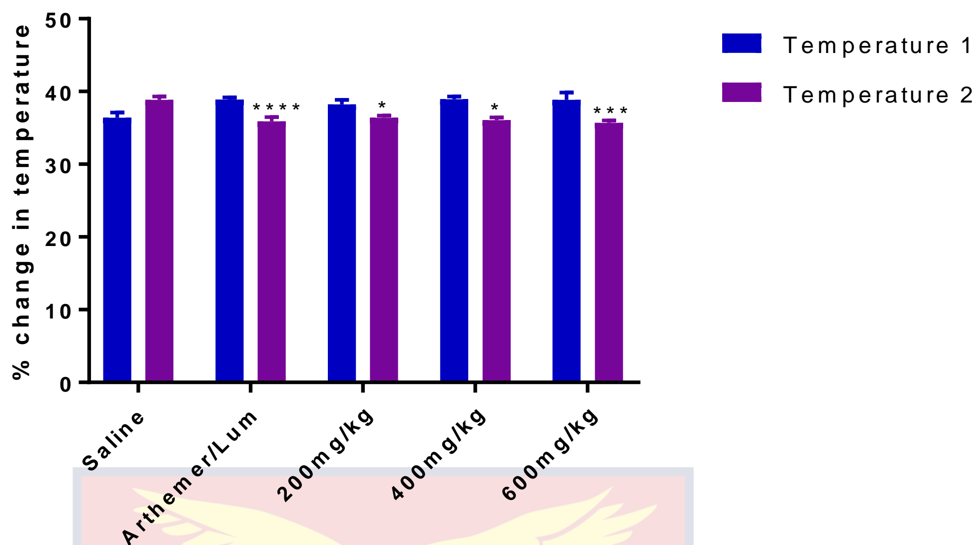

Data are expressed as mean±SEM (n=5); a, normal saline (control); b, art/lum; c, 200 mg/kg; d, 400 mg/kg; e, 600 mg/kg; [1]p< 0.0121 *, [2]p< 0.0003 ***, [3]p< 0.0001 ****, Temperature 1= Temperature before treatment; Temperature 2 = Temperature after treatment.

Figure 19: Temperature during Prophylactic Test before and after administration of ethanol leaf extract of *C. procera* against *P. berghei* infected mice

Figure 19 shows the rectal temperature (Temperature 1) of the animals when infection had been established. From the above figure, it was realised that after infection, the temperature of animals was high. After treatment, it was revealed that 200 mg/kg was significantly high (P< 0.0121). Dose 400 mg/kg increased significantly (P< 0.0121). At dose 600 mg/kg the extract significantly (P<0.0003) increased the temperature of the animals. For that of the arthemeter/lumefantrine, the level of significance (P<0.0001) was high.

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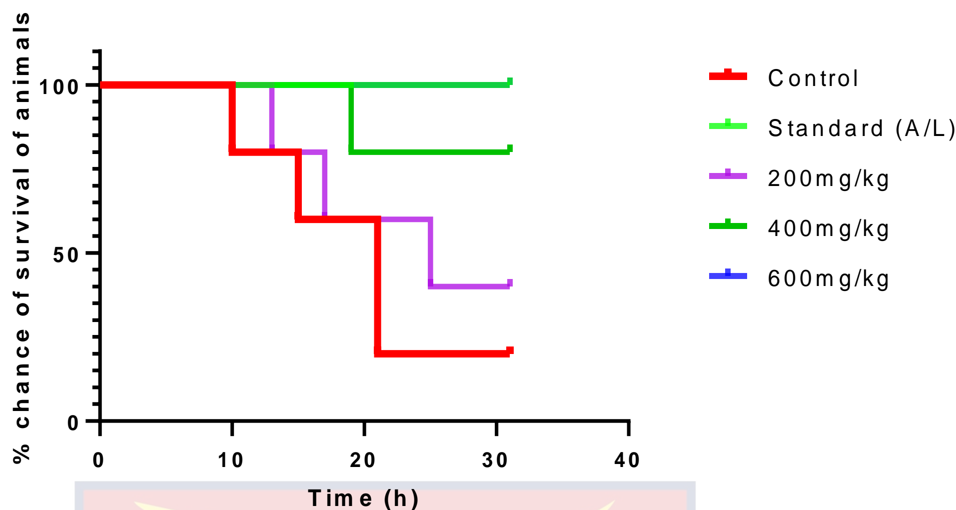

Figure 20: Crude Extract of *Calotropis procera* on the survival of mice in Prophylactic Test

Figure 20 shows the chance of the animals to survive after treatment is done. A period of 30 days was used to monitor the animals to elucidate their chances of surviving. It was realised that with the control, at Day 10, an animal died. Between Days 10-20 some animals also died, then from Days 20-30, the rest of the animals died. At dose 200 mg/kg, the animals' survived till after Day 10 when an animal died. Between Days 18–25 another animal died. At dose 400 mg/kg, all the animals survived till Day 20-30 when an animal died. At dose 600 mg/kg, all the animals survived throughout the period. The standard group as well survived within the period of 30 days when they were being monitored. Survival time of the infected mice pretreated with the crude extract indicated that only the highest dose was capable of significantly prolonging survival time compared to control.

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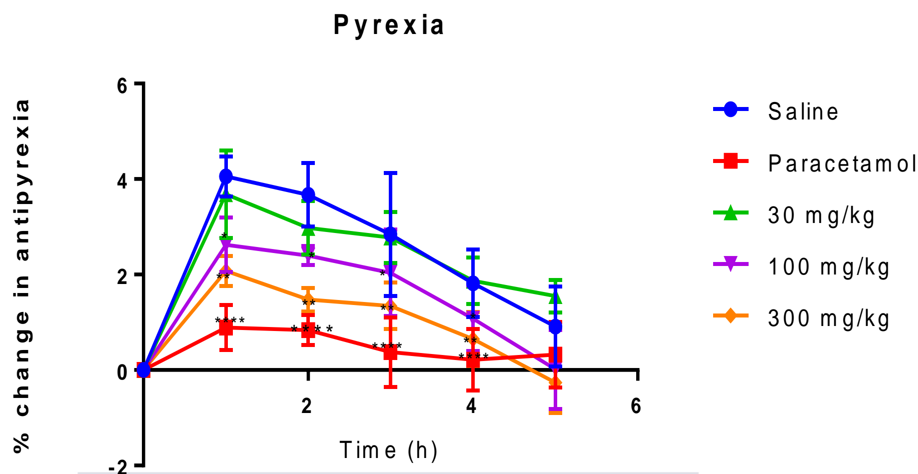

Data are expressed as mean±SEM (n=5); a, normal saline (control); b, art/lum; c, 30 mg/kg; d, 100 mg/kg; e, 300 mg/kg; [1]p< 0.0673 ns, [2]p< 0.0224*, [3]p<0.0002***, [4]p< 0.0001 ****

Figure 21: Antipyretic activity of *Calotropis procera* on Baker's yeast-induced pyrexia in mice

From figure 21, administration of Baker's yeast produced an increased pyrexia in the mice from zero (0) percentage to four (4) within the first hour of yeast injection. At doses 100 mg/kg, 300 mg/kg and paracetamol, the pyrexia significantly from 2 hours to 5 hours. *Calotropis procera* antipyretic activity at doses of 100 mg/kg showed slight level of signficance (P< 0.00224) and 300 mg/kg was significantly high (P< 0.0002). The crude extractat dose 30 mg/kg showed no significance difference (P< 0.0673). However, the standard drug, paracetamol 150 mg/kg demonstrated a higher (P< 0.0001) antipyretic activity.

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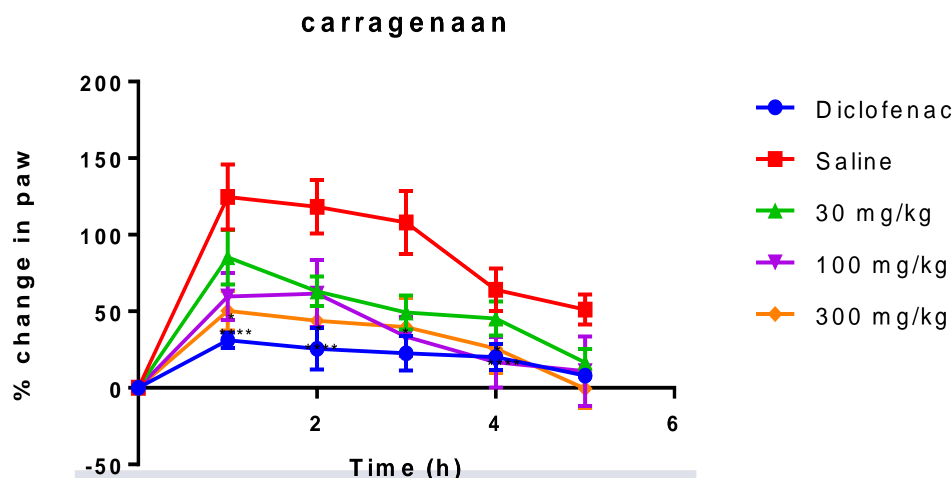

Data are expressed as mean±SEM (n=5); a, normal saline (control); b, art/lum; c, 30 mg/kg; d, 100 mg/kg; e, 300 mg/kg; [1]p< 0.0810 [ns], [2]p< 0.0144*, [3]p< 0.0001 ****

Figure 22: Effect of ethanolic leave extract of *C. procera* on carrageenan-induced oedema in mice

Figure 22 showed that inflammation increased gradually with all doses in the first hour. At dose 30 mg/kg the crude extract could not reduce inflammation significantly (P< 0.8107). At dose 100 mg/kg, there was no level of significance (P< 0.5098) but at dose 300 mg/kg the ethanolic leave extract decreased inflammation significantly (P< 0.0144). However, the standard drug, diclofenac 100 mg demonstrated the excellent anti-inflammatory activity (P< 0.0001).

Acute toxicity of *C. procera* on the brain, kidney, liver and spleen

In the *in vivo* acute toxicity studies of the plant extract, there were no gross physical and behavioural changes such as rigidity, sleep, diarrhoea, depression, abnormal secretion and hair erection for 24 h. All the mice survived within the 2-week observation period.

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Histopathological Studies

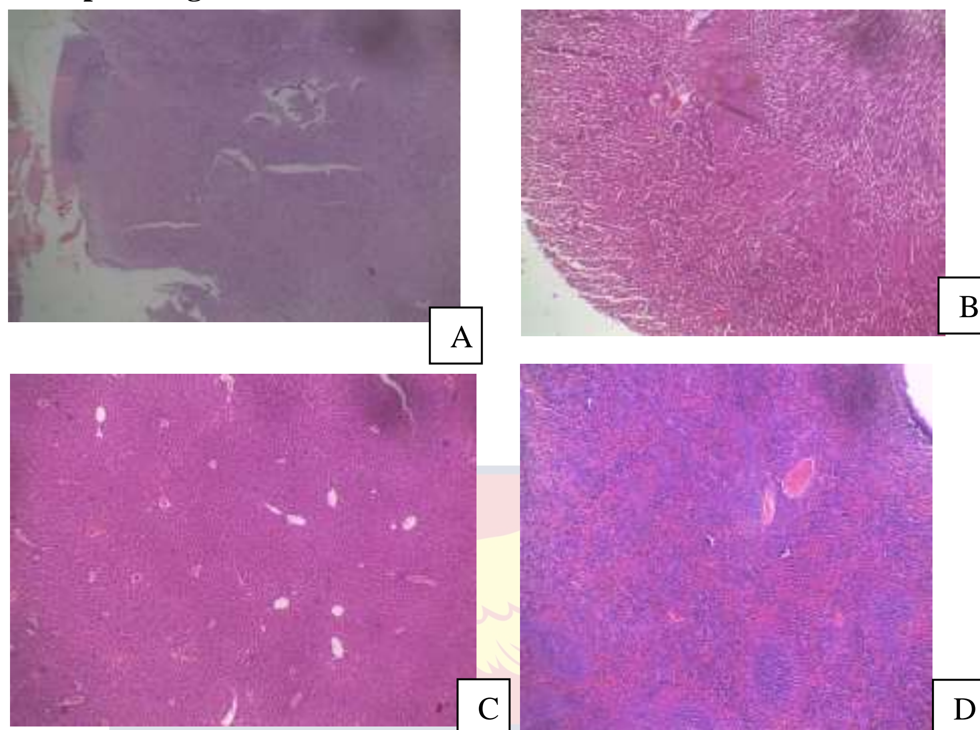

Figure 23(I) Photomicrographs of H and E stained sections of organs of mice treated with Artemether/Lumefantrin (Positive Control); (A) Brain, (B) Kidney, (C) Liver and (D) Spleen showing no signs of mild inflammatory changes with no tissue damages or injury at 400× magnification.

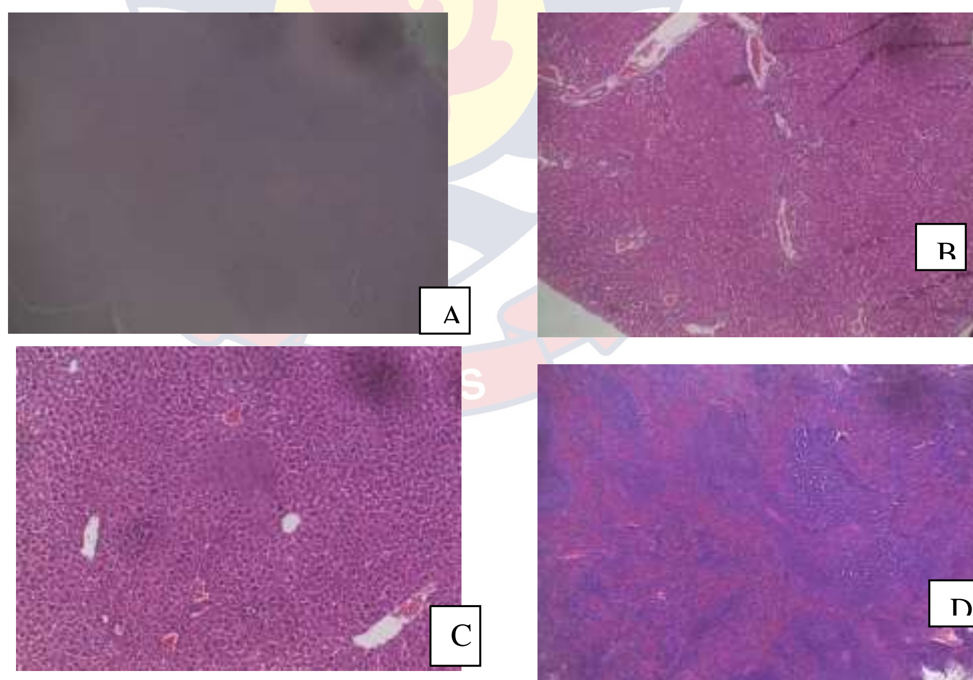

Figure 23 (II) Photomicrographs of H and E stained sections of organs of mice treated with Saline (Negative Control); (A) Brain, (B) Kidney, (C) Liver and (D) Spleen showing no mild inflammatory changes as well as no\tissue damages or injury at 400× magnification.

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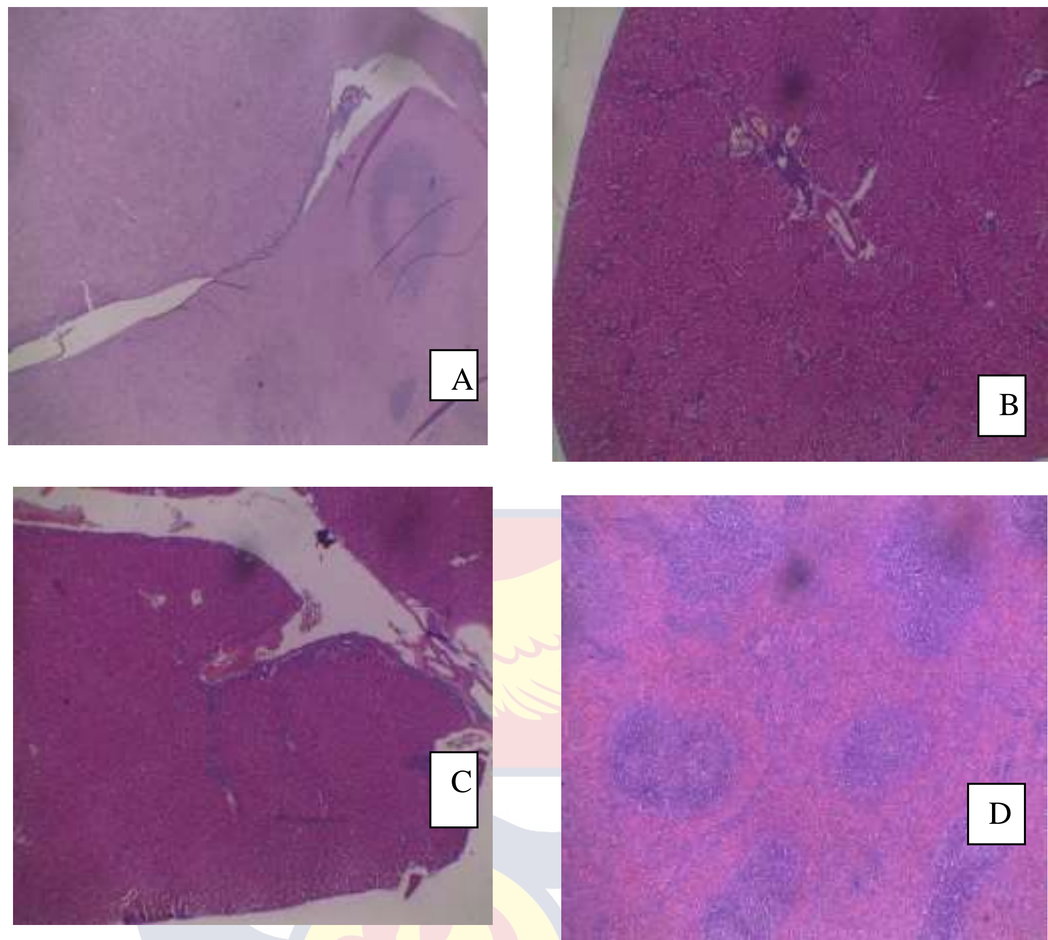

Figure 23 (III) Photomicrographs of H and E stained sections of organs of mice treated with *C. procera*. (A) Brain, (B) Kidney, (C) Liver and (D) Spleen showing mild inflammatory changes with no tissue damages or injury at dose 200 mg/kg, at 400× magnification.

→ Indicates congestion; → Indicates mild inflammation

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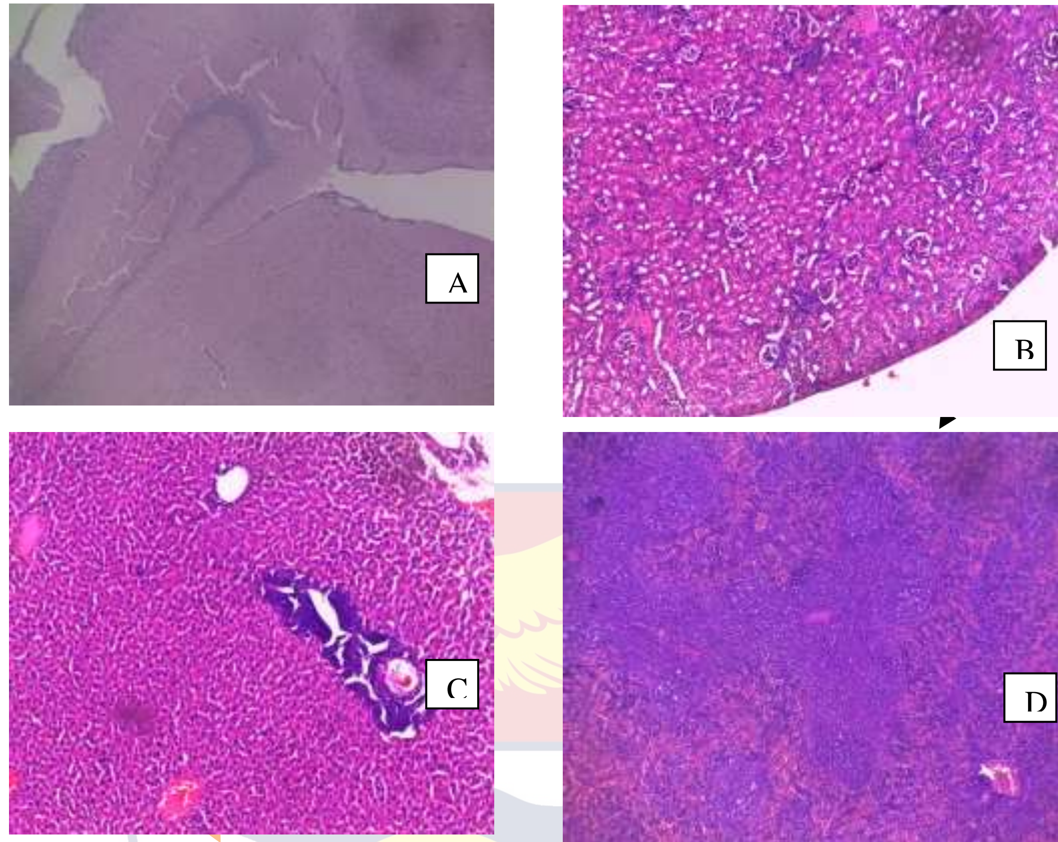

Figure 23 (IV) Photomicrographs of H and E stained sections of organs of mice treated with *C. procera*. (A) Brain, (B) Kidney, (C) Liver and (D) Spleen showing mild inflammatory changes with no tissue damages or injury at dose 400 mg/kg, at 400× magnification. → Indicates congestion; → Indicates mild inflammation

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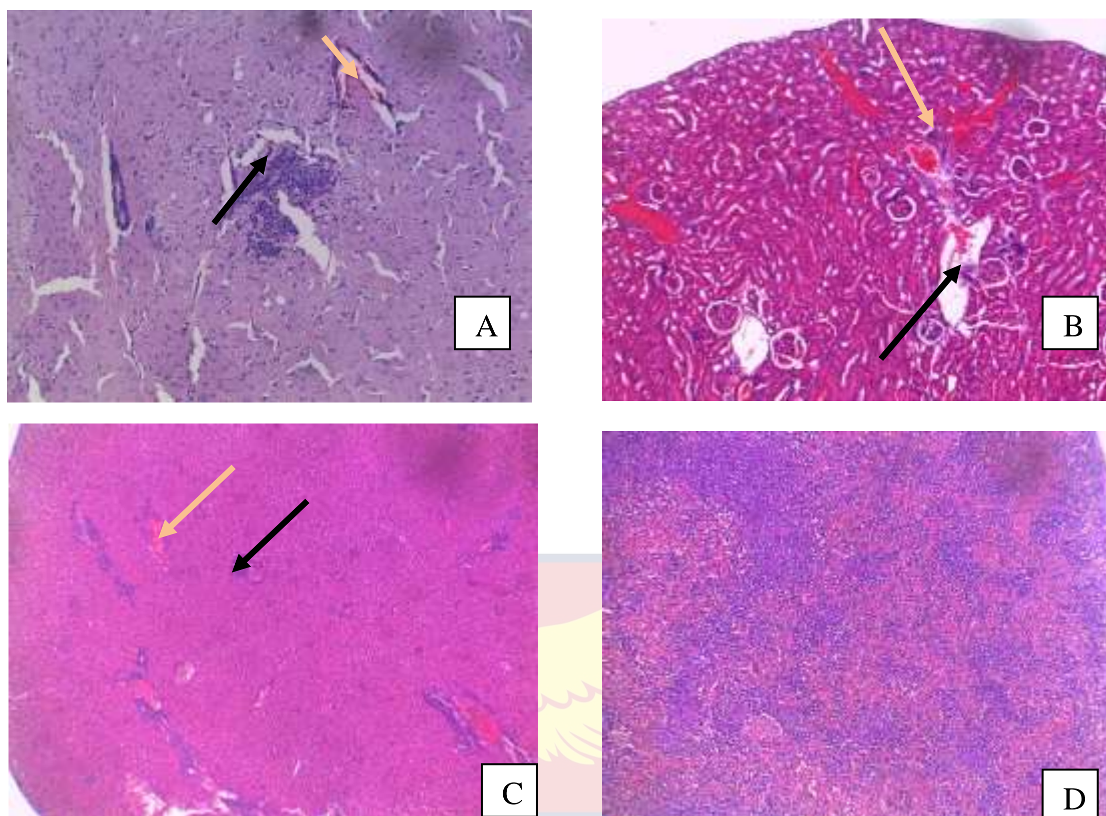

Figure 23 (V).Photomicrographs of H and E stained sections of organs of mice treated with *C. procera*. (A) Brain, (B) Kidney, (C) Liver and (D) Spleen showing mild inflammatory changes with no tissue damages or injury at dose 600 mg/kg, at 400× magnification. → Indicates congestion; → Indicates mild inflammation

From the figure 23 (I), the result shows that those that was administered with artemether/lumefantrin (positive control) had no mild inflammatory changes or any form of congestions as well as no tissues damages or injury to their brain, kidney, liver or spleen.

From the figure 23 (II), the result shows that the animals took the normal saline (negative control) and also had no mild inflammatory changes or any form of congestions as well as no tissues damages or injury to their brain, kidney, liver or spleen.

From the figure 23 (III), the result shows that at dose 200 mg/kg, the brain had no signs of inflammation and congestion. There was no tissue damage associated with the organ as well. At the dose 200 mg/kg, the kidney

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showed mild inflammatory change which is evident by lympohocytic infiltrate. The kidney did not exhibit tissue damage or injury. At dose 200 mg/kg the liver showed mild inflammatory change which is evident by accumulation of red blood cells within the microvascular. There was no tissue damage. The spleen did not show any sign of inflammatory changes and tissue damages.

From figure 23 (IV), at dose 400 mg/kg the results showed that the brain had no signs of inflammation and congestion. There was no tissue damage associated with the organ as well. The kidney showed mild inflammation which is evident by periglomerular chronic inflammatory cells. The kidney also had congestion with no change in tissue architecture. At the same dose 400 mg/kg, the liver exhibited mild inflammatory change which is evident by periportal inflammation. There was congestion with no change in tissue architecture. The spleen did not show any sign of inflammatory changes and tissue damages.

From figure 23 (V), the result shows that at dose 600 mg/kg the brain showed signs of mild inflammatory changes and congestion. There was no tissue damage associated with the organ. At the same dose, the kidney exhibited mild inflammatory change and congestion. The kidney had no tissue damage or injury. The liver showed exhibited mild inflammatory change as well as congestion. There was periportal inflammatory change in the liver. There was no significant tissue damage. The spleen did not show any sign of mild inflammatory changes nor tissue damages.

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Discussion

In vitro antiplasmodial activity of *C. procera* on *Plasmodiun falciparum* in mice

From figure 12, it was realised that the A/L and crude leaves extract exhibited antiplasmodial activity since IC_{50} values were <100 µg/mL. The parasitaemia suppressive effect of the plant could be attributed to the presence of alkaloids, flavonoids, saponins, and terpenoids. The suppressive effect of the plant is similar to other species of the same genus such as *C. gigantea.* A previous study also reported that flavonoids and steroids isolated from *C. gigantea* flower showed an *in vitro* antiplasmodial activity with IC_{50} between 50 and 100 µg/mL against *P. falciparum* strain. The chloroform extract of leaves and methanol extract of the stem did not show any antiplasmodial activity since the IC_{50} value were >100 µg/mL (Ochieng, Midiwo, Owuor, 2010). The differences in the results of the chloroform extract of leaves and methanol extract of the stem of *C. gigantea* to that of ethanol extract of *C. procera* might be due to the methodology used.

Wong et al (2011) assessed the antiproliferative (APF) and antiplasmodial (APM) activities of five selected Apocyanaceae species including *C. procera*, wherein it was found effective against 3D7 strain,which correlates with this study.The excellent *in vitro* antiplasmodial activity of the plant extracts in the present study might be due to the presence of strong phytochemical constituents such as alkaloids, phenols and flavonoids.

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***In vivo* antiplasmodial activity of *C. procera* on *Plasmodiun berghei* in mice**

The 4-day suppressive test, which mainly evaluates the antimalarial activity of extracts on early infections, was used in the study for determination of percent suppression of parasitaemia. As shown in figure 13, the 4-day suppressive study revealed that the crude extract exhibited a significant reduction of parasitaemia but at dose 200 mg/kg, there was no significance. This means the plant possesses antiplasmodial activity because as the dose was increased, the percentage parasitaemia reduced. This implies that leaf extract of *C. procera* has very high level of significance ($P<0.0001$) and dose-dependent chemosuppression. It indicates that the test compound is active in standard screening studies as revealed by Wong, Yau, Noor and Fariza, reported in 2011. The results with the high level of significance ($P<0.0001$) concord with results obtained by Bantie et al., (2014), when methanol leaf extract was used at a high dose. This could mean that the extract works best at high dose. Again, the results was compared with the results from the extracts of *Cassia occidentalis*, *Morinda morindoides*, and *Phyllanthus niruri* at dose of 400 mg/kg, the most active extract that was from *Morinda morindoides* reduced parasitaemia with the $P< 0.0066$ (Tona, Mesia, & Ngimbi, 2001). This results obtained had a similar P value at the same dose. This indicates that the plants extracts could have active compounds at that dose for antiplasmodial activity. These active compounds could include the presence of alkaloids, flavonoids, saponins (Nethathe, & Ndip, 2011).

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According to Krettli, Adebayo, and Krettli (2009), a sample must suppress parasitaemia by 30% in order to be considered active. Based on this assertion, the crude ethanolic leaf extract is active against malaria infection.

Anaemia is a common symptom of malaria-infected mice (White et al., 2014). Thus an ideal antimalarial agent obtained from plants is expected to prevent loss of red blood cells in infected mice due to the rise in parasitaemia. The PCV was measured to evaluate the efficiency of the ethanolic extract in preventing haemolysis due to escalating parasitaemia level. The fundamental cause of anaemia includes the following mechanisms: the clearance and/or destruction of infected RBCs, the clearance of uninfected RBCs, and erythropoietic suppression and dyserythropoiesis (White, 2017). Each of these mechanisms is concerned with both human and mouse malarial anaemia (Lamikanra, Brown, Potocnik, Casals-Pascual, Langhorne, Roberts, 2007). In both figures 14 and 15, there was an increase in PCV and haemoglobin respectively after treatment. In the PCV, the doses 400 mg/kg and 600 mg/kg were significant with the $P< 0.0117$ and the level of significance occurred in the positive control (arthemeter/lumifantrin) $P< 0.0112$. This means that the crude extract was able to significantly prevent PCV reduction. The tested plant extracts did show preventive effect on PCV in 4-day suppressive test.

For haemoglobin, the results revealed that the extract dose-depedently increased PCV and for that matter haemoglobin level. Reduction in PCV and haemoglobin are cardinal signs of malaria-infected mice (Moore et al., 2006). Hence, ideal plant extracts with antimalarial activity are expected to prevent malaria-associated reduction of PCV and haemoglobin due to rise in parasitaemia. The highest dose of *C. procera* showed a remarkable increment

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in PCV and haemoglobin compared to the infected but untreated mice. This activity might have resulted from the overall improvement in PCV and haemoglobin, and parasite clearance among treated mice (Suleman et al., 2018).

In the present study, the crude ethanolic leaf extract of *C. procera* reduced the level of parasitaemia. The present observation is supported by Chandel and Bagai (2010) study, which reported that the ethanolic extract of the leaves of *Ajuga bracteosa* reduced the number of *Plasmodium* parasites in a mouse model.

Ogbuehi, et al. (2014), have reported the suppressive, repository and therapeutic activity of the methanolic root extracts of *Anthocleista noblis*, *Nauclea latifolia* and *Napoleona imperialis* from medicinal plants in Nigeria responsible for reducing parasitaemia.

In the present study, the tested plant extracts did show an increment in the PCV and haemoglobin in 4-day suppressive test. This findings contradicts with previous studies on ethanolic root extract of *Dodonaea angustifolia* which did not show protection against reduction in PCV (Amelo, Nagpal, & Makonnen, 2014). This could be because of red cell destruction or spleen reticuloendotelial cell action (Bronte, 2013). This findings are in agreement with the report of Abdela, et al., 2014, who indicated that the extract and fractions of *G. ternifolia* significantly prevented PCV reduction in a dose-dependent manner. The increment in the PCV and haemoglobin might have emanated from the ability of the plant extract to reduce parasitaemia which resulted in an improvement in PCV and haemoglobin in the treated mice. The findings of Satish, et al., 2017 also showed that *Calotropis gigantea* plant

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extracts did not show any preventive effect on PCV reduction in 4-day suppressive test. However, the reduction of PCV was in slight variation when compared to the controls. This did not match the finding of this study and it might be due to the low dose of the extract.

The result in figure 16 revealed that the crude ethanolic extract exerted significant suppression of parasitemia. All treated groups brought about reduction of parasitemia at high doses. Since it is desirable to have curative activities in a phytodrug, it may be possible to consider this plant as a potential source of antimalarial agents. Hence from the result, it proves that *C. procera* has antiplasmodial activity to reduce parasitaemia. The result confirms that which was obtained by Balogun, et al., (2010). Again, similar results were achieved by Bero, Ganfon and Jonville (2009), when compared to the result of this study. The results obtained by Bero et al (2009) was due to the presence of alkaloids, phenolic compounds, and terpenoids in these extracts which could be responsible for their antimalarial activity. The alkaloids possess antiplasmodial properties.

Body weight loss is a common symptom of malaria-infected mice. So after treatment, as presented in figure 17, the results showed that the weight of the body of the animals increased due to reduction in parasitaemia. Thus ideal antimalarial agents obtained from plants are expected to prevent body weight loss in infected mice due to the rise in parasitaemia. Yen (2010), had a similar result despite the fact that in his work, the increase in weight was not consistent with increase in dose, the crude extracts of tested plants significantly prevented weight loss associated with the decrease in parasitaemia level in the curative test. The preventive effect of extract might

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be due the presence of saponins, flavonoids, glycosides and phenolic compounds found in the crude ethanolic extract. The study showed that the weight increase of the crude extract of *C. procera* was dose-dependent. This is similar to the finding of Bantie-Mekonnen (2015), who also showed that an increase in weight was not consistent with an increase in the dose, though the fruit extract of *Croton macrostachys* significantly prevented weight loss and this was associated with an increase in the parasitemia level, but that of the root extract of the same plant prevented weight reduction in a dose-dependent manner. This finding may indicate the presence of appetite-suppressant secondary metabolites in the fruit extract, rather than in the root extract, or some other difference. It was reported by Nureye, et al., (2018), that the highest doses of butanol and chloroform fractions showed a remarkable increment in body weight compared to the infected but untreated mice. This activity might have resulted from the parasite clearance among treated mice.

The results in figure 18 showed that the crude extract of 400 mg/kg and 600 mg/kg significantly suppressed the parasite when compared to the negative control. Although complete eradication was not achieved, maximum suppression of parasitemia was noted by the positive control. This could also mean that when the extract is taken before infection is established, there is the potential to suppress the parasite just as the standard would do. The study of Nureye, et al. (2018) produced similar $P < 0.001$ at maximum suppression. The results obtained concords that of the findings of the Nyandwaro, et al., (2020), in which the peptide extract exhibited dose-dependence suppressive activity. This might be due to the presence of flavonoides in the extract.

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In the prophylactic test, *C. procera* resulted in better efficacy than the negative control. The probable mechanism to produce prophylactic activity on *P. berghei* infection might be inhibiting proliferation of parasites due to direct cytotoxic effect (Golenser, Waknine, Krugliak, Hunt, & Grau, 2006) and modulation of the membrane of the erythrocytes preventing parasite invasion (Hansen, 2012). In the prophylactic test, the crude ethanolic extract significantly suppressed the parasitaemia level in the mice at high doses . The active compounds such as flavonoides, triterpenoids and steroids could have helped to suppress the parasites.

Again, reduction in body temperature is a cardinal sign of malaria-infected mice. Hence, ideal plant extracts with antimalarial activity is expected to prevent malaria-associated reduction of temperature due to the rise in parasitaemia (Amelo et al., 2014). There was a significant improvement in the body temperature after treatment with the extract and this might be due to parasite clearance among treated mice. The result obtained is in agreement with the findings of previous studies on aqueous fraction of other plants (Amelo, Nagpal, & Makonnen, 2014; Abdela, et al., 2014). The findings also support the findings of Nureye et al. (2018), that all doses of the crude *Gardenia ternifolia* extract and middle and larger doses of butanol and chloroform fractions prevented the decrease in rectal temperature associated with infection. Overall, this activity might probably indicate the ability of plants to ameliorate some pathological processes of malaria that cause reduction in body temperature. In another study, crude leaf extract of *Ajuga remota* prevented body temperature drop at 50 and 100 mg/kg doses on day 4 as compared to day 0 and this may indicate that the plant at low dose has

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activity to reduce rectal temperature compared to that of *C. procera* (Nardos, Makonnen, 2017).

According to Nardos and Makonnen (2017), the crude extract of *Ajuga remota* increased the survival time of mice at all dose levels. That of the *C. procera* was dose–dependent. This could mean that the crude extract of *Ajuga remota* might have activity for suppression of parasitaemia at low dose and hence enhance the survival of the mice. The findings of Amelo et al., 2014 showed that the n-butanol and chloroform *Dodonaea angustifolia* fractions prolonged the mean survival time of the study mice. This confirms the results of the study. This may indicate that the extract suppressed *P. berghei* and reduced the overall pathologic effect of the parasite on the study mice. But all doses from aqueous *Dodonaea angustifolia* fraction were not able to significantly prolong the mean survival time as compared with control group. This contradicts the findings of the study as that of the *C. procera* was dose-dependent. This may be due to the less schizoticidal activity of the fraction as compared to the other two fractions.

From figure 21, the result showed that *Calotropis procera* at doses 100 mg/kg and 300 mg/kg and paracetamol reduced the body temperature significantly from 2 hours to 5 hours. The antipyretic activity of the plant extract at doses of 100 mg/kg ($P< 0.00224$) and 300 mg/kg ($P< 0.0002$) was significantly higher than that of control. Though at dose 30 mg/kg it is seen that there is reduction in high temperature yet it was not significantly ($P< 0.0673$) noticed. This might be due to the fact that at low doses the extract is not able to reduce high temperatures. The study showed that crude ethanolic leaf extract of *C. procera* possessed significant antipyretic activity in Baker's

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yeast-induced pyrexia at doses 100 mg/kg and 300 mg/kg. The standard (paracetamol) achieved maximum antipyretic activity in 3 hours. On the other hand, maximum antipyretic activity for *C. procera* at dose 300 mg/kg occurred at 4 hours, indicating slow but steady absorption of the drug from the GIT; this may have been responsible for the prolonged action of the extract. The antipyretic activity of the extract was dose-dependent with the higher dose producing greater activity. The result obtained is in agreement with that which was reported by Dewan, Suresh and Vijay, (2000)

Rang, Dale and Ritter (2001), assert that paracetamol possesses potent antipyretic and analgesic activities with minimal anti-inflammatory activity. It may selectively inhibit specific COX isoform in the CNS to inhibit prostaglandin synthesis to achieve its antipyretic effect but does not influence body temperature when it is elevated by factors such as exercise or increase in ambient temperature (Goodman, & Gilman 2001). Phytochemical compounds such as steroids, carbohydrates, tannins, triterpenoids, flavonoid and glycosides were found to be present in the extract during phytochemical screening. The antipyretic potentials of steroids, tannins, triterpenoids, flavonoid and glycosides have been reported in various studies (Kim, Son, Chang, & Kang, 2004; Niazi, Gupta, Chakarborty, Kumar, 2010). Therefore, the antipyretic activity of *C. procera* may be due to its contents of steroids, tannins, triterpenoids, flavonoid and glycosides.

Furthermore, indirect evidence seems to support the influence *C. procera* of on the biosynthesis of prostaglandin (PGE 2) which is a regulator of body temperature; this may also partly account for its antipyretic activity in yeast-induced pyrexia model (Dewan,Suresh, & Vijay 2000).

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Body defense mechanism, commonly known as inflammation, is a response to many physiological conditions such as infection and thermal and/or physical injuries (Lintermans et al., 2014). Inflammatory response is necessary for the survival against environmental pathogens and harms. Inflammation is categorised into five cardinal signs which are known as redness, swelling, heat, pain and loss of function (Purnima, et al., 2010). Prostaglandins are produced by the cells which are involved in the production of pain, fever and inflammation. Several enzymes such as cycloxygenase including COX-1, COX-2, and COX-3 are responsible for the production of prostaglandin (Purnima, et al., 2010). COX-2 is responsible for promoting pain, inflammation and fever by producing the prostaglandin. Hence by inhibiting the cyclooxygenase enzyme, prostaglandin production can be blocked (Patrignani, 2005). Nonsteroidal anti-inflammatory drugs (NSAIDs) are usually indicated in order to relieve the symptoms. By using the NSAIDs and other opiods, many side effects can occur such as gastric lesion; so the uses of these drugs are not successful (Patrignani, 2005.

From figure 22, the result from the study showed that at doses 30 mg/kg and 100 mg/kg, the extract was not able to reduce inflammation. This could mean that at low doses the extract may not work effectively on inflammation. At dose 300 mg/kg, the ethanolic leave extract of the *C. procera* was found to decrease inflammation. This result differs from what was obtained by Purnima et al., 2010. In that study, the anti-inflammatory activity at the dose of 100 mg/kg of extract of *Mimusops elengi* was evaluated by measuring the average volume of the paw oedema at different time intervals. This might be due to the

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presence of terpinoids in the extract and this is a good anti-inflammatory compound.

In carrageenan-induced oedema, crude ethanolic extracts of *C. procera* showed a significant effect at the early stage of inflammation, which could be attributed to the involvement of histamine, kinins, and serotonin release, while later, there was a further reduction in paw oedema, which could be attributed to the release of prostaglandins. Involvement of flavonoids in the reduction of inflammation is reported (Jude, Anwanga, Samuel, & Louis, 2012).

The results of the acute toxicity of *C. procera* revealed that there was no mortality observed up to the maximum dose level of 600 mg/kg body weight of the extract administered orally, where single high dose is recommended for testing acute toxicity (OECD, 2008).

Liver, kidney and spleen are organs of metabolism and excretion which are likely affected by potentially toxic agents (Kripa, Chamundeeswari, & Thanka, 2011). Some phytochemical contents from *C. procera* extracts such as cardenolides and alkaloids are known to have toxic effect (Al-Snafi, 2015). Their accrual after repeated dose administration might affect the thrombopoiesis and produce harmful effects on spleen, liver and the kidney function (Hayashi, 2014). The result (H & E) revealed that administration of *C. procera* caused mild inflammatory changes on the kidney and liver without tissue damage at all doses. There were chronic inflammatory cells appearing in the treatment groups. This result confirms a previous study conducted by Shahat and Shihata (2012) who reported similar disturbance in biochemical and hematological markers after long term administration of *C. procera* extract and suggested harmful effects.

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At all doses, the spleen showed congestion which was as a result of the extract taken. The spleen represents an important clearance site for some chemicals (Cataldi et al., 2017). An accumulation of these substances leads to alterations and changes in spleen histology (Cataldi, et al., 2017). However, liver and splenic congestion may be due to difficulty in venous drainage, infiltration of elements produced by metabolic disorders or haematological cancer as confirmed by Petroianu, (2007). But since the spleen did not experience any form of congestion, then it may be due the fact that it was having hyperglycaemia.

From figure 23 (I, II and III) at doses 200 mg/kg, 400 mg/kg and 600 mg/kg, the kidney showed signs of congestion. The congestion of the kidneys suggest the failure of the kidney to excrete potassium, leading to increased levels of potassium in the extracellular fluid and blood urea(Morag, 1989).

At doses 200 mg/kg and 400 mg/kg, the brain exhibited no signs of mild inflammation and congestion. At dose 600 mg/kg, the brain exhibited signs of mild inflammation and congestion. This could mean that the extract at low doses do not have adverse toxic effect on the brain but would show some toxic effect on the brain.

Chapter summary

The result of this study showed that the crude ethanolic leaf extract of *Calotropis procera* has antiplasmodial activity on chloroquine-sensitive *Plasmodium falciparum* (3D7 strain). Again in the *in vivo* work carried out, it came to light that there was antiplasmodial activity of the plant extract on *Plasmodium berghei*.

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The toxic effect of the plant on the organs of the animals was not so severe though the plant is deemed to be that poisonous.

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CHAPTER FIVE

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

Introduction

Malaria is one of the highest recorded deadly diseases of the world of which Ghana is not an exception. The *Plasmodium* parasite is now building resistance to lot of the currently available orthodox drugs. This has necessitated the need to research into plant medicines which will help to drastically reduce the multiplication of the parasite or eliminate the parasite once found in the host.

Calotropis procera is a medicinal plant has been found to cure lots of diseases but few studies have been carried out on its ability to work on the malaria parasite. This study seeks to assess the efficacy of the *Calotropis procera* on *Plasmodium berghei* in mice.

Summary of Study

The purpose of the study is to determine the efficacy of *Calotropis procera* extracts on *Plasmodium berghei* in Swiss albino mice. The study focused specifically on: (a) the *in vitro* antiplasmodial activity of the crude ethanol leaf extract of *Calotropis procera* against chloroquine-sensitive *Plasmodium falciparum* (3D strain), (b) the *in vivo* antiplasmodial activity of the crude ethanol leaf extract of *Calotropis procera* against *Plasmodium berghei* (ANKA strain), (c) to investigate the toxic effect of crude ethanol leaf extract of *Calotropis procera* on the brain, kidney, liver and spleen.

Swiss albino mice were purchased from the animal house near Korle-bu Teaching Hospital in Accra, Ghana. Mice were maintained in the laboratory under standard condition (temperature of 22 ± 3^0C, relative

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humidity of 40 - 50% and 12 h light/12 h dark cycle) with a commercial food and water *ad libitum*. Mice were acclimatised for one week before the study. Purposive sampling technique was used to select the mice. The sum of 195 mice was used in the study.

The data was collected and statistically analysed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test for *in vitro* antiplasmodial test, Rane's test, 4-day suppressive test and prophylactic test. Two-way analysis of variance (ANOVA) followed by Tukey–Kramer's multiple comparison test was used to analyse data on rectal temperature, body weight, carragenaan, pyrexia, haemoglobin level and PCV. Data on the survival curve was carried out using chi square (log-rank test). Results were deemed statistically significant if $P < 0.05$ at 95% confidence intervals.

Key Findings

The *in vitro* study carried out revealed a mean $IC_{50} = 83.91 \pm 0.375$, which shows that there is antiplasmodial activity of the plant extract on the *Plasmodium falciparum*.

The *in vivo* study carried out with respect to the 4-day suppressive test, Rane's/curative test and the prophylactic test also showed positive results. In the 4-day suppressive test, quite a significant percent of the parasites were killed by the crude extract at high dose. The PCV after treatment though increased, did not exhibit any level of significance except at the high dose which showed slight level of significance. Similarly there was an increase of hemoglobin in mice treated with *C. procera* extract. In the Rane's test, there was significant drop in the parasitaemia since the extract is dose-dependent. In

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the test, the weight of the animals significantly increased as the dose was increased. The Prophylactic test also revealed that there was significant suppression in parasites as the dose was increased. Again there was significant increase in temperature after treatment. This enhanced the survival of the animals. The antipyretic effect of the plant extract also significantly reduced pyrexia at high dose. The extract slightly reduced inflammation at high dose and this shows that the extract activity on inflammation is minimal.

Histopathological studies revealed that as the dose increases, there was mild inflammation and congestion on the kidney, liver and spleen with no change in tissue architecture. The brain did not have any of such inflammations and congestion.

Conclusions

The study has shown that there is high prevalence of reported malaria cases in various health facilities of which most deaths have been recorded. The study also revealed that there is an increasing level of resistance to most drugs by the malaria parasite. Though some refined drugs remains fairly active, an increase in resistance to newly made drugs by the parasite is a cause of concern.

The results obtained from the study shows that the plant crude ethanol extract had antiplasmodial activity to the parasite. But from the study it was revealed that the plant has toxic effect on the organs of animals. Hence in its refinery the compound that makes it so should be eliminated so as to enhance its potency to treating malaria without any further problems.

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Recommendation

As malaria is one of the deadly diseases that developing countries (of which Ghana is no exception) have been struggling with in recent times, it is therefore necessary for the government to create funds for research institutions to carry out a series of work on other plants to elucidate its potency in treating the malaria disease.

Suggestions for Future Work

Although the study provides insight into the curative nature of the plant, no work was done about its efficiency at the molecular level. Future perspectives could include;

The study was exploratory in nature and in order to accept or refute the findings of the study and generalise them for the usage in research institutions, it is suggested that the study is replicated by other researchers.

The potency of the plant at the molecular level should also be considered and worked on if need be.

Further research can also be conducted to confirm the toxicity of the plant since the findings of the study showed mild inflammation on some vital organs of the animals.

VERITAS LUMEN NOBIS

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APPENDICES

Table 1: Media, reagents and solutions

Media, Reagents and Solutions	Preparation	Manufacturer, town/country
Normal Saline	0.9g/100mL of water	Oxoid, England
Mission Hb Haemoglobin Testing System	Haemoglobin and Haematocrit in whole blood	Acon, USA
Serological pipette 5mL	Individually wrapped, 50 per bag, 6 per carton, 300 pieces	VWR, Taiwan
Serological pipette 10mL	Individually wrapped, 50 per bag, 4 bags per carton, 200 pieces	VWR, Taiwan
Serological pipette 25mL	Individually wrapped, 50 per bag, 4 bags per carton, 200 pieces	VWR, Taiwan
Pipette tips 2-200uL	Bulk, yellow,1000 pieces	VWR, Taiwan
Pipette tips 50-1000mL	Bulk, blue, 1000 pieces	VWR, Taiwan
Pipette tips 0.1 – 10uL	Bulk, 1000 pieces	VWR, Taiwan
15mL centrifuge tubes	Sterile, with screw caps, 50/rack	VWR, Taiwan
50mL centrifuge tubes	Sterile, with screw caps, 50/rack	VWR, Taiwan
25cm^2 culture flask	Plug-seal, Non treated, Pyrogen-Dnase free	VWR, Taiwan
96 well plate	Flat-bottom plate, sterile	VWR, Taiwan
1.5mL microcentrifuge tube	Sterile, colourless with attached lid	VWR, Taiwan
Reagent reservoir	Individually wrapped, Bulk, 100/pack	VWR, Taiwan
Cryogenic tubes	Sterile,1.8mL	VWR, Taiwan
Complete filtration unit/ Bottle top filter	PVDF, 0.22um	VWR, Taiwan
Nitrile Examination Gloves	Medium size, 100/box	VWR, Taiwan
IMDM powder	L-glutamine, phenol red, sodium pyruvate	Gibco, USA
Gentamicin	50mg/ 100mL	Sigma, Japan
Taylor Wharton Liquid Nitrogen Tank	35L Open Dewar	Taylor Wharton, Belgium
DMSO		Fisher Scientific, UK
RPMI 1640 medium, powder	with L-glutamine and 25 mM HEPES, without sodium bicarbonate	Sigma, Japan
Vacutainer	ACD-A 1.5mL	BD Vacutainer, USA
Invitrogen SYBR Green 1 Nucleic Acid Gel	10000X concentrate in DMSO	Invitrogen, Carlsbad,USA

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Stain		
Albumax II	Lipid-rich bovine serum albumin	Gibco,USA
BD Vacutainer ACD Tubes	Pack of 100, yellow closure	Fisher Scientific, UK
Mixed gas	O_2, N_2, CO_2	Airliquide, Paris, France
Single Channel pipette	Volume 0.5uL – 10uL. D-10	Sigma, Japan
Single Channel pipette	Volume 2uL – 20uL. D-20	Sigma, Japan
Single Channel pipette	Volume 20uL – 200uL. D-200	Sigma, Japan
Single Channel pipette	Volume 100uL – 1000uL. D-1000	Sigma, Japan
Multi 12 Channel pipette	Volume 10mL – 200uL. D-200	Sigma, Japan
Viroskan multimode microplate reader	Absorbance, Fluorescence intensity, Luminescence	Sigma, Japan
Leave and stem bark mill	and Time VLOLOTDO Serial no 981109	Glen Creston,Canada Fisher Scientific Int.,
Formaldehyde solution	10-14% methanol added as a stabilizer	UK TEK, China
Tissue processor		RTS, Ger
Rotary Microtome	Tissue processing	Leica, Germany
Cold plate	RM2125	Leica, Germany
Heating area	EG1150C	Leica, Germany
Themostat oven	EG1150H	Leica, Germany
Stainer	DHG-9035A	Boekel Scientific, Ger.
Water bath	XL	Cos bio,Delhi
Liquid haematoxylin		Cosmos biomedical,D
compound	5.35mL acetic acid	Huida, China
Eosin	1% Aqueous	Bliss GVS Pharma,
Microscope slides	1”×3”, 1mm-1.2mm thick	India
Lonart DS	Artemether 80mg, Lumefantrine 480mg,	Greenlife Medics, Canada
Digital thermometer	excipient q.s	Adam equip, UK
	Mercury, graduated seal	Olympus, Japan.
Weighing scale	tube	Oneonta, USA
Microscope		
Venier calliper		

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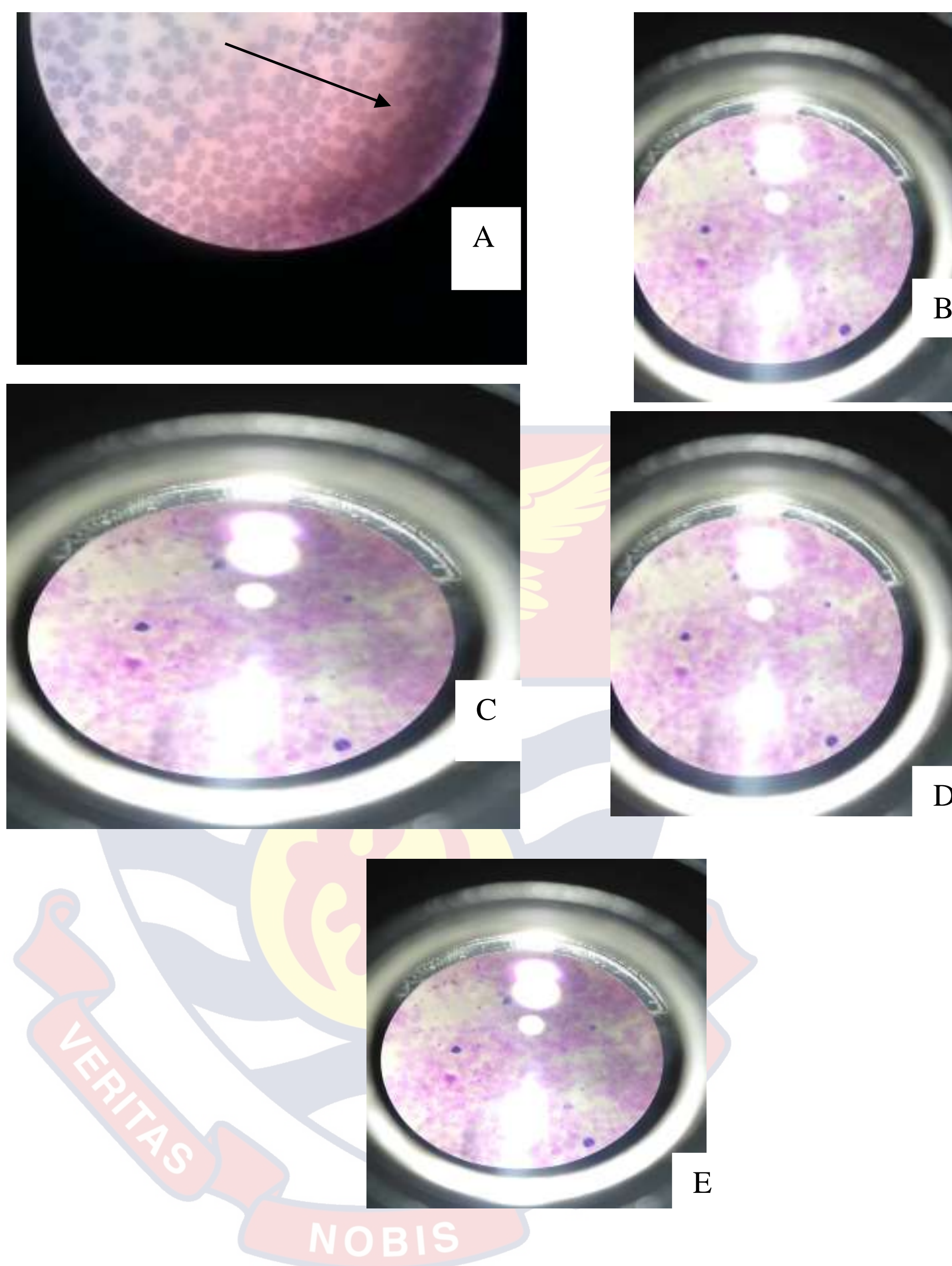

Fig. 1: Microscopic observations of *P. berghei* inhibition after treatment with (a) Artemether/Lumefantrine (Standard); (b) Saline (Control); (c) *C. procera* leaf extracts at concentration (200 mg/kg); (d) *C. procera* leaf extracts at concentration (400 mg/kg); (e)*C. procera* leaf extracts at concentration (600 mg/kg); withGiemsa stain at 400× magnification showing the ring stage for 4-days Suppressive Test

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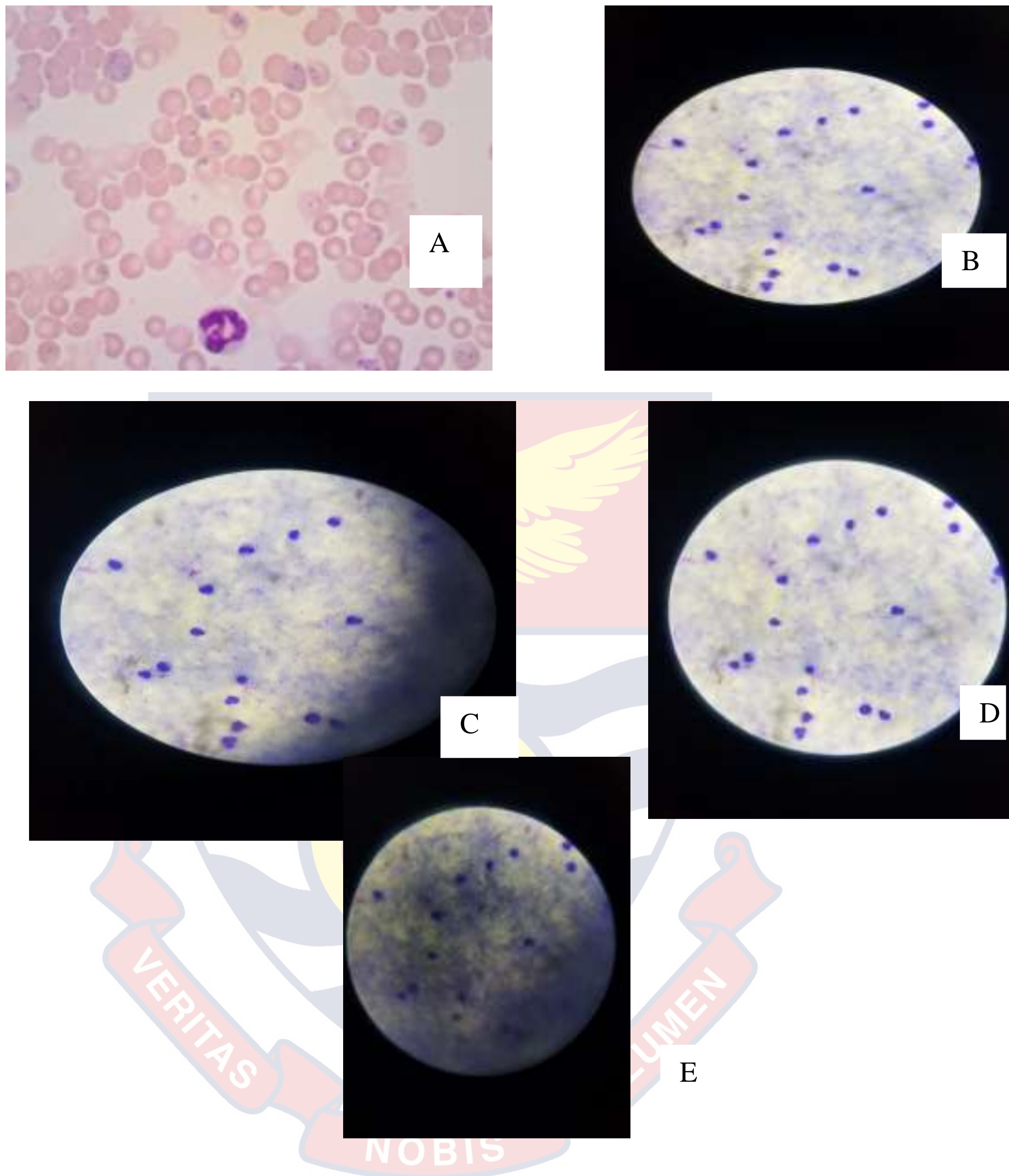

Fig. 2: Microscopic observations of *P. berghei* inhibition after treatment with (a) Artemether/Lumefantrine (Standard);(b) Saline (Control); (c) *C. procera*leaf extracts at concentration (200 mg/kg); (d) *C. procera* leaf extracts at concentration (400 mg/kg); (e)*C. procera* leaf extracts at concentration (600 mg/kg); withGiemsa stain at 400× magnification showing the ring stage for Curative/Rane's test.

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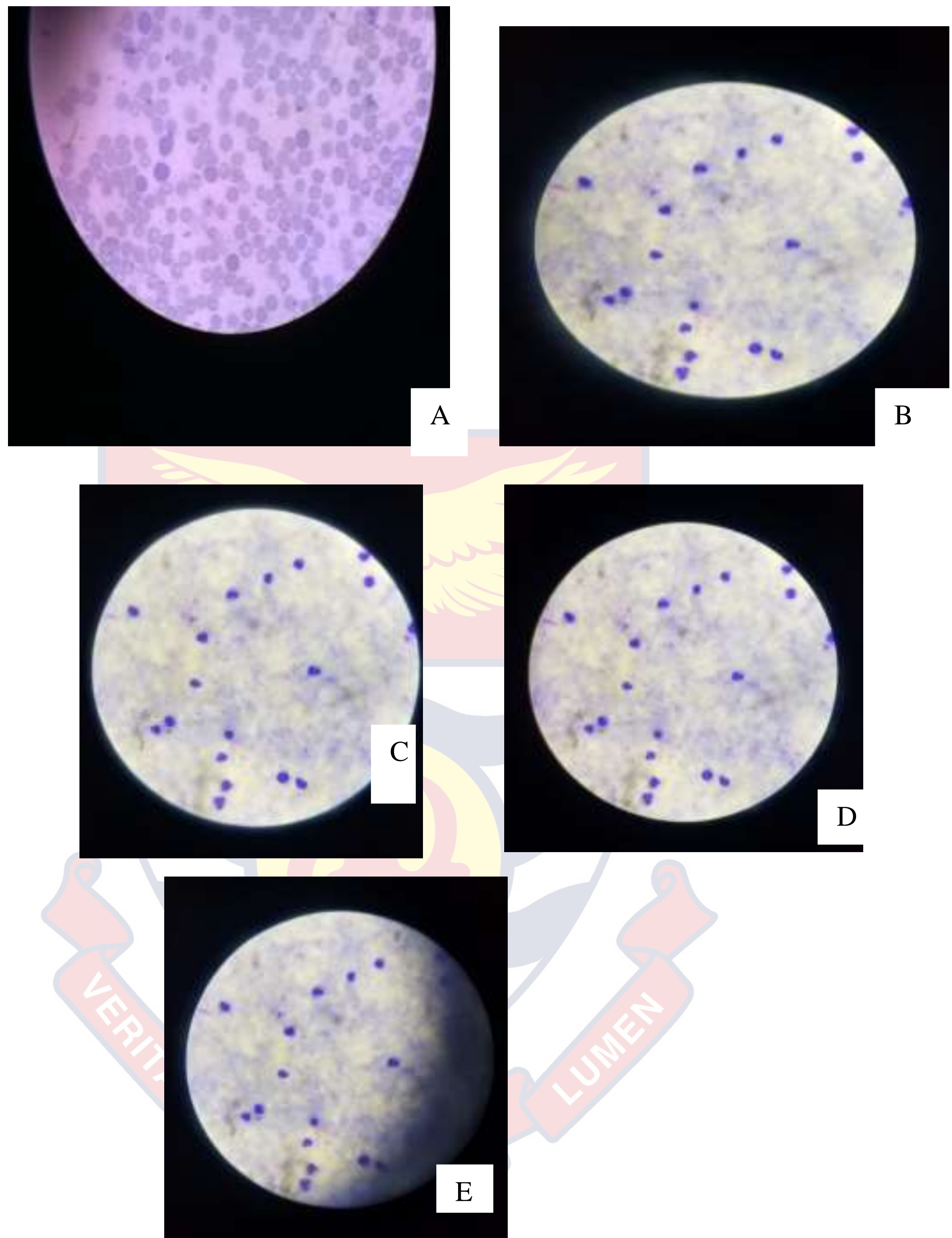

Fig. 3: Microscopic observations of *P. berghei* inhibition after treatment with (a) Artemether/Lumifantrin (Standard); (b) Saline (Control); (c) *C. procera* leaf extracts at concentration (200 mg/kg); (d) *C. procera* leaf extracts at concentration (400 mg/kg); (e)*C. procera* leaf extracts at concentration (600

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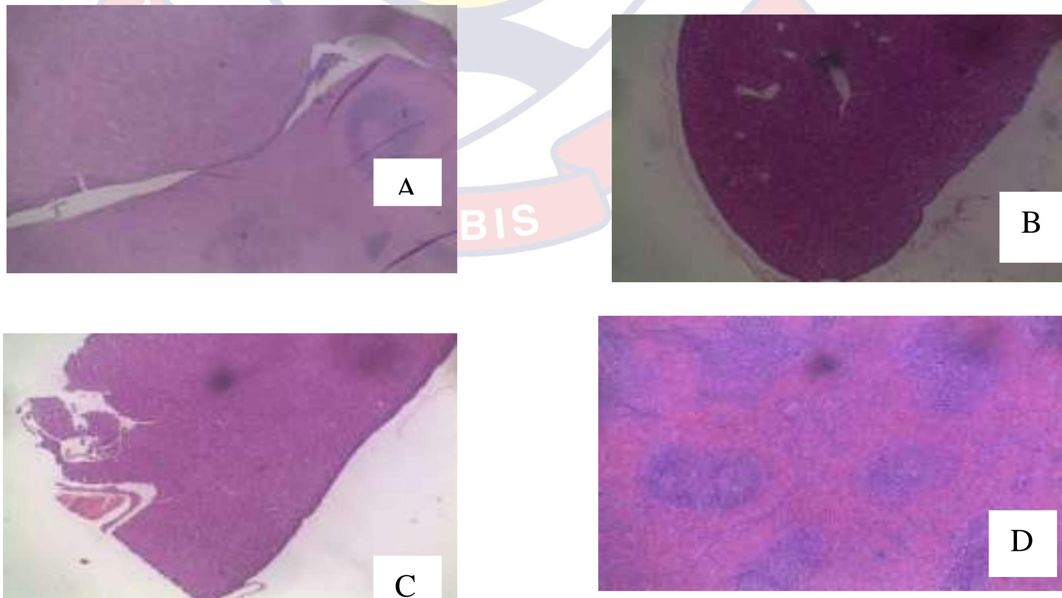
mg/kg); with Giemsa stain at 400× magnification showing the ring stage for Prophylactic test.

Calotropis procera crude ethanol extract

Histopathological Slides



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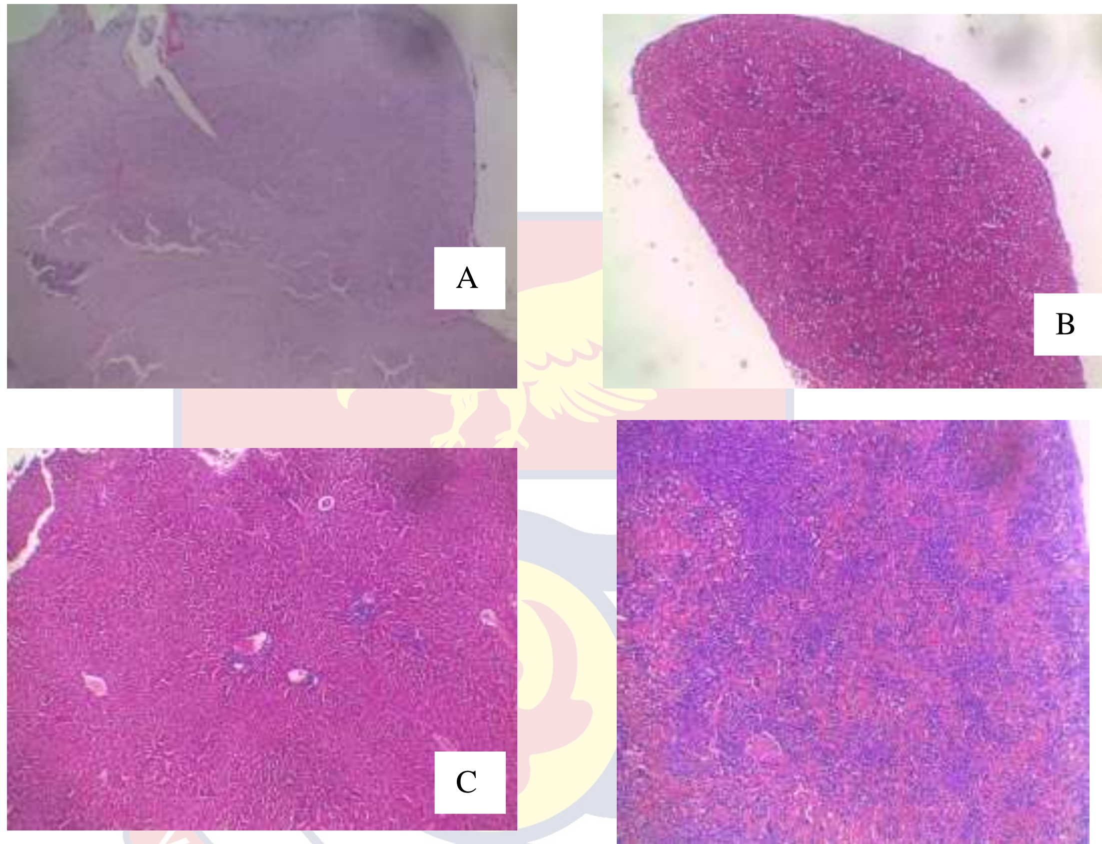
Photomicrographs of H and E stained sections of organs of mice treated with *C.procera*. (A) Brain, (B) Kidney, (C) Liver and (D) Spleen showing mild inflammatory changes with no tissue damages or injury at dose 200 mg/kg, at 400× magnification.



Photomicrographs of H and E stained sections of organs of mice treated with *C.procera*. (A) Brain, (B) Kidney, (C) Liver and (D) Spleen showing mild inflammatory changes with no tissue damages or injury at dose 400 mg/kg, at 400× magnification.

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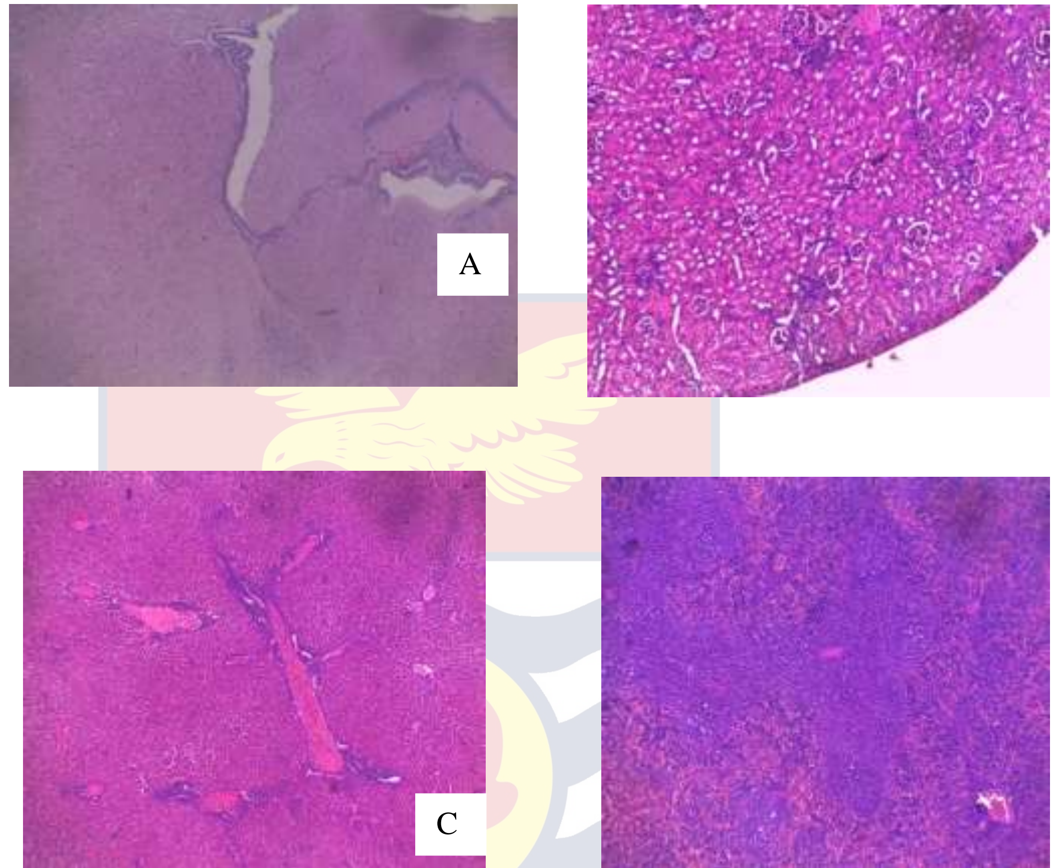

Photomicrographs of H and E stained sections of organs of mice treated with *C.procera*. (A) Brain, (B) Kidney, (C) Liver and (D) Spleen showing mild inflammatory changes with no tissue damages or injury at dose 600 mg/kg, at 400× magnification.

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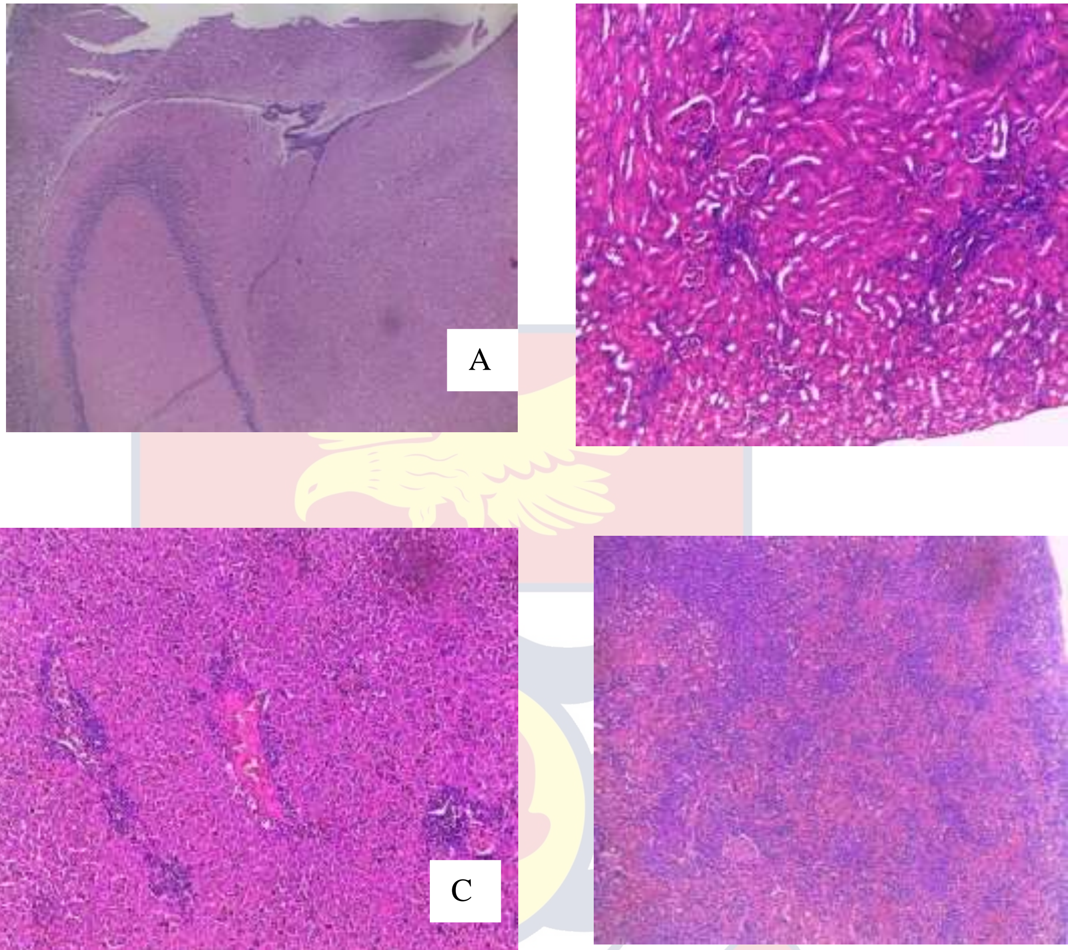

Photomicrographs of H and E stained sections of organs of mice treated with *C.procera*. (A) Brain, (B) Kidney, (C) Liver and (D) Spleen showing mild inflammatory changes with no tissue damages or injury at dose 600 mg/kg, at 400× magnification.

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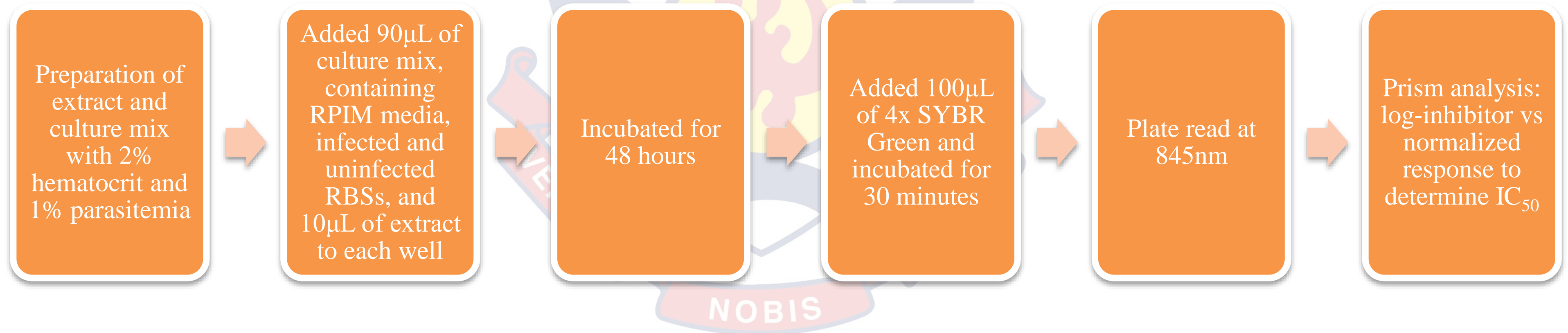
Methods – SYBR Green Assay

- SYBR Green assay uses stained parasite DNA to visualize and quantify parasite levels in vitro



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Methods – SYBR Green Plate Set-Up

	1	2	3	4	5	6	7	8	9	10	11	12
		Artesunate			Calotropis procera			Calotropis procera			Artesunate	
A		100 ng/mL			100 µg/mL			100 µg/mL			100 ng/mL	
B		50 ng/mL			50 µg/mL			50 µg/mL			50 ng/mL	
C		25 ng/mL			25 µg/mL			25 µg/mL			25 ng/mL	
D		12.5 ng/mL			12.5 µg/mL			12.5 µg/mL			12.5 ng/mL	
E		6.25 ng/mL			6.25 µg/mL			6.25 µg/mL			6.25 ng/mL	
F		3.125 ng/mL			3.125 µg/mL			3.125 µg/mL			3.125 ng/mL	
G		iRBCs			1.56 µg/mL			1.56 µg/mL				
H		RBCs			0.78 µg/mL			0.78 µg/mL				

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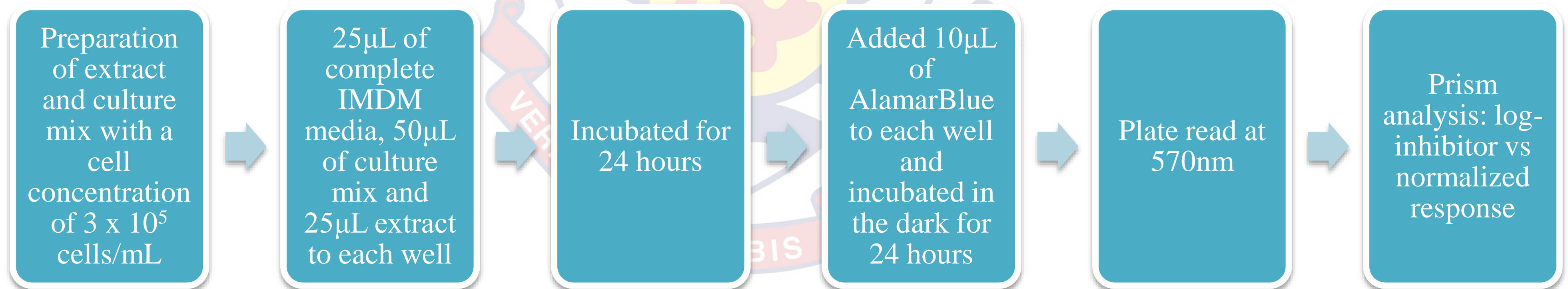
Methods – AlamarBlue

- AlamarBlue assay uses a cell viability indicator to visualize and quantify live parasite concentration
- Living cells reduce resazurin to resorufin, changing the solution from blue to pink in color



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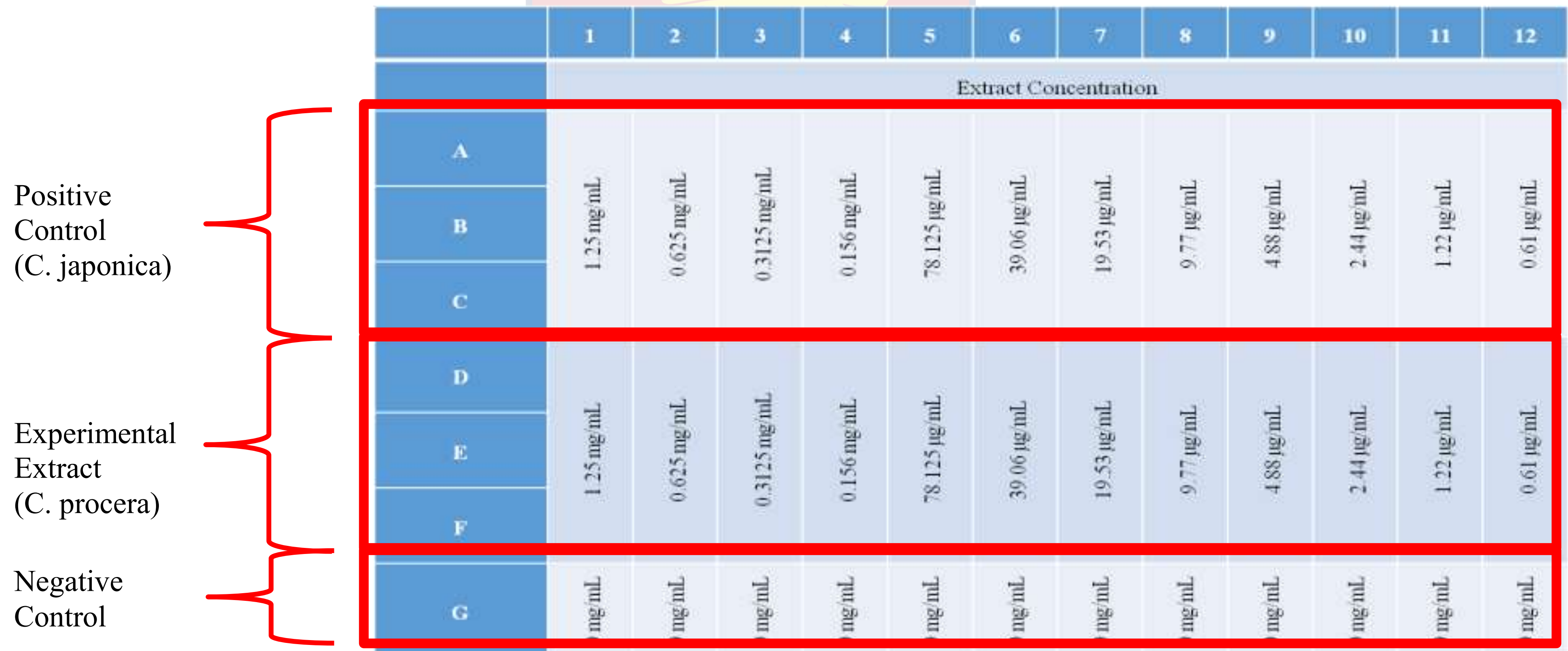
Methods – AlamarBlue Plate Set-Up



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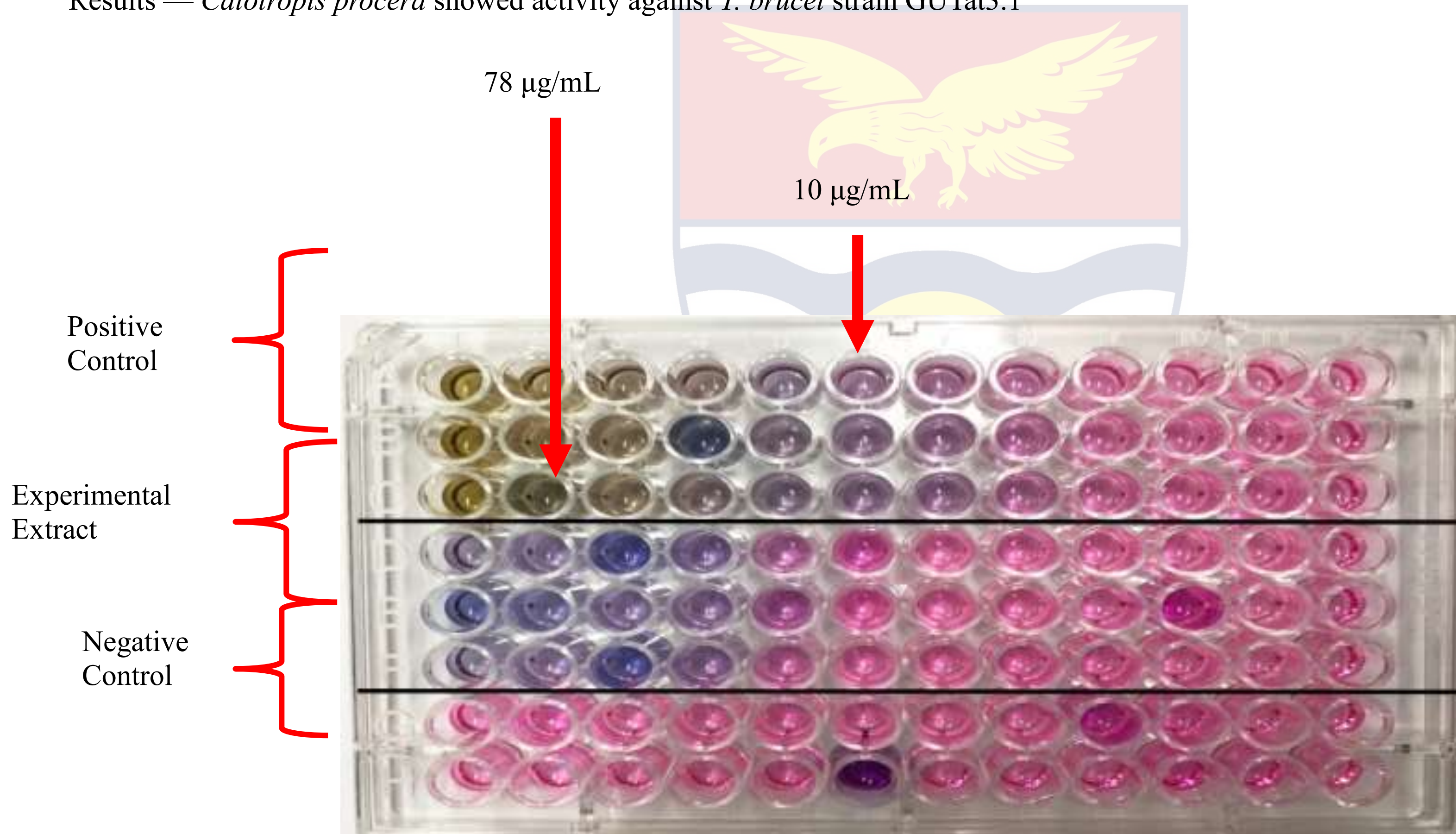
Results — *Calotropis procera* showed activity against *T. brucei* strain GUTat3.1



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