EVALUATION OF ANTIMALARIAL POTENTIAL OF CYSTEINE PROTEASE INHIBITORS FROM *CALOTROPIS PROCERA* LINN AND *MORINGA OLEIFERA* LAM ON *PLASMODIUM BERGHEI* INFECTED MICE

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ZARIA, NIGERIA

OCTOBER 2017

DECLARATION

I declare that the work in this thesis entitled " **Evaluation of Antimalarial Potential of Cysteine Protease Inhibitors from *Calotropis procera* Linn and *Moringa oleifera* Lam on *Plasmodium berghei* Infected Mice** " has been carried out by me in the Department of Biochemistry under the supervision of Prof. I. A. Umar, Prof Sani Ibrahim and Prof .E. Onyike. The information derived from the literature has been duly acknowledged in the text and list of references provided. No part of this thesis was previously presented for another degree or diploma at this or other institute.

ABDULLAHI ABDULKADIR _____ _____

Name of Student **Signature** **Date**

CERTIFICATION

This Thesis entitled, "**Evaluation of Antimalarial Potential of Cysteine Protease Inhibitors from *Calotropis procera* Linn and *Moringa oleifera* Lam on *Plasmodium berghei* (Nk 65) Infected Mice** " by Abdullahi Abdulkadir meets the regulations governing the award of the Degree of Doctor of Philosophy (Ph.D) in Biochemistry of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

In thankfulness, this work is dedicated to Almighty ALLAH and entire scientific community.

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ABSTRACT

Cysteine protease of Plasmodium parasite plays a major role in the survival of the organism inside its host. Various extraction media were used to evaluate different parts of *Calotropis procera* and *Moringa oleifera* for Cysteine Protease inhibitor (CPI) activity. The latex of *C.procera* and *M.oleifera* seeds extracts with higher CPI activity were concentrated by cold acetone precipitation, further subjected to purification using ammonium sulphate fractionation and Phenyl sepharose column and characterized. Subchronic toxicity of the extracts was evaluated in mice after intraperitoneally (ip) administering different doses for 21 days. A 4-day suppressive antimalarial test was carried out using *Plasmodium berghei* NK65-infected mice, to which the extracts were administered at different doses intraperitoneally. The partially purified CPIs from *C.procera* and *M.oleifera*, exhibited inhibitory activities of 31.47 units/mg proteins and 11.23 units/mg protein respectively. The partially purified inhibitors have optimum temperature at 60^0C and 50^0C for *C.procera* and *M.oleifera* respectively. The pH optimal for *C.procera* and *M.oleifera* are 6.0 and 7.0 respectively. The partially purified inhibitors from both plants were stable at temperatures below 60^0C for 6 hours and were active at wide range of pH values from 2 to 12. Chemical modification of the some key amino acids present in the binding site of CPI, revealed the presence Cysteine and Histidine for *C.procera* latex and Lysine for *M.oleifera* seeds.The kinetic analysis revealed a mixed type of inhibition for both inhibitors against *Plasmodium berghei* cysteine protease enzyme. The fifty percent inhibitory concentration (IC_{50}) values of 23.10±1.2 µg/ml and 17.2±0.3 µg/ml with inhibitor binding constant (K_i) values of 8.0µg/ml and 20.0µg/ml for both inhibitors suggested high affinity between inhibitors and enzymes. Serum biochemical parameters evaluated which included Alanine Aminotransferase (ALT),

Aspartate Aminotransferase (AST) and Creatinine for subchronic toxicity were not significantly (P<0.05) altered at all doses of the extract used. There was significantly (P<0.05) elevated serum glucose at 30 and 50mg/kg BW/day for crude CPI extract of *C.procera* latex. A significantly (P<0.05) reduced Triacylglycerol level was observed at all doses of crude CPIs extract from *M.oleifera* seeds treated group compared to the control group. However, the partially purified CPI extract of *C.procera* latex reduced parasitaemia by 77% at a dose of 50 mg/kg. BW, while partially purified CPI extract of *M.oleifera* seeds reduced parasitaemia by 57% at a dose of 70 mg/kg BW on day 8-posttreatment.The crude and purified CPIs extract from both plants improved the survival of animals compared to control group. The disease-induced decreases in packed cell volume (PCV) were ameliorated by treatment with CPIs extract. It can be concluded from the results obtained in this study that partially purified CPI from the plants extracts at the dose and route used could be possible antidote against malaria.

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LIST OF ABBREVIATIONS

ACT- Artemisinin –based Combination Therapy

ALT- Alanine Aminotransferase

ANOVA- Analysis of Variance

AST- Aspartate Aminotransferase

BAPNA - α-N-benzoyl-DL-arginine-p-nitroanilide

BSA- Bovine Serun Albumin

CPI- Cystein Protease Inhibitor

DNA- Deoxyribonucleic Acid

EDTA - Ethylene Diamine Tetra Acetic acid

IC_{50} -Concentration of an inhibitor at which the response is reduced by half

kDa - KiloDalton

*K*i - Dissociation Constant

K_m - Michaelis-Menten Constant

PBS- Phosphate Buffer Saline

PCV- Packed Cell Volume

PPI- Plant Protease Inhibitors

RBC- Red Blood Cells

SDS- PAGE - Sodium Dodecyl Sulphate Polyacrylamide gel Electrophoresis

USAID- United State of Agency for International Development

V_{max} - Maximal Velocity

WHO- World Health Organization

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

Malaria remains a major and growing threat to the Public health and economic development of countries in the tropical and subtropical regions of the world (USAID, 2014; WHO, 2016). More than 60% of the world live in malaria endemic regions with an estimated 900 million cases (Snow *et al*., 2005; Bero *et al.*, 2009) and up to 0.5-0.7 million deaths each year (Walker *et al*., 2015; WHO, 2016). Most of whom are children of less than 5 years of age mainly from Africa with an estimated cost of $12 billion annually (Duvvuru, 2006). In addition, pregnant women, non–immune travelers, refugees, and displaced persons are at significant risk of exposure to malaria (USAID, 2005).

Nigeria loses more than 300,000 people annually from malaria (Christopher *et al.*, 2012; USAID, 2014) and an estimated $4 billion in the cost of treatment and prevention (Adebayo and Krettli, 2011). Despite extensive control efforts, the incidence of malaria is increasing in most endemic areas of the World (Mendis *et al.*, 2009). A major reason for the persistence of severe malaria problem is the limited success in the development of malarial vaccines, the increasing resistance of the mosquito vector to currently available insecticides and the resistance of the parasite to available chemotherapeutic agents (Fidock, 2010). Resistance to chloroquine and its analogue, the most widely used antimalarial in the last 5 decades and artemisinin-based combination therapies (ACTs) is now very common (Dondorp *et al.*, 2009), and other available antimalarial are limited by resistance, high cost and toxicity (Satoru *et al.*, 2010), hence a need to search for new drugs.

Parasitic organisms perpetuate themselves within their hosts by triggering the expression of many molecules that activate survival mechanisms (Pereira *et al.*, 2011). Among these molecules, the proteases are crucial for the life cycle of the parasite and are responsible for their virulence and pathogenicity in host organisms (James *et al.*, 1999; Pirta *et al.*, 2016). These enzymes have been implicated in many adaptation mechanisms for survival in the host, including modulation of the host immune system, invasion and destruction of host tissues, parasite dissemination. They are also involves in acquisition of essential nutrients that assure survival and proliferation to sustain the infection (Rosenthal *et al.*, 1999; Pirta *et al.*, 2016) and therefore are attracting attention as drug targets (Kim *et al.*, 2009).

Naturally occurring proteins and peptides (Gideon, 2003; Marcos *et al*., 2008; Chamy *et al*., 2016) as well as several chemically synthesized inhibitors of these proteases are now being licensed as drugs (Lemuel *et al.*, 2009). Lead compounds have also being developed that are effective against these proteases from important pathogenic organisms such as Human Immunodeficiency Virus (HIV), *Aspergillus* and *Candida spp* (Coomb and Mottran 1997; Johnson and Pellecchia, 2006; Arun *et al.*, 2016). Some of the HIV protease inhibitors approved by the Food and Drug Administration (FDA) include Amprenavir (Agenerase), Fosamprenavir (Lexiva), Indinavir (Crixivan), Lopinavir/ritonavir (Kaletra), Ritonavir (Norvir), Saquinavir (Fortovase), and Nelfinavir (Viracept). Aprotinin is another protease inhibitor that has uses in biochemical research, as a clotting agent to reduce blood loss in heart surgery and potentially in agriculture and is sold under the name of Trasylol (Arun *et al.*, 2016).

The cysteine proteases play an important role in the infection, replication, and metabolism of protozoan parasites (Mottram *et al.*, 1998; 2004; Brito *et al.*, 2016).

Trypanosomes Leishmania and *Entamoeba histolytica* contain an abundance of cysteine proteases (Hide *et al.,* 1997; Rosenthal *et al* 2003; Mirela *et al.,* 2012) that are members of the papain family. The possibility that inhibitors of cysteine proteases could be effective antiparasitic agents has been recognized for some time (McKerrow *et al.,* 1995; Robertson *et al.,* 1996; Selzer *et al.,* 1997).

Erythrocytic stages of *Plasmodium* parasite are responsible for the clinical stage of Malaria (Rosenthal., 1998; 2011.) causing symptoms that include anaemia, fever, chills, nausea, and flu-like illness and in severe cases of coma and death. Cysteine proteases of *Plasmodium* mediate hepatocyte invasion by sporozoites, liver stage development (Rennenberg *et al.,* 2010) and are also critical for the invasion of red blood cells,which initiates the erythrocytic cycle and rupture of erythrocyte by the mature parasite(Amlabu *et al.,* 2011_a ; Musyoka *et al.,* 2016).

Rupture of host erythrocyte and red blood cells invasion by the mature parasite were inactivated by cysteine protease inhibitors (Rockett *et al*., 1990; Asawamahasakda *et al*., 1994; Belinda *et al.,* 2003; Pedro *et al*., 2009). Cysteine Proteases are also involved in the crucial process of Hemoglobin degradation pathways by intracellular parasites, which provide amino acids as nutrients for their own growth and maturation (Rosenthal, 1998; Klemba *et al*., 2004).

Report by Ch'ng *et al*. (2010) using cysteine protease inhibitors, demonstrated that cysteine proteases of *P. falciparum* are also involved in chloroquine-mediated programmed cell death. The best characterized *Plasmodium* cysteine proteases are the falcipains (FP), papain family enzymes FP-1, FP-2 and FP-2', and FP-3. Among the four, only falcipain-2 and falcipain-3 appear to be the principal food vacuolar hemoglobinases (Pandey and Rajnikant, 2012). FP-2 is also responsible for the activation of aspartic protease (plasmepsin

2) by cleaving off the propeptide (Verma *et al.,* 2016; Drew *et al*., 2008). Furthermore, FP-2 has been shown to hydrolyze erythrocyte cytoskeleton and might, therefore, play a role in the escape of the parasite from the erythrocyte (Guido *et al.,* 2011). Several homologs of falcipain which includes vivapain 2 and 3 (VP-2 and VP-3) of *P. vivax*, bergheipain of *P.berghei*, knowlesipain 2 and 3 (KP-2 and KP-3) of *P. knowlesi* have already been confirmed (Musyoka *et al*., 2016).Undegraded hemoglobin accumulates in the food vacuole, when falcipain-2 gene was disrupted, confirming that this enzyme participates in haemoglobin hydrolysis.

Falcipain-2 and falcipain-3 share 67% sequence identity and contribute more or less equally to the digestion of hemoglobin (Ch'ng *et al*., 2010). Cysteine protease inhibitors that inhibit FP-2 and FP-3 consistently block hemoglobin hydrolysis and parasite development (Mane *et al*., 2013). Several *in vitro* and *in vivo* studies using inhibitors such as peptidyl fluoromethyl ketones, vinyl sulfones, aldehydes and nonpeptidic inhibitors supported the development of cysteine protease inhibitors as anti-malarial drugs (Rizzi *et al.,* 2011; Mane *et al*., 2013).

Similarly, a model rodent *Plasmodium* parasite also contains several cysteine proteases, bergheipain 2 (BP-2) of *P.berghei*, chabaudipain 2 (CP-2) of *P. chabaudi* and yoelipain 2 (YP-2) of *P. yoeli* (Cheryl *et al*., 2005; Musyoka *et al*., 2016). Low and high molecular weigth cysteine protease of both *Plasmodium falciparum* and *Plasmodium berghei* had earlier been isolated (Smith *et al*., 2014; Liu *et al*., 2015; Salas and Faculty, 2015). Since the degradation of Haemoglobin is vital for parasite survival, as the parasite has a limited capacity for synthesis of amino acid and the quantity of free amino acid within erythrocyte is not sufficient for parasite needs (Rosenthal *et al*., 1988; 1993), the inhibition of this

degradation pathway offers a valid approach by which to develop novel chemotherapeutic agents.

Traditional medicinal remedies for several diseases abound in most endemic region (Antoniana, 2009; Cláudio and Lucia, 2011; Lawal *et al.*, 2015). Protease inhibitors from plants potently inhibited the growth of a variety of pathogenic bacterial and fungal strains (Kim *et al*, 2009: Sharma *et al.*, 2011; Ali *et al*., 2014; Sousa *et al.*, 2015).

Cysteine protease inhibitor have been identified and studied in many plant sources such as rice (Abe *et al.*, 1987), maize (Abe *et al.*, 1994), soybean (Misaka *et al.*, 1996), cowpea potato, Chinese cabbage (Lim *et al.*, 1996) and carrot (Ojima *et al.*, 1997). Thiol protease inhibitors present in the plant system perform a variety of functions, including their role in storage proteins (Xavier-Filho, 1992), as regulators of endogenous proteolytic activity (Ryan, 1990) and as participants in the mechanism of programmed plant cell death (Solomon *et al.*, 1999). Furthermore, protease inhibitors are expressed in abiotic stress (Franco and Melo, 2000) and in plant defence processes against insect (Ryan, 1989) and pathogens attack response (Ceros and Carbonell, 1993).

Cysteine protease inhibitor present in cereal seeds like rice and maize have been used to prevent certain types of cancer (Schelp and Pongpaew, 1988). Cysteine protease inhibitor extracted from plants has been reported to cure many parasitic diseases(Doyle *et al*., 2007; Kang *et al*, 2012; Bunyatang and Chirapongsatonkul, 2013; Costa and Lima, 2015) but only few have been identified with anti-malaria activities.

Calotropis procera and *Moringa oleifera* are widely distributed in West Africa. Various reports are available on their protein content (José *et al*., 2010; Fabiano *et al.*, 2011), proteases (Asrar *et al.*, 2012; Abdullahi and Isah, 2009; Onyike and Abdullahi, 2006;

Cleverson *et al.*, 2007) and the antimalarial activities of different part of *Calotropis procera* (Mudi and Bukar, 2011).

Despite numerous pharmacological properties of the leaves and latex of *Calotropis procera* (Larhsini *et al.*, 1997; Kumar and Roy, 2009; Bharti *et al.*, 2010; Begum *et al*., 2010; Cristina, 2011; Kumar *et al*., 2014; George *et al*., 2016; Sayed *et al*., 2016) and presence of protease inhibitor from the closely related plants, *Calotropis gigantea* (Kaladhar *et al*., 2013). Information concerning the identification of protease inhibitor in the plant is lacking.

A number of medicinal properties attributed to different parts of *Moringa* have been recognized by many authors (Fahey, 2005; Thurber and Fahey, 2009; Oliveira *et al.*, 2010; Mahajan and Meht, 2010; Suaib *et al.*, 2012; Dasgupta *et al.*, 2016). Antiplasmodial effect of *Moringa oleifera* has been reported (Shuaibu *et al.*, 2008; Adebayo and Krettli, 2011; Lawal *et al*., 2015). Presence of protease enzyme (Satish *et al.*, 2012) serine protease inhibitor from *Moringa oleifera* with potential therapeutic applications (Bijina *et al.*, $2011_{a;}$ 2011_{b}) has been demonstrated. No report on the cysteine protease inhibitor with antimalarial activities has been demonstrated from this plant. This project has been designed to extract Cysteine protease inhibitor from *C.procera* and M.*oleifera* and evaluate them for antimalarial activities.

1.2 Statement of Research Problems.

Plasmodium parasite resistance to currently available drugs continues to undermine various malaria control efforts. Cysteine proteases of *Plasmodium* parasite are not targeted by the existing drugs. The enzymes are implicated in parasite survival in both liver and blood stages in the host. The blood stages of these parasites are responsible for the clinical symptoms of malaria. *Plasmodium* cysteine proteases play important roles in critical parasite-

specific processes during the erythrocytic cycle such as hemoglobin digestion, merozoite release from infected erythrocytes and invasion of fresh erythrocytes by the released merozoites. Effort to develop other drugs that target different mechanism of action is highly necessary. Hence, the extension of search for inhibitors of Plasmodium Cysteine proteases from natural sources especially from plant sources.

Consenquently any phytomedicine that potentially inhibit this enzyme could be exploited as a source of antimalarial agents.

1.3 Justification

The potential and efficacy of non-protein inhibitor from plants and synthetic peptide cysteine protease inhibitors in treatment of *Trypanosoma cruzi*, *Plasmodium falciparum* , *Leishmania major* , antiviral, and anticancer is documented (Johnson and Pellecchia, 2006), but few reports are available on effects of plant protein cysteine protease inhibitor on these organisms.

Antiplasmodium activity of different parts of *Calotropis procera* and *Moringa oleifera* has been reported (Shuaibu *et al.*, 2008; Mudi and Bukar, 2011; Adebayo and Krettli, 2011; Lawal *et al.*, 2015). Serine protease inhibitor with various therapeutic activities have been reported in various part of *Moringa oleifera* (Bijina *et al.*, 2011_a; 2011_b), but literature on protease inhibitor from *C. procera* are scanty despite many reports on presence of this molecule from plants of related family. Ramos *et al*. (2010) and Oliveira et *al*., (2012) described peptide inhibitor from *C. procera* latex with insecticidal activity and protection against *Salmonella Typhimurium* respectively. There is dearth on the antiplasmodial activity from protease inhibitor of both *C. procera* and *M.oleifera*. Thus Inhibition of the malaria

parasites' cysteine proteases with the inhibitor isolated from these plants become paramount and will contribute to chemotherapy of Malaria infection.

1.4 Research Aim and Objectives:

1.4.1 Aim of the Study

To extract, purify and characterize active Cysteine Protease Inhibitor (CPI) with antimalarial activities from *Calotropis procera* and *Moringa oleifera.*

1.4.2 The Specific Objectives are:

I. Screen for Cysteine Protease Inhibitors (CPIs) from extracts of different parts of the plants

II. Partially Purify and Characterise the CPI

III. Determine Median lethal dose (LD_{50}) and Subchronic Toxicities study of Crude and partially Purified CPI fraction(s) in Mice

IV. Partial purification of Cysteine Protease (CP) of *Plasmodium berghei* and study on its inhibitory effect by partially purified fraction of both plants

V. To evaluate antimalarial activities of both Crude and Partially Purified CPI extracts in Mice

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1. Global Incidences of Human Malaria

Malaria is one of the infectious diseases contributing to worldwide mortality and morbidity (Breman, 2001). The burden of malarial infections is borne by young children and pregnant women in sub-Saharan Africa, where the disease is progressively associated in social and economic hardship (WHO, 2014; Breman, 2001). Malaria caused an estimated 214 million clinical episodes, and 455,000 deaths (WHO, 2016). An estimated 90% of deaths in 2015 were in the African Region, followed by 6% in the South-East Asian Region and 3% in the Eastern Mediterranean Region. About 86% of deaths globally were in children. A half of the world's populations (3.2 billion people) live in areas at risk of malaria transmission in over 95 countries and territories (WHO, 2016).

About 60 million Nigerians, have malaria more than once in a year, with pregnant women and children (under 5 years) being more susceptible to the attack due to their low resistance and therefore constitute 92% of the prevalence (Lawal *et al.*, 2015). Five species of *Plasmodium* are responsible for human malaria: *Plasmodium falciparum, P.malariae, P. vivax, P. ovale* and *P. knowlesi* (Schofield and Grau, 2005). However, the above-mentioned species of *Plasmodium* infect human, the effects of *P. knowlesi* and *P. malaria* are less marked when compared to *P. falciparum* and *P. vivax.* Hence, *P. falciparum* has received a lot of consideration mainly because it is the most fatal and is responsible for the most malarial infections (Guerra *et al*., 2007).

Plasmodium vivax has strong preferences for reticulocytes during invasions and is relatively non-lethal (Chiang *et al*., 2006). It causes up to 80 million cases per year with 15% in Africa and 85% outside Africa (Mendis *et al*., 2009). *Plasmodium ovale* is an uncommon parasite confined to tropical Africa and accounts for 10% of all malarial infections in Africa but is less common in other malarial endemic regions (Winstaley *et al*., 2004). *Plasmodium malariae* is milder than other types of malaria and usually found in older red blood cells (Chiang *et al*., 2006). It is found in most endemic areas, throughout sub-Saharan Africa, but is much less common than the other species (Guerra *et al*., 2007).

The *Anopheles* mosquito is key vector for the malaria parasites. In Africa, the *Anopheles gambiae* is the most efficient in transmitting the disease (Holt *et al.*, 2010). Malaria is also transmitted through blood transfusions, from mother to unborn baby (i.e. congenitally), as well as by sharing contaminated hypodermic needles (Heelan *et al.*, 2002). The transmission of malaria is prevalent with the rainy season, which provides breeding sites for the *Anopheles* vector. The *Plasmodium* parasite is only carried by these mosquitoes in warm, humid and wet climates but disappears over winter. Larvae control is therefore, imperative in the control of this epidemic disease (Holt *et al.*, 2010).

Other factors that contribute to the increase of malaria incidences include travelling to and from epidemic areas. Because of intensity of the disease and failure of malaria control policies implemented in the past, there is a general agreement that significant reduction in the malaria problem will involve the coordinated use of numerous strategies. These includes artemisinin based combination therapies (ACTs), integrated vector management (IVM), insecticide-treated nets (ITNs) and better diagnostic and effective treatment (McKenzie *et al*., 2002).

2.1.1 Rodent malaria parasites

Rodent malaria is caused by four species, *Plasmodium berghei*, *Plasmodium chabaudi, Plasmodium yoelii* and *Plasmodium vinckei,* which was isolated from wild thicket rats in Africa and have been adapted to grow in laboratory rodents (Smith and Parsons, 1996). *Plasmodium chabaudi chabaudi* exhibits many features associated with the pathogenesis of human malaria, such as rosetting, sequestration and antigenic variation (Stephens *et al*., 2012).

In the absence of sufficient similarity to human parasite, primate model exist but pharmacokinetic evaluations are much more expensive in monkey models that can sustain infection by *P. falciparum*. Housekeeping genes and biochemical processes are conserved between rodent and human parasites (Smith and Parsons, 1996).

Resistance in some rodent parasite has shown resemblances to resistance in human parasites. For instance, Sulfadoxine-Pyrimethamine (SP) resistance in *P. chabaudi* and atovaquone resistance in *P. berghei* have shown correlation to resistance in *P. falciparum* (Mueller *et al*., 2009). *Plasmodium berghei* was originally discovered in 1948 by Vincke and Lips, they infects hamsters, rats and mice (Janse and Waters, 2006). At least five isolates (strains) of *P. berghei* have since been isolated namely, NK65, ANKA, SPII, WK6S, and K173 (Janse and Waters, 2006).

All different isolates have a similar morphology, life cycle stages and duration of development. The strains also show a similar sensitivity to antimalarial drugs (Janse and Waters, 2006). *Plasmodium berghei* has successfully been used in drug testing research and

is probably the best practical model for experimental studies of human malaria (Janse and Waters, 2006). Thus, in this study, *P. berghei* NK65 was used as experimental model.

2.1.2 The life cycle of *Plasmodium*

The parasite is spread to human hosts via the *Anopheles* mosquito vector (Gardner *et al*., 2003). All *Plasmodium* species causing human malaria cases exhibit similar life cycles but a few variations occurred between various species (Wellems *et al*., 2009). *P. falciparum* is the most virulent *Plasmodium* species as its infections are accompanying with high levels of parasitemia. The other four *Plasmodium* species cause minor infections. The *Plasmodium* life cycle occurs between the mosquito vector and the human host. The transition between the cold-blooded mosquito host and the warm-blooded human host occur during sexual and asexual stages respectively. The asexual phase is further divided into two stages: the liver (pre-erythrocytic) stage and the blood (erythrocytic) stage (Figure 2.1).

The malaria parasite life cycle begins with bite of the vertebrate host by a female *anopheles* mosquito. As a result of the biting, sporozoites get into the host (Wellems *et al*., 2009). The sporozoite then enters the circulatory system where it invades the liver cells. At this stage the parasite, go through an asexual replication stage known as the exo-erythrocytic schizogony. Merozoites are then released into the blood circulation, where they entered into the erythrocytes and instantaneously undergo a trophic phase causing in the development of the parasite.

The early trophozoite stage is called the ring form, a name indicating to the structure of the parasite at this stage. After the development of the trophozoites, the parasite metabolism turn out to be activated; they feed on the host cytoplasm and digest host

hemoglobin into amino acids (Dorin-Semblat *et al*., 2008). Rupture of the infected erythrocyte, an event claimed to be proteolytic process released the merozoites into circulation (Rosenthal *et al*., 1988; Rosenthal *et al*., 1996). Red blood cells invasion by the merozoites results in another round of erythrocytic replicative stage. However, the schizonts differentiate into male and female gametocytes.

The gametocytes need to be picked by another mosquito to finish the life cycle, in which they mature into a zygote. The zygote elongates and become motile (ookinetes). Ookinetes invades the midgut wall of the mosquito and develop into oocysts. Sporozoites develop from grown and ruptured oocysts (Dorin-Semblat *et al*., 2008). The sporozoites move to the mosquito's salivary glands. The *Plasmodium* life cycle is then indefinitely continued by another infected mosquito biting a human during a blood meal.

2.1.3 Clinical features and pathology

Clinical features of malaria start from no apparent illness (uncomplicated), to the typical symptoms of malaria, to severe disease (Schofield and Grau, 2005). The pathogenesis of malaria fever is caused by rupturing of schizonts which release of merozoites, malarial pigment, parasite proteins and cellular debris which stimulate human mononuclear cells to release tumor necrosis factor (TNF) and other pyrogenic cytokines (Lou *et al*., 2001).

Plasmodial glycosylphosphatidylinositols (GPI) released from rupturing of red cells induce proliferation of pro-inflammatory cytokines, interferon-γ(INF-γ), tumor necrosis factor(TNF-α), interleukin (IL-1), IL- 12 and macrophage colony-stimulating factor, and lymphotoxin, as well as superoxide and nitric oxide implicated in development of cerebral malaria (CM) (Angulo and Fresno, 2002; Fujioka and Aikawa 2002). Some features of severe

malaria are common to all infected species, with hypoglycemia and lactic acidosis occurring in the terminal phase of avian, rodent, simian and human malaria (Angulo and Fresno, 2002).

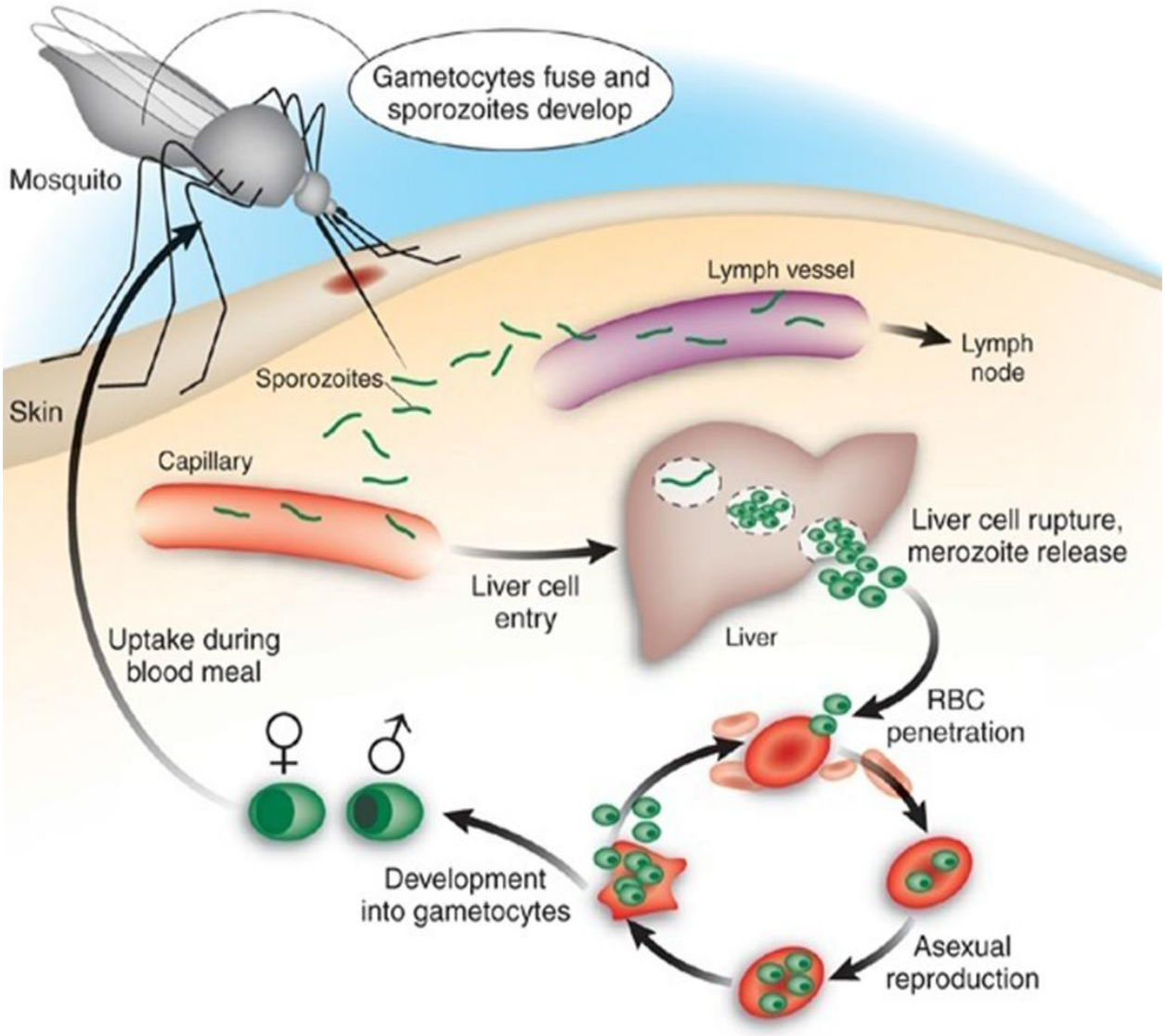


Figure 2. 1: The *Plasmodium* Life Cycle. The Sexual Stages In The Mosquito Vector and Asexual Stage in Human Host (Teixeira *et al*., 2011).

Plasmodium falciparum erythrocyte membrane protein-1(PfEMP-1) a major export product from the parasite to the surface of the infected erythrocyte causes the infected erythrocyte to stick to the endothelial cells that line blood vessels (Webster and Hill, 2003). Sequestration occurs mainly during the second half of the intra-erythrocytic asexual growth phase of the parasite, following the cytoadherence of mature parasites to endothelial cells through electron-dense knobs on the pRBC surface, hence, blocks cerebral capillaries causing ceberal malaria (CM) (Deitsch and Hviid, 2004). Sequestrations play a vital role in many of the life threatening complications typical of severe malaria such as metabolic acidosis, seizures, and hypoglycemia (Bell and Molyneux, 2007).

Parasitized erythrocytes sequestration also occurs in rodent CM although in a less conspicuous manner than in humans (Hearn *et al*., 2000). Sequestration of *Plasmodium falciparum* is a distinctive pathologic feature that often resulted into fatal human cerebral malaria. Sequestration of parasitized erythrocytes causes clogging of the minute capillaries, venules, and arterioles culminating in damage of vital organs like the brain, kidneys, lungs, liver, and gastrointestinal tract which resulted to cerebral malaria, renal failure, pulmonary dysfunction among others (Hearn *et al*., 2000).

In contrast, the *P. vivax* species is termed benign malaria because it is moderately non-lethal, but is often go together with debilitating conditions (Mueller *et al.*, 2009). High fever is usually accompanied with headache, shivering, intense perspiration and other symptoms such as abdominal pain, diarrhea, slight jaundice, cough, enlarged liver and spleen, and vomiting (Chiang *et al*., 2006). The clinical features and pathogenesis of severe malaria include shock, impaired consciousness, respiratory difficulty, anemia (destruction of erythrocytes), acidosis, and hypoglycemia (Mueller *et al.*, 2009).

2.1.4 Malaria diagnosis

In most countries of sub-Saharan Africa where malaria is endemic, the standard for laboratory confirmation of a clinical malaria diagnosis is a peripheral blood film, examined microscopically (Batwala *et al*., 2010). The quantitative buffy coat method (QBCTM), uses micro-haematocrit tubes pre-coated with fluorescent acridine orange stain to highlight malaria parasites. Rapid Diagnostic Test (RDT)) is a third diagnostic approach involving the rapid detection of parasite antigens using rapid immuno-chromatographic technique based on the detection of the Histidine-rich protein 2 (HRP-II) of *P. falciparum*. Compared with light microscopy and QBC, this test yields rapid and highly sensitive diagnosis of *P. falciparum* infection. Parasite nucleic acids are detected using polymerase chain reaction (PCR). PCR is most useful for species confirmation of the parasite after the diagnosis has been established by either smear microscopy or RDT (Ceusters and Smith, 2009; Batwala *et al*., 2010).

2.1.5 Malaria control

Control vector remains the most largely effective way to avoid malaria transmission, and as such it is one of the four basic practical elements of the global malaria control strategy which consist; the Indoor residual spraying (IRS) with insecticides and use of Insecticide-treated nets (ITNs) (Kappe *et al*.,2010). The prevalent of the insecticide resistance raises concerns for vector control implementation and sustainability particularly for the control of the main vector of human malaria (Breman, 2001; Enayati *et al*., 2010).

Understanding parasite biology and malaria epidemiology, has greatly advanced, yet a safe and effective malaria vaccine for use in humans remains elusive (Breman, 2001).

However, new vaccines that prevent malaria infection have been recently reported. These vaccines known as RTS, S/AS01B and RTS, S/AS20B have passed through third stage clinical trials, and now on pilot stage (WHO, 2016).

2.1.6 Malaria chemotherapy

Antimalarial agents can be grouped and assessed based on the stages of the *plasmodium* life cycle that they target, their chemical structure, molecular targets, half-life or cost (Daily, 2006; Schlitzer, 2007; Huthmacher *et al*., 2010). Antimalarial drugs may act on different stages of the malaria parasite, due to variation in stage specific biology. For example, tissue schizonticides kill hepatic schizonts and prevent invasion of erythrocytes, thereby stopping the establishment of clinical malaria.

Hypnozoiticides kill persistent intra-hepatic stages (occurring in *P. vivax and P. ovale* infections) thus prevent relapses from these dormant stages of infection. Among the antimalarials in clinical use, only primaquine is available for the elimination of hypnozoites (Cogswell, 1992; Daily, 2006). Antifolates attack all developing stages of the malaria parasite (Chiang., 2006).Gametocytocides eliminate the intraerythrocytioc sexual forms (gametocytes), and thus prevent human to human transmission (Schlitzer, 2007).

Primaquine, artemisinin, and its derivatives are reported to have gametocytocidal activity (Chiang *et al*., 2006). Most antimalarials in clinical use are blood schizonticides, which work by eliminating the asexual erythrocytic parasite form (Schlitzer, 2007). As there are no dormant hepatic stages in *P. falciparum* malaria, contrarily to *P.vivax,* blood schizonticides are sufficient to manage infections. Based on chemical structure, antimalarial

drugs are classified as; aminoquinolines, arylalcohols, quinoline alcohols, antifolates, antibiotics and artemisinins (Robert *et al*., 2001).

For molecular targets, the erythrocytic stage of the parasites hydrolyses between 60-80 % of the haemoglobin into haem and globin. The haem is said to be toxic to the parasites, thus neutralize the haem by the process of polymerization to haemazoin, which is not harmful to the parasite. This polymerization is inhibited by antimalarials such as chloroquine mefloquine, halofantrine, lumefantrine and artemisinins; resulting in the accumulation of haem and eventual death of the parasites (Schlitzer, 2007).

Quinoline antimalarials such as chloroquine, for example, act by inhibiting the synthesis of the hemozoin pigment when they get trapped in the digestive vacuole of the parasite resulting to the extra proton they gain due to the acidic environment of the vacuole (Jiang *et al*., 2006). Hemoglobin hydrolysis by the Plasmodium parasite is mediated by a number of classes of proteases enzymes. These proteases appear to mediate the invasion and rupture of infected erythrocytes as well, possibly to get through the erythrocyte cytoskeleton, a complex network of proteins (Rosenthal *et al*., 1998; Alida *et al*., 2005).

Atovaquone inhibits complex III of the plasmodium respiratory chain (Huthmacher *et al*., 2010). Synthesis of dihydrofolate is inhibited by antifolate such as pyrimethamine and sulfonamides, which is necessary in the biosynthesis of pyrimidines. Iron chelators, however, are active by preventing iron from entering parasites metabolic pathways, as well as combining with iron, forming toxins to the parasites (Gavigan *et al*., 2001).

Free radicals produced by some antimalarial drugs including artemisinin-related compounds are toxic to plasmodium. Artemisinin inhibits the polymerization of haem to

haemazoin by alkylation (Mishra *et al*., 2009). The haem or Fe^{2+} cleaves the peroxide bridge in the artemisinin compound leading to the formation of free radicals. These radicals then alkylate one of the pyrazole rings in the haem nucleus, hence inhibiting the formation of haemazoin.

Among potential targets for new modes of chemotherapy are malarial proteases. Cysteine and aspartic protease inhibitors are now under study as potential antimalarials. The increased understanding of the mode of action of this compound will contribute in the mitigation in the rapid spread of resistance against presently used antimalarials (Gavigan *et al*., 2001).

2.2 Enzymes of *Plasmodium* Parasite

Many *Plasmodium* enzymes play key role in the parasite survival. *Plasmodium* parasites are very much dependent on glucose and very sensitive to oxidative stress. Preuss *et al*. (2012) have identified glucose-6-phosphate dehydrogenase (G6PD), a key enzyme of Pentose phosphate pathway (PPP) as a drug target. Major differences between parasite and human G6PD could be exploited for antimalarial drug design (Preuss *et al*., 2012).

Protein kinases of *Plasmodium* are another class of enzyme important to the parasite metabolism. Most key enzymes in the inositol pathway are phosphorylated, which strongly suggests additional levels of regulation and crosstalk with other protein kinases that coregulate different biological processes (Talevich *et al*., 2011). Protein kinase A and phosphatidylinositol-signaling are involed in the regulation of host cell invasion (Lasonder *et al*., 2012). In addition, the second messenger cAMP has been shown to be important for the parasite's ability to infect the host's liver. The *P. falciparum* genome encodes two adenylyl

cyclases (PfACα and PfACβ), the enzymes that synthesize cAMP (Salazar *et al*., 2012). The PfACβ plays an important role during the erythrocytic stage of the *P.falciparum* life cycle. Biochemical characterization of PfACβ revealed a marked pH dependence, and sensitivity to a number of small molecule inhibitors that kill erythrocytic parasites (Salazar *et al*., 2012).

Histone deacetylases (HDACs) are another class of *Plasmodium* enzymes. They effect post-translational modifications of proteins by altering the acetylation state of lysine residues (Andrews *et al.,* 2012). HDACs control epigenetic changes that trigger cell transformation and proliferation of transformed cells associated with many diseases and they are thought to be validated drug targets for some types of cancer and are promising therapeutic targets for a range of other diseases, including malaria (Andrews *et al*., 2012). HDAC inhibitors are emerging as a promising new class of antimalarial drugs with potent and selective action against *Plasmodium* parasites *in vitro* (Andrews *et al*., 2012).

Another metabolic pathway crucial for the survival and replication of the parasite is the synthesis of the purine nucleoside monophosphates (Jersey *et al*., 2011).They are vital for the production of DNA and RNA molecules. A key enzyme in this pathway is the 6-oxopurine phosphoribosyltransferase (PRTase). The identification and characterization of inhibitors of these enzymes as antimalarial drugs have been reviewed (Jersey *et al*., 2011).

Plasmodium parasites also possess a single pyruvate dehydrogenase (PDH) enzyme complex that is localized to the plastid-like organelle known as the apicoplast. However, unlike most eukaryotes, *Plasmodium* parasites lack a mitochondrial PDH. The PDH complex catalyses the conversion of pyruvate to acetyl-CoA, an important precursor for the tricarboxylic acid cycle and type II fatty acid synthesis (FAS II) (Pei *et al*., 2010).

Malaria parasites adapt to the oxidative stress during their erythrocytic stages with the help of vital thioredoxin redox system and glutathione redox system. Glutathione reductase, thioredoxin reductase and gamma-glutamylcysteine synthetase (gamma-GCS) are important enzymes of these redox systems that help parasites to maintain an adequate intracellular redox environment (Kapoor and Banyal, 2009). Activities of glutathione reductase and thioredoxin reductase in *P. berghei*-infected host erythrocytes were found to be higher than those in normal host cells. These enzymes being mainly confined to the cytosolic part of cell-free *P. berghei* may promise advances in chemotherapy of malaria (Kapoor and Banyal, 2009).

Proteases of *Plasmodium* are a class of enzymes that has received a lot of research attention both in the prevention and management of malaria infection. These enzymes have been implicated in the life cycle of these parasites in both insect and its mammalian host (Rosenthal, 1998). Cysteine proteases (Facipains) and aspartic proteases (Plasmepsin) of *Plasmodium falciparum*, in particularly, have been extensively worked upon. The two proteases offer a potential promise as target of antimalarials agent (Rosenthal, 1998; Rosenthal, 2004).

2.3 Proteases

Proteases are a group of enzymes present in all organisms (Shindo and Van Der Hoorn, 2008; Sadij and McKerrow, 2002). They hydrolyse the peptide bonds, which link amino acids in a protein molecule or a polypeptide chain. Proteases play important functions in organisms, beginning from microbes, fungi, plants and animals. These enzymes are vital

for the survival of all categories of organisms, and are encoded by approximately 2% of all genes (Barrett, 2001).

They also play a significant role in many complex physiological and pathological processes such as DNA replication and transcription, activation of zymogens, protein catabolism, tissue arrangement, cell growth, blood coagulation, immune response, cell signalling, and migration. Futhermore morphogenesis in development, tumour growth, inflammation, metastasis apoptosis, release of pharmacologically active peptides and hormones from precursor proteins, and transport of secretory proteins across membranes requires this enzyme (Sadij and McKerrow, 2002).

In general term, extracellular proteases digest large proteins to smaller molecules for subsequent take up by the cell, while, intracellular proteases executed a serious task in the regulation of metabolism. Besides being essential from the physiological point of view, proteases are potentially dangerous to their proteinaceous location and the separate cell or organism and their activity must precisely be control. When unrestrained, proteases can be responsible for serious diseases. The control of proteases is usually accomplished by regulated expression/secretion and/or activation of proproteases, by the inhibition of their proteolytic activity and by degradation of mature enzymes (Barrett *et al*., 1998).

Parasites propagate themselves inside their hosts by activating the expression of many molecules that initiate their survival machinery (Pereira *et al*., 2011). Proteases are crucial for the life cycle of the parasite and are responsible for their virulence and pathogenicity in host organisms (James *et al*., 1999; Sadij and McKerrow, 2002; Pirta *et al.*, 2016). These enzymes have been involved in many adaptation mechanisms for survival of parasite in the

host, including invasion and destruction of host tissues and modulation of the host immune system. They are involves in parasite dissemination, acquisition of essential nutrients that assure its existence and proliferation to sustain the infection (Hide *et al.*, 1997; Rosenthal *et al*., 1999; Sadij and McKerrow, 2002; Pirta *et al*., 2016) and are thus attracting consideration as drug targets (Kim *et al*., 2009).

The proteases are classify into specific groups usually based on the amino acid residue at the active site, thus for cysteine proteases, cysteine (CYS) is used for catalytic activity. Serine (SER), aspartate (ASP), threonine (THR) and glutamic acid (GLU) are the amino acids used for the catalytic activities of proteases respectively (Rawlings and Barret 1993). Specificity for substrate and the catalytic mechanism of peptide hydrolysis is additional basis on which proteases are classified. The similarities in amino acid sequences, the optimum pH ranges for activity and inhibitor binding/similiarity (Bode and Huber, 1992) are some of the other features used for classifying proteases in specific groups (Rawlings *et al.*,2010).

2.4 Cysteine Proteases

Mammalian Cysteine proteases are usually found inside the lysosomes. The most important of these are the papain-like enzyme, known as Cathepsins B, H, L, and S (Barrett *et al.*, 1998; Lalmanach *et al*., 2015). Lysosomal cysteine proteinases are important in intracellular protein turnover, antigen presentations and processing events (Henskens *et al*., 1996; Mckerrow et *al*., 1999). They are also implicated in muscle protein turnover and cleavage of several precursor proteins (Lalmanach *et al*., 2015).

Under pathological conditions, lysosomal human cysteine protease are secreted or released by autolysis, the activity of these enzymes can result to permanent damage,

(Henskens *et al*., 1996). Alzheimer disease, inflammation, tumor growth and metastasis are classical examples of pathological processes resulting from disproportions between proteases and their physiological inhibitors (Blankenvoorde *et al*., 1996). A study of the physiology and pathology of mammalian cysteine proteases has aided the design of selective therapeutic agents (Lecaille *et al*., 2002). Mamalian papain-like cysteine proteases have also been recognised as viable drug targets for diseases such as osteoporosis, cancer, immune-related diseases and arthritis (Lalmanach *et al*., 2015).

Cysteine proteases of many diseases causing organism catalyse the entrance of host tissues, and in many bacterial infections are likewise involved in food digestion (Mckerrow, 1999). Parasite cysteine proteases play central roles in immune evasions, activation of enzymes, emergence and molting of parasite (Sajid and McKerrow, 2002). Viral cysteine proteases are implicated in the proteolytic cleavage of precursor proteins for virus replication and in the production of new viral particles (Coombs *et al.,* 2001).

Protozoal cysteine proteases are also involved in the cell invasion of the host and in the metabolism of host proteins for their existence (Mckerrow et *al*., 1999). They are also implicated in the degradation of host immune molecules. They induce diseases such as Chagas' diseases, malaria and other parasitic infections (Redzynia *et al*., 2009).

2.4.1 Catalytic mechanism of cysteine proteases

The cysteine proteases active sites consist of the conserved cysteine, histidine and asparagine residues (papain numbering CYS25, HIS159and ASN175). The proteolytic process of this enzyme is initiated with the formation of an ion pair between CYS and HIS, which is stabilized by hydrogen bond from ASN (Lecaille *et al*., 2002). The subsequent close proximity between CYS and HIS enhances the nucleophillic attack of CYS, which stabilized the cysteine residue prior to substrate binding. They react with substrates and peptides (inhibitors) by three key processes (which are shown in Figure 2.2): hydrolysis (A), acylation (B) and deacylation (C).

After enzyme interaction with a substrate or peptide, the nucleophilic thiolate cysteine attacks the carbonyl carbon of the substrate or peptide scissile bond (Teixeira *et al*., 2011). This forms a tetrahedral intermediate, which is stabilized by the oxyanion hole. The tetrahedral intermediate is then transformed into an acyl enzyme (enzyme-substrate thiol ester) and the C-terminal portion of the substrate or peptide is released in a process called acylation (B) (Lecaille *et al.*, 2002). Subsequently, the acyl enzyme is hydrolyzed by water (A) and it forms a second tetrahedral intermediate, which splits into the free enzyme and N-terminal protein of the substrate in a process called deacylation (C). The active site of this enzyme are generally known to be the representative prime targets for therapeutic intervention (Lecaille *et al*., 2002).

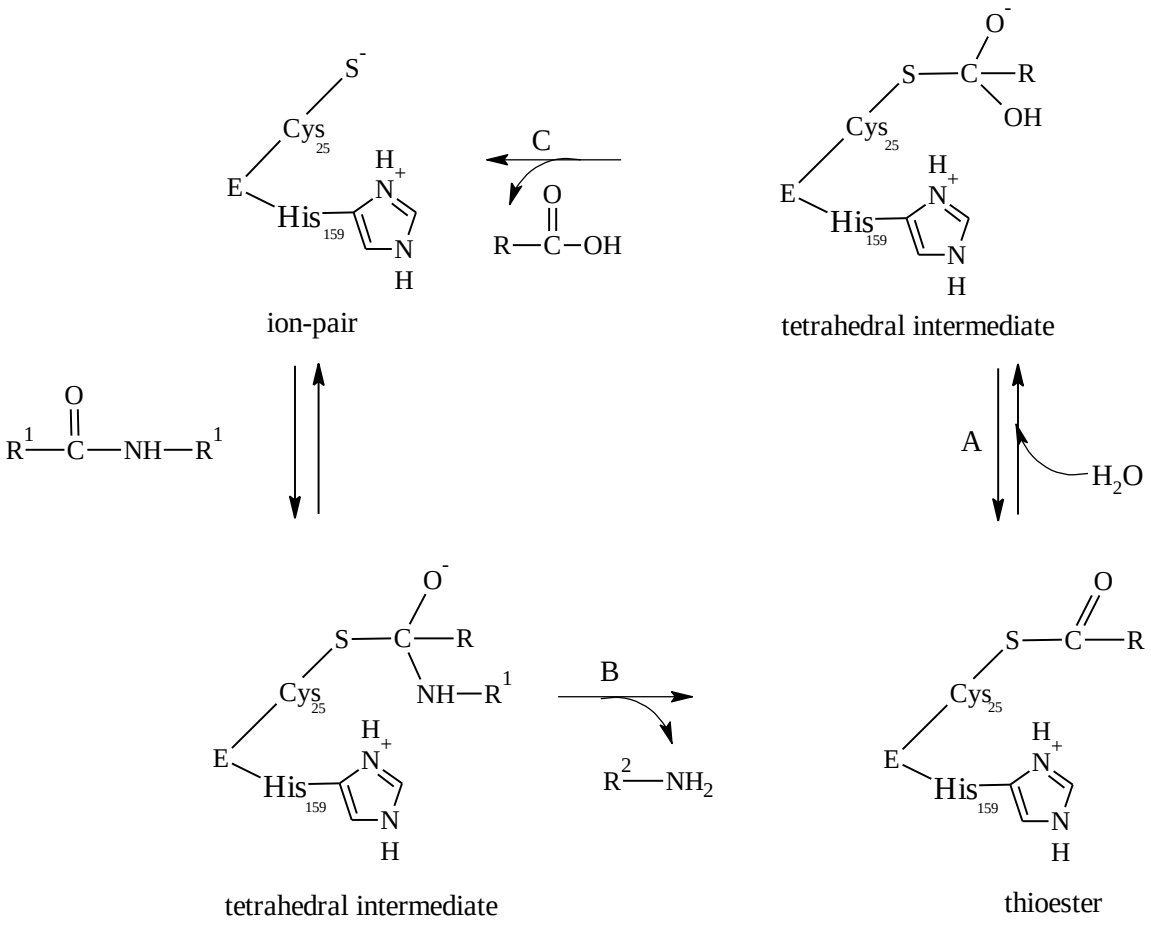


Figure 2. 2: Catalytic Mechanism of Cysteine Proteases.

A show the hydrolysis process, **B** the acylation, **C**-deacylation and **E** shows the Enzyme and **R** the side chain of Peptide Substrate (Lecaille *et al*., 2002).

2.4.2 Cysteine proteases of *Plasmodium*

There have been extensive *in vivo* and *in vitro* studies regarding the roles of *Plasmodium* cysteine proteases using their specific inhibitors. These studies have identified three key functions of *Plasmodium* cysteine proteases: hemoglobin hydrolysis, erythrocyte rupture and erythrocyte invasion by malaria parasites (Rosenthal, 2004). Analysis of the *P. falciparum* genome has resulted in the identification of three papain-like cysteine proteases: falcipains, dipeptidyl peptidase, a calpain homolog, and serine-repeat antigens (Pandey *et al.*, 2004). Falcipains are the most studied cysteine proteases of *P. falciparum* (Pandey *et al* 2004). The falcipains are all expressed during the erythrocytic stage of the parasite (Wegscheid-gerlach *et al*., 2010).They share a number of features and sequence identity with papain-like cysteine proteases. There are four falcipains: falcipain-1 (FP1), two nearly identical proteases falcipain-2 (FP2) and falcipain-2' (FP2') and falcipain-3 (FP3) (Hanspal *et al*., 2002; Singh *et al*., 2006; Jeong *et al.*, 2006). FP1 is encoded on chromosome 14, while FP2, FP2' and FP3 are located on the chromosome 11. FP2 shares 96% sequence identity with FP2', 68% identity with FP3 and 38% identity with FP1 (Rosenthal, 2004).

Hemoglobin degradation is an essential process for amino acids release for the parasite survival within its host. Inhibition of this degradation has been one of the focuses for drug development against malaria (Shenai *et al*., 2000). FP2 is the most abundant and widely studied of all the cysteine proteases (Wang *et al*., 2006) and it often been called the principal hemoglobinase (Pandey *et al*., 2005). The falcipains have an acidic pH optimum, they require reducing conditions for optimal activity, and are inhibited by typical cysteine protease inhibitors (Rosenthal *et al*., 2002).

2.4.3 Erythrocyte invasion and rupture

The invasion of erythrocyte by free merozoites and rupture of erythrocyte by mature schizonts are carried out by Cysteine protease enzyme (Rosenthal *et al*., 1993; Karthik *et al*., 2014). In the process of red blood cell invasion, numbers of surface membrane proteins of merozoites are proteolytically process before binding to receptors of RBC membrane. For example, merozoite surface protein 1(MSP1) is processed in a manner usually inhibited by CPI and resulted in the blockage of RBC invasion by the merozoite. Cysteine proteases of *Plasmodium* are thought to activate the aspartic proteases that are responsible for proteolytic processing of merozoites proteins (Rosenthal *et al*., 1993. Cysteine protease inhibitors irreversibly block the rupture of host cell membranes, thereby preventing the parasite from invading fresh erythrocytes (Glushakova *et al*., 2007).

Falcipain-2(FP2) is implicated in the degradation of erythrocyte membrane proteins such as ankyrin, the band 4.1 protein and thus destabilizes the erythrocyte membrane structure, which leads to rupturing of the host cell and eventual release of the merozoites (Shenai *et al*., 2000; Dahl and Rosenthal, 2005). Some Cysteine protease involved in the invasion and rupture of RBC include, a 68kDa Protease from schizont and merozoite of many plasmodium species, serine repeat antigen (SERA) and related protein, serine repeat protein H (SERP H) (Goldberg *et al*., 1991; Shenai *et al*., 2000) from mature schizont.

2.4.4 Hemoglobin degradation

Most abundant protein in the RBC, Hemoglobin, is completely degraded after parasite entry (Goldberg *et al*., 1991; Teixeira *et al*., 2011). Degradation of host hemoglobin provides amino acids for the production of proteins by the parasite since parasite has a limited

capacity to synthesize its own amino acids (Goldberg *et al*., 1991). Infected erythrocytes have a higher concentration of amino acids than uninfected erythrocytes, and the hemoglobin content of infected erythrocytes decreases significantly (25-75%) during the life cycle of the erythrocytic parasites. The degradation of hemoglobin releases the heme, which is detoxified by polymerization into a crystalline pigment called the hemozion (Goldberg *et al*., 1991).

The haemoglobin degradation process occurs predominantly during the trophozoite stage of the erythrocytic parasite life cycle and is accompanied by erythrocyte cytoplasm ingestion (Rosenthal, 1998). In order to obtain the free amino acids from hemoglobin digestion, the parasites take up erythrocyte cystol via cytostome organelle and transport this material by vesicular trafficking to the food vacuole (Goldberg *et al*., 1991). The food vacuole is an acidic lysosome-like organelle, which contains three enzymes: aspartic, cysteine and metallo proteases, all of which are involved in hemoglobin degradation (Lew *et al.,* 2003). These acidic proteases have been partially purified from the parasite extracts of the different *Plasmodium* species (Rosenthal, 1998). The degradation of hemoglobin has been analyzed by Sodium dodecyl sulphate-polyacrylamide gel Electrophoresis (SDS-PAGE) in which the disappearance of the substrate (hemoglobin) was demonstrated (Francis *et al*., 1997, Shenai *et al*., 2000).

Evaluation of the parasite biochemistry has resulted in the observation that aspartic (Francis *et al*., 1997), cysteine (Sijiwali *et al*., 2001; 2002) and metalloprotease (Eggleson *et al*., 1999; Gavigan *et al*., 2001) are involved in the digestion of hemoglobin in an orderly fashion. In this process (Figure 2.3) the breakdown of hemoglobin is initiated by two aspartic acid proteases, plasmepsin (PM) I and II. Secondary acidic proteases, PM IV and histo-aspartic protease (HAP) and cysteine proteases, Falcipains, FP2, FP2'and FP3 (Subramanian

et al., 2009) follow the cleavage of hemoglobin by PM I and II by cleaving the unravelled hemoglobin even further. Metalloproteases, falcilysin then cleave the hemoglobin fragments from secondary cleavage into individual amino acids (Goldberg *et al*., 1991).

Plasmodium genome analysis shows the presence of 10 aspartic proteases PM (Coombs *et al*., 2001), namely PM I, II, HAP, IV, V, VI, VII, VIII, IX and X. Only three (PM VI, VII and VIII) of the 10 aspartic proteases are not expressed at the intra-erythrocytic stage, and are thus likely to be involved in the insect or exo-erythrocytic stage of the parasite life cycle (Banerjee *et al*., 2002). The other 7 aspartic proteases are expressed during the erythrocytic stage of the parasite but PM V, IX and X do not seem to have any functional role in the food vacuole (Banerjee *et al*., 2002). Only two of the 10 aspartic proteases, PM I and II, initiate haemoglobin degradation (Coombs *et al*., 2001). PM I and II appear to have specificity towards native hemoglobin (Francis *et al*., 1997).

Evidences suggests that both aspartic and cysteine proteases cleave native hemoglobin (Liu *et al.*, 2006, Drew *et al*., 2008). Additionally, the latter argument proposes that cysteine proteases should be pursued as key drug targets for the epidemic malaria because they play a major role in hemoglobin degradation (Teixeira *et al.*, 2011; Shah *et al*., 2011). It is also suggested that cysteine proteases FP2, FP2' and FP3 are involved in the initial and subsequent cleavage of hemoglobin.

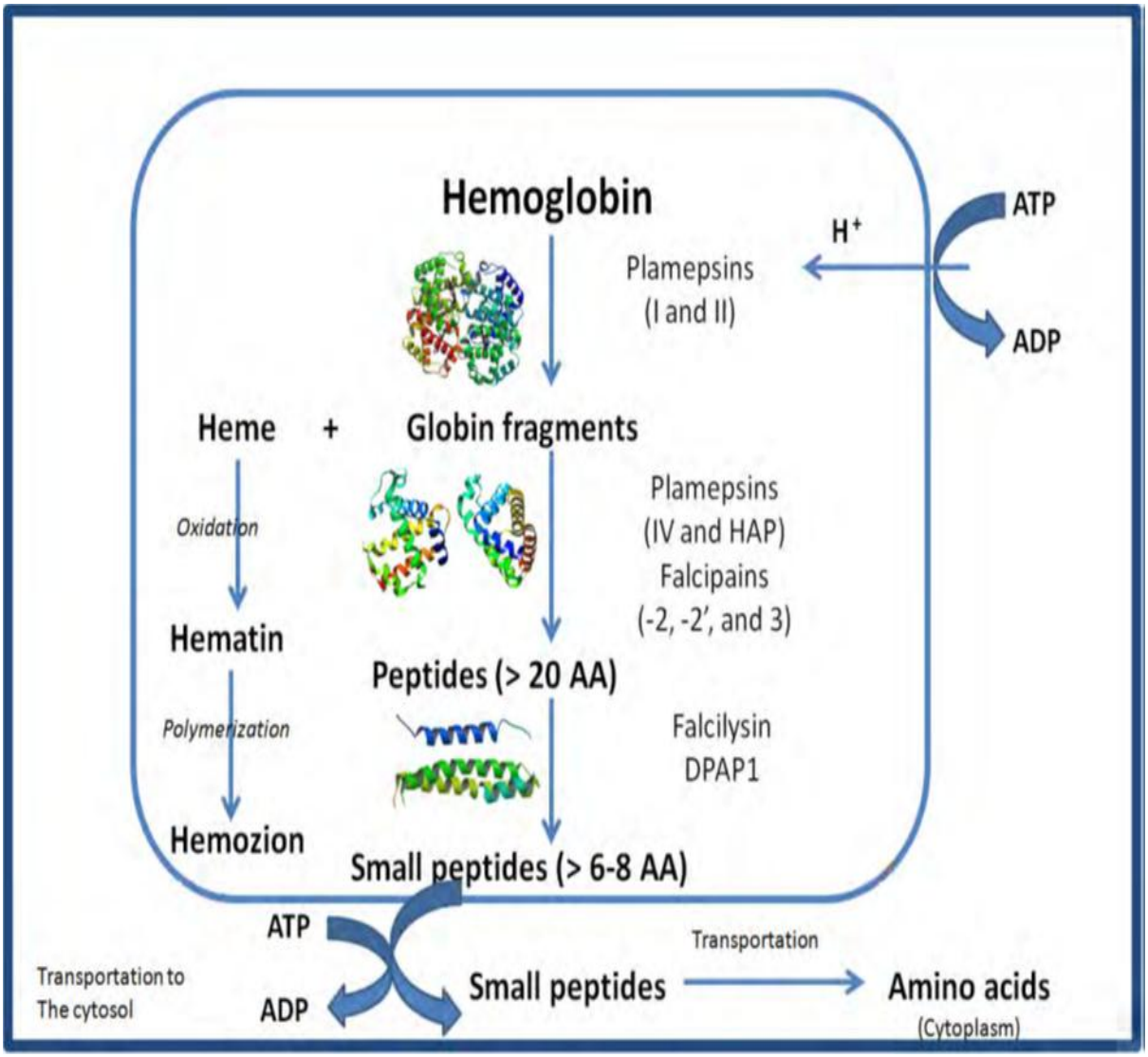


Figure 2. 3: The Pathway for Hemoglobin Hydrolysis

The pathway is initiated by aspartic acid proteases (plasmepsins), and then cleaved by cysteine proteases (falcipains). The peptides are then further cleaved to small peptides and to amino acids by metalloproteases, falcilysins (Ettari *et al*., 2009)

Experimental studies by Salas *et al.*, (1995) showed that FP2 was able to cleave both native and denatured hemoglobin, and validated that hemoglobin cleavage was blocked by cysteine protease inhibitors and not by inhibitors from other classes of proteases. Liu *et al.* (2006) also demonstrated that blockage of plasmepsins using the potent inhibitor, pepstatin, had negligible effect on the parasite, but FP2 knockout killed the parasite.

The incubation of cysteine protease and its inhibitor L-transepoxy-succinyl-leucyl-amido-(4-guadino)-butane (E-64) resulted in buildup of undigested erythrocyte cytoplasm in the parasite food vacuole *in vivo* (Rosenthal, 1998). Leupeptin and E-64 are both non-specific inhibitors of cysteine proteases. Non-specific inhibitors of aspartic acid and cysteine proteases eradicate the parasites both *in vivo* and *in vitro,* whereas inhibitors only specific to cysteine proteases prevented parasite maturation *in vivo* and *in vitro,* thus providing justification that only cysteine proteases should be pursued as promising drug targets (Liu *et al*., 2006).

Individual knockout of plasmepsins and a combination knockout together with plasmepsin IV/I only resulted in a slight reduced growth of the parasite (Liu *et al*., 2006). FP2 knockout markedly diminished the activity of cysteine proteases and blocked the hydrolysis of hemoglobin, which was indicated by a swollen, dark stained food vacuole (Shenai *et al.*, 2000; Sijiwali and Rosenthal, 2004). The effect of aspartic acid protease inhibitors is increased when used in combination with cysteine proteases inhibitors or in falcipain-2 knockout parasites (Sijiwali and Rosenthal, 2004; Liu *et al*., 2006).

However, the disruption of FP2 gene indicated that the loss of FP2 alone is not enough to kill the parasite, suggesting that other cysteine proteases are involved in parasite invasion

and growth within the erythrocyte (Alida *et al.,* 2005). Therefore, the parasite can be killed by deletion of multiple cysteine proteases. Falcipains, except for FP1, have a hemoglobin degrading function and are involved in the conversion of proplasmepsins into active aspartic acid proteases (Drew *et al*., 2008; Pandey *et al.,* 2009). Since FP2 is expressed at the early trophozite stage, it might function as an enzyme needed for maturaion, as FP2' and FP3 are produced at the late trophozoite/early schizont stage (Hogg *et al*., 2006).

2.5 Protease Inhibitors

Protease inhibitors (PIs) are widely distributed and most abundant type of proteins that are found in all living forms, which includes: microorganisms, plants and animals. These inhibitors are involved in regulation of enzyme activity in which there overexpression usually resulted in to diseases (Majumdar, 2013). Protease inhibitors are of many types, which are categorized based on the specificity, amino-acid sequences, localization of the reactive sites, disulfide bridge topology, mechanism of action and three-dimensional structure (Rawlings *et al*., 2004). The different types of Protease inhibitors include aspartate protease inhibitors, serine protease inhibitors, cysteine protease inhibitors and metalloprotease inhibitors.

Protease inhibitors find application in different areas like pharmacology, agriculture and biotechnology. Use of natural protease inhibitors could be an effective means of extending the shelf life of proteinaceous foods including seafood, meat and milk, by retarding or preventing hydrolysis caused by the action of endogenous and exogenous proteases during food processing and preservation (Weng and Delisu, 2002; Bijina *et al*., 2011_b). Specific protease inhibitors are effective tools for inactivating the target proteases which are involved in pathogenic process of human diseases like arthritis, pancreatitis, hepatitis, cancer, AIDS,

thrombosis, emphysema, high blood pressure, muscular dystrophy (Johnson and Pellecchia, 2006). Protease inhibitors are one of the prime molecules with highly proven inhibitory activity against insect's pests and are used as bioinsecticides to developed transgenic plants (Bijina *et al*., 2011_a).

Besides inhibitors that are proteins, certain bacteria and fungi synthesize peptides and derivatives of peptides that are active peptidase inhibitors. Possibly the best known of these is leupeptin (N-acetyl-L-leucyl-Lleucyl D, L-argininaldehyde) from *Streptomyces exfoliates* which inhibits a wide range of serine, cysteine and threonine-type peptidases, including trypsin, calpain, clostripain (Shamsi *et al*., 2016). Other small molecule inhibitors produced by actinomycetes include bestatin and amastatin (inhibitors of aminopeptidases) and tyrostatin, which inhibit different types of protease.

The cysteine protease specific inhibitor, E-64, initially isolated from *Aspergillus japonicus* has been studied extensively as a potential antitumour agent in cell culture and animal models (Bijina *et al*., 2011_a). General-purpose cysteine peptidase inhibitors include N-ethylmaleimide, iodoacetate, iodoacetamide and p-chloromercuribenzoate (PCMB). General-purpose serine peptidase inhibitors include diisopropyl fluorophosphates (DFP), 3, 4-dichloroisocoumarin (DCI), and phenylmethanesulfonyl fluoride (PMSF). Metallo-peptidase inhibitors include the metal chelators EDTA (ethylene diamine tetraacetic acid), ethylene glycol tetraacetic acid (EGTA) and 1, 10-phenathroline.

Bikunin, a Kunitz-type protease inhibitor, found in human amniotic fluid, serum and urine is a serine protease inhibitor with anti-metastatic activity. α2-macroglobulin (α2-M) a major serum glycoprotein with four identical subunits of approximately 185 kDa each is

another nonspecific protease inhibitor (Ryan, 1990). A thrombin inhibitor, Hirudin is the final effector in coagulation, which converts soluble fibrinogen into insoluble fibrin. Hirudin is a 65 amino acid peptide isolated from the salivary glands of the medicinal leech with a Ki of 0.2 pM (Bijina *et al.*, 2011_b).

2.5.1 Plants protease inhibitors

Plants defend themselves against herbivores and pathogenic organisms by producing bioactive secondary metabolites and defensive proteins (Montesinos, 2007). Production of such defensive agents may be constitutive or induced by wounding or pathogen infection. Several non-protein and protein defensive bioactives inhibit plant protein digestion by the attacking organisms. The destructive potential of proteases means that their activity has to be tightly regulated to prevent autolysis of protease-producing cells (Kuhar *et al.*, 2014). Therefore, the quest for novel protease inhibitors offers development of ecologically friendly pest and pathogen management strategies (Majumdar, 2013).

Plant protease inhibitors are widely distributed among different botanical families, which inhibit different types of proteases. In plants, 1 to 10% of their total proteins are protease inhibitors, mostly found in storage organs such as seeds and tubers. The physiological significance of protease inhibitor on the digestive physiology of animals, occurrence, classification, and the role of inhibitors as defensive proteins in plants against insects and microorganisms has been extensively studied (Ryan, 1990).

Plant proteinase inhibitors (PPIs) play an important role by interacting with their proteases forming protein complexes that modulate enzyme activity. The significance of PPIs as a relevant element controlling plant physiology is evident by their tremendous distribution

among different species. PPIs are represented by 19 families in the inhibitors section of MEROPS database (Rawling *et al*., 2010).

2.5.2 Cysteine protease inhibitor

Cysteine protease inhibitor (CPI) also called Cystatins are the natural inhibitors of cysteine proteinases (Henskens *et al*., 1996; Dubey *et al*., 2007). Chicken egg white in 1968 was the first cystatin. These ubiquitous inhibitors of Cystiene proteases play several important roles in plants, from the control of various physiological and cellular processes in plant to the inhibition of exogenous Cysteine proteases secreted by arthropods and pathogens to digest plant tissues (Dubey *et al*., 2007; Costa *et al*., 2014). Cystein protease inhibitors from different parts (seeds, root, fruits and leaves) of monocotyledonous and dicotyledonous plants were studied and reviewed by Mosolov and Valueva (2005).

The inhibitors of this enzyme are divided into structurally related families: cystatins, stefins, kininogens and phytocystatin. However, the classification of the first three CPIs was based only on animal tissues. A fourth group belonging to the cystatin superfamily found only in the plant tissues emerged called Phytocystatin. The first plant cystatin was established in rice seeds (oryzacystatin) by Abe *et al.,* (1987). Several other plant CPI were discovered in potato, soy, wheat, corn, cowpea, ragweed, avocado, and papaya (Dubey *et al.,* 2007).

Homology searches show that phytocystatins are similar to family 102 cystatins of animal origin, but lack disulfide bonds like family 1 cystatins (Abe and Arai, 1987). Despite the high protein sequence homology, the gene organization of oryzacystatins is markedly different to that seen in animal cystatins. Therefore, it has been suggested that a

new cystatin family exists, namely the phytocystatins (Brown *et al*., 2000). Cysteine protease inhibitors have been shown to play a key role against viruses, bacteria, and parasites, in the control of tumor growth and metastasis, in the protection against tissue destruction (Henkens *et al*., 1996; Sousa *et al*., 2015). Many viruses require proteolytic cleavage to become infectious. Cystatins possess activity against a variety of viruses such as poliovirus, rhinovirus, coronavirus, and herpes simplex virus (Blankenvoorde *et al*., 1996).

Most cystatins are competitive inhibitors of cysteine proteinases, which form equimolar complexes with their target enzymes (Blankenvoorde *et al*., 1996). Their mode of action is based on three domains that show highly conserved amino acids sequences. These are important for the inhibitory activity. These domains consist of 10 amino acid residues in the amino terminus, a β-hairpin loop containing the conserved QVVAG residues, and a second β-hairpin loop containing the conserved residues Leu102, His 104 in family 1 and Trp104 in family 2 cystatins (Zindel *et al*., 2013). This segment penetrates and covers the active site in such a fashion to block the papain or other cysteine proteinase's active site cysteine residue (Blankenvoorde *et al*., 1996; Schechter and Berger, 1967). Alterations to the proteinase inhibitors-cysteine proteinase ratios contribute to the progression of several pathological processes.

Shamsi *et al.*, (2016) have reported antitumor activity of cysteine proteinase inhibitors, E-64 and leupeptin, by selective reduction of the growth of transformed cells and reduction of the occurrence of cancer in animal models. Cysteine proteinases activate proinflammatory mediators, and catalyze tissue degradation. Periodontitis and rheumatoid arthritis are inflammatory diseases catalyzed by cysteine proteinases. Smoking is associated with lower cystatin activity during gingival inflammation (Blankenvoorde *et al*., 1996).

Cathepsins B and L are enzymes associated with the onset of rheumatoid arthritis and higher levels of the enzymes are found in synovial tissues and fluids of arthritis patients (Goldberg *et al*., 1991). Therefore, cathepsin B seems to be a good target for pharmacological intervention. Cysteine proteinase inhibitors anti-inflammatory and anti-rheumatic drugs successfully reduce cysteine proteinases that catalyze tissue destruction in rheumatoid arthritis (Blankenvoorde *et al*., 1996). The potential exists to utilize CPI in food and pharmaceutical formulations as inhibitors of enzymes associated with the onset and/or progression of a wide range of pathological processes.

2.5.3 *Plasmodium* and cysteine protease inhibitor

General cysteine protease inhibitors have been used to analyze their roles both *in vitro* and *in vivo* (Ramjee *et al*., 2006). For the *in vivo* studies, animal models of malaria were tested in order to confirm that inhibitors have potent antiparasitic activity, oral bioavailability, safety and pharmacokinetic properties (Rosenthal *et al*., 2002). The major challenge encountered from *in vivo* analysis is that *P. falciparum* can only be studied in a few primate species and therefore only murine models have been used so far. However, despite the limitations of murine models, cysteine protease inhibitors demonstrated *in vivo* antimalarial effects. Fluoromethyl ketone was needed in high doses but it cured malaria in 80% of *P. vinkei* infected mice (Rosenthal *et al*., 1993), while vinyl sulfone cured 40% of mice when infected by *P. vinkei* (Sijiwali and Rosenthal, 2004). These two compounds are relatively poor inhibitors of vinkepain-2, as compared to their inhibition of falcipain-2; therefore, it can be assumed that improved inhibitors will generate better success (Rosenthal and Nelson, 1992; Rosenthal *et al*., 1993).

Peptidyl aldehyde and α-ketoamide are amongst the most promising cysteine protease inhibitors to be used for chemotherapeutic treatment (Rosenthal, 2003). These inhibitors block the activity of falcipain-2 and falcipain-3, which prevents parasite development because it prevents hemoglobin degradation (Lee *et al*., 2003). Some of the small molecule inhibitors targeting the falcipains and other homologous cysteine proteases in other *Plasmodium* species include fluoromethyl ketones, vinyl sulfone, chalcones and phenothiazines (Rosenthal *et al*., 1996; Kerr *et al*., 2009). The compounds mentioned above have been argued to have inhibitory activity against the falcipains, the main support of this argument was provided by the evidence of undegraded hemoglobin and parasite development was halted during *in vivo* antimalarial activity assays (Rosenthal *et al*., 2002; Lee *et al*., 2003).

It should be noted that during the process of inhibitor design, two critical factors must be taken into consideration: (1) the side effects of the inhibitor and (2) the complimentary of the inhibitor to the target protease active site. Therefore based on these approaches of inhibitor design it has been observed that despite the extensive *in vivo* and *in vitro* work that have been done on cysteine protease inhibitors, some could not access the food vacuole and were therefore rendered ineffective (Singh and Rosenthal, 2001; 2004). Some cysteine protease inhibitors have been found to be effective against five strains of *P. falciparum* that differ widely in their sensitivities towards standard antimalarial agents, this observation suggest that they will not result in multidrug resistant parasites (Rosenthal *et al.*,1991;Singh and Rosenthal, 2001).

2.5.4 Purification of protease inhibitors

Purification of protein to homogeneity is a vital requirement for the characterization and property studies. There are several reports on purification of protease inhibitor from plants, animals and microbes. Protein purification methods like ammonium sulfate precipitation, gel filtration chromatography, ion exchange chromatography, hydrophobic chromatography, affinity chromatography, and RP-HPLC were regularly employed. The homogeneity is confirmed by methods like SDS-PAGE, Isoelectric focusing, MALDI-TOF, etc. Ammonium sulphate precipitation of the inhibitors was employed commonly to precipitate proteins. Alternatively, protease inhibitors were precipitated using acetic acid, ethanol, acetone, or combination of ethanol and acetone (Jui *et al* 2002; Karthik *et al*., 2014). Most purification protocols require more than one-step to achieve the desired level of product purity. High purity and yield can be achieved by adding or repeating steps.

Ion exchange chromatography is one of the chromatographic procedures employed in purification of protease inhibitors, wherein anion or cation exchangers are used according to the binding characteristic of protease inhibitor on the ion exchange resin. DEAE cellulose have been employed for the purification of a novel bi-functional protein proteinase/amylase inhibitor from the dietary leguminous pulse *Phaseolus aureus Roxb* (Majumdar, 2013). Cation exchange chromatography on SP-Sepharose and anion exchange chromatography on Q-Sepharose are employed for the purification of trypsin-chymotrypsin inhibitors from *Vigna mungo* (Shamsi *et al*., 2016). Hydrophobic interaction chromatography on phenyl sepharose column (Gupta *et al.,* 2005) used in the present research is another type of chromatography with few reports on their applications

2.5.5 Characterization of protease inhibitors

Most protease inhibitors are stable in the presence of chemical and physical agents, such as reducing agents, temperature and pH. Intra molecular disulphide bridges are presumably responsible for the functional stability of these protease inhibitors (Bijina *et al.*, 2011_a). Extreme pH conditions will change the structure of the inhibitor such that they no longer bind with the enzymes or with their substrates. Under strong acidic or alkaline conditions, the proteinaceous inhibitors get denatured and therefore they lose their activity partially or completely.

The majority of the protease inhibitors exhibiting anti-feedent properties reported so far are active against the neutral serine proteases such as trypsin and chymotrypsin (Ryan, 1990). Insects that feed on plant material possess alkaline guts and depend predominantly on serine proteases for digestion of food material. Protease inhibitors from soyabeans with wide ranges of temperatures and pH stability have been reviewed by Jack *et al*., (2004).

Metal ions enhance the structural stability of a protein in a conformation needed for its biological function. Metal ions have a key role in upholding the structural integrity of protease inhibitor (Cordt *et al*., 1994). The side chain carboxylates of aspartate and glutamate residues can contribute in binding of divalent cations to metalloproteins. The main amino acid residues that serve as ligands to divalent ions are Aspartate, Glutamate, Threonine and Histidine (Majumdar, 2013).

Modification of amino acid by side chain-specific reagents is an important tool for studying structure and functional relationship of biologically active proteins. Amino acid side chain specific chemicals like N-Bromosuccinanmide (NBS), N-Ethylmaleimide, PMSF,

trinitrobenzene-sulfonic acid (TNBS) and diethylpyrocarbonate (DEPC) are commonly used. The amino acid of pearl millet cysteine protease inhibitor is modified using amino acid specific chemical modifiers. Modification of histidine using 10mM Diethylpyrocarbonate and arginine with 10 mM p-Nitrophenylglyoxal enhanced protease inhibitory activity, whereas cysteine, glutamic/aspartic acid resulted in abolition of the anti-fungal activity of CPI.

The role of amino acids Lys, Arg and His in the structure and stability of the legume Bowman-Birk inhibitor was studied by chemical modifications. The presence of these amino acids and their association influence the physiological and functional role of these inhibitors (Bijina *et al*., 2011_a). Modification studies of *Lentinus* proteinase inhibitor, purified from the fruiting bodies of the edible mushroom, suggest involvement of one or more arginine residues in the inhibition of trypsin. Trypsin inhibitor purified from the seeds of *Peltophorum dubium* was inactivated by lysine and arginine modifying reagent (Shamsi *et al*., 2016).

The inhibitor constant, K_i, is an indication of how potent an inhibitor to its target enzyme. Enzyme inhibition studies are routinely conducted to assess the presence and magnitude of protein-drug interactions. Characterization of inhibitor includes understanding the nature of inhibition process (i.e., competitive, noncompetitive, or uncompetitive) and determination of the inhibition constant (K_i). A given inhibitor may inhibit several proteases, and potency of an inhibitor is often described as IC_{50} (the molar concentration of the inhibitor that gives 50% inhibition of the target enzyme activity) (Shamsi *et al*., 2016). Recombinant cnispin (rCnp) from *Basidiomycete Clitocybe nebularis*, which inhibits trypsin, with a Ki value of 3.1 nM, was shown to be a fast-acting and tight-binding inhibitor of this enzyme (Shamsi *et al*., 2016).

2.6 *Calotropis procera*

2.6.1 Ethnopharmacology

Calotropis procera Linn (Plate I) is a wild plant growing in tropical regions. It belongs to Family *Asclepiadaecae*. It is known as Swallow wort and Sodom apple in English, various vernacular names, Tunfafia in Hausa and Bomubomu in Yoruba. In traditional medicine, this plant has been advocated for a variety of disease conditions (Al-snafi, 2015) including ulcers, piles (Warret, 1997), leprosy and tumours (Qureshi *et al*., 2007). Leaf and root of this plant have been used to relive joint pain, toothache, skin disease, rheumatism, migraine and aches (Larhasini *et al*., 1997). The milky latex is used in the treatment of leprosy, eczema and inflammation (Fahey, 2005). Antidiarrhoeal, antioxidant and antihyperglycemic activity is also reported from the latex (Kumar and Roy, 2009). The latex part of the plant has been reported to cure cutaneous infections, syphilis, and malarial infection (Mudi and Bukar, 2011). The leaf has been used as an anti-inflammatory and anti-microbial agent (Fahey, 2005).

The root of *C.procera* is used as a hepatoprotective agent, against colds and coughs, syphilis and elephantiasis, as an anti-inflammatory, analgesic, antimalarial and antimicrobial agent (Mudi and Bukar, 2011). The flower of the plant have been found to be cytostatic, abortifacient, antimalarial, antiasthma and relieve piles (Mudi and Bukar, 2011; Kazeem *et al*., 2016).



Plate I: *Calotropis procera* Leaves and Flowers

2.7 *Moringa oleifera*

2.7.1 Ethnopharmacology

Moringa oleifera Lam is called wonderful plant (Plate II) it belongs to family *Moringaceae*. In traditional medicine various parts of the tree are used therapeutically, including for the treatment of rheumatism, venomous bites, and as cardiac and circulatory stimulants, cholera, scurvy, respiratory ailments, tumours and they are also applied externally to cure inflammatory swellings (Shuaib *et al*., 2008). Juice extracted from the leaves has antibacterial and antimalarial properties (Adebayo and Krettli, 2011; Lawal *et al*., 2015).

In Nigeria, it is locally used as tonic and aphrodisiac, and in the treatment of intestinal worms and asthma. Studies on the various therapeutic effects of *M.oleifera* has been reported among which includes: antibiotic (Fahey, 2005), anticancer (Dasgupta *et al*., 2016), anti-ulcerogenic, analgesic, antiurolithiatic, antitrypanosomal and larvicidal activities (Anwar *et al*., 2007). Fresh root of the young tree (also the root bark) is used internally as stimulant, diuretic and anti-lithic and externally applied as a plaster or poultice to inflammatory swellings (Dasgupta *et al*., 2016).



Plate II: ***Moringa oleifera*** **Leaves, Flowers and Seeds**

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals:

Benzoyl-DL-argininyl-p-nitroanilide (BapNA), Papain (EC3.4.22.2 from *Carica papaya* latex) Phenyl-Sepharose CL-B, Trypsin,Chemotrypsin, Pepsin, Elastase, Ammonium sulphate, Triton X-100, all reagents used in electrophoresis, buffer components, nitrocellulose membrane (dialysis bag), cysteine-HCl, were from Sigma Chemical Co.(USA). Reagent kit for glucose is from Randox, triacylglycerol and creatinine kits were obtained from AGAPE laboratory, while kits for liver functions were obtained from Spectrum and Dialab. All other reagents were of analytical grade.

3.1.2 Plant material

The plants were collected between periods of November to January in Samaru, Zaria (Latitude 11.15^0N and Longitude 7.65^0E). The plants were identified in the Department of Biological Sciences, Ahmadu Bello University, Zaria, Kaduna State with voucher number 900219 and 571 for *Calotropis procera* and *Moringa oleifera* respectively. Different plant parts including seeds, leaves, latex, flowers, roots and stem bark were used for the study.

3.1.3 Animals

Healthy Swiss albino mice of either sex, about 4 - 6 weeks old and weighing between 20-30g each were obtained from Ahmadu Bello University, Zaria. The mice were housed in well-ventilated cages and are allowed free access to commercial food pellets and water ad

libitum. Experiments were conducted in strict compliance with internationally accepted principles for laboratory animal use and care as contained in the Canadian Council on Animal Care Guidelines and Protocol Review (CCAC, 1997).

3.1.4 Parasite

Plasmodium berghei berghei NK65, chloroquine sensitive strain was obtained from National Institute of Pharmaceutical Research and Development (NIPRD) Abuja, Nigeria and maintained in laboratory by serial passage in mice.

3.2. Methods

3.2.1 Extraction of protease inhibitor

Fresh parts (25g each of); leaves flowers, latex, roots and stem barks of *C. Procera* and seeds, leaves, flowers, roots and stem barks *of M. oleifera* was used. Each parts were blended with 100 mL each of sodium chloride 15% (w/v) , sodium hydroxide 0.2% (w/v), hydrochloric acid 0.05M, phosphate buffer 0.1 M (pH 7) and distilled water as described by (Wu and Whitaker, 1990). The clear supernatant obtained after homogenization and centrifugation (10,000 rpm, 15 min, 4 ^{0}C) represent the crude extract, and was assayed for protease inhibitor activity and protein content. The extract with highest activity was used for further studies.

3.2.2 Acetone precipitation

Four volumes of cold acetone were added with stirring to the crude extract. After 20 min stirring, the precipitate was collected by suction filtration, washed with cold acetone. The precipitate was air dried and stored at 4°C under desiccation. The acetone precipitate was

suspended in 100mM phosphate buffer, pH 7.6. This was designated as the crude protein acetone extract (Rao *et al.*, 1983).

3.2.2.1 Inhibitory Effect of Acetone Precipitate on Papain Activity

Different concentrations of protein precipitate of *C.procera* and *M.oleifera* (ranging from 0.1-3.0mg/ml) were incubated with 1mg/ml papain, prepared in 100mM phosphate buffer, pH 6.8, containing 0.3mM EDTA and 2 mM cysteine-HCl. The reaction was initiated with 200µL of 1.25mM BApNA solution for 1 hour at 37^0C. The reaction was terminated with 30% acetic acid (Wannapa *et al.*, 2006). Activity of inhibitor was assayed as described below.

3.2.3 Protease inhibitor assay

Protease inhibitor activity was assayed according to the method of Wannapa *et al.*, (2006). Protease activity was assayed using N-Benzoyl-DL-argininyl-p-nitroanilide (BapNA) as substrate.

Assays were performed by adding 290µL of 50mM Tris–HCl (pH 7.6) and 200µL of 1.25mM BA*p*NA solution to the previously preincubated cysteine protease (1mg/ml papain prepared in 100mM phosphate buffer, pH 6.8, containing 0.3 mM EDTA and 2 mM cysteine-HCl) with 0.1mg/ml of inhibitor extracts for 10 min, at 25^0 C. After 30 min at 37^0C, the reaction was stopped by adding 150µL of acetic acid (30%) with total reaction mixture of 2 ml. The resulting color was measured spectrophotometrically at 410 nm. All assays were performed in triplicate.

One unit of enzyme activity was defined as 1 µmol of *p*-nitroaniline (*p*NA) released per min from the hydrolysis of BA*p*NA under the reaction conditions.

One unit of inhibitory activity corresponds to the amount that is required to inhibit the release of 1 µmol of p-nitroaniline per mL per min.

3.2.4 Protein estimation

Protein content was determined according to the method of Bradford (1976) using Bovine Serum Albumin (BSA) as the standard and the concentration was expressed in milligram per milliliter (mg/mL).

3.2.5 Purification of crude extracts from *C.procera* and *M.oleifera*

3.2.5.1 Ammonium sulphate fractionation

Crude protein extract from section **3.2.2** was precipitated at different percentages of ammonium sulphate saturation (10-80%).The precipitated proteins were separated by centrifugation at 10,000 rpm at 4°C for 20 min. Active protein pellets for each precipitation were dissolved in phosphate buffer and dialysed against the buffer.The dialysed samples from both plants were used for further purification.

3.2.5.2 Hydrophobic chromatography of inhibitor on phenyl-sepharose column.

Hydrophobic interaction chromatography was performed on a Phenyl Sepharose(Gupta *et al* .,2005).The column (1 cm×6.5 cm) was equilibrated with 100mM sodium phosphate buffer, pH7.2 containing 1M ammonium sulphate . The active proteins from step **3.2.5.1.**were mixed with buffer (20.0 ml, containing 1M ammonium sulphate, pH adjusted to 7.2) and was loaded onto the column at a flow rate of 0.7 ml min^{-1}. The bound

proteins were then eluted from the column using 0.1M sodium phosphate buffer, pH 7.2 containing a decreasing step gradient of ammonium sulphate (0.5 M, 0.2 M, and 0.1 M). The final fraction was eluted with 0.1M sodium phosphate buffer, pH 7.2 containing no ammonium sulphate. Fifty fractions (2ml each) and 30 fractions for *C.procera* and *M.oleifera,* respectively, were collected and tested for papain inhibitory activity and protein concentration.

3.2.6 Characterization of purified inhibitor

3.2.6.1 Effect of pH on activity of purified inhibitor

Purified inhibitors were prepared in different buffer systems of pH 2–12 at 37 ^{0}C incubated for 15min. The buffers used are: 100mM; acetate buffer (pH2-6), phosphate buffer (pH6-8), and tris buffer (pH9-12). Protease inhibitor assay was conducted as describe in section **3.2.3**.

3.2.6.2 Effect of pH on the stability of the inhibitor

The purified inhibitors were incubated with 0.1 ml of different solutions of buffers for 72 h at 4^{0}C .The pH was adjusted by using 100mM of acetate buffer (pH2-6), phosphate buffer (pH6-8), and tris buffer (pH9-12). After this treatment, 0.4 ml of 0.2 M phosphate buffer, pH 7.6 was added and aliquots were assayed for antipapain activity.

3.2.6.3 Effect of temperature on activity of purified inhibitor

The activity of the purified inhibitor was determined as a function of temperature. For this purpose 0.5 ml of the purified inhibitors were added to 0.3 ml of the substrate and incubated at varying temperatures; 10^0, 20^0, 30^0, 40^0, 50^0, 60^0, 70^0, 80^0, 90^0 and 100^{0}C after 60 minutes of incubation, the reaction was stopped and the activity determined.

3.2.6.4 Effect of temperature on the stability of the inhibitor

Thermal stability was determined by pre-incubating 1.0mL of the purified inhibitor for 30 min at varying temperatures; 30^0C, 40^0C, 50^0C, 60^0C, 70^0C, 80^0C, 90^0C and 100^0C. The tubes were cooled to room temperature, substrate added to the reaction mixture and incubated at 37^0C for 2 Hours and assayed for residual inhibitory activity.

3.2.6.8 Effect of chemical modification of amino acids on inhibitor activity

Chemical modifications of amino acids of purified inhibitor were carried out using different chemical modifiers under their respective reaction conditions. Hundred microlitre of purified inhibitor (0.1 mg/mL) was used for this study. After the incubation with 25mM of each modifier (Iodoacetamide, Succinic anhydride, Diethyl pyrocarbonate, Phenyl methyl sulphonyl fluoride and *N*-Bromosuccinamide for Cysteine, lysine, Histidine, Serine and Tryptophan respectively) in 0.1M Tris buffer pH 7.0 at 30^0C for 60min, the sample dialysed against phosphate buffer and residual inhibitory activity measured(Krishnan and Murugan, 2015).

3.2.6.9 Effect of concentrations on the activity of the purified inhibitor.

Different concentrations of purified protease inhibitor (ranges:10-80µg/ml) were preincubated with 0.5mg/ml of papain prepared in 100mM phosphate buffer, pH 6.8, containing 0.3mM EDTA and 2 mM cysteine-HCl at 37^0C for 60 minutes. The inhibitor activity was assayed for as described in section **3.2.3.**

3.2.7 Toxicological studies

3.2.7.1 Determination of median lethal dose (LD_{50})

The experiment was carried out in two phases. In phase one, three groups comprising of three mice each were treated intraperitoneally (ip) with 10, 100 and 1000mg/kg body weight (BW) respectively of crude extracts from both plants. Phase two experiment had four groups of three mice each. They were intraperitoneally (ip) treated with 200,400,600 and 800mg/kg body weight (BW) respectively of crude extracts from both plants (Lorke, 1983). A control group was given phosphate buffer saline (PBS). Mice were observed over 72 h and mortality was recorded. The LD_{50} value was determined by calculating the geometric mean of the animals that survived.

$LD_{50} = \sqrt{}$ (Highest non-lethal dose) × (Lowest lethal dose)

3.2.7.2 Subchronic toxicity test

Subchronic toxicity was determined according to the method of Aniagu, *et al.,* 2004). Mice (45) were divided into nine groups (1-9) of five mice each; the first group (1) served as control. The animals in control were given only 0.2ml/kg.bw phosphate buffer saline (PBS), while the groups (2-4) received 20mg/kg.bw, 30mg/kg.bw and 50mg/kg.body weight respectively of crude extracts of latex from *C.procera,* while group 5 was given 50mg/kg.bw of purified extract from latex of *C.procera* intraperitonealy (i.p) for 21 days.

Groups(6-8) were given 30mg/kg.bw,50mg/kg/bw and 70 mg/kg.bw respectively of crude extracts from seeds of *M.oleifera,* while group 9 was given 70mg/kg.bw of purified extract from seeds of *M.oleifera* intraperitonealy (i.p) for 21 days. Packed cell volume (PCV)

of each groups were compared before and after 21 days period. On the 22nd day mice were sacrificed, blood samples collected by cardiac puncture and allowed to clot, centrifuged at 4000 rpm for 10 minutes and serum separated, then diluted twofold and used for analysis.

3.2.8 Determination of packed cell volume (PCV)

Blood was collected into heparinized capillary tubes and drawn up by capillary action to about three quarters of the tubes length with one end of the tube sealed. The blood was centrifuged using a hematocrit centrifuge at a speed of 5000rpm for five minutes. Results were taken using a hematocrit reader (Coles, 1980).

3.2.9 Biochemical assays

3.2.9.1 Determination of Serum Alanine Aminotransferase (ALT)

PRINCIPLE: L-alanine reacted with α-ketoglutarate in a transamination reaction catalysed by alanine transaminase to form pyruvate and L-glutamate. The pyruvate formed then reacted with 2, 4-dinitrophenylhydrazine. The activity of Alanine transaminase in the sample is proportional to the concentration of pyruvate hydrazone formed, which was measured spectrophotometerically at 546nm (Rietman and FrankeL, 1957). The equation of the reaction is shown below.

L-Alanine + α-ketoglutarate $\xrightarrow{\text{ALT}}$ Pyruvate + Glutamate

Pyruvate + 2, 4-dinitrophenylhydrazine $\longrightarrow$ Pyruvate hydrazone

METHOD: In the first tube (reagent blank), 0.5ml of solution R1 (Phosphate buffer, L-alanine and α-ketoglutarate) and 0.1ml of distilled water were pippeted. To the second tube

(sample), 0.5ml of solution R1 was added to 0.1ml of serum. The mixture were shaken properly and left to incubate for 30 minutes at 37^0C. Then 0.5ml of solution R2 (2, 4-dinitrophenylhydrazine) was pipetted into the two tubes. The mixtures were left to incubate for 20 minutes at 25^0C. Finally, 5.0 ml of sodium hydroxide was added into the tubes. After 5 minutes, the absorbance was taken using spectrophotometer at a wavelength of 546nm (Rietman and FrankeL, 1957).

ALT activity (unit/L) = (ΔA/min) x1746

3.2.9.2 Determination of Serum Aspartate Aminotransferase (AST)

PRINCIPLE: L-aspartate in the buffer reacted with α-ketoglutarate in a transamination reaction catalysed by aspatate transaminase in the sample to form oxaloacetate and L-glutamate. The oxaloacetate formed was then reacted with 2, 4-dinitrophenylhydrazine to form oxaloacetate hydrazone. The activity of aspartate transaminase in the sample was proportional to the concentration of oxaloacetate hydrazone formed, which was measured spectrophotometerically at 546nm (Rietman and FrankeL, 1957). The equation of the reaction is shown below.

L-Aspartate + α-ketoglutarate $\xrightarrow{\text{AST}}$ Oxaloacetate + L – Glutamate

Oxaloacetate + 2, 4-dinitrophenylhydrazine $\longrightarrow$ Oxaloacetate hydrazone

METHOD: To the first tube (reagent blank), 0.5ml of solutionR1 (Phosphate buffer, L-aspartate and α-ketoglutarate) and 0.1ml of distilled water was pipetted. To the second tube (sample), 0.5ml of solution R1 and 0.1ml of serum were added. They were shaken properly and left to incubate for 30 minutes at 37^oC. To two tubes, 0.5ml of Solution R2 (2, 4-

dinitrophenylhydrazine) was added and were left to incubate for 20 minutes at 25ºC. Finally, 5.0ml of sodium hydroxide was added into the tubes. After 5 minutes, the absorbance was taken using spectrophotometer at a wavelength of 546nm (Rietman and FrankeL, 1957).

AST activity (Unit/L) = (ΔA/min) x1746

3.2.9.3 Determination of Serum Alkaline Phosphatase (ALP)

Principle: p-nitrophenyl phosphate was hydrolysed to p-nitrophenol and inorganic phosphate. The rate at which p-nitrophenyl phosphate is hydrolysed was directly proportional to the alkaline phosphatase activity measured at 405nm (Rosalki *et al*, 1993).

P-nitrophenyl phosphate + H_2O $\xrightarrow{ALP}$ P-nitrophenol + Inorganic phosphate

METHOD: Working reagent was prepared by mixing 4ml of reagent 1 (Dieethanol amine Buffer, pH 10.2 and Dieethanol amine Buffer, pH 10.2 and Magnessium choride) with 1ml of reagent 2(p-nitrophenyl phosphate). 1000µl of the working reagent was pipetted into a test tube, 20 µl of the sample was added, mixed and incubated for one minute at 37ºC. Absorbance was measured at 405 nm against the reference blank.

ALP activity (U/L) = (ΔA/min) x 2750.

3.2.9.4 Determination of Serum Creatinine

PRINCIPLE: Creatinine present in the serum reacts with alkaline picrate to form a colored complex. The rate of formation of colored complex is directly proportional to creatinine concentration. This rate of reaction (intensity of orange color produced) is measured

colorimetrically at 500nm and is compared with that of the standard (Bartels and Bohmer 1973).

Creatinine + Picric acid ⟶ Creatinine picrate

METHOD: Equal volume of the prepared standard reagents were mixed with sodium hydroxide and picric acid at 37 ^{0}C. Two test tubes were used with 1ml standard reagent as blank and remaining tube with 0.1ml sampls. It was mixed thoroughly and the absorbance was recorded at 500nm after 30 sec as A1 and at interval of 90 sec as A2.

Creatinine conc. (mg/dl)= (A2-A1 sample)/(A2-A1 Std) x Standard conc. x sample DF.

DF=dilution factor

3.2.9.5 Determination of Serum Glucose

PRINCIPLE: Glucose was determined after enzymatic oxidation in the presence of glucose oxidase. The hydrogen peroxide formed reacts with phenol and 4-aminophenazone in the presence of Peroxidase to form a red-violet quinoneimine, which is measured at 510nm (Trinder, 1969).

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone} + \text{Phenol} \xrightarrow{\text{Peroxidase}} \text{quinoneimine} + 4H_2O$$

METHOD: The solutions of Working reagents(1ml) were pipetted out into clean, dry test tubes and labelled as blank (B), standard (S), and test (T). Distilled water was considered as the blank solution. To 1.0 ml of the reagent, 10 µl of standard glucose (100 mg/ dl) was added and incubated for 15 min at 37 ^{0}C. The glucose concentration was estimated by adding 10 µl of the serum sample to 1.0 ml of the reagent, mixed well, and incubated at 37^{0}C for 15 min. This incubated mixture was removed and the absorbance was recorded against a reagent blank at 510 nm.

Glucose conc. (mg/dl) = (Absorbance of sample)/(Absorbance of standard) x Standard conc.

3.2.9.6 Determination of Serum Triacylglycerol

PRINCIPLE: - This involved the calorimetric determination of quiononeimine, a color compound formed after the enzymatic hydrolyses of triglyceride in the sample at 500nm (Tietz, 1990).

Triacylglycerol $\xrightarrow{\text{Lipase}}$ Glycerol + Fatty Acid

Glycerol + ATP $\xrightarrow{\text{Glycerol Kinase}}$ Glycerol -3- Phosphate +ADP

Glycerol-3-phosphate + O_2 $\xrightarrow{\text{Glycerol phosphate Oxidase}}$ Dihydroxyacetone phosphate + H_2O_2

H_2O_2 + Aminoantipyrine + 4-Chlorophenol $\xrightarrow{\text{Peroxidase}}$ Quinoneimine + HCl + $4H_2O$

METHOD:-Working reagent (1000µl) was added to tubes containing 10µl blank, standard and sample respectively. The tubes were mixed and incubated for 5 min at 37^0C. The absorbance of sample and standard were measured against the reagent blank. The concentration of triglyceride was calculated.

Triacylglycerol conc. (mg/dl) = (Absorbance of sample)/(Absorbance of Std) x Std conc.

3.2.10 Studies on *P.berghei* cysteine protease

3.2.11 Inoculation of mice and parasite count

A donor mouse with rising parasitemia of 20 % was sacrificed and blood was drawn in a Heparinised syringe and diluted in phosphate buffered saline. Infection was initiated by 0.2ml needle passage of the parasite preparation from a donor mouse to healthy mice via intraperitoneal route (Klemba *et al*., 2004). Parasitemia was monitored by microscopic

Giemsa-stained thin blood smears. The number of parasitized erythrocytes in about 10 fields was counted twice and the average was computed to give the parasitemia of each mouse. Percent parasitemia was calculated as:

$$\% \text{ Parasitiemia} = \frac{\text{Number of parasitized RBC}}{\text{Total number of RBC}} \times 100$$

3.2.12 Preparation of parasite extracts

Blood obtained from 28 infected mice at the peak of parasitemia was incubated with 0.01% saponin in 100 mM phosphate buffer saline (PBS) pH 6.0 at 37^0C for 10min to lyses Red Blood Cell (RBC) and this was washed four times in ice cold PBS(10,000 x g,5min). The released parasite was lysed using the protocol described by Amlabu *et al.* (2011_a). Briefly, 0.5 % Triton X-100 in Tris-buffer saline was added to the parasites and incubated at 4^oC for 90 min. The supernatant was collected after an initial centrifugation at 10,000 x g for 30 min at 4^oC and the pellets from preparation was reextracted twice .The extracts were then pooled together as crude enzyme sample.

3.2.13 Assay of parasite cysteine protease

Crude enzyme extract(0.5mg/ml) was prepare in 100mM phosphate buffer, pH 6.8, with 0.3mM EDTA and 2 mM cysteine-HCl. Assay was performed by adding 290µL of 50mM Tris–HCl (pH 7.6) and 200µL of 1.25mM BApNA solution to the parasite extract for 10min. at 25^0 C. After 30 min at 37^0C, the reaction was stopped by adding 150µL of acetic acid (30%) of total reaction volume of 2ml. The activity was measured at 410nm colometrically.

3.2.14 Partial purification of cysteine protease from *Plasmodium berghei*

The crude enzyme was partially purified using phenyl sepharose column as previously described in section (**3.2.5.2**).

3.2.15 Inhibitory potential of partially purified extracts against cyseine protease from *Plasmodium berghei*

Five different concentrations of purified inhibitor from both plant extracts (10-50µg/ml) were mixed with 0.5mg/ml *P.berghei* cysteine protease extract prepared in 100mM phosphate buffer, pH 6.8, with 0.3mM EDTA and 2 mM cysteine-HCl. These were incubated at 37^0C for 15 min, before addition of substrate (1.25mM BApNA as substrate) to start the reaction. After 30 min at 37^0C, the reaction was stopped by adding 150µL of acetic acid (30%). Residual activity of protease was determined spectrophotometrically at 410nm.

3.2.16 Kinetics of inhibitory activity against cyseine protease from *Plasmodium berghei*

The degree of inhibition against Cysteine protease extracts from *Plasmodium berghei* was determined at different substrate concentrations (ranges; 0.1-2µM) and at fixed concentrations of inhibitor (40µg/ml and 60µg/ml, for *C.procera* Latex and 20µg/ml and 40µg/ml, for *M.oleifera* seeds). The data obtained were used to plot the Michaelis menten curve to show the effect of substrate and inhibitor concentrations on enzyme activity. Michaelis Menten curve was then transformed to Lineweaver–Burk plots, in which the inverse of the initial velocity was plotted against the inverse of the substrate concentration in the absence of an inhibitor and in the presence of an inhibitor. K_m, V_{max} and inhibition constant (K_i) from secondary plot were calculated.

3.2.17 *In vivo* antiplasmodial suppressive test of Extracts

3.2.17.1 Suppressive test

Extract from different part of the plants showing good CPI activity which was phosphate buffer extract from *C.procera* latex and *M.oleifera* seeds were used for in *vivo* 4-day suppressive test (Peters, 1965). Sixty eight mice were grouped into groups (1,2a&b,3a&b,4,5,6 a&b,7 a&b,8 a&b , 9 a&b,10 a&b and 11 a&b) of three(3) mice each respectively. They were infected intraperitoneally (i.p) with parasitized erythrocytes from a donor mouse with 0.2ml of diluted infected blood each. Those in group, 1, 2 and 3 are normal groups (not infected but treated with PBS) and treated intraperitoneally with *C.procera* latex extracts and *M.oleifera* seeds extracts respectively. Treatment of mice commenced 2hours after infection and repeated daily for three days consecutively (i.e 2, 24, 48, and 72 h after inoculation).

3.2.11.2 Treatment Groups

Group1: Not infected but treated with Phosphate buffer saline (PBS) as normal control.

Group2a: Not infected but treated with 30mg/kg of body weight of crude *C.procera* latex extracts.

Group2b: Not infected but treated with 30mg/kg of body weight of purified *C.procera* latex extracts.

Group3a: Not infected but treated with 50mg/kg of body weight of crude *M.oleifera* seeds extracts.

Group3b: Not infected but treated with 50mg/kg of body weight of purified *M.oleifera* seeds extracts

Group4: Infected and was administered PBS **(**negative control group)

Group5: Infected treated with Arthemisinin, 5mg/kg body weight (positive control group)

Group6a: Infected and treated with 20mg/kg of body weight of crude *C.procera* latex extracts.

Group6b: Infected and treated with 20mg/kg of body weight of purified *C.procera* latex extracts.

Group7a: Infected and treated with 30mg/kg of body weight of crude *C.procera* latex extracts.

Group7b: Infected and treated with 30mg/kg of body weight of purified *C.procera* latex extracts.

Group8a: Infected and treated with 50mg/kg of body weight of crude *C.procera* latex extracts.

Group8b: Infected and treated with 50mg/kg of body weight of purified *C.procera* latex extracts.

Group9a: Infected and treated with 30mg/kg of body weight of crude *M.oleifera* seeds extracts.

Group9b: Infected and treated with 30mg/kg of body weight of purified *M.oleifera* seeds extracts.

Group10a: Infected and treated with 50mg/kg of body weight of crude *M.oleifera* seeds extracts.

Group10b: Infected and treated with 50mg/kg of body weight of purified *M.oleifera* seeds extracts.

Group11a: Infected and treated with 70mg/kg of body weight of crude *M.oleifera* seeds extracts.

Group11b: Infected and treated with 70mg/kg of body weight of purified *M.oleifera* seeds extracts

Twenty-four hours after the last treatment blood smears from all animals were prepared, fixed with methanol and Giemsa stain. The Parasitemia level was determined by counting average number of parasites in 10 fields of at least 1000 erythrocytes. The parasitized red cells were recorded as the percentage of the total RBCs as against the control.

The parameters evaluated included: Packed cell volume, percentage parasitemia and percentage survival for each experimental group.

% Parasite inhibition of the extract:

% Suppression = APC – APT / APC X 100.

APC = Average Parasitaemia in the Negative Control, APT = Average Parasitaemia in the Test group

3.2.12 Statistical analysis

All Data obtained were expressed as Mean ± standard deviation. The results were analysed statistically using one-way analysis of variance (ANOVA) followed by Duncan

multiple test. The level of significance was set at $P < 0.05$.Graph pad prism software was used to calculate IC_{50}.

CHAPTER FOUR

4.0 RESULTS

4.1 Screening for CPI Activity from the Different Parts of Plants

Crude extracts from different parts of *C. procera* and *M. oleifera* obtained with variuos solvent media were screened for cysteine protease inhibitory activity. The recovery in terms of protein content (mg/ml), papain inhibitory activity (units/ml) and specific activity (units/mg) was determined and are shown in Figures 4.1-4.3 for *C. procera* extracts and Figures 4.4 - 4.6 for *M. oleifera* extracts respectively.

All the solvents used were able to solubilize various amounts of proteins (between 0.06-3.03 mg/ml) from parts of both plants. However, latex of *C. procera* showed higher protein contents compared to other parts of the plants (Figure 4.1). In addition, the leaf and seed of *M. oleifera* showed higher protein contents compared to other parts of the plants (Figure 4.4).

The results for inhibitory activity of the different parts of the plant extracts with papain enzyme (Figures 4.2 and 4.5) revealed that 0.1M phosphate buffer soluble protein extracts from latex of *C.procera* and seeds of *M. oleifera* possessed higher inhibitory activity of 22.43 and 21.23units/ml respectively. Inhibitory activity was not observed when sodium hydroxide (0.2%NaOH) was used for the extraction of *C. procera* flower (Figure 4.2) and when sodium chloride (15%NaCl) was used for the extraction of the stem bark of *M. oleifera* (Figure 4.5).

The Phosphate buffer extracts for *C. procera* latex had higher specific activity of 31.47units/mg protein (Figure 4.3) when compared to other parts of the plant and solvents

used. Similarly, the seeds of the *M. oleifera* phosphate buffer extracts showed higher specific activity of 11.23 units/mg protein when compared to other parts of the plant (Figure 4.6).

Table 4.1 summarizes the percentage recovery yield of acetone-precipitated proteins from the latex of *C. procera* and seeds of *M. oleifera*. The percentage yield of 12.8% and 9.8% was recorded for seeds of *M. oleifera* and latex of *C. procera* respectively.

Figures 4.7 and 4.8 show dose dependent inhibitory activity of different concentrations of acetone precipitated protein for latex of *C. procera* and seeds of *M. oleifera,* respectively. The fifty percent inhibitory concentration (IC_{50}) for crude latex extract of *C. procera* was 1.35mg/ml. and crude seeds extract of *M. oleifera* showed IC_{50} of 1.22 mg/ml.

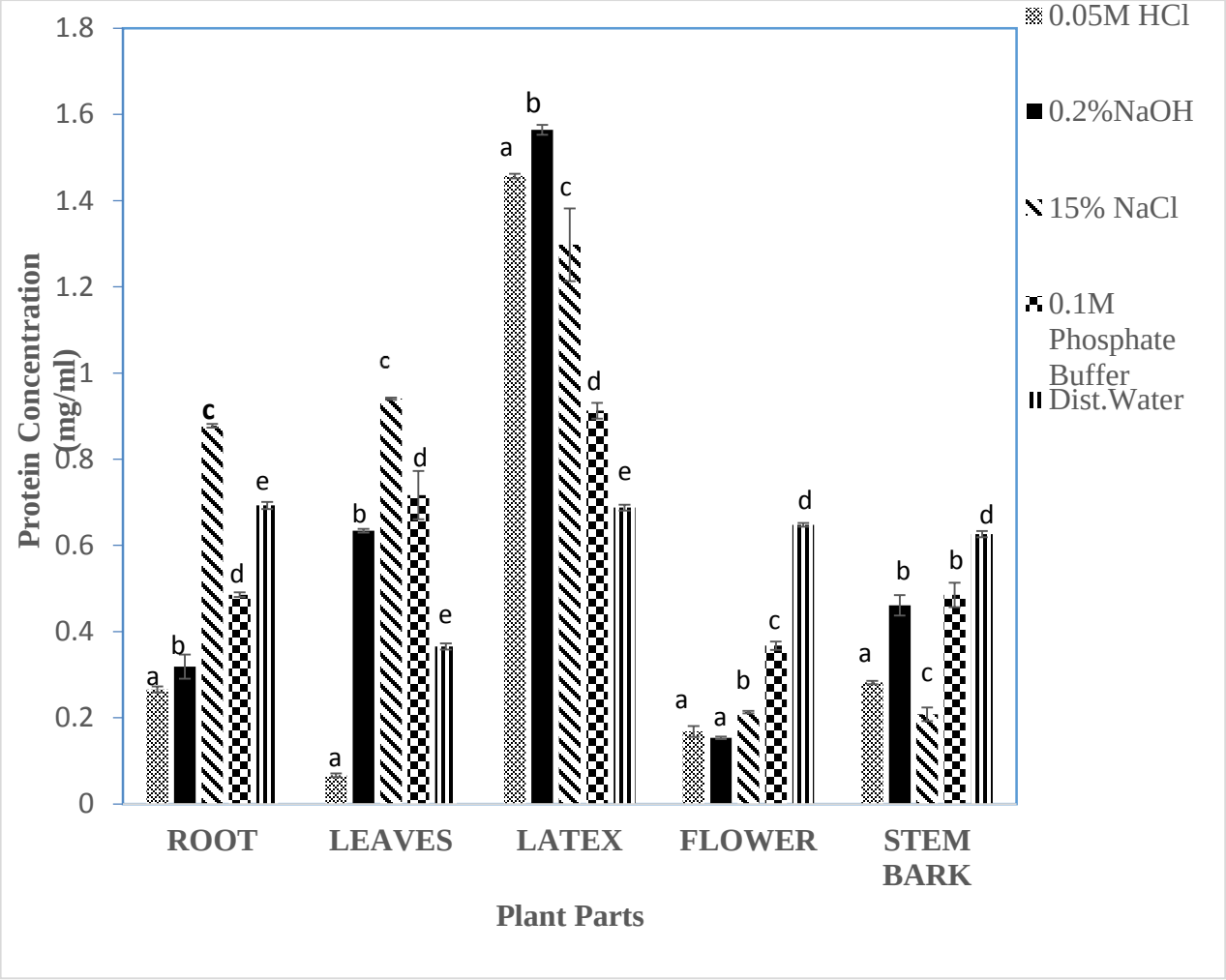


Figure 4. 1: Protein Concentration in Different Solvents Extracts of *C.procera* Parts

Values are Mean ± SD. (n=3)

Bars with different letters in the same plant part differ significantly ($p<0.05$)

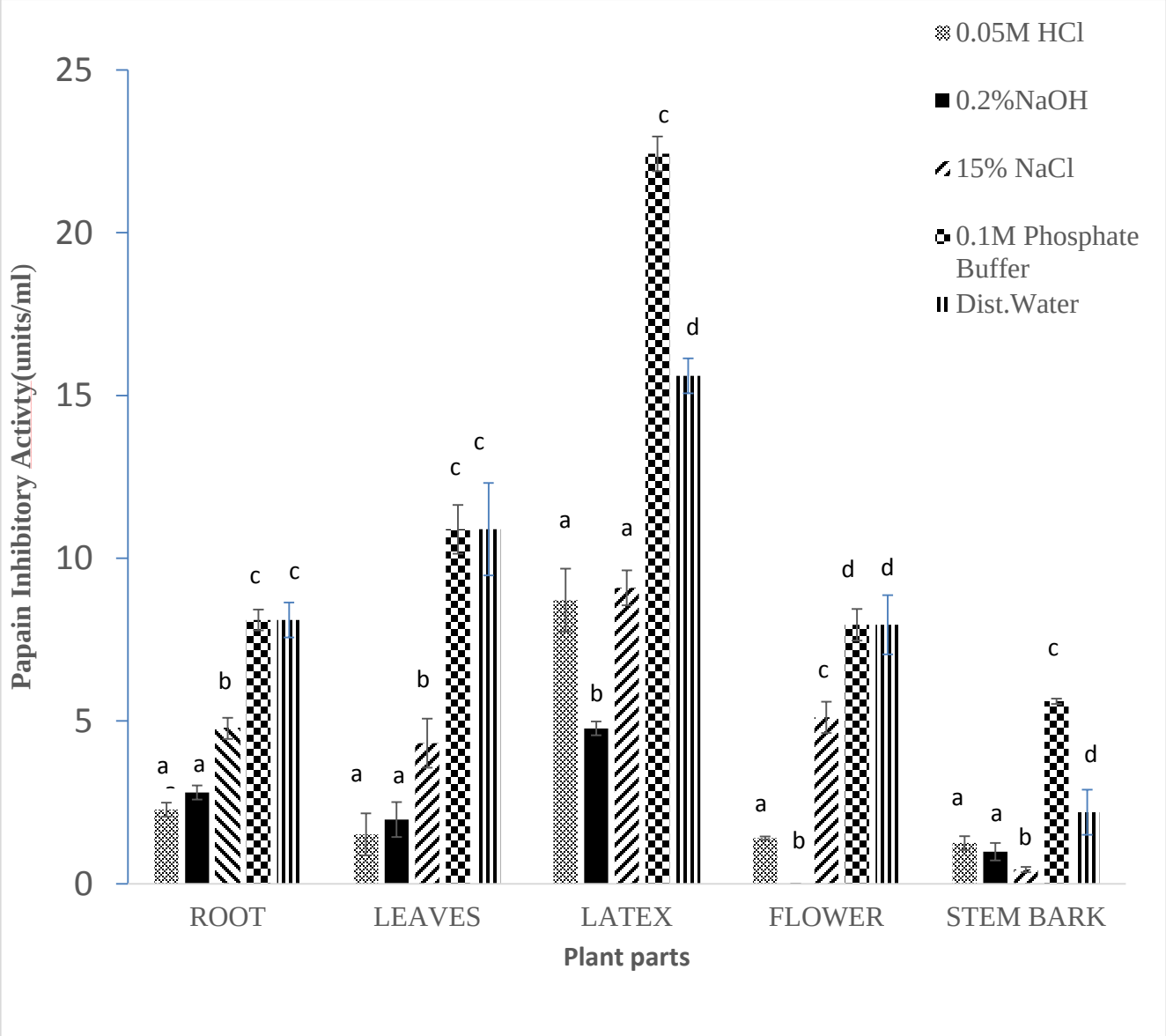


Figure 4. 2: Protease Inhibitory Activity of Different Solvents Extracts from *C.procera* Parts

Values are Mean ± SD. (n=3)

Bars with different letters in the same plant part differ significantly ($p<0.05$)

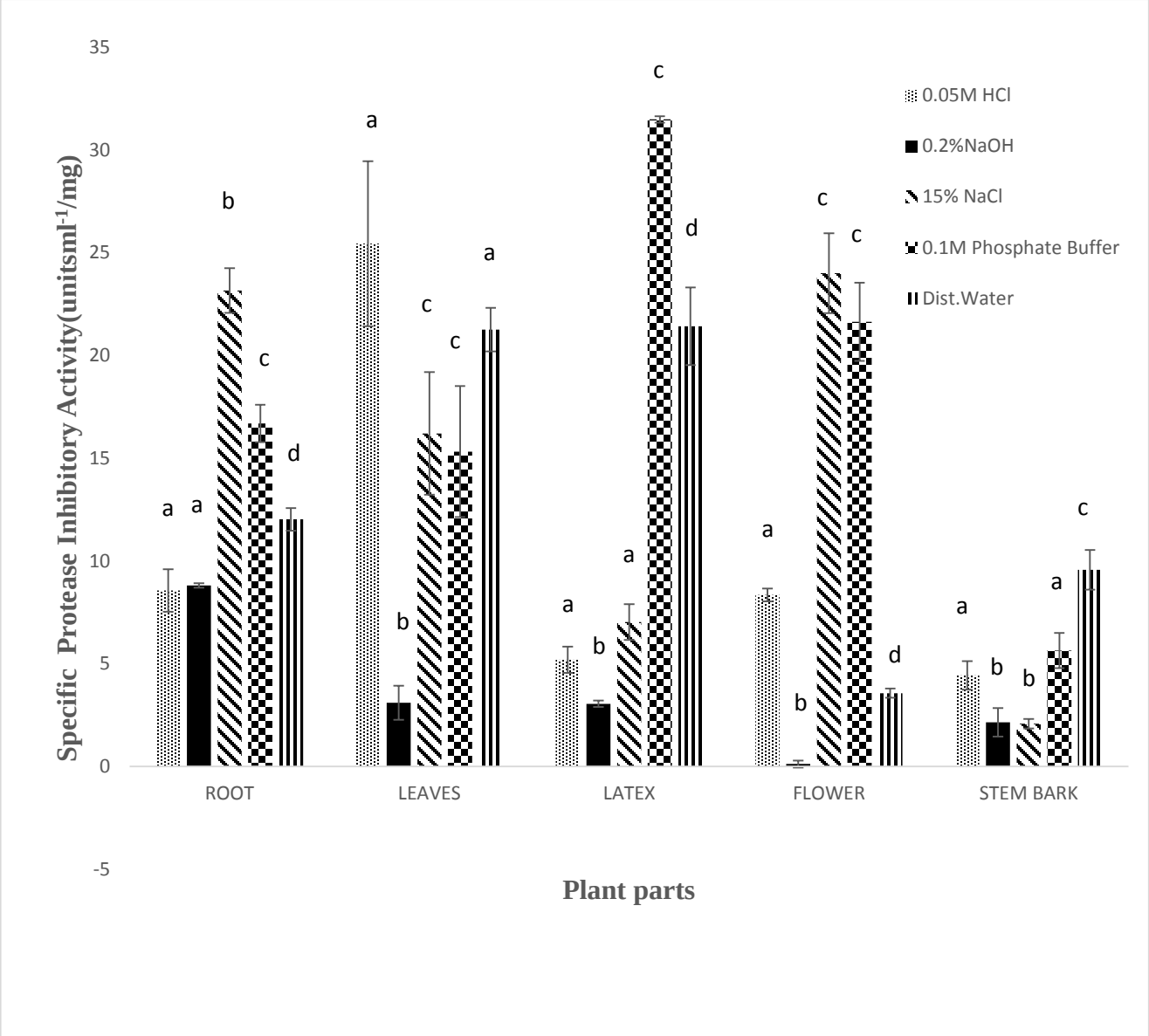


Figure 4. 3: Specific Protease Inhibitory Activity of Different Solvents Extracts from *C.procera* parts

Values are Mean ± SD (n=3)

Bars with different letters in the same plant part differ significantly ($p<0.05$)

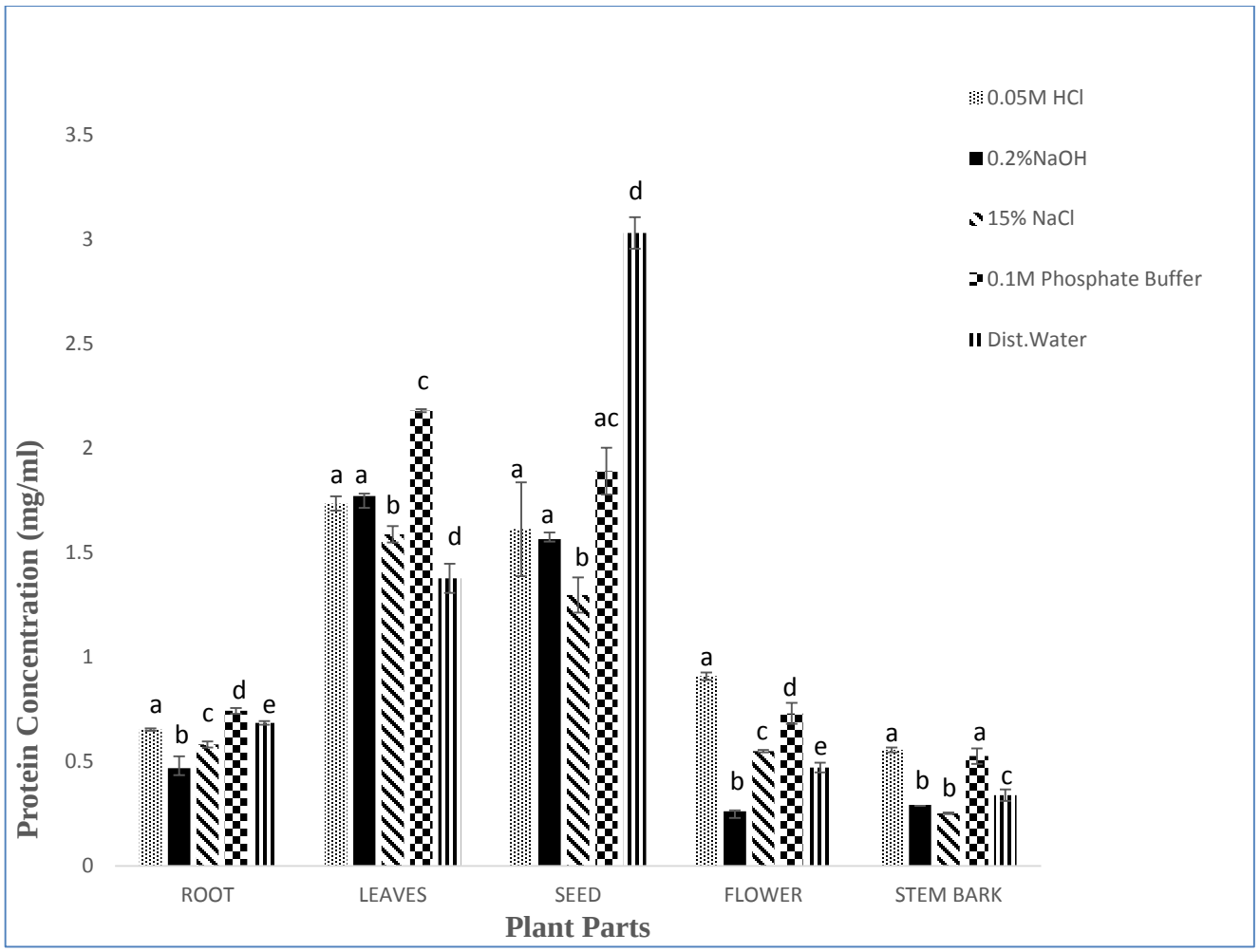


Figure 4. 4: Protein Concentrations in Different Solvents Extracts of *M.oleifera* Parts.

Values are Mean ± SD. (n=3).

Bars with different letters in the same plant part differ significantly ($p<0.05$)

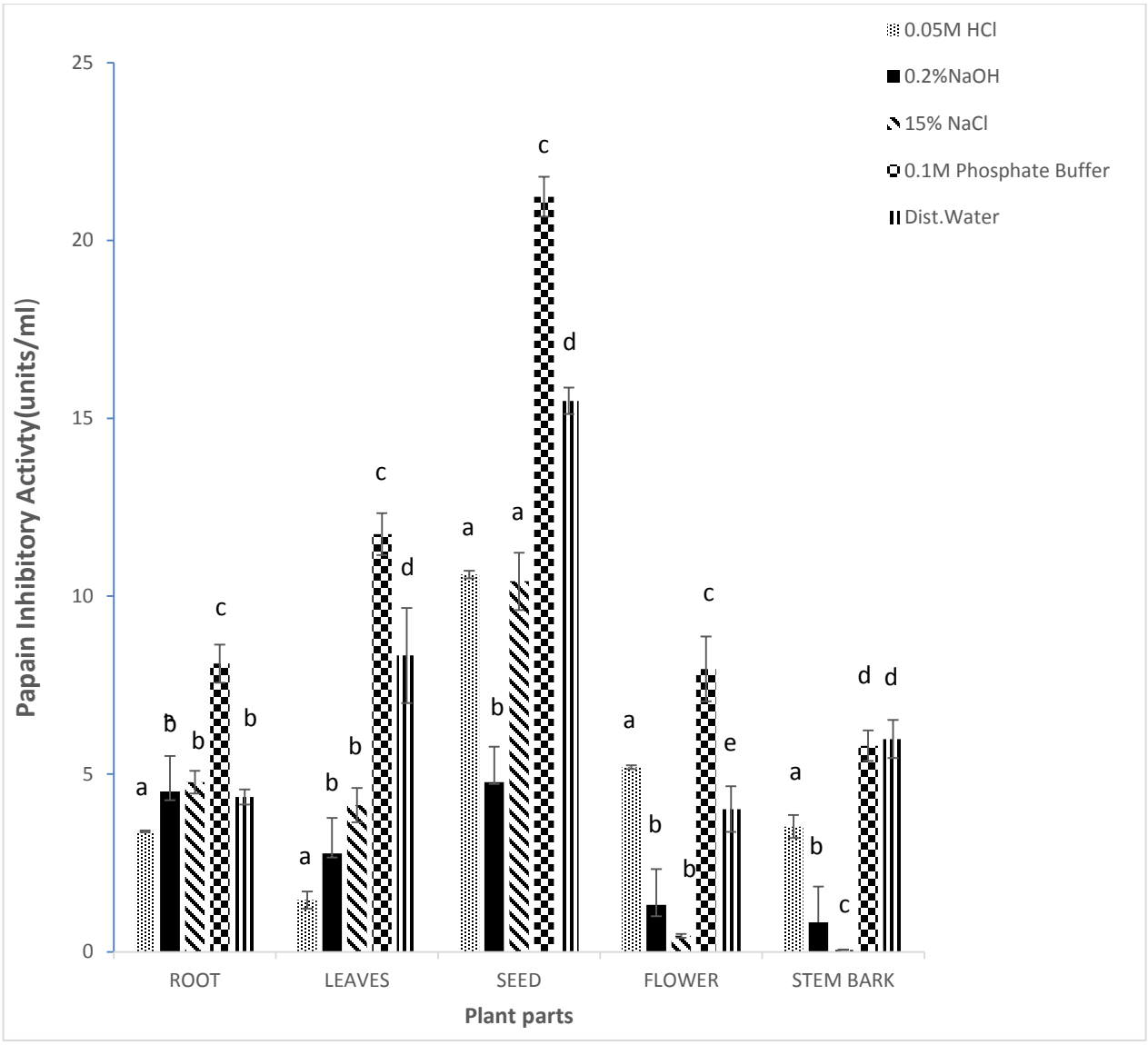


Figure 4. 5: Protease Inhibitory Activity of Different Solvents Extracts from *M.oleifera* Parts

Values are Mean ± SD. (n=3).

Bars with different letters in the same plant parts differ significantly ($p<0.05$)

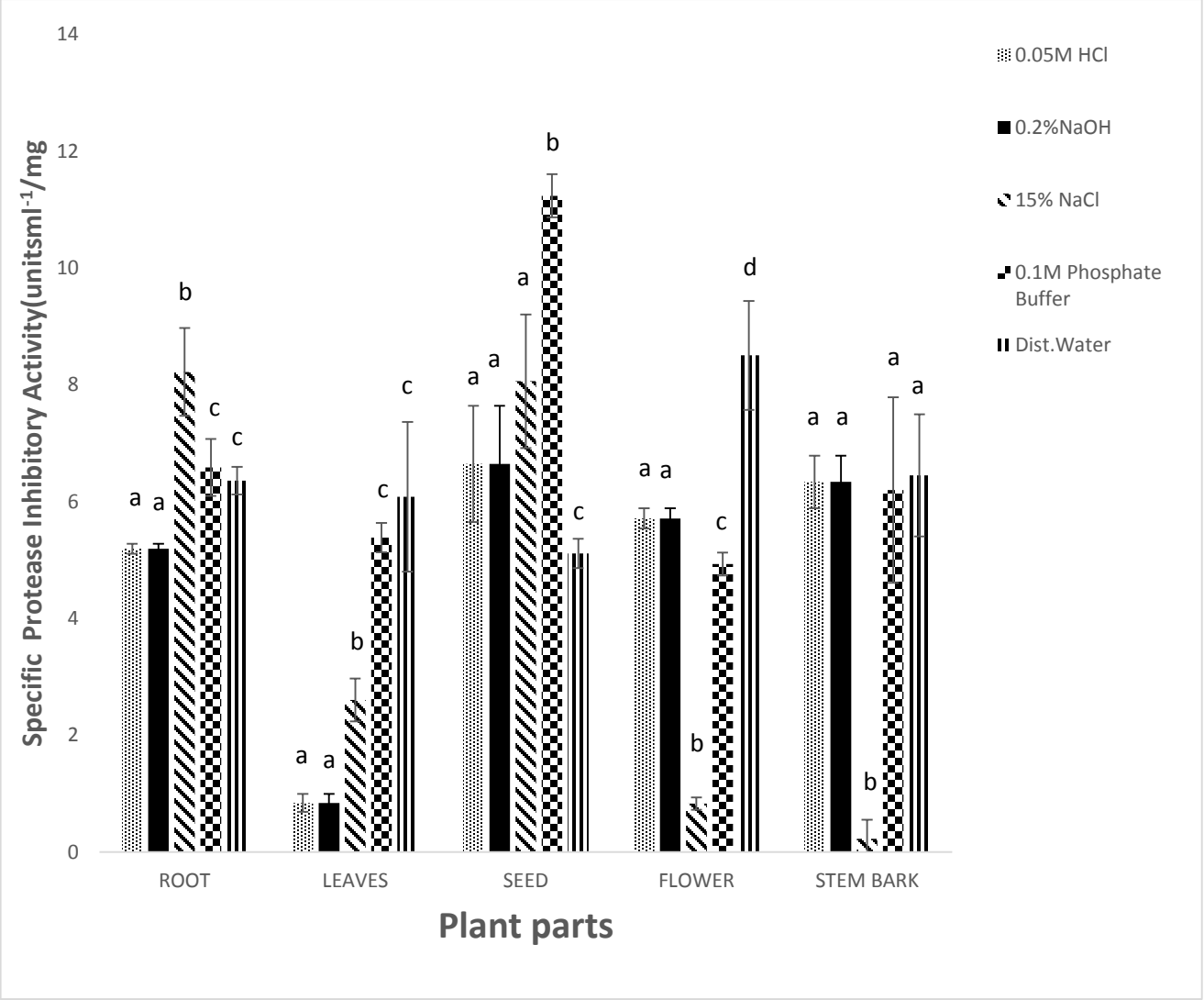


Figure 4. 6: Specific Protease Inhibitory Activity of Different Solvent Extracts from *M.oleifera* Parts

Values are Mean ± SD (n=3)

Bars with different letters in the same plant parts differ significantly ($p<0.05$)

Table 4. 1: Percentage Yield of Cold Acetone Precipitate of Extracts of *C.Procera* Latex and *M.oleifera* Seeds

Plant	**Amount of plant material (g)**	**Yield(g)**	**% Yield**
***Calotropis procera* (Latex)**	25.00	2.45 ± 0.57	9.80 ± 2.30
***Moringa oleifera*(seeds)**	25.00	3.50 ± 1.41	12.80 ± 5.70

Values are Means ± SD (n=3)

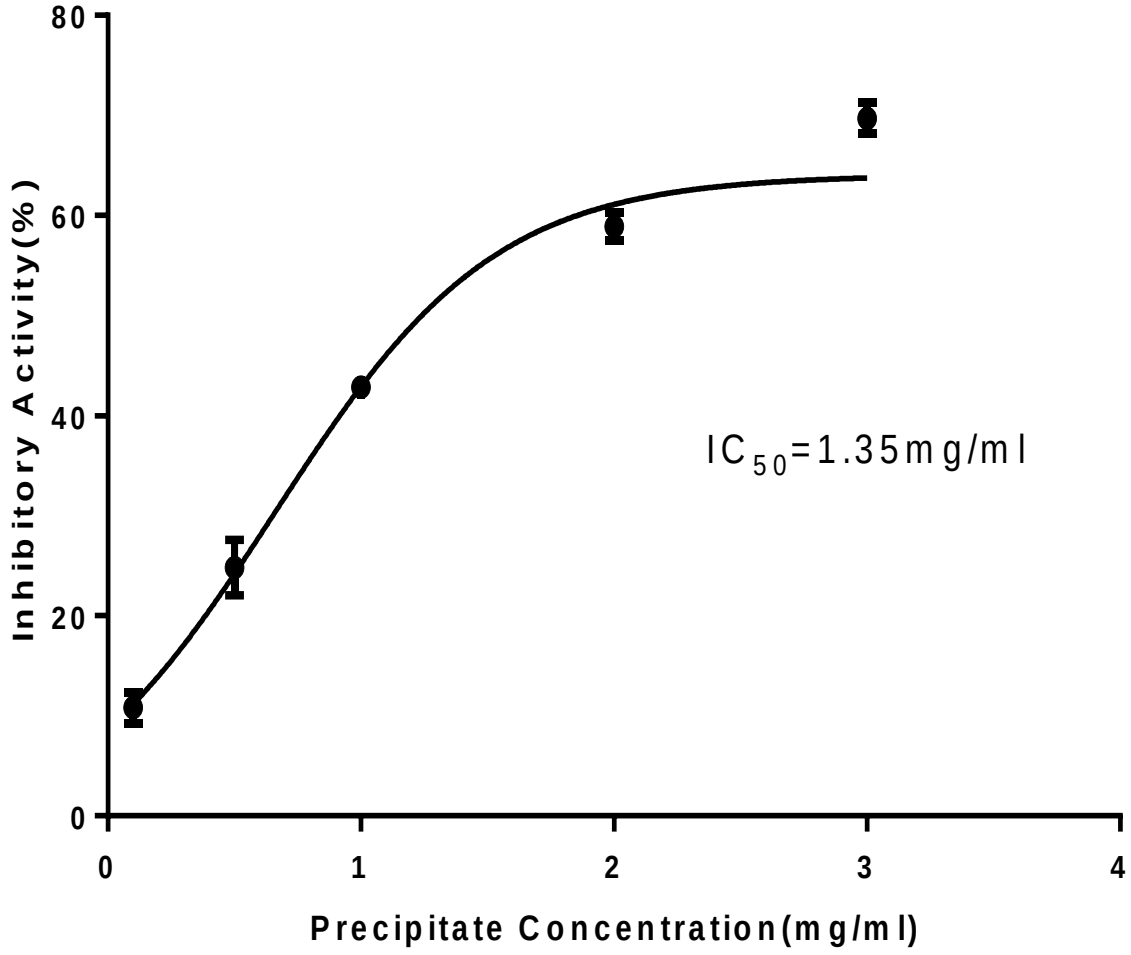


Figure 4. 7: Protease Inhibtory Activity of the Acetone Precipitated Extracts of *C.procera* Latex

Values are Mean ± SD (n=3).

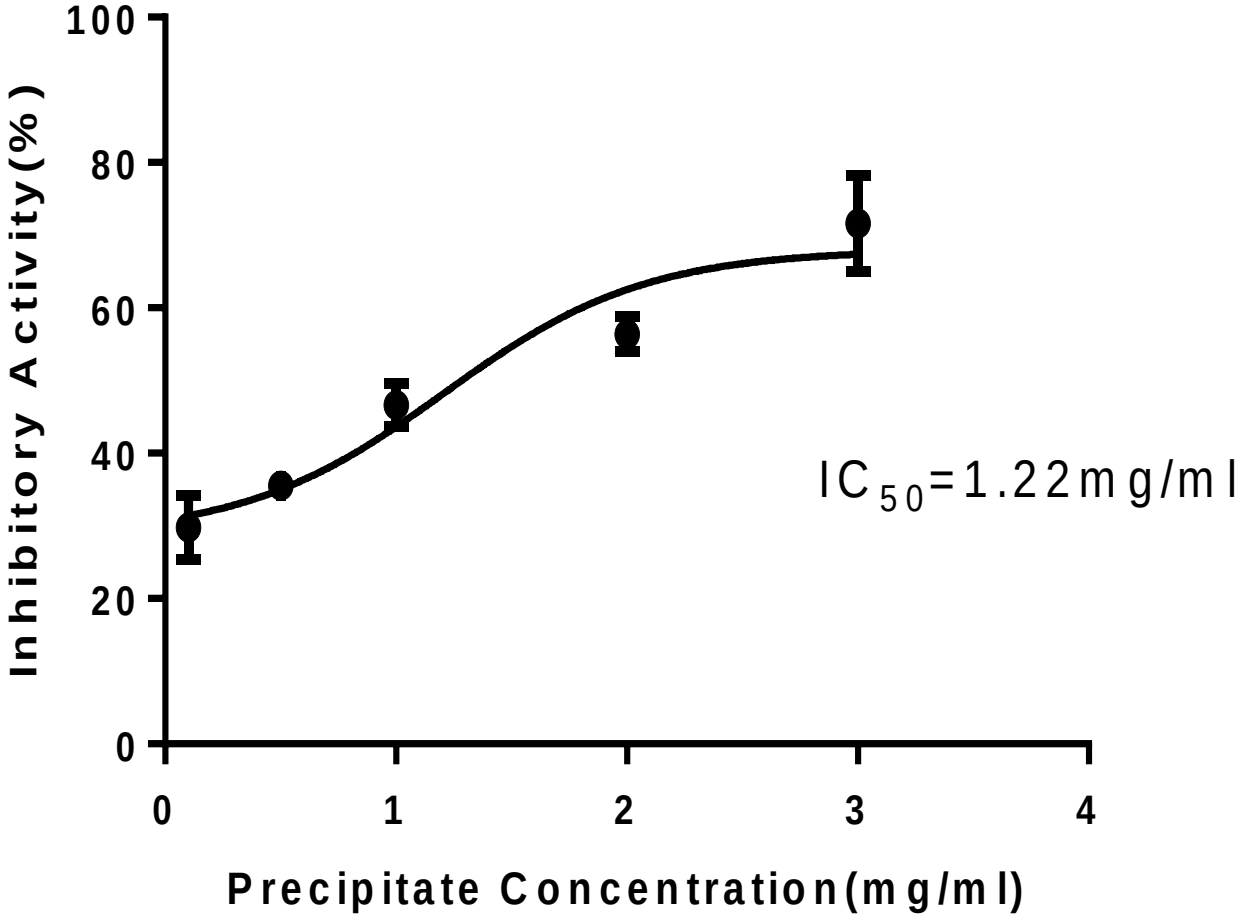


Figure 4. 8: Protease Inhibtory Activity of the Acetone Precipitated Extracts *M.oleifera* Seeds.

Values are Mean ± SD (n=3).

4.2. Purification and Characterisation of the Inhibitor

4.2.1 Ammonium sulphate fractionation of crude extracts

Crude acetone precipitate from *C. procera* latex and *M. oleifera* seeds were subjected to ammonium Sulphate precipitation between 10-80% saturation. *C.procera* latex precipitated proteins showed maximal specific protease inhibition between 20-60% saturation of ammonium sulphate with maximum activity recorded at 50% saturation (Figure 4.9). *M. oleifera* seeds precipitated proteins also showed maximal specific protease inhibition between 40-70% ammonium Sulphate with maximum activity recorded at 60% saturation (Figure.4.10).

4.2.2 Hydrophobic chromatography on phenyl sepharose column

The elution profiles of fractionated proteins from *C. procera* latex and *M. oleifera* seeds on phenyl sepharose column at a flow rate of 0.7 ml min^{-1} are shown (Figure 4.11 and 4.12).Several proteins peaks fractions were eluted from both plants extracts. The bound active proteins fractions from *C. procera* latex extract were eluted with buffer containing 0.1 M ammonium sulphate at fraction numbers 17 to 25. The highest peak of activity was observed in fractions 19 and 20 (Figure 4.11). The buffer containing 0.2 M ammonium sulphate eluted active proteins fractions from *M. oleifera* seeds extract at fraction number 7 to 17 with high activity recorded at fraction number 12 (Figure 4.12).

The protein yield of cysteine protease inhibitor extracted from the *C. procera* latex and *M.oleifera* seeds are summarized in Tables 4.2 and 4.3, respectively. The protein yield of protease inhibitor obtained from *C. procera* latex from cold acetone precitation, $(NH_4)_2SO_4$ precipitation, and phenyl sepharose chromatography, were 35.71, 31.40 and 12.3 percent

respectively (Table:4.2).For *M. oleifera* seeds inhibitor the protein yield from cold acetone precitation, $(NH_4)_2SO_4$ precipitation, and phenyl sepharose chromatography, were 67.12, 58.80 and 29.32 percent respectively.

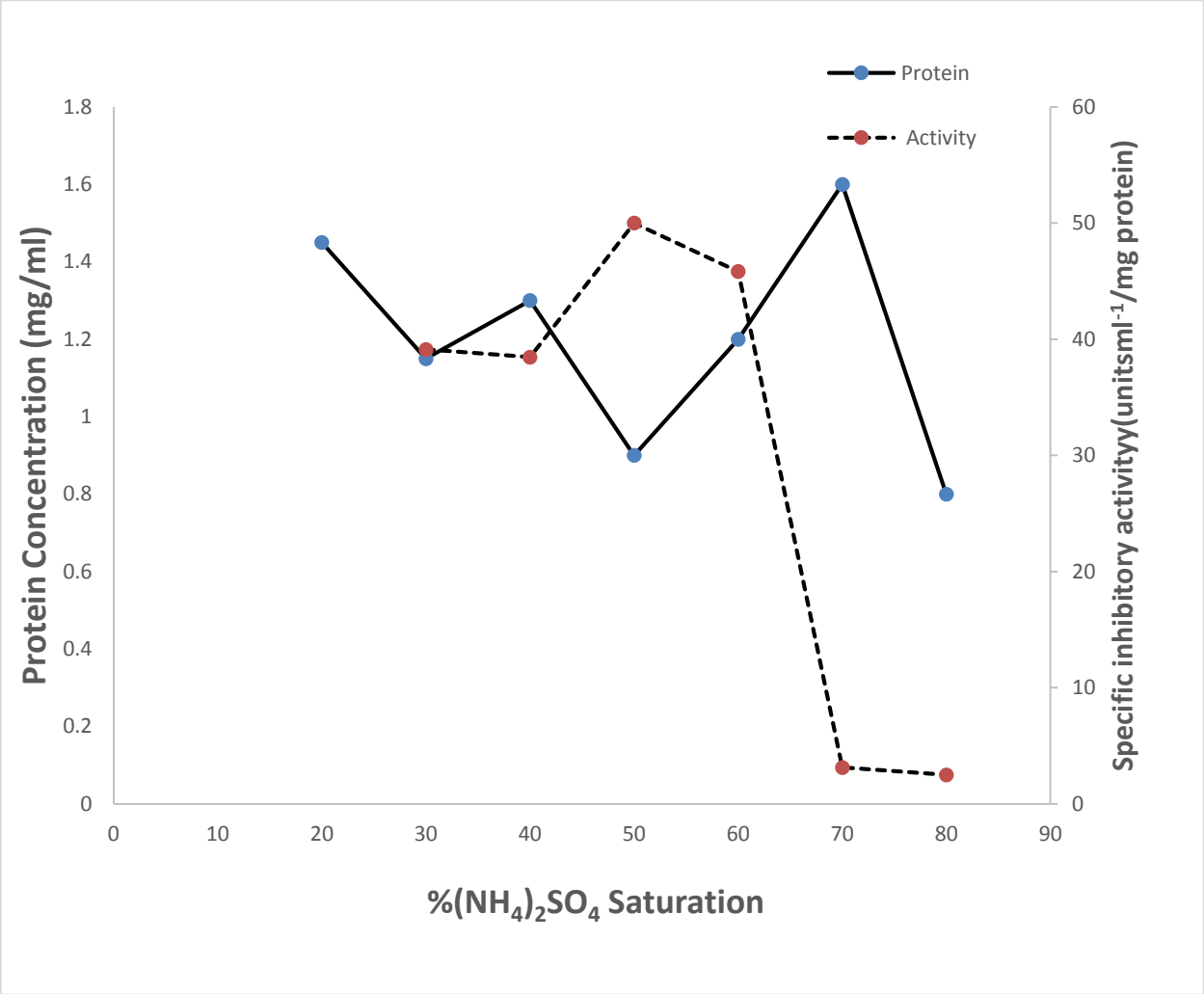


Figure 4. 9: Ammonium sulphate Profile of Protease Inhibitor from Extracts of *C. procera* Latex

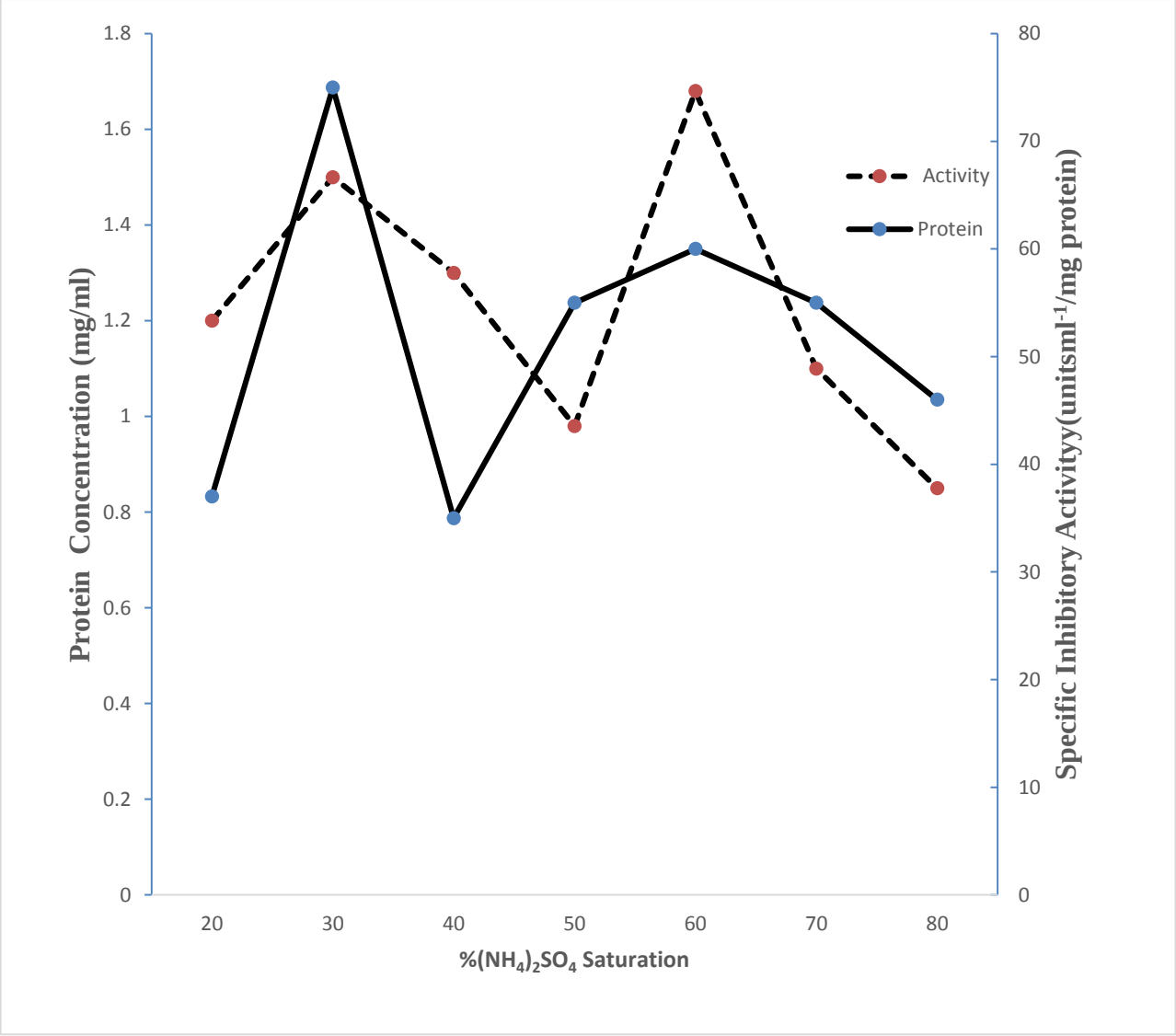


Figure 4. 10: Ammonium sulphate Profile of Protease Inhibitor from Extracts of *M.oleifera* Seeds

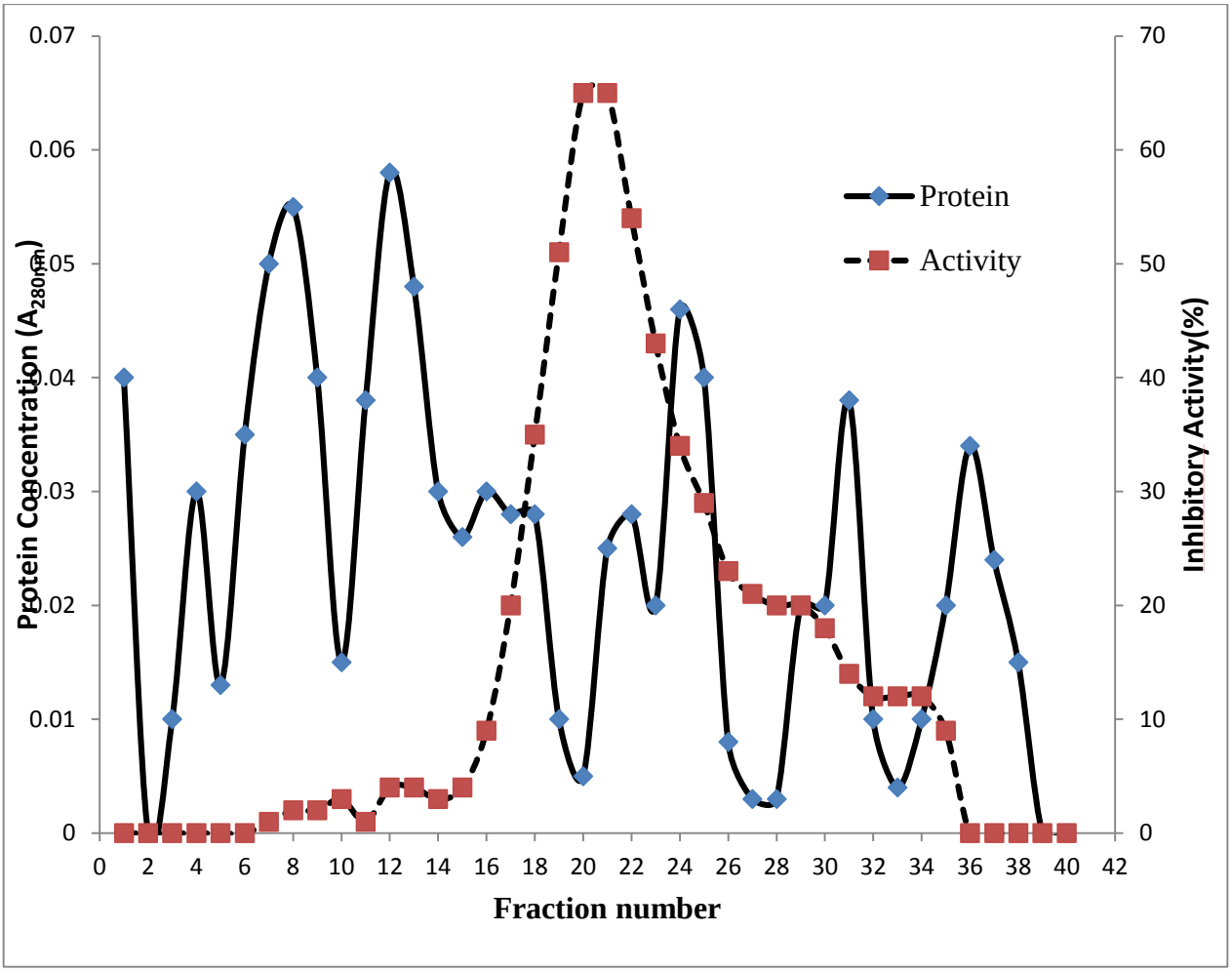


Figure 4. 11: An Elution Profile for the Hydrophobic Interraction Chromatography of *C.procera* Latex Inhibitor on Phenyl-Sepharose Column

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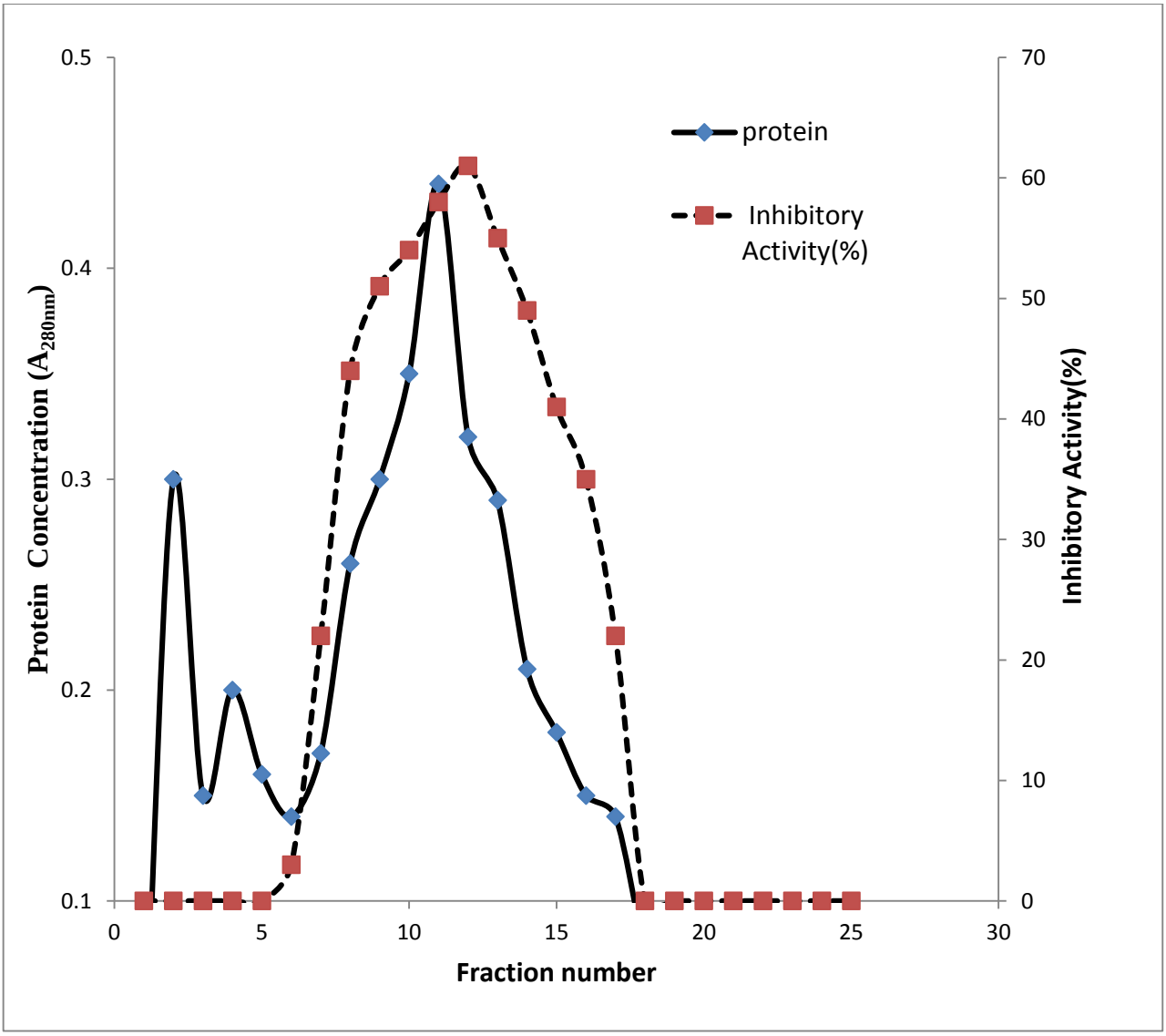


Figure 4. 12: An Elution Profile for the Hydrophobic Interaction Chromatography of *M.oleifera* Seeds Inhibitor on Phenyl-sepharose Column

Table 4. 2: Protein Yield for the Partially Purified Extracts from *C.procera* Latex

		Volume (mL)	Protein (mg/ml)	Total protein (mg)	Yield (%)
1	Crude extract	70	3.48	243.60	100
2	Acetone precipitation	20	4.35	87.00	73.40
3	$(NH_4)_2SO_4$ Fraction (20-60%)	16	4.78	76.48	58.29
4	Hydrophobic interaction Chromatography (Phenyl Sepharose)	12	2.50	30.00	48.72

.

Table 4. 3: Protein Yield for the Partially Purified Extracts from *M.oleifera* Seeds

		Volume (mL)	Protein (mg/ml)	Total protein (mg)	Yield (%)
1	Crude extract	150	6.48	972	100
2	Acetone precipitation	45	4.35	195.75	94.68
3	$(NH_4)_2SO_4$ Fraction (10-80%)	38	3.81	144.78	79.57
4	Hydrophobic interaction Chromatography (Phenyl Sepharose)	17	1.9	32.3	42.34

4.3 Characterization of the Partially Purified Inhibitors

4.3.1. pH optima and stability studies of inhibitor

The pH effect on the activity profile of the inhibitor is shown in Figure 4.13. Inhibitors from the plants were active over a narrow range of pH of 5-8. *Calotropis procera* inhibitor had optimal activity at acidic pH of 6.0. Maximum inhibitory activity at neutral pH of 7.0 was observed for *M. oleifera*.

The result of stability studies is presented in Figure 4.14. It was observed that both inhibitors from *C. procera* and *M. oleifera* were stable over a broad pH range of 4.0-10.0.

4.3.2 Temperature optima and stability studies of inhibitor

Results presented in Figure 4.15 suggest that Inhibitor from *M. oleifera* was most active at 50^0C, while *C. procera* inhibitor had optimal temperature of activity at 60^0C. The inhibitor activity declined at temperatures above 60°C, and the proteins lost most of their activity at 90°C.

The temperature stability profiles of the protease inhibitor are presented in Figure 4.16. The partially purified inhibitor from *C. procera* was heat stable at temperature between 30-80^0C, there was a sharp declined in activity after preincubation at temperatures above 80^0C. *M. oleifera* seeds inhibitor showed a similar trend in stability for two hours.

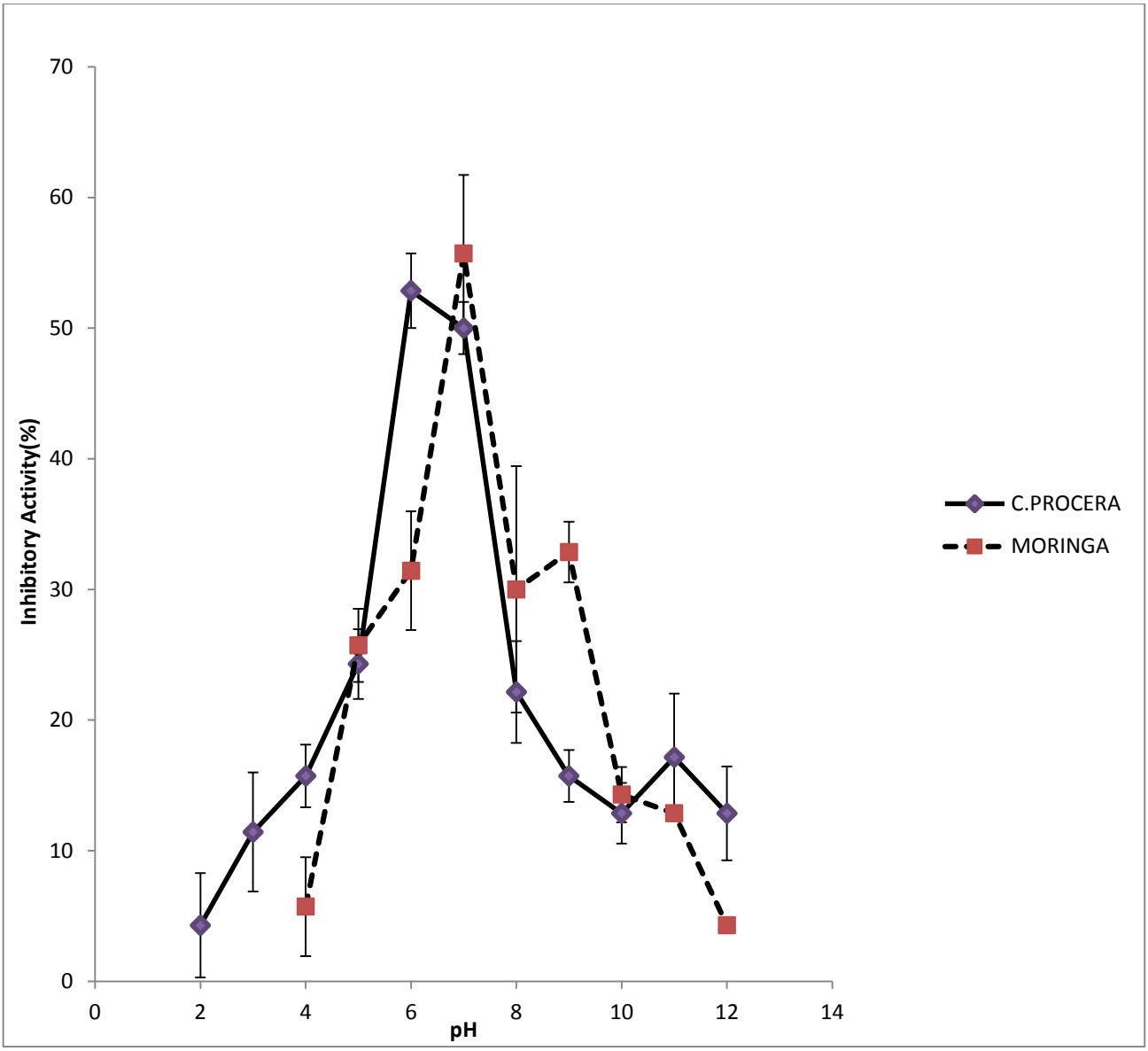


Figure 4. 13: The Effect of pH on Inhibitory Activity of Purified Extracts from *C.procera* Latex and *M.oleifera* Seeds

Values are Mean ± SD. (n=2)

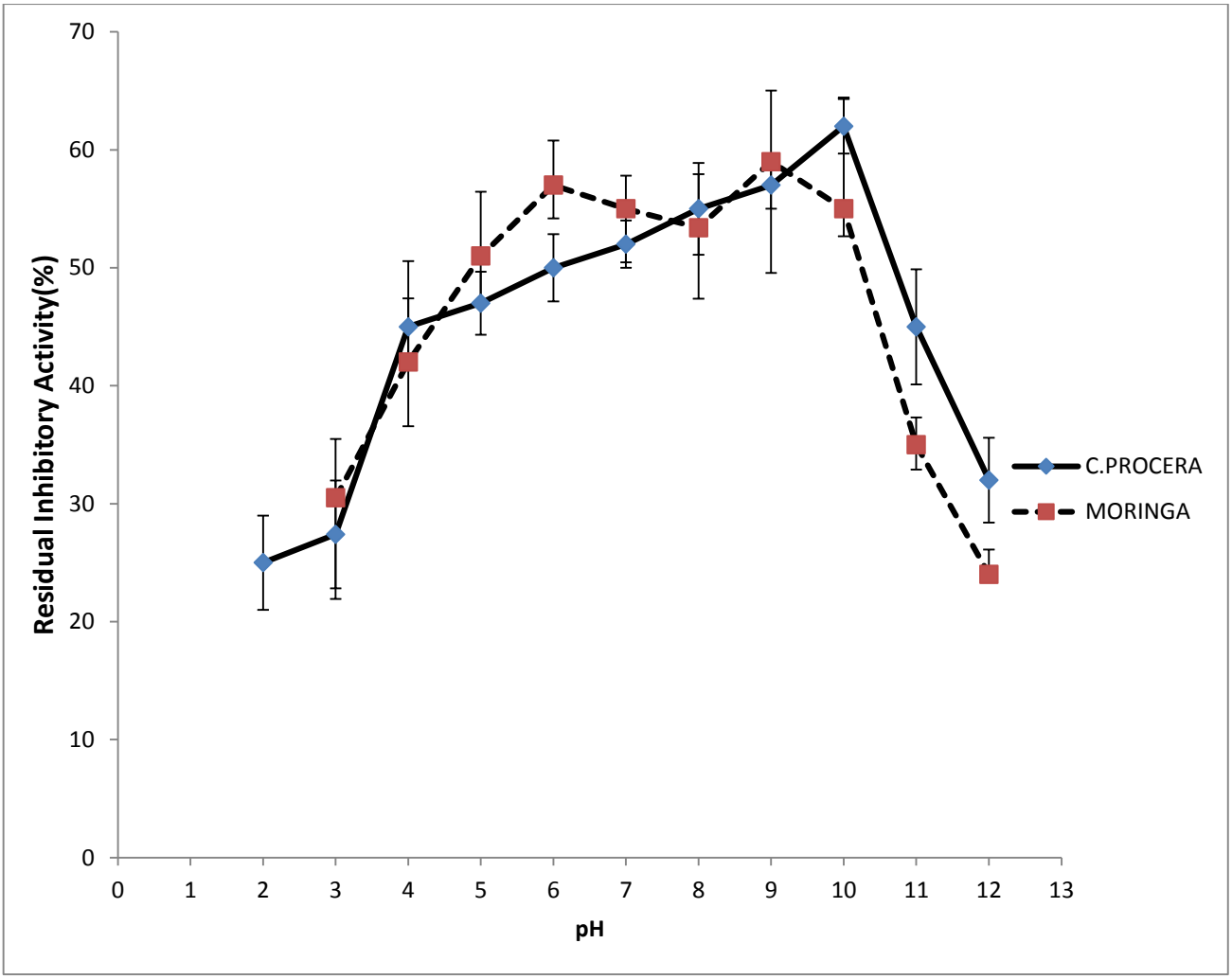


Figure 4. 14: The Effect of pH on the Stability of Purified Extracts from *C.procera* Latex and *M.oleifera* Seeds

Values are Mean ± SD. (n=2)

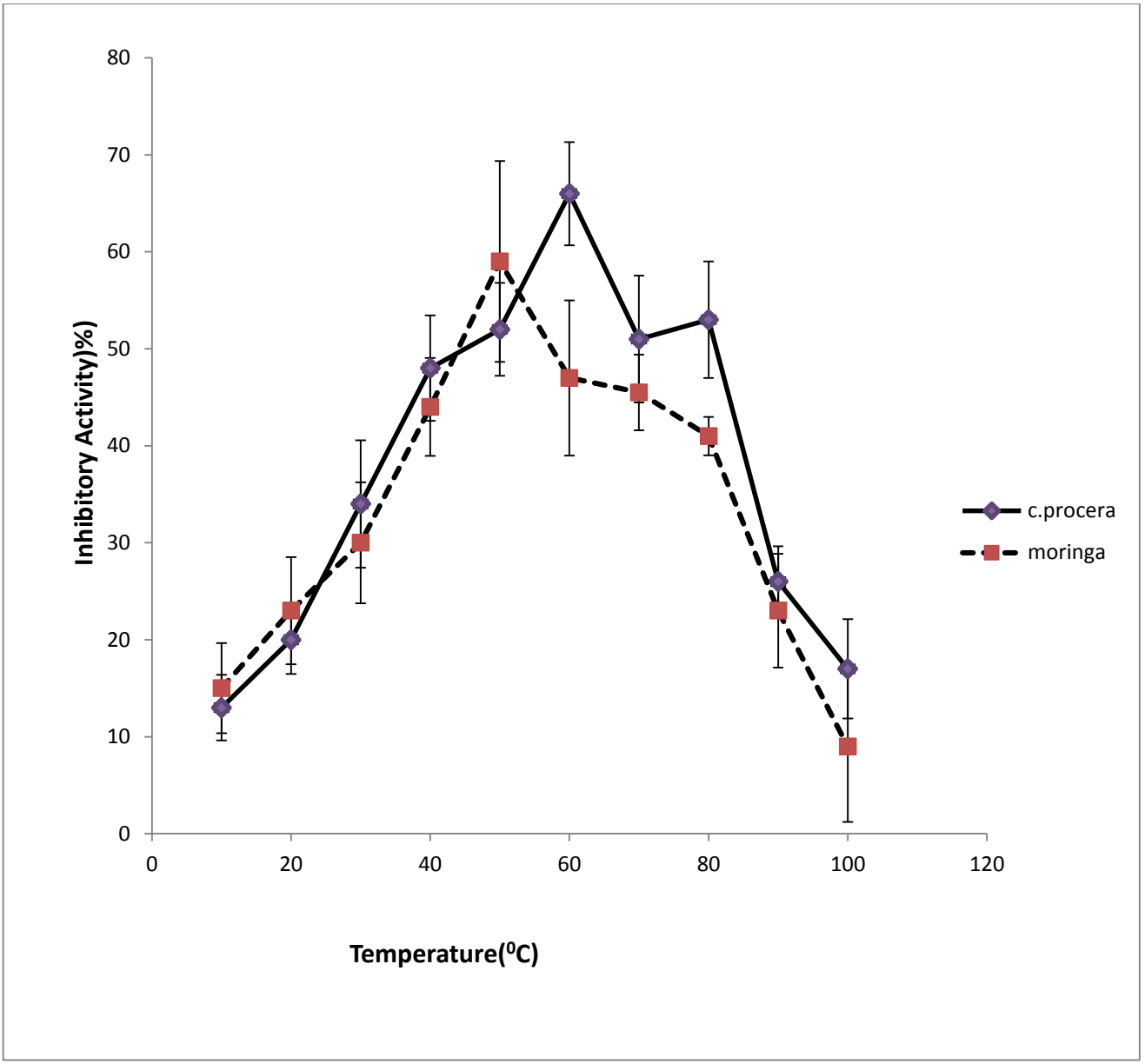


Figure 4.15: The Effect of Temperature on Activity of Purified Extracts from *C.procera* Latex and *M.oleifera* Seeds.

Values are Mean ± SD. (n=2).

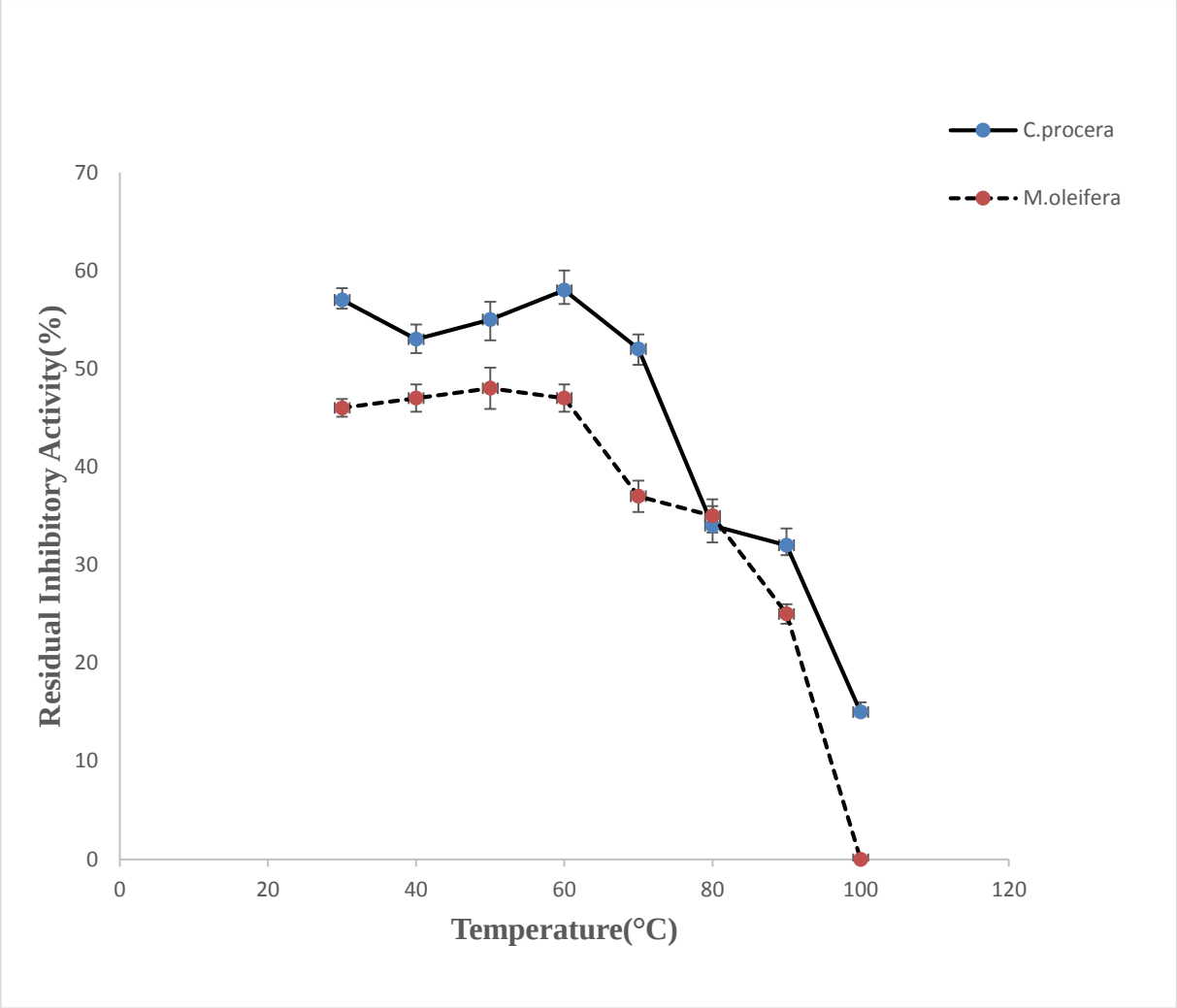


Figure 4. 16: Thermostability Profiles of Purified Extracts from *C.procera* Latex and *M.oleifera* Seeds at Different Temperatures

4.3.3 Effect of the amino acids modification on the activity of inhibitor

Figure 4.17 shows that chemical modification of lysine and serine of partially purified inhibitor from *C. procera* latex protein resulted in activity enhancement. Modification of Tryptophan using N-Bromosuccinamide for partially purified CPI from *M. oleifera* seeds led to an enhancement in the inhibitory activity as compared to control. Furthermore, Cysteine, histidine and serine modification did not affect CPI activity from *M. oleifera*. In contrast, lysine modification in *M. oleifera* was observed to result in loss of CPI activity (Figure 4.17). In addition, both cysteine and Histidine modification for *C.procera* led to drastic loss in activity.

4.3.4 Effect of concentrations of purified inhibitor on the activity of protease

Inhibitory activity of partially purified CPI at different concentrations of purified inhibitors from *C. procera latex* and *M. oleifera* seeds are presented in Figures 4.18 and 4.19. Partially purified CPIs from both plant extracts inhibited papain in dose dependently (Figures 4.18 and 4.19). Fifty percent inhibitory concentration (IC_{50}) of C. *procera latex* was estimated from the curve (Figure 4.18) to be 22.3±0.60µg/ml. An IC_{50} of 18.5±0.8µg/ml was estimated for partially purified CPI from *M. oleifera* seeds.

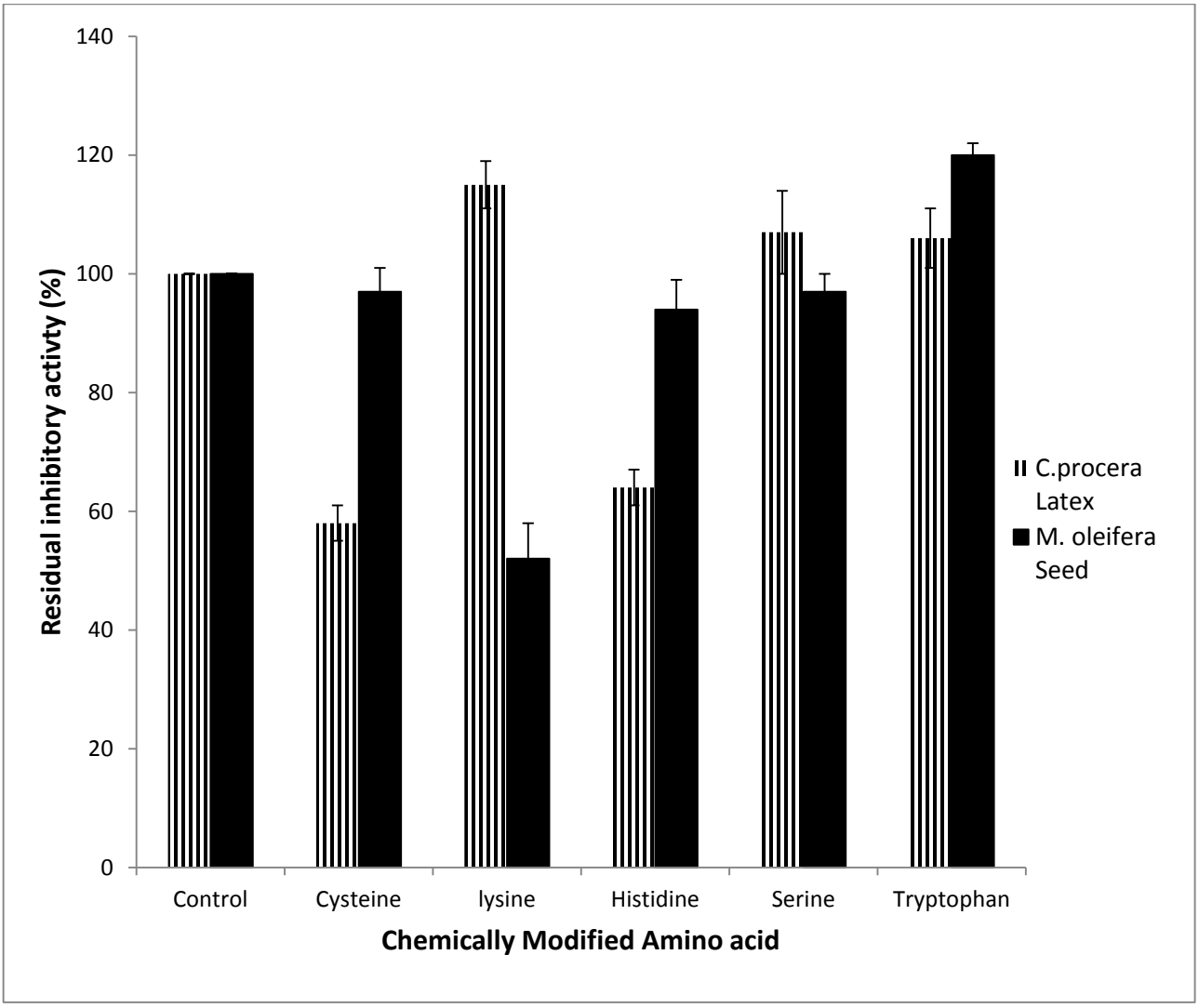


Figure 4. 17: Effect of Chemical Modification of Amino acids on the Inhibitory Activity of *C.procera* Latex and *M.oleifera* Seeds

Values are Mean ± SD. (n=2)

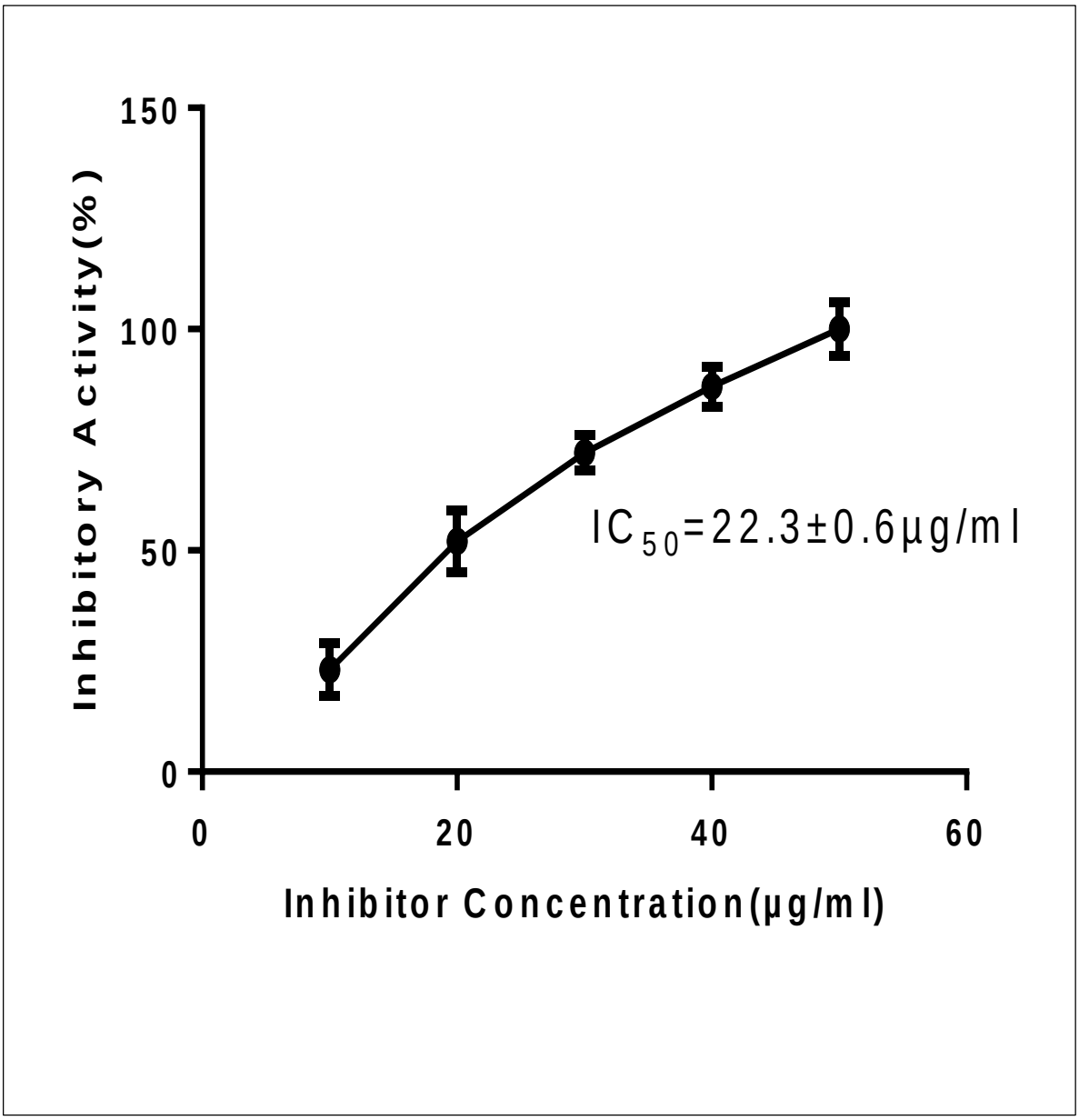


Figure 4. 18: Protease Inhibitory Activity of the Purified Extracts of *C. procera* Latex

Values are mean± SD

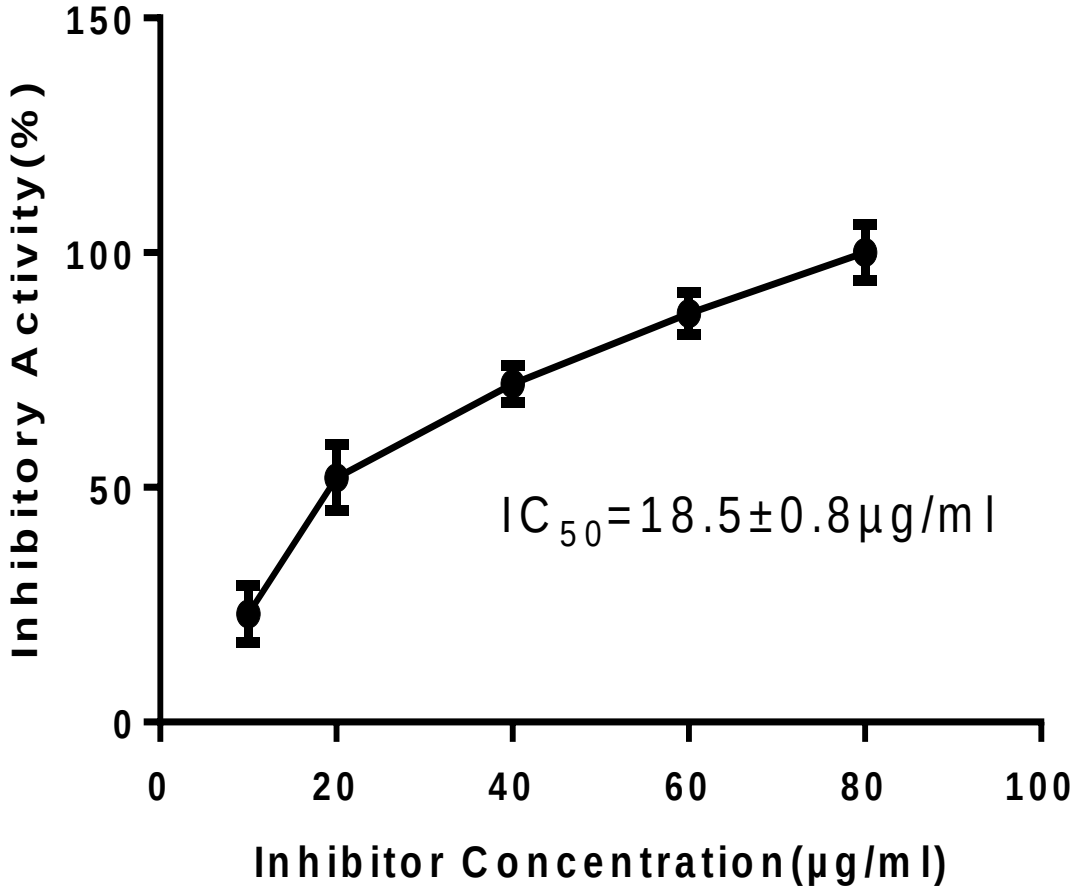


Figure 4. 19: Protease Inhibitory Activity of the Purified Extracts of *M.oleifera* Seeds.

Values are mean± SD

4.4 Determine LD_{50} and Subchronic Toxicities of Crude and Purified Inhibitors

4.4.1. Determination of LD_{50}.

Median lethal dose (LD_{50}) of crude acetone protein extracts of *C. procera* latex and *M. oleifera* seeds were determined in mice and the results are presented in Appendix IV. In the first phase of treatment, mice administered the crude protein extract at 1000mg/kg of both plant showed clinical signs of toxicity and all died after 24 hours. There were no clinical signs of toxicity observed with mice treated with 10mg/kg and 100mg/kg body weight. In the second phase, animals administered 200mg/kg and 400mg/kg of both crude extract showed no toxicity, while two-third of mice administered 600mg/kg crude protein extract of *C. procera* latex died. Crude extract of *M. oleifera* seeds at 800mg/kg killed two-third of the animals. From this result LD_{50} for latex of *C. procera,* latex was calculated to be 490mg/kg body weight while the LD_{50} for *M. oleifera* seeds was 690mg/kg body weight.

4.4.2 Subchronic toxicity studies

The packed cell volume (PCV) of mice treated with *C. procera* latex and *M. oleifera* seeds for twenty-one days are presented in Table 4.4. The PCV of mice treated with different concentrations of *C. procera* latex and *M. oleifera* seeds showed no significant differences ($P<0.05$) compared to control group post treatment.

Serum biochemical indices of mice treated with extracts from *C. procera* latex are summarized in Table 4.5. Animals treated with crude extracts of *C.procera* showed a significant elevation ($P<0.05$) in serum Glucose level as compared to control. Furthermore, a slight reduction which was not significant ($P<0.05$), compared to control was observed in the levels of serum Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT),

Triacylglycerol and Alkaline phosphate (ALP) in mice treated with crude extracts of *C.procera* latex. In addition, serum creatinine level of mice treated with *C.procera* latex showed no significant changes ($P<0.05$) compared to control. The partially purified inhibitor treated group at the dose of 50mg/kg body weight showed no significant changes ($P<0.05$) compared to control in all biochemical parameters measured.

Table 4.6 shows serum biochemical values of mice treated with extracts from *M.oleifera* seeds. Animals treated with crude extracts of *M.oleifera* seeds showed no significant changes ($P<0.05$) in AST, ALP and creatinine levels as compared to control. However, an elavated levels of Glucose and decreased in Triacylglycerol levels was observed for animals treated with extracts from *M.oleifera* seeds which were not significant ($P<0.05$). Serum biochemical values of animals treated with partially purified inhibitor from *M.oleifera* seeds showed no significant alteration ($P<0.05$).

Table 4. 4: Mean Packed Cell Volume of Experimental Mice Treated with Extracts from *C.procera Latex* and *M.oleifera* Seeds for 21 Days.

***C.procera* (latex)**				***M.oleifera* (seeds)**			
Groups	Pretreatment PCV%	Posttreatment PCV%	%change	**Groups**	Pretreatment PCV%	Posttreatment PCV%	%change
Control (PBS)	47.56±1.53	48.96±0.90	3.02±1.5[a]	**Control (PBS)**	48.52±1.20	49.56±0.91	2.10±1.2[a]
20mg/kg Crude	47.82±0.88	48.86±0.17	2.20±1.8[a]	**30mg/kg Crude**	48.18±2.25	49.12±1.35	1.91±1.3[a]
30mg/kg Crude	48.7±94	49.9±37	2.52±1.3[a]	**50mg/kg Crude**	47.38±0.64	49.52±0.77	4.32±1.5[b]
50mg/kg Crude	471.5.88±1.09	49.04±0.83	2.47±1.1[a]	**70mg/kg Crude**	49.2±1.709	49.42±1.56	0.45±0.3[c]
50mg/kg purified	46.8±1.8	48.4±0.45	3.31±1.7[a]	**70mg/kg purified**	47.7±1.4	48.8±0.7	2.25±1.3[a]

Values are Mean ± SD (n=5)

Values with different superscript letters within the same column are significantly different ($P < 0.05$)

Table 4. 5: Serum Biochemical Parameters of Mice Treated Extracts from *C.procera* Latex

	Glucose (mg/dl)	Triacylglycerol (mg/dl)	AST(U/L)	ALT(U/L)	ALP(U/L)	Creatinine (mg/dl)
Control (PBS)	95.73±0.42[a]	150.88±2.34[a]	36.56±0.47	39.24±0.39[a]	129.88±0.63[a]	0.96±0.23
20mg/kg crude CPI	93.28±0.42[a]	145.54±1.25[ab]	36.40±0.80	40.64±0.71[ab]	128.94±0.74[a]	0.96±0.15
30mg/kg crude CPI	103.02±2.00[b]	140.66±0.67[b]	37.32±.94	38.50±0.31[a]	128.04±0.34[ab]	0.95±0.30
50mg/kg crude CPI	108.04±1.07[c]	148.12±2.69[a]	37.64±1.44	42.82±1.43[b]	126.70±0.73[b]	1.09±0.43
Purified 50mg/kg	96.34±1.42[a]	148.58±2.34[a]	37.83±0.77	39.74±0.56[a]	128.58±0.83[a]	0.97±0.33

Values are Mean ± SD (n=5)

Values with different superscript letters within the same column are significantly different ($P < 0.05$)

Table 4. 6: Serum Biochemical Parameters of Mice Treated Extract from *M.oleifera* Seeds

	Glucose (mg/dl)	Triacylglycerol (mg/dl)	AST(U/L)	ALT(U/L)	ALP(U/L)	Creatinine (mg/dl)
Control (PBS)	96.35±0.55^{a}	150.56±2.84^{a}	38.36±0.47	39.34±0.49^{a}	129.66±0.63	0.95±0.13
30mg/kg crude CPI	97.40±0.88^{a}	146.80±1.41^{b}	41.50±1.44	38.50±1.45^{b}	128.50±0.57	0.98±0.25
50mg/kg crude CPI	103.62±1.27^{b}	144.32±1.93^{b}	37.64±1.44	39.48±0.49^{ab}	128.44±0.82	1.05±0.30
70mg/kg crude CPI	103.64±0.97^{b}	141.88±1.80^{b}	36.20±0.91	42.58±1.51^{ab}	128.00±1.11	1.00±0.43
Purified 70mg/kg	98.10±1.88^{a}	148.80±1.41^{a}	40.50±1.54	39.51±0.39^{ab}	128.20±1.21	0.94±0.33

Values are Mean ± SD, (n=5)

Values with different superscript letters within the same column are significantly different

($P < 0.05$)

4.5 Purification of Cysteine Protease from *Plasmodium berghei*

Elution profile of *Plasmodium berghei* cysteine protease by hydrophobic interaction Chromatography on a Phenyl Sepharose column is shown (Figure 4.20). The active proteins were eluted with buffer containing 0.5, 0.2, 0.1 and 0.0 M ammonium sulphate. The fractions with protease activity were pulled together for further studies.

The protease activity yield of 35.1% and purification fold of 3.67 were observed for the cysteine protease enzyme (Table 4.7).

4.6. Kinetics Properties of *P.berghei* Protease with Purified Inhibitors

Figure 4.21 shows the effect of the purified inhibitor of *C.procera latex* and *M.oleifera* seeds on the activity of *Plasmodium berghei* cysteine protease.The IC_{50} of purified inhibitor from *C.procera latex* and *M.oleifera* seeds were 23.10±1.2µg/ml and 17.2±0.3µg/ml respectively.

Effect of Substrate and Inhibitor Concentrations on the Activity of *P.berghei* Cysteine protease are presented in Figures 4.22 and 4.23.The reciprocal values of substrate concentrations, initial velocities of enzyme obtain from Figures 4.22, and 4.23 were used to plot figures 4.24 and 4.25 respectively.

Double reciprocal plots (Lineweaver–Burk plot) presented in Figures 4.24 and 4.25 for purified inhibitor from C.*procera latex* and *M.oleifera* seeds, to deduce their mechanism of inhibition, yielded a mixed pattern of inhibition for both extracts. From the plot, K_m and V_{mav} of the Enzyme are calculated to be 1.1µM and 9.0µmol/min respectively. Inhibition binding constant (K_i) were found to be 8.0µg/ml and 20.µg/ml (Appendix IV) for purified CPI of *C. procera latex* and *M.oleifera* seeds respectively.

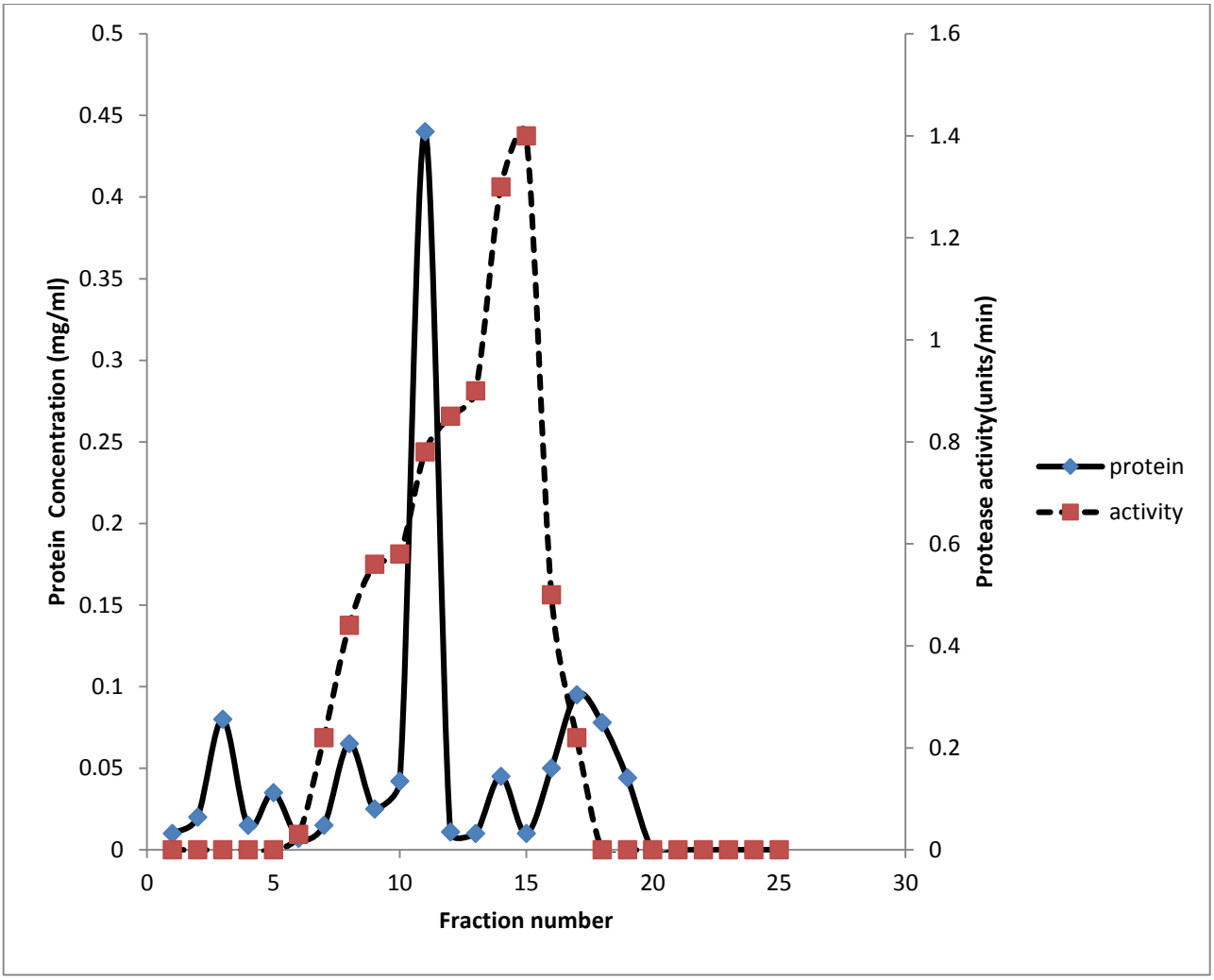


Figure 4. 20: An Elution Profile for the Hydrophobic Chromatography of *P.berghei* Cysteine Protease on Phenyl-Sepharose Column.

Table 4. 7: Purification Table of *P.berghei* Cysteine protease

		Volume (mL)	Protein (mg/ml)	Total protein (mg)	Protease activity (Units)	Specific Protease activity (Units)/mg protein	Purification Fold	Yield (%)
1	Crude extract	20	1.46	29.2	470	16.09	1	100
2	Hydrophobic interaction Chromatography (Phenyl Sepharose)	3	0.93	2.79	165	59.13	3.67	35.1

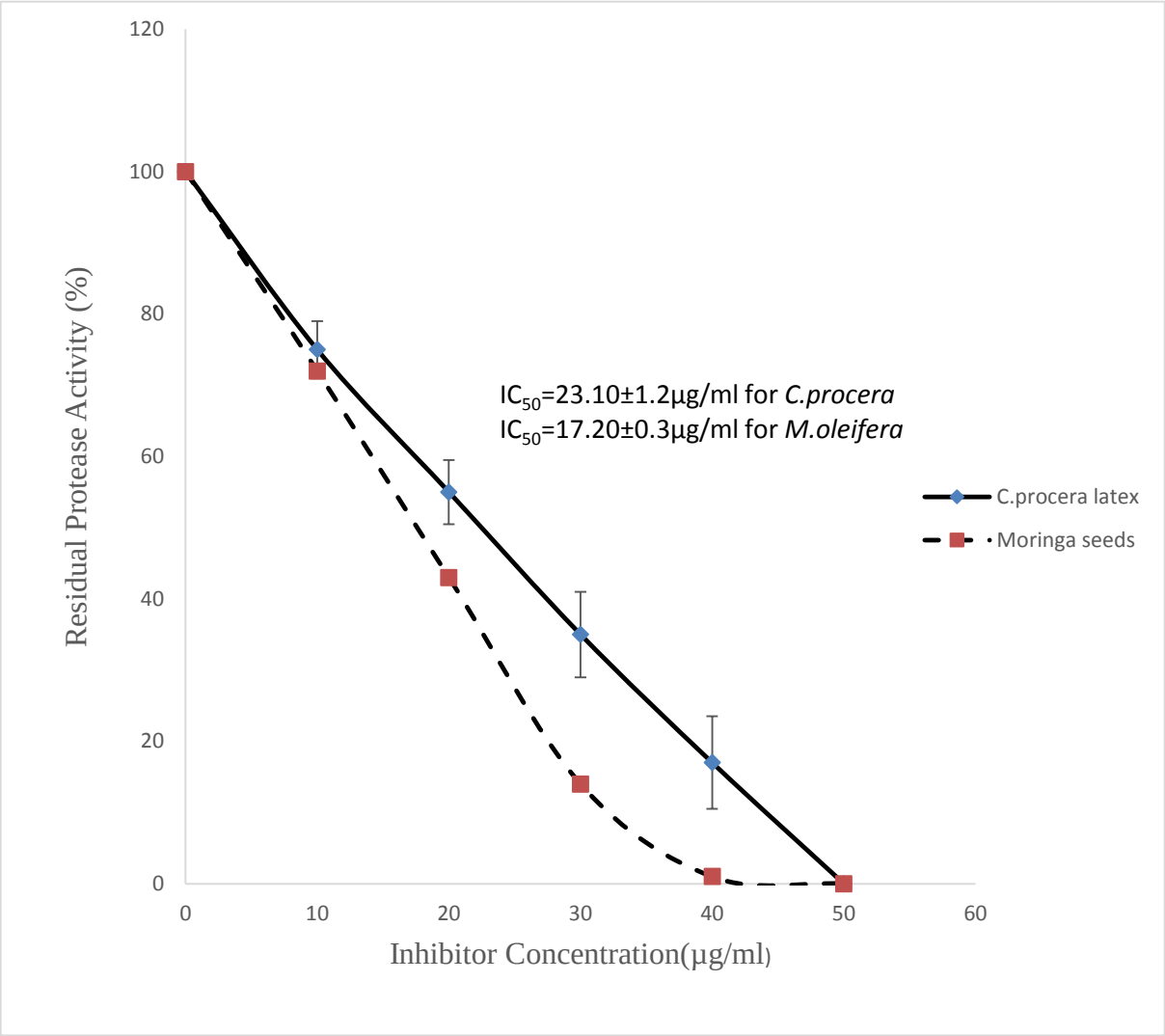


Figure 4. 21: Effects of the Purified Extracts from *C. procera latex* and *M.oleifera Seeds* on *P.berghei* Protease activity.

Values are mean± SD (n=3)

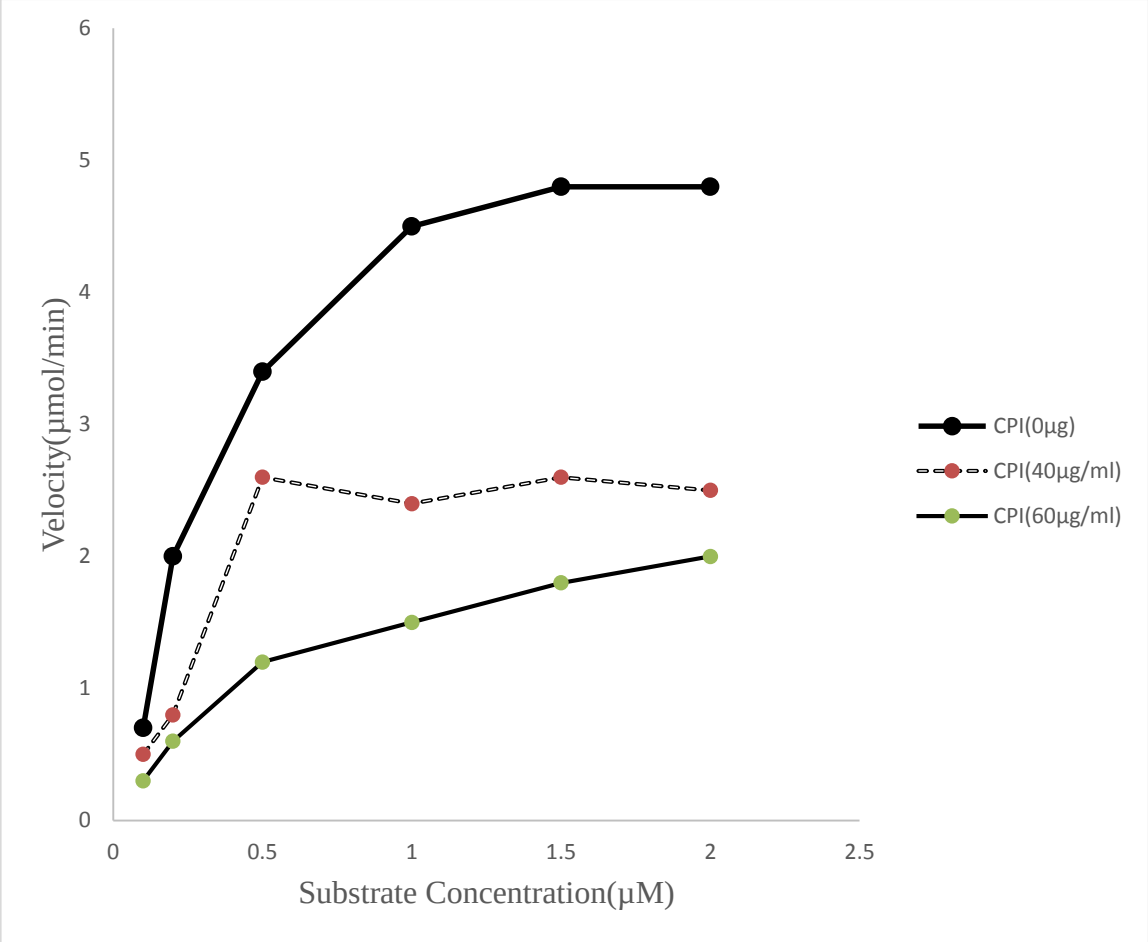


Figure 4. 22: Effect of Substrate and *C.procera* Latex Inhibitor Concentration on the Activity of *P.berghei* Cysteine Protease

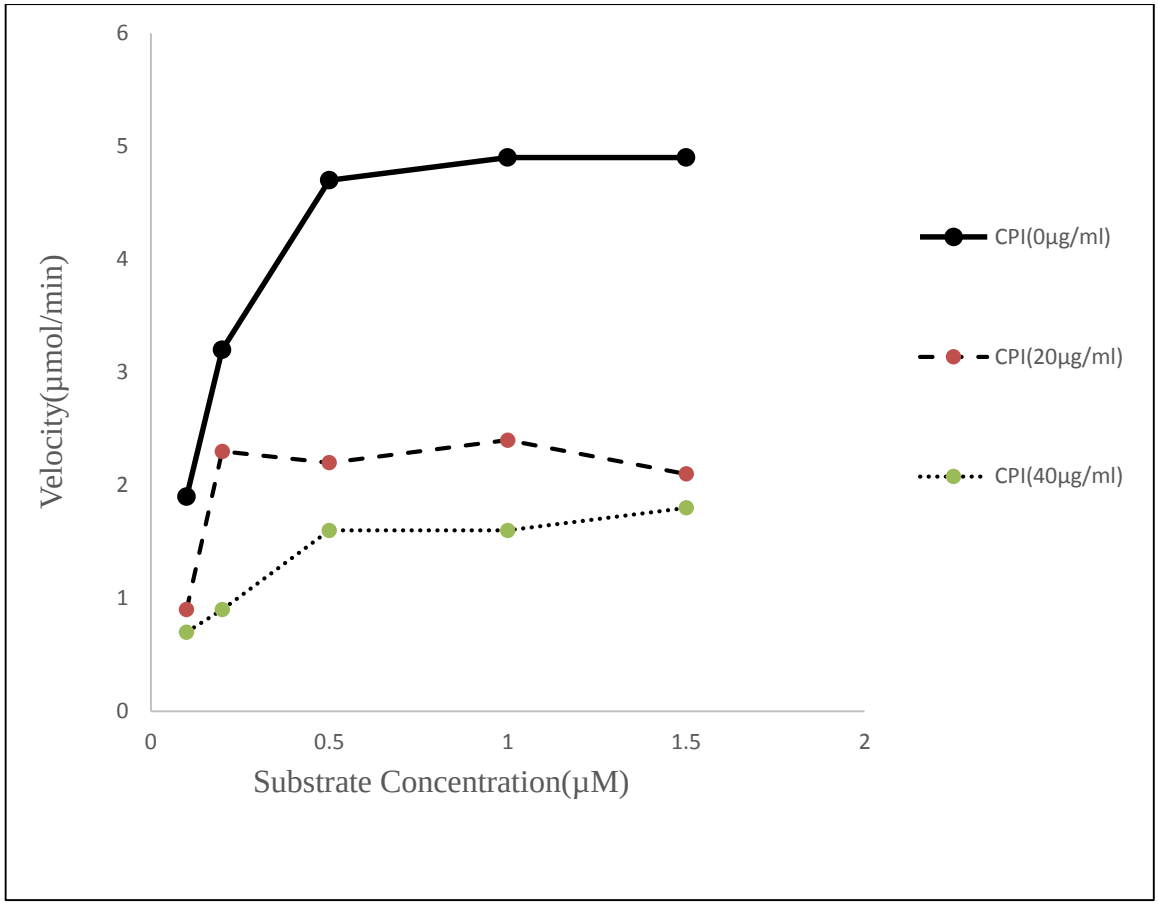


Figure 4. 23: Effect of Substrate and *M.oleifera* Seeds Inhibitor Concentrations on the Activity of *P.berghei* Cysteine Protease

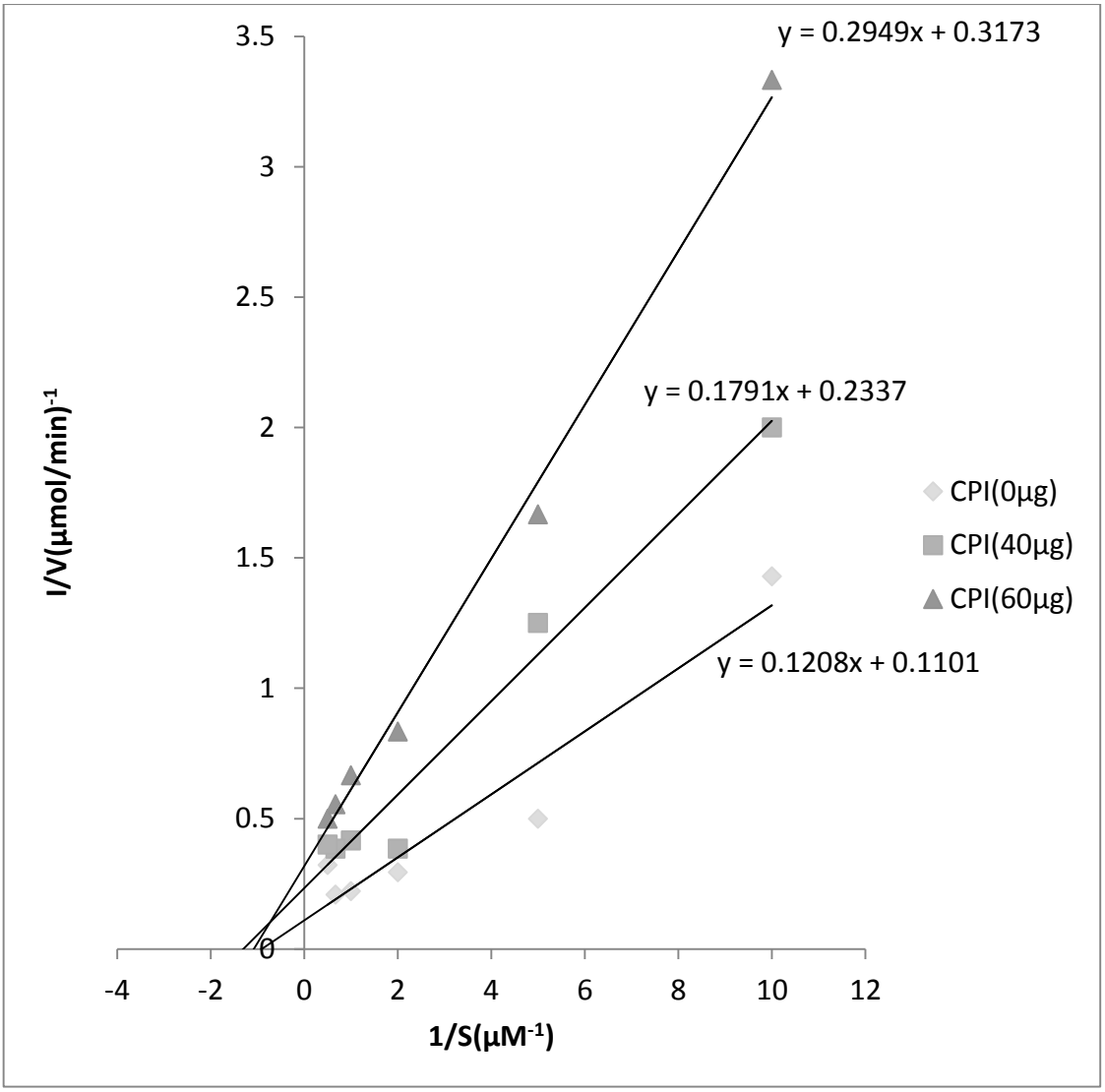


Figure 4. 24: Lineweaver-Burk Plots of Initial Velocity Data for the Determination of Inhibition Pattern on *P.berghei* Cysteine Protease by *C.procera* Latex inhibitor

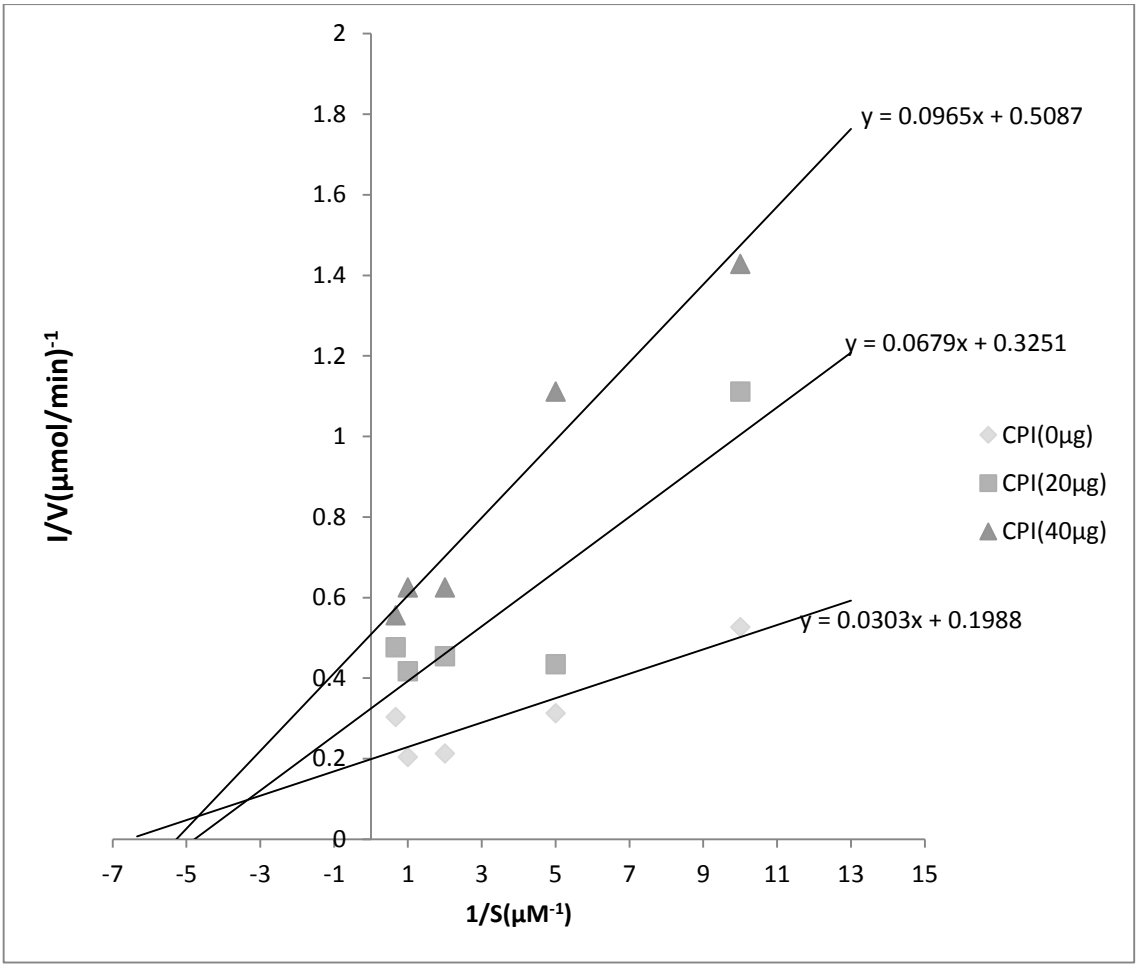


Figure 4. 25: Lineweaver-Burk Plots of Initial Velocity Data for the Determination of Inhibition Pattern on *P.berghei* Cysteine Protease by *M.oleifera* Seeds inhibitor

4.7 *In-vivo* Antimalarial Activities of Crude and Purified Protease Inhibitors Extracts

4.7.1 Effect of the extracts on parasitaemia level of mice infected with *P.berghei*

The course of parasitaemia for animals treated with inhibitor extracts are shown in Figures 4.26-4.29. A dose dependent decrease in parasitaemia was observed on mice treated with the crude extracts of *C.procera* at days 8 and 10 post-treatment compared to placebo control. By day 12 post-treatment, parasitaemia continued to increase in all surviving animals. Mice treated with positive control drugs (artesunate) showed a reduction in parasitaemia level and parasite was cleared in circulation at day 14 post-treatment (Figures 4.26).

All groups treated with different doses of purified extract of *C. procera* (Figure 4.27) had significantly lower level of parasitaemia compared to mice in the placebo group which had consistent increase in parasitaemia until they succumbed to infection.

Experimental animals treated with crude extracts of *M.oleifera* seeds (Figure 4.28) had a drop in parasitemia level at days 8 and 10 post-treatment compared to placebo group. Parasitaemia in-group treated with the crude extract, continued to increase until all died. At day 30 post-treatment, a mouse treated with 70mg/kg body weight had a 53.6% parasitaemia level.

Lower parasitemia level was also observed in all groups treated with purified extracts of *M.oleifera* seeds compared to the placebo group (Figure 4.29). Both purified plant extracts could not cleared the parasite but suppressed parasitaemia level and delayed the unset of infection compared to infected untreated animals.

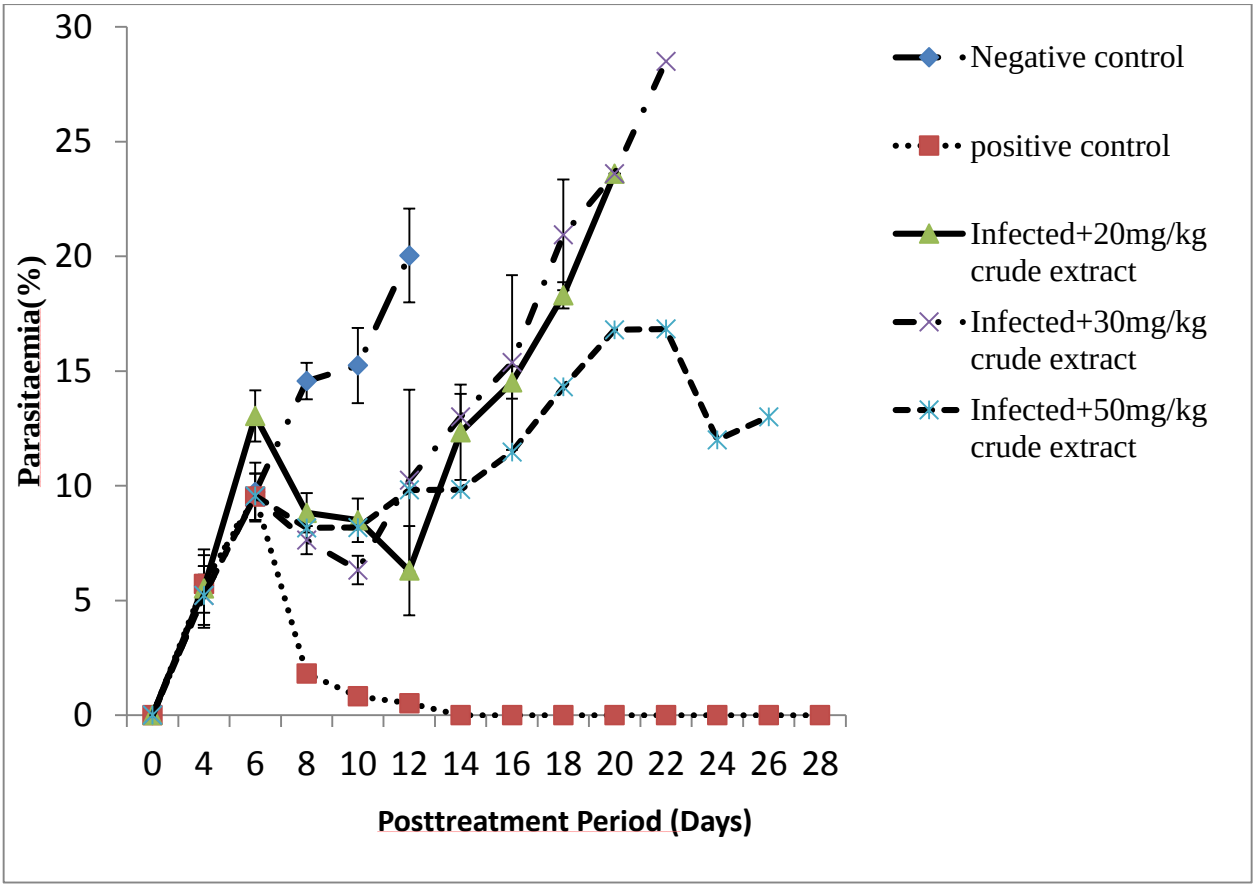


Figure 4. 26: Course of Parasitaemia in Experimental Mice Infected with *P.berghei* and Treated with Crude Extract from *C.procera* Latex.

Values are Mean ± SD. (n=5)

Negative control- Infected+Phosphate buffer saline (PBS)

Positive control- Infected+ artesunate.

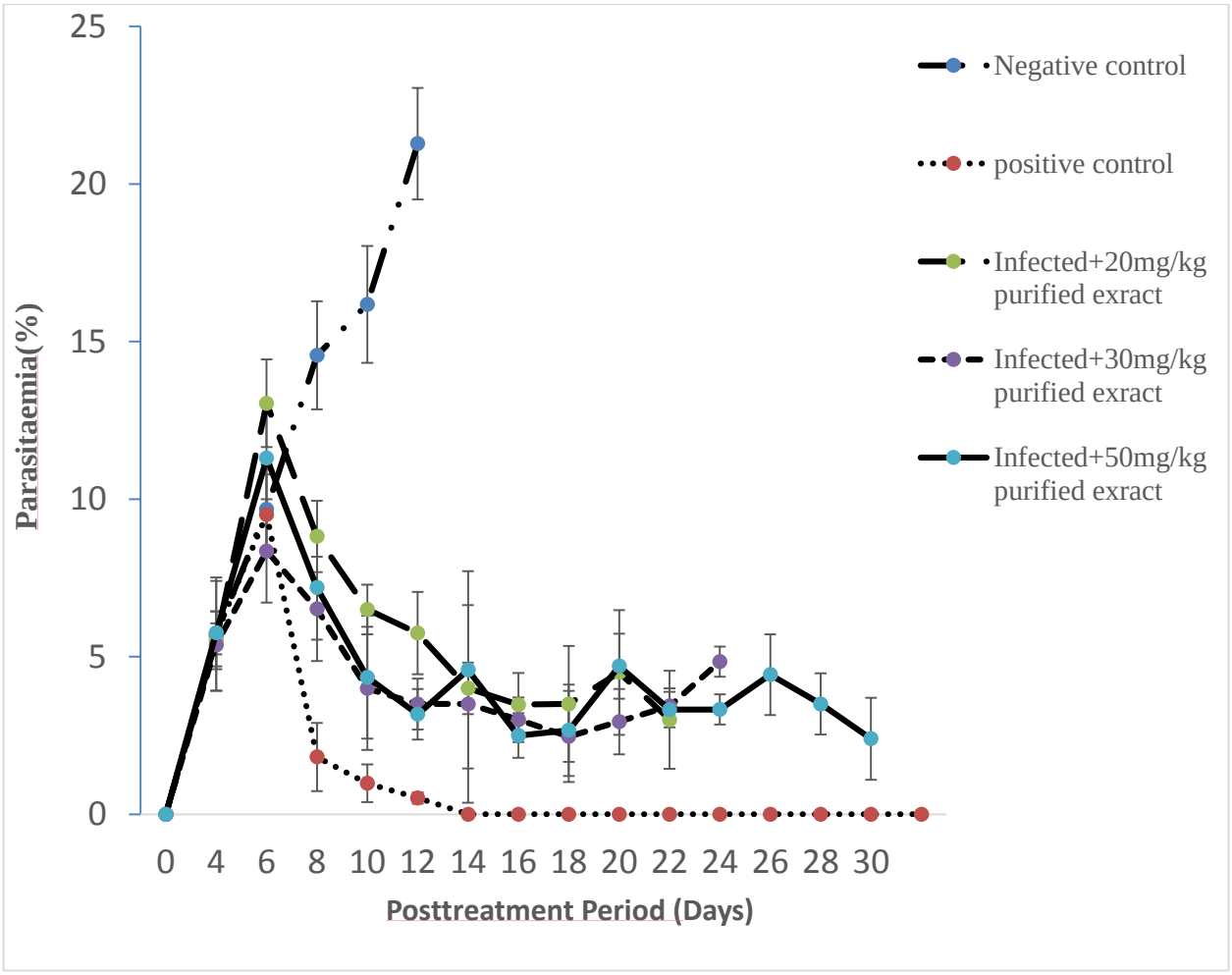


Figure 4. 27: Course of Parasitaemia in Experimental Mice Infected with *P.berghei* and Treated with Partially Purified Extracts from *C.procera* Latex.

Values are Mean ± SEM. (n=5)

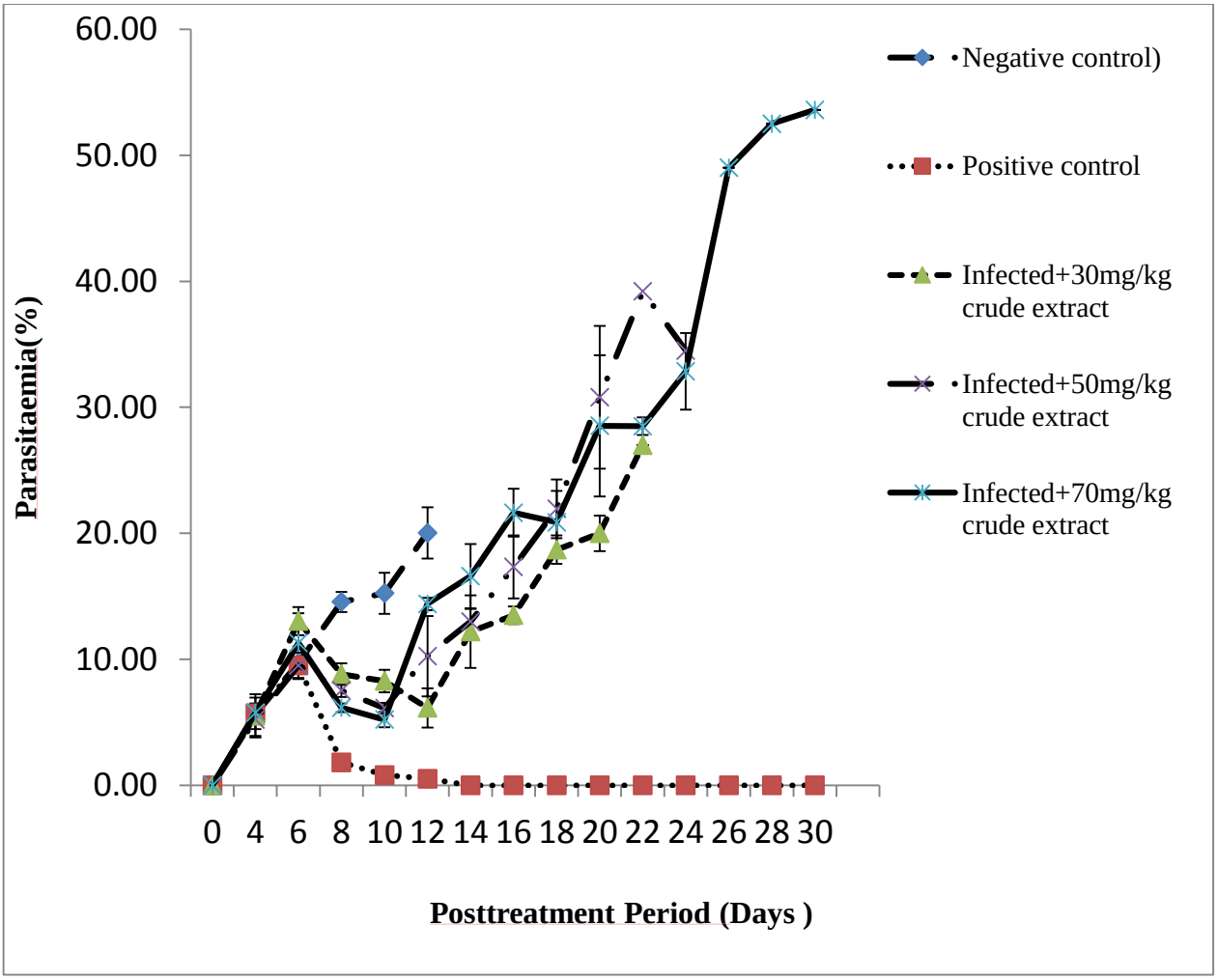


Figure 4. 28: Course of Parasitaemia in Experimental Mice Infected with *P.berghei* and Treated with Crude Extracts from *M.oleifera* Seeds.

Values are Mean ± SD, (n=5)

Negative control- Infected + Phosphate buffer saline (PBS).

Positive control- Infected + artesunate.

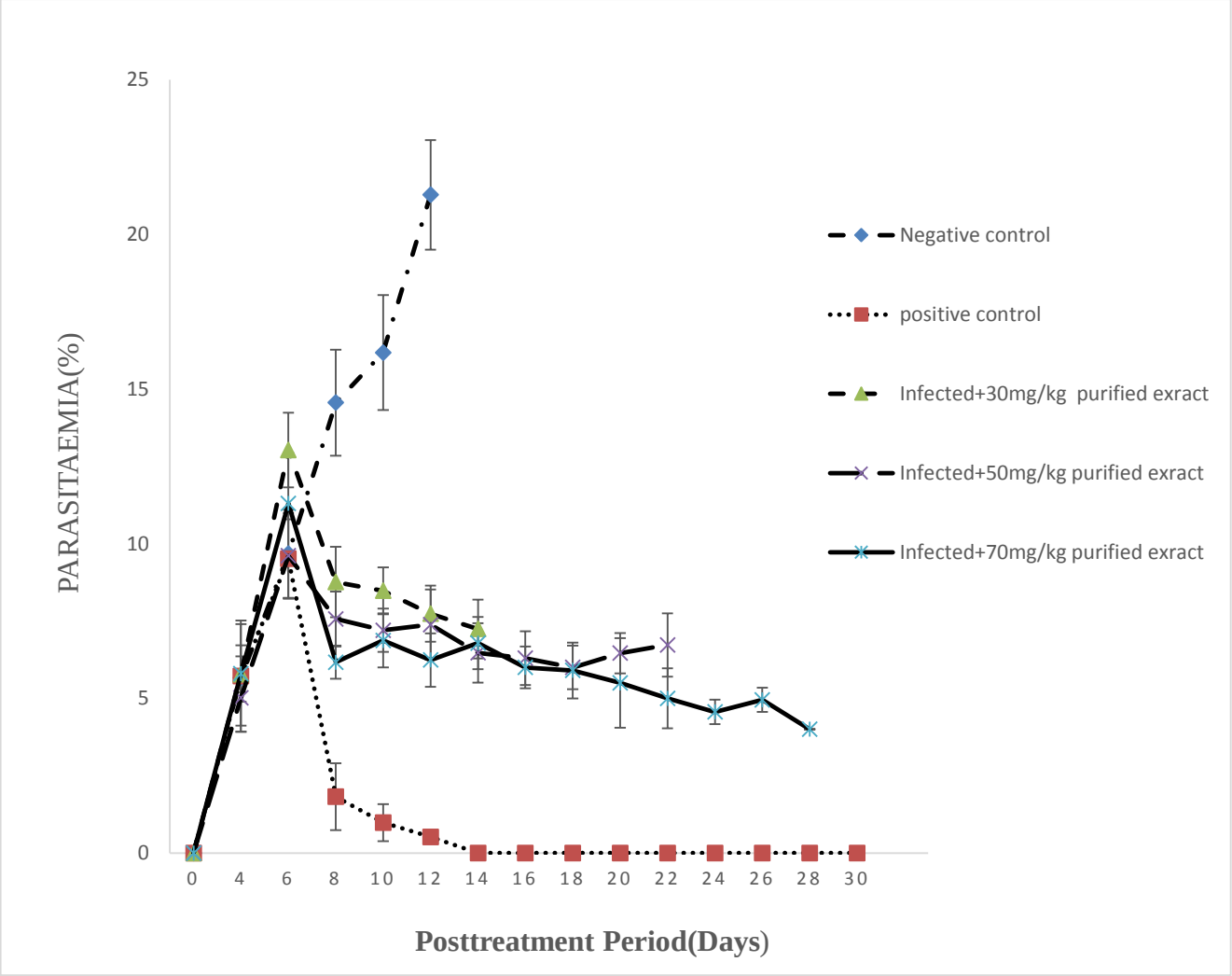


Figure 4. 29: Course of Parasitaemia in Experimental Mice Infected with *P.berghei* and Treated with Partially Purified Extract from *M.oleifera* Seeds.

Values are Mean ± SD (n=5)

4.7.2 Effect of extracts on percentage survival of the mice infected with *P.berghei*

Figures 4.30-4.33 show the percentage survival of animals treated with inhibitors extracts. Treatment of animals with increasing doses of extracts from *C.procera* and *M. oleifera* resulted in significantly increased survival ($P<0.05$) of experimental animals compared to placebo control. Mice in the placebo control died between days 9-12 posttreatment. All Mice in the positive control and normal group survival more than 30 days. However, 4 of 5 mice treated with inhibitor extract from *C.procera* latex survived up to 30 days of experimental period. Three mice in the group treated with crude extract of *M. oleifera* seeds treatment group survived beyond day 32 post-treatment. All animals treated with purified extract of *M.oleifera* seeds survived more than 32 days posttreatment period.

4.7.3 Effect of extract on the parasite suppression

A summary of four days parasite suppressive test by the extracts are presented in Table 4.8 and 4.9. The result of the four days suppressive test of animals treated with crude and purified extracts from *C.procera* latex showed a significant ($P<0.05$) increase in percentage parasitaemia suppression at day 8 posttreatment compared to placebo control group. Animals treated with the highest dose (50mg/kg.bw) of crude and purified extract of *C.procera* latex gave percentage suppression of 48±0.3 % and 77.00 ± 0.32% respectively. A suppression of 94.60 ± 0.24% was observed for positive (artesunate) control group (Table 4.8) at the same period.

The parasitaemia suppression of animals treated with crude and purified extracts from *M. oleifera* seeds shown a significant ($P<0.05$) increase in parasite suppression at 8 days post-treatment compared to untreated control. Animals treated with the highest dose

(70mg/kg.bw) of crude and purified extract of *M. oleifera* gave percentage suppression of 65.61±0.34 and 57.34±0.32% respectively (Table 4.9). However, the positive control groups had a high significant (P<0.05) suppression compared to treated mice.

4.7.4. Effect of extracts on the packed cell volume (PCV) of mice infected with of *P.berghei* at day 8 post infection

Packed cell volume of *P.berghei* infected and treated with extracts from *C.procera* latex and *M. oleifera* seeds at day 8.posttreatment are presented in Figures, 4.34 and 4.35 respectively. The packed cell volume (PCV) of untreated Mice were significantly (P<0.05) lower than those of either the normal control group or those given different doses of both plant extracts (*C.procera* latex and *M.oleifera* seeds) and that of positive control (infected and treated with artesunate). There was however, no significant difference among infected treated mice with either plant extracts or normal control groups.

Comparison of administration of different doses of crude and purified extracts of both plants on packed cell volume of the mice, showed that the low and medium doses (30mg/kg.bw and 50mg/kg.bw) of purified inhibitor extracts from *M.oleifera* seeds cause a significant reduction in PCV compared to normal control group at day 8 posttreatment.

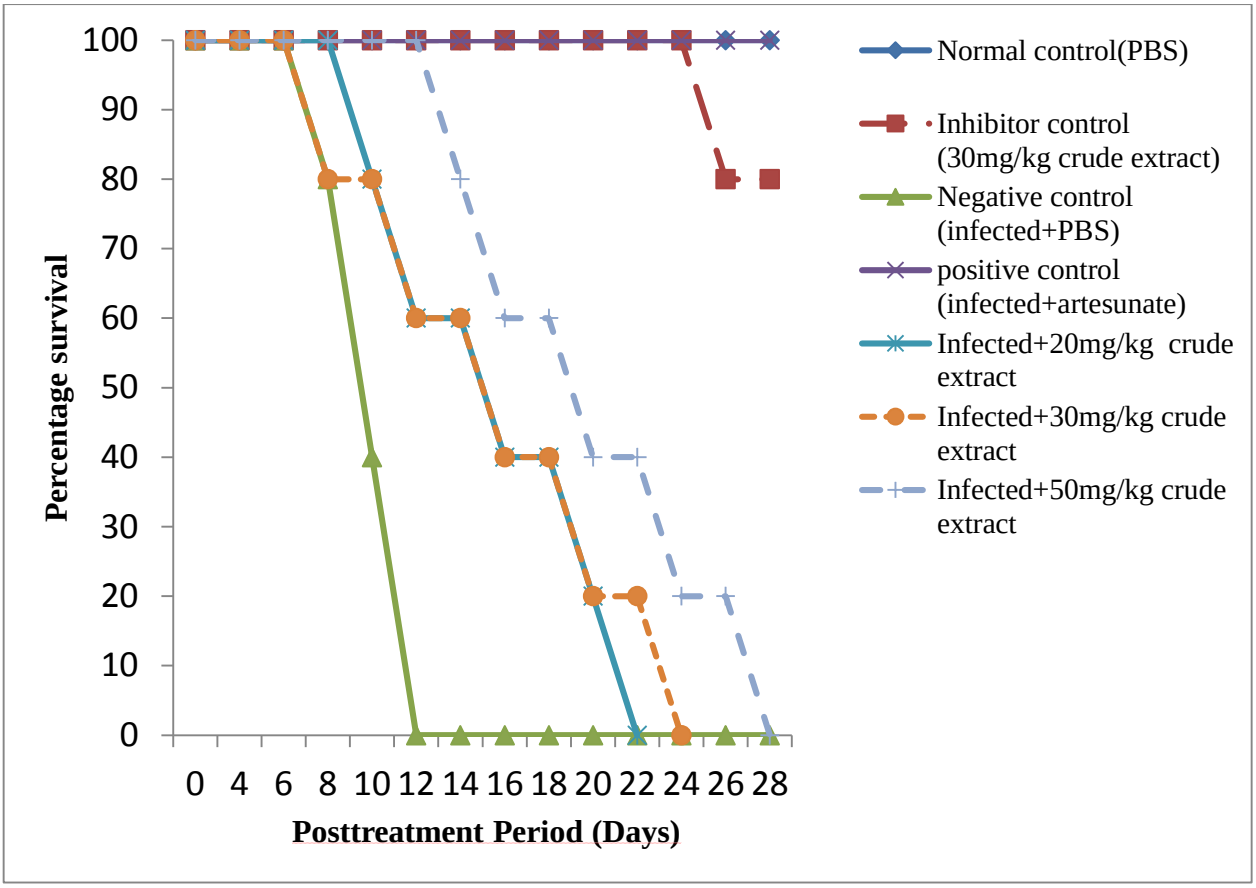


Figure 4. 30: Percentage Survival of the Experimental Mice Infected with *P.berghei* and Treated with Crude Extracts from *C.procera* Latex

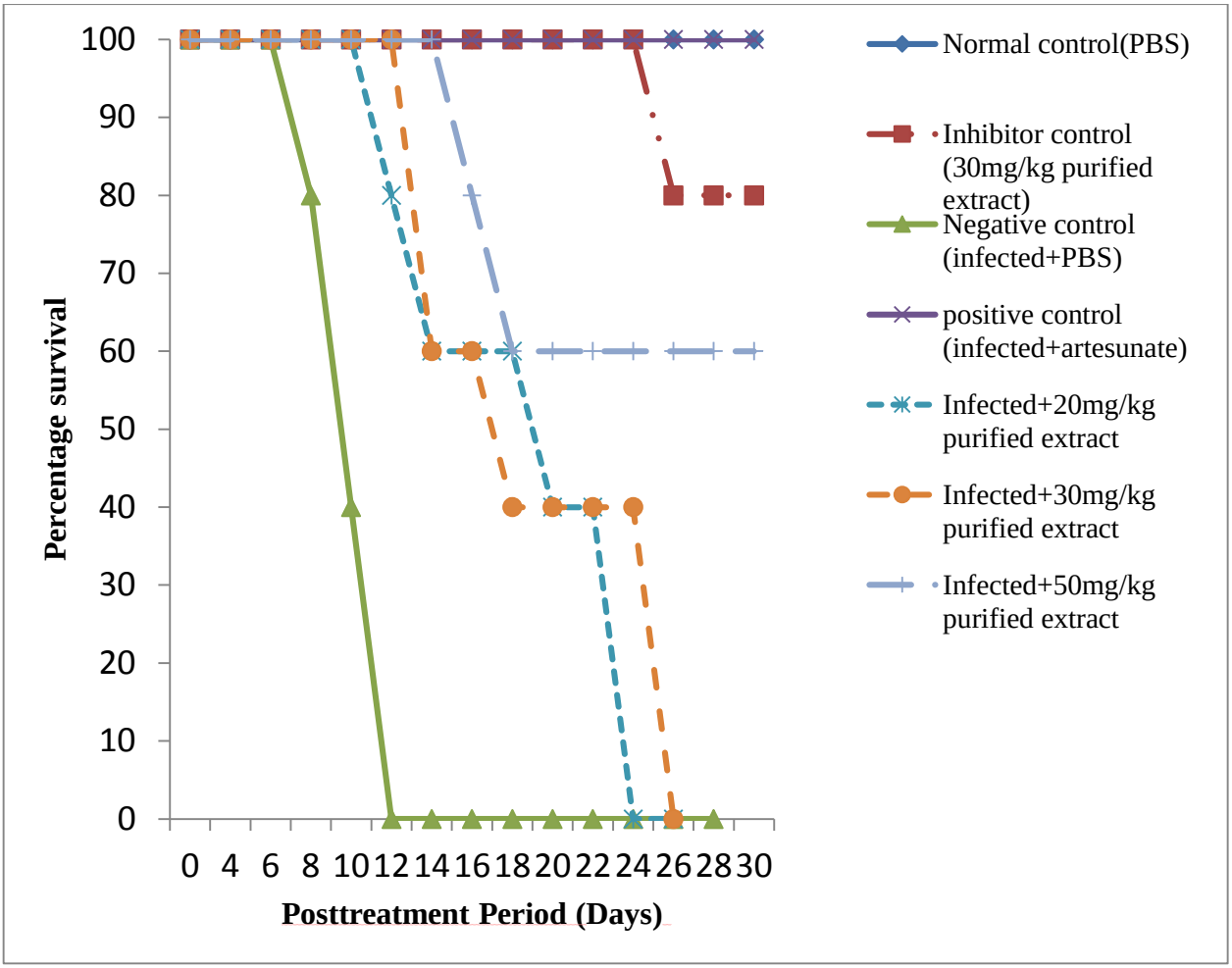


Figure 4. 31: Percentage Survival of the Experimental Mice Infected with *P.berghei* and Treated with Partially Purified Extract from *C.procera* Latex.

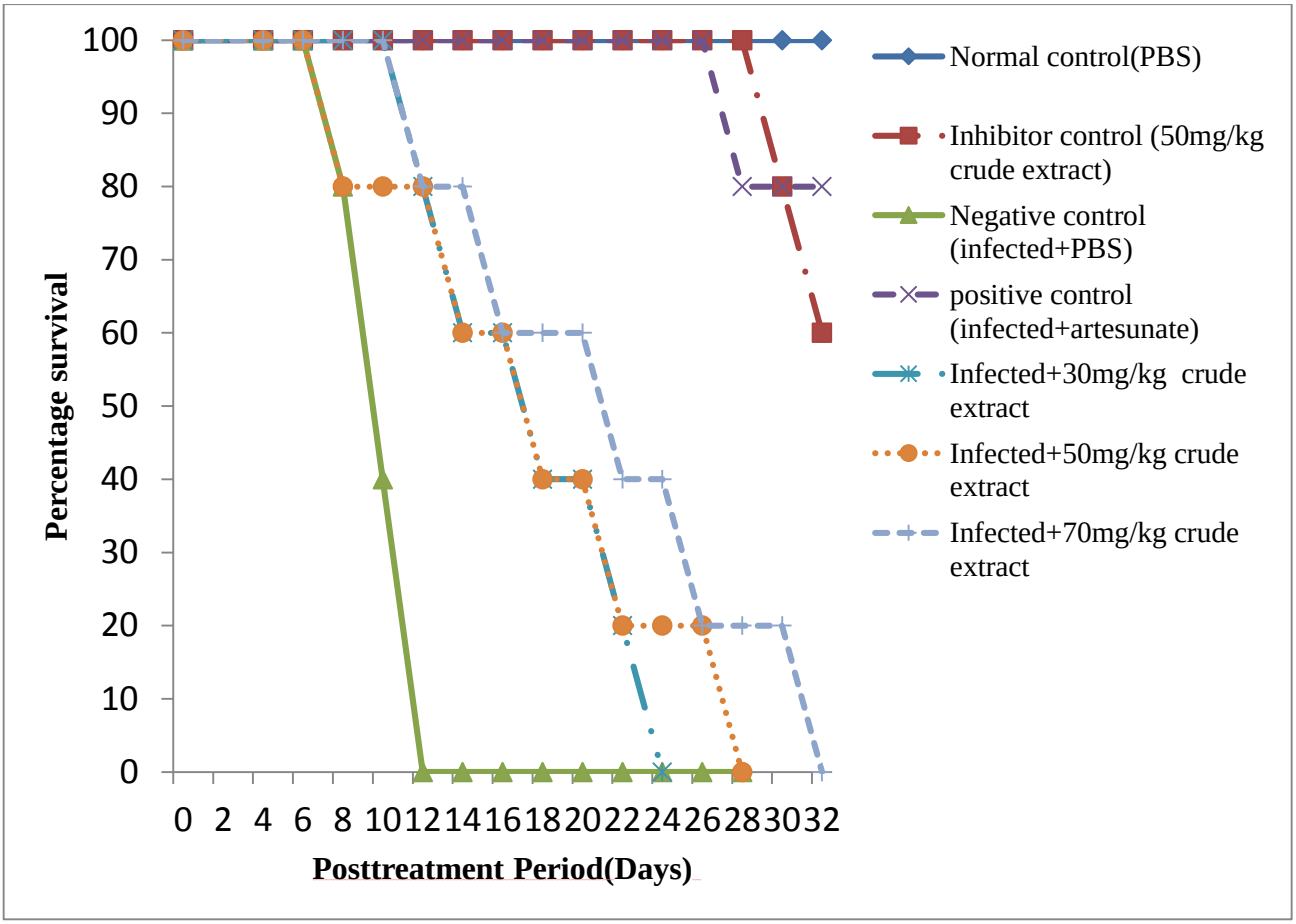


Figure 4. 32: Percentage Survival of the Experimental Mice Infected with *P.berghei* and Treated with Crude Extract from *M.oleifera* Seeds.

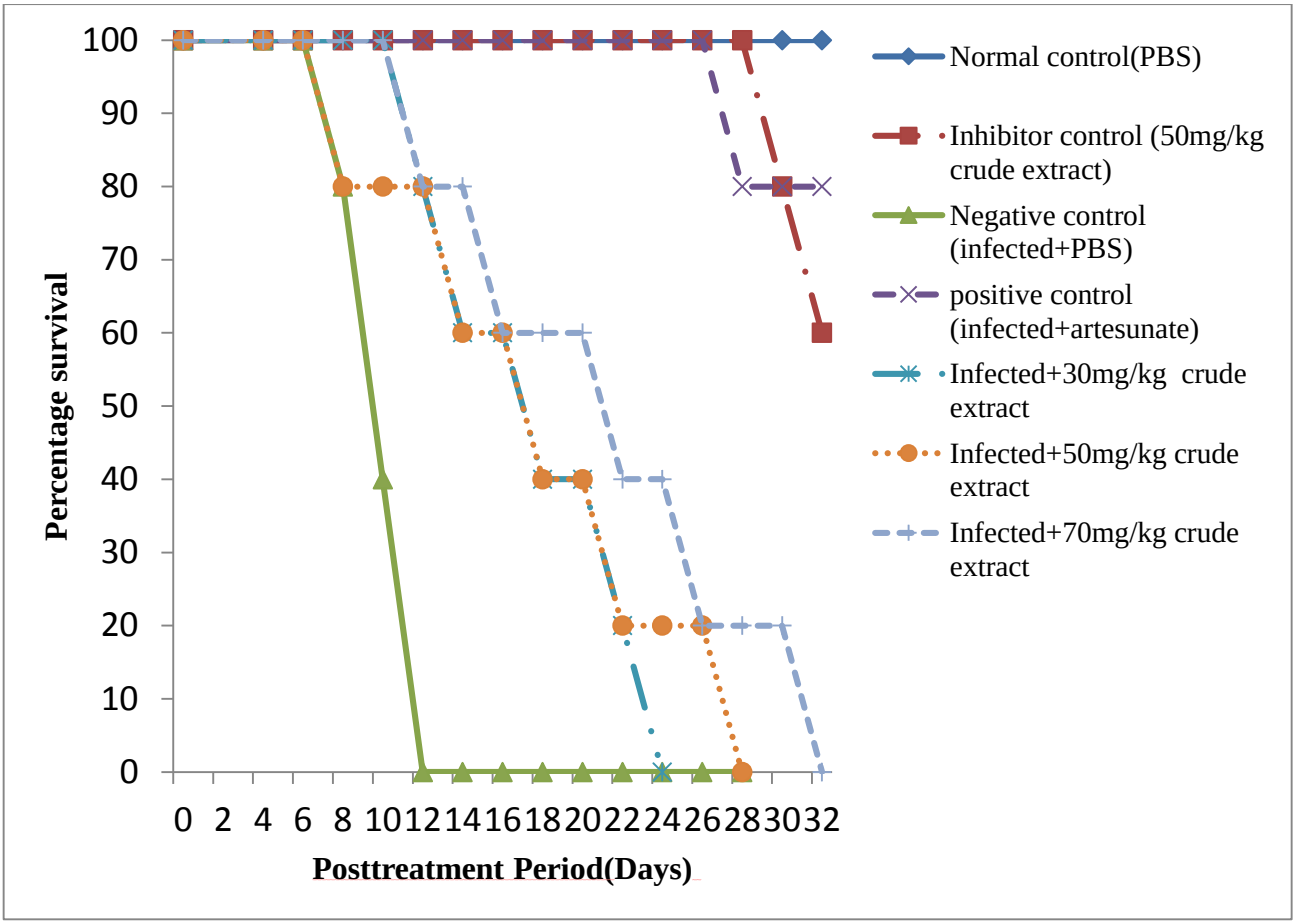


Figure 4. 33: Percentage Survival of the Experimental Mice Infected with *P.berghei* and Treated with Crude Extract from *M.oleifera* Seeds.

Table 4. 8: Antimalarial Suppressive Test of *C.procera* Latex Extracts Treated Mice

	Crude *C.procera* latex		**Purified *C.procera latex***	
	%Parasitaemia	%Suppression	%Paracetaemia	%Suppression
Infected+PBS (negative control)	15.24±0.70 [a]		15.24±0.70	
Infected+artesunate (positive control)	0.82±0.34	94.60±0.24[c]	0.82±0.13	94.60 ±0.24[a]
Infected+20mg/kg	8.5±.86	39.42±0.42[a]	6.5±0.45	57.34±0.40[b]
Infected+30mg/kg	6.3± 0.62	58.66±0.52[b]	4.0±1.30	73.75±0.54[c]
Infected+50mg/kg	7.58±0.35	48.00±0.32[b]	3.5±1.3	77.00±0.32[c]

Values are Mean ± SD

Values with different superscript letters are significantly different ($P < 0.05$)

Table 4. 9: Antimalarial Suppressive Test of *M.oleifera* Seeds Extracts Treated Mice

	Crude *M. oleifera* seeds		**Purified *M. oleifera* seeds**	
	%Parasitaemia	%Suppression	%Paracetaemia	%Suppression
Infected+PBS(negative control)	15.24±0.70		15.24±0.70	
Infected+artesunate (positive control)	0.82±0.32	94.60±0.24[a]	0.82±0.32	94.60±0.24[a]
Infected+30mg/kg	6.13±0.86	59.42±0.23[a]	8.5±1.45	44.22±0.21[b]
Infected+50mg/kg	7.52± 0.54	50.60±0.25[b]	7.2±1.40	52.75± 0.26[b]
Infected+70mg/kg	5.22± 0.32	65.61±0.34[c]	6.5±1.2	57.34± 0.32[b]

Values are Mean ± SD

Values with different superscript letters are significantly different ($P < 0.05$)

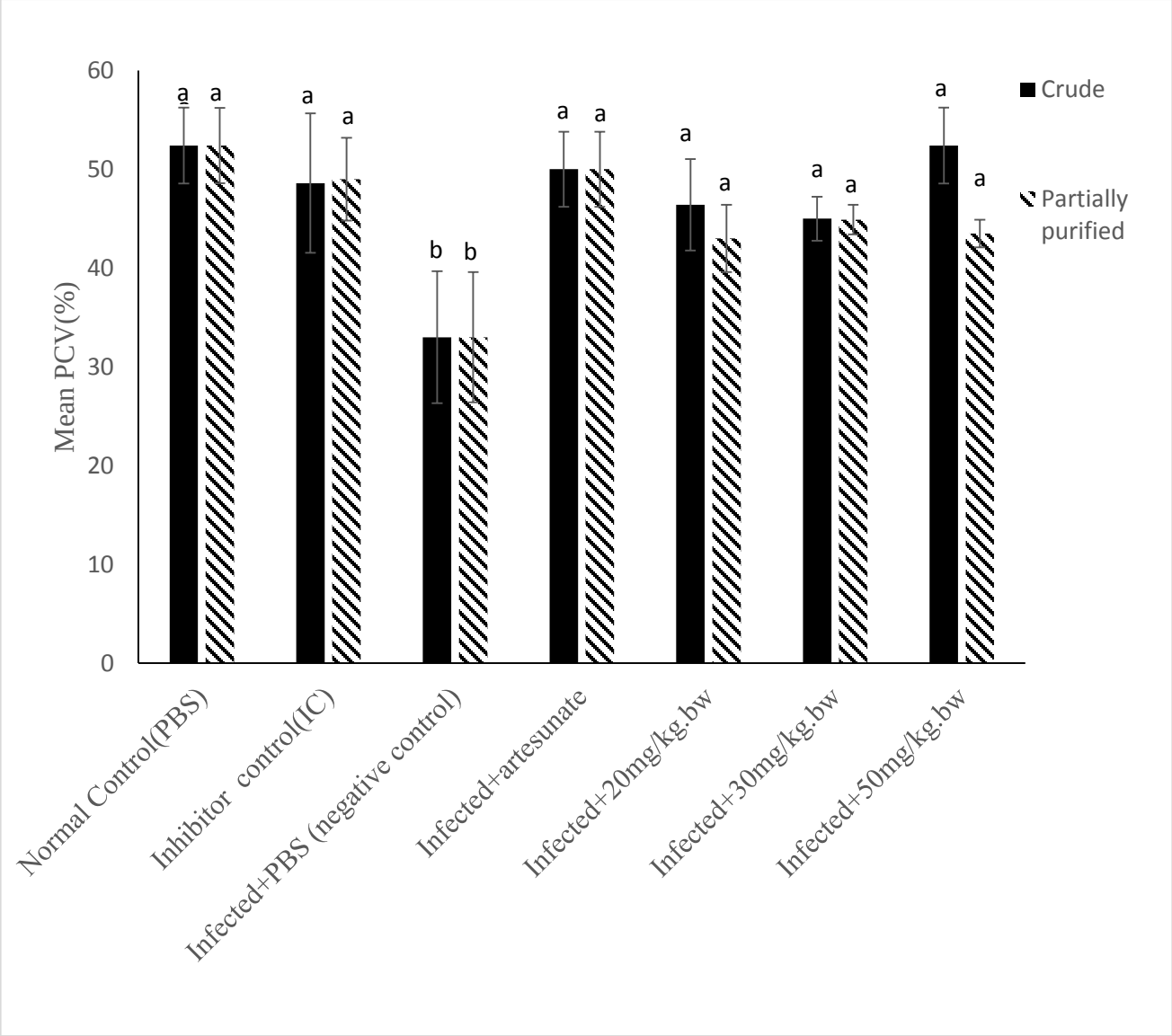


Figure 4. 34: Packed Cell Volume of Experimental Animals Infected with *P.berghei* and Treated with Crude and Purified Extract of *C.procera* Latex

Values are Mean ± SD

Bars with different letters differ significantly ($p<0.05$)

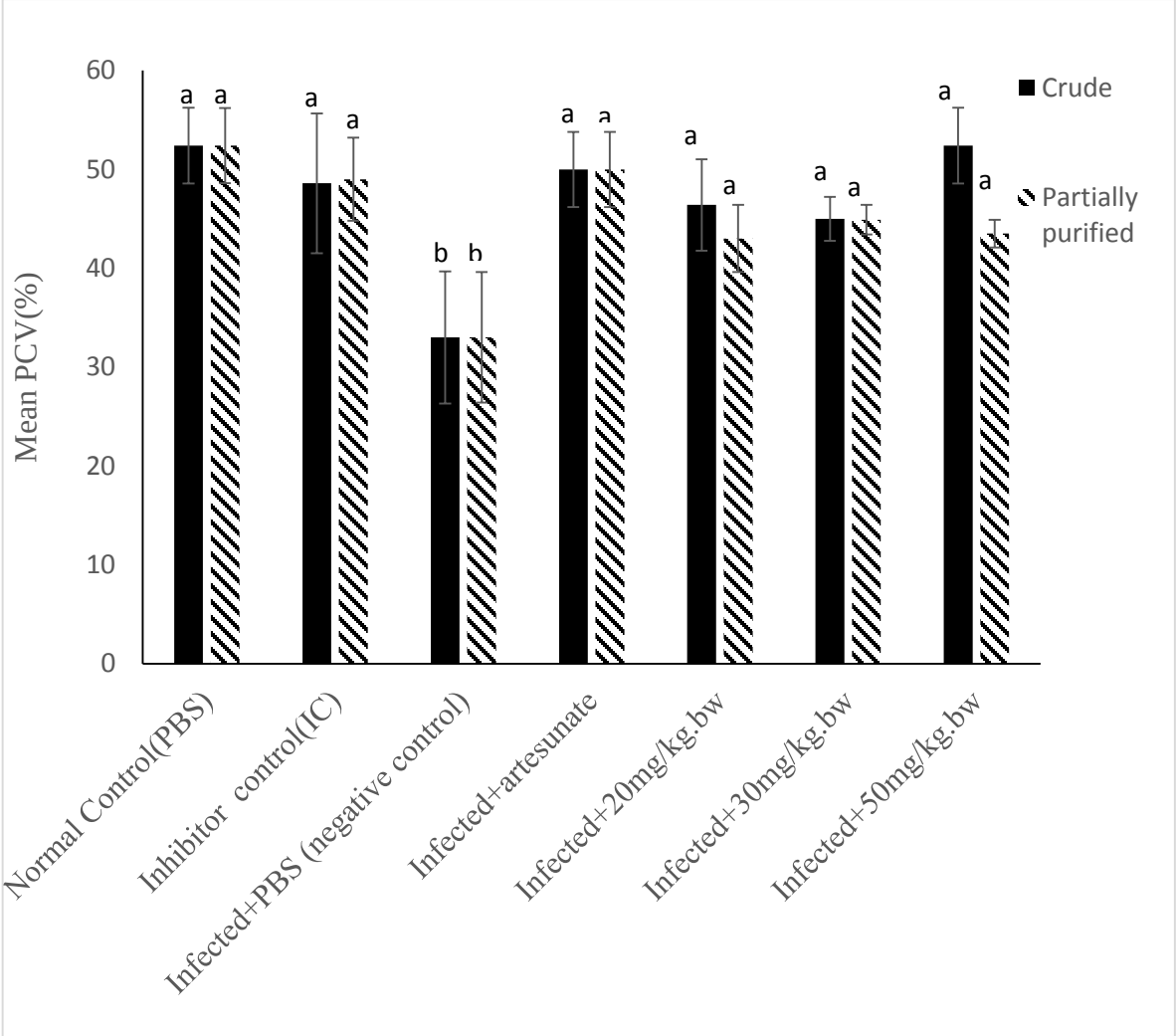


Figure 4. 35: Packed Cell Volume of Experimental Animals Infected with *P.berghei* and Treated with Crude and Purified Extract of *C.procera* Latex.

Values are Mean ± SD

Bars with different letters differ significantly ($p<0.05$)

CHAPTER FIVE

5.0 DISCUSSION

Several parasites causing mammalian diseases are dependent on cysteine proteases for their life cycle. The central role of cysteine proteases in the pathogenesis and survival of plasmodia parasites in the host erythrocytes makes them attractive antimalarial drug targets (Rosenthal, 2011). Inhibition of these proteases by natural proteins/peptides that will not have off target binding could be useful in the treatment of these parasitic diseases especially in *Plasmodium* infections (Karthik *et al*., 2014; Rosenthal, 2011). Plants produce protease inhibitors in order to modulate the activity of self-proteases, but these molecules can also confer to them resistance to other exogenous proteases by pathogens and insect pests attack. Consenquently taking advantages of plants that potentially inhibit this enzyme could be another source of antiparasitic agents and in extention antimalarial agents.

C*alotropis procera* and *Moringa oleifera* are widely distributed in subtropical world and can survive many environmental unfavourable conditions. Numerous bioactive proteins were isolated from these plants and their pharmacological effects are documented (Ramos *et al*., 2010; Al-snafi, 2015; Lawal *et al*., 2015).

In the present investigation, significantly higher specific cysteine protease inhibition was exhibited by phosphate buffer extract from the latex of *C.procera* and seeds of *M. oleifera* compared to other solvents, thus, phosphate buffer were selected as the effective extraction medium for maximal extraction of protease inhibitor without any loss in activity. This is based on the fact that reports on solubilization of therapeutic proteins using various solvent system indicated that phosphate buffer extraction protect the protein structural

integrity and conferred stability to these agents as these molecules are subjected to various forces aimed at isolating them for investigation (Bijina *et al*., 2011_a). Saline extract, which contain proteins from the latex of C.procera, was reported to have anticancer activities (Oliveira *et al*., 2010). Other researchers used 0.01 N NaOH (Marconi *et al*., 1993), distilled water (Jung *et al*., 2012) and 0.1 M phosphate buffer, pH 7.5 (Bijina *et al*., 2011_a) as extraction media for various therapeutic proteins. Phosphate buffers at concentrations of 0.1 - 0.5 M are known for their solubilizing properties.

The crude phosphate buffer extracts from the latex of *C.procera* and seeds of *M. oleifera* were concentrated using cold acetone precipitation. The crude acetone protein pricipitate exhibited dose dependent inhibitory activity with papain enzyme with an IC_{50} value of 1350 µg/ml and 1,230 µg /ml for *C.procera* and *M.oleifera* respectively. These findings are more than hundred fold those reported by Abd El-latif (2015) with an IC_{50} value of 21.04 µg/ml on papain by inhibitor exracted from *Callosobruchus maculatus.*

Partial purification of crude acetone precipitated proteins from latex of *C.procera* and seeds of *M.oleifera* via ammonium sulphate precipitation followed by hydrophobic chromatography on phenyl sepharose column were carried out. The maximum inhibitory activity was recorded at 50% and 60 % ammonium sulphate saturation for latex of *C.procera* and seeds of *M.oleifera* respectfully. The ammonium sulphate solution is one of the commonest salts medium used in the initial steps in the protein purification. It has advantage over other solvents in stabilization of protein of interest. Krishnan and Murugan (2015) reported 20-90 % ammonium sulphate saturation for *Solanum aculeatissimum* protease inhibitor. This step of purification yielded a higher fold of purification compared to crude acetone precipitate protein in the case of *M.oleifera* seeds and a low purification fold for *C.procera* latex. The low purification fold observed for *C.procera* latex may be attributed to

presence of activated protease enzyme or to some impurities interfering in this stage of purification, or it could also be due to stability factor.

Further purification of both inhibitors by phenyl sepharose chromatograph yielded several proteins fractions with a single activity peak fractions for both plants with a purification fold of 3.95 and 3.19 and activity yield of 48.7% and 42.34% for latex of *C.procera* and seeds of *M.oleifera* respectively. Phenyl-Sepharose as an adsorbent for hydrophobic interaction chromatography has been reported to be effective for the purification of some proteases, and with purification factors in the range of 2.9 - 60 and activity yields in excess of 88% was obtained (Gupta *et al.,* 2005). The increase in activity of up to 48.7% and 42.34% recovery for both inhibitors in this investigation is an indicative of exclusions of endogenous protease and an improved stability of the inhibitors.

The presence of Cysteine protease from *C.procera* have been reported by Onyike and Abdullahi (2006) and Abdullahi and Isah (2009) and serine protease inhibitor from *M.oleifera* (Bijina *et al*., 2011_a). After several attempt in using ion exchange chromatography to purify the inhibitor without success, we therefore decided to explore differences in the hydrophobic interaction of protease and their inhibitor on phenyl-sepharose column. Karlsson *et al*. (1985) successfully separated calpain and their inhibitors in one single step on phenyl-sepharose column.

The partially purified extract of *C.procera* latex and *M.oleifera* inhibited *plasmodium* cysteine protease with the IC_{50} value of 23.50 µg/ml and 17.5 µg/ml indicating that the extracts bind to parasite protease at low concentration. Similarly, a peptide from *Streptomyces* sp LK3 extract showed inhibition of serine protease from *Plasmodium berghei* with an IC_{50} of 25.78 µg /ml (Karthik *et al*., 2014). Kamkumo *et al.,* (2012) reported that cysteine proteases of *Plasmodium falciparum*(Falcipain-2) was inhibited by fraction and

purified compound (2,3,6-trihydroxy methyl benzoate) from *Sorindeia juglandifolia* with IC_{50} of an 11.6 μg/ml and 21.9 μg/ml respectfully.

Furthermore, a biflavone (fukugetin) isolated from *Garcinia brasiliensis* inhibited major cysteine protease of *Trypanosoma cruzi* (Cruzain) with an IC_{50} of 7.0 μg/ml (Assis *et al*., 2013). *Leishmania mexicana* cysteine protease activity was also inhibited by another biflavone (quercetin) with an IC_{50} of 18.03μg/ml (Sousa *et al*., 2015). Our results demonstrated that both inhibitors are potent inhibitor of *Plasmodium berghei* cysteine protease.

The protease inhibitor from both plants had broad activity at pH range of 4.0-10.0, and was completely inactive at the extreme acidic and basic pH. The broad pH activity exhibited by these inhibtors is comparable with broad pH 2-10 reported for protease inhibitor from *Solanum aculeatissimum* (Krishnan and Murugan, 2015). The extreme pH conditions may possibly have totally altered the tertiary structure of the inhibitor making them completely inactive to bind with the enzymes. Under strong acidic or alkaline conditions, the proteinaceous inhibitors get denatured and consequently they lose their activity partially or completely. In general, all the protease inhibitors isolated from these plants have a wide pH range of 2.0-10.0. The intramolecular disulphide bridges are presumably responsible for the functional stability of protease inhibitors as result of extreme temperature and pH. Inactivation is due to unfolding of proteins by heat, as a result of the disturbance in the covalent and non-covalent interactions. Similar stability of serine protease inhibitor isolated from *Solanum aculeatissimum* toward temparature and pH was reported (Krishnan and Murugan, 2015).

Effect of amino acids chemical modifiers on the activity of inhibitor from *C.procera* attest that, modification of amino acids, tryptophan, enhanced the inhibitory activity. In

contrast, cysteine and Histidine modification led to drastic loss of activity. In addition, modification of lysine from *M.oleifera* inhibitor reduced its activity. The loss of activity after modification of some amino acids might be due to the alteration in the structural conformation of the inhibitor molecule by the modifying agents.

It can, therefore, be inferred from the results that the *C.procera* contained Cysteine and Histidine, while *M.oleifera* inhibtor contained lysine that are responsible in binding to the enzyme. Modification of protease inhibitor from Solanum *aculeatissimum* revealed involvement of lysine and tryptophan as the amino acids responsible for binding to the enzyme (Krishnan and Murugan, 2015). Knowledge of the reactive molecule(s) and the functional group(s) involved for a particular biological activity is a valuable tool for designing novel protease inhibitors as drugs for blocking specific protease actions and thus make protease inhibitors as a target for the pharmaceutical industry.

Modifications allow us to understand the chemical group involved in specific protein-protein interactions. In chemical modification, a reagent binds covalently to specific amino acid side chains of a protein and may produce changes in the activity of a protein (Krishnan and Murugan, 2015; Shamsi *et al*., 2016).

Median lethal dose (LD_{50}) of 490mg/kg.bw and 690 mg/kg.bw intraperitoneally for extract of *C. procera* latex and *M. oleifera* seeds is classified as moderately toxic according to Corbett *et al*. (2013). Lorke, (1983) also roughly classifies substances according to their LD_{50} as follows: very toxic (LD_{50} < 1.0mg/kg), toxic (LD_{50} up to 10mg/kg), less toxic (LD_{50} up to 100mg/kg), only slightly toxic (up to 1000mg/kg) and substances with LD_{50} values greater than 5,000mg/kg are practically non-toxic. Median lethal dose (LD_{50}) values greater than 5,000mg/kg.bw of aqueos extract of *C. procera* leaves (Mudi and Bukar, 2011) and *M. oleifera* (Fahey, 2005) have been reported.

In the subchronic toxicity experiment, crude and purified extracts from *C. procera* latex and *M. oleifera* seeds did not produce any significant changes ($P<0.050$) in packed cell volume (PCV) of the treated animals in all the doses used when compared to the control group. This suggests that the extracts may have no toxicological effects on the red blood cell forming tissues.

The Liver is the major organ involved in drug biotransformation. Levels of serum liver biomarker enzymes are assayed to evaluate any toxic effects on the liver (Mukinda and Syce, 2007). Increases in the level glucose observed for both plant extract inhibitors may be attributed to the roles of host proteases (calpains) played in the secretion of insulin (Sreenan *et al*., 2001). The inhibitors may be inhibiting the activity of calpain thus prevent it from playing its roles of proteolytic processing for secretion of insulin. There were no significance ($P<0.05$) alteration of the activities of other serum biochemical parameters of treated mice from both extracts of the plant measured as compared to control group.

The partially purified cysteine protease from *Plasmodium berghei* extract gave a percentage yield of protease activity of 35.1% and purification fold of 3.67, using phenyl sepharose column. One-step purification of the enzyme on passage through phenyl sepharose column chromatography could suggest that the enzyme contain high quantities of hydrophobic amino acids in its side chain. Amlabu *et al*. (2011_a) had earlier reported partially purified *Plasmodium berghei* cysteine protease using ion-exchange chromatography with molecular weight ranges 14-40kDa.

The low IC_{50} values (23.10±1.2µg/ml for *C.procera* latex and 17.2±0.3 µg/ml for *M.oleifera* observed toward *Plasmodium berghei* cysteine protease indicated that the extracts is binding to the enzyme at very low concentration. Kinetic analysis of purified inhibitor from both plant extracts on the enzyme revealed a mixed type of inhibition, indicating that the

inhibitors can bind to the enzyme and to enzyme substrate complex. The kinetic binding constants (K_i) are 8.0µg/ml and 20.0 µg/ml for *C.procera* latex and *M.oleifera* seeds respectively. A serine protease inhibitor isolated from Marine *Actinobacteria* demonstrated a competitive type of inhibition with kinetic binding constant (K_i) of 18.4 µg/ml toward *Plasmodium berghei* protease (Karthik et *al.*, 2014).

In the *in-vivo* antimalarial studies, animals treated intraperitoneally with crude and purified cysteine protease inhibitors from both plants extracts markedly lowered the parasitaemia, as evident from the 4-days chemosuppressions test. The parasite suppressive activities of both plant extracts were enhanced by further purification.

However, increases in the parasitaemia level observed for animals treated from crude extracts of *C.procera* latex and *M.oleifera* seeds after 8 days post-treatment compared to groups treated with standard drug, could be as a result of the fact that the extract at the dose administered had not accumulated sufficiently to bring about considerable chemosuppression. This could also be as result of extracts delay in access to infected erythrocytes and finally to the parasite's food vacuole where degradation of haemoglobin will be inhibited to prevent amino acid for parasite protein synthesis and growth. Interestingly, despite the rising parasitaemia of the extract-treated mice, these animals survived longer than placebo control group. The higher dose from crude extracts of both plants clearly delayed progression of parasitemia, as treated groups lived longer than the untreated groups. The long survival of groups of animals receiving treatment despite high parasitemias compared to untreated group at point of death, signifies that the extracts might be reducing pathological effects on the animals, which causes the early death seen in the *P. berghei* infections. Another reason that might explain this slower growth of the parasite in

the presence of the extracts inhibitor is that the extracts may be modulating the immune response of the host and thereby alters the outcome of the infection by preventing the cascade of events that leads to early death seen with *P. berghei* infection (Feng *et al*., 2012).

Furthermore, it is also possible that initial suppression of the parasite by extracts and post-treatment rise may be due to the fact that crude extract is inhibiting cysteine protease secreted by the mature parasite (trophozoite, schizont and merozoite), but had no effect on the ring stages (younger parasites), leaving them to replicate and grow. The ring stage is known to produce little or none cysteine protease enzyme and might be escaping the effect of inhibitor extracts.

The extract may also be inhibiting proteolytic processing of circumsporozoite protein by a parasite cysteine protease, thereby preventing sporozoite invasion of host cells (Adebayo and Krettli, 2011). The findings indicate the preventive potential of the extract, since cysteine protease activity is involved in the invasion of RBC and degradation of immunoglobulins of the host.

Several *in vitro* and *in vivo* studies using cysteine protease inhibitors such as peptidyl fluoromethyl ketones, vinyl sulfones, aldehydes and non-peptidic inhibitors impedes cysteine proteases activity of *Plasmodium* parasites and show the curative effect in mice infected with *Plasmodium berghei* parasite (Rosenthal, 2011; Mane *et al.,* 2013).

The purified extracts from both plants at the dosage tested, significantly decreased percentage parasitaemia in a dose dependent manner, most active at the higher doses and thougth not significantly compared with the stardard control. Partialy purified protein inhibitors retain their antimalarial activity with the high percent suppression compared to crude extract but could not cure the animals of infection. This result is in contrast with that of Rosenthal *et al.,* (1993) who reported 4 days doses of synthetic CPI MU-Phe-Hphe-

CH_2F) cured 80% of infected mice , indicating that CPI from both plant are not as potent as synthetic inhibitor probably due to their protein nature which may be digested by the host enzyme (Feng *et al*., 2012). Soares *et al*. (2015) also reported cysteine protease inhibitor from *jatropha curcas* seeds with potent inhibitory activity against *trypanosoma gondii*. Similarly, Karthik *et al.* (2014) demonstrated a serine protease inhibitor isolated from Marine Actinobacteria that cured *Plasmodium berghei* infected mice.

Cysteine protease enzyme is essential at all stages of the parasite's life cycle. Inhibitors of plasmodium cysteine protease display considerable antiplsmodial activity and some have been shown to cure infection in mouse models. Since resistance limits currently available therapeutics, efforts have been made with new therapeutic targets, among which the proteolytic apparatus of *plasmodium* parasites are promising (Rosenthal, 2011).

Survival time of mice treated with the extracts of these plants was longer compared to the respective negative control group, confirming that the crude extracts from both plant extracts suppressed *P. berghei* and probably reduced the overall pathogenic effect (damages to orgarns) of the parasite on the infected mice.

Malaria is characterized by haematological changes, which extremely influence the pathogenesis of the disease. The disease is linked with decline in the haematocrit or packed cell volume (PCV) in the infected hosts, confirming that anaemia is a critical feature in the pathogenesis. The PCV was measured to evaluate the effectiveness of the crude extract and partially purified extracts in preventing hemolysis due to the rising parasitemia level.

The underlying cause of anemia includes the following mechanisms: the clearance and or destruction of infected RBC. The clearance of uninfected RBC by erythrophagocytosis of microphages. The erythropoietic suppression and dyserythropoiesis, which is associated with cytokines that initiates the diversion of iron necessary for the synthesis of hemoglobin

to the storage compartments of macrophages in the reticuloendothelial system resulting in limited erythropoiesis. Thus, less iron is made available for heme synthesis. Each of these mechanisms has been implicated in human and in mouse malarial anemia. The increase in PCV of infected treated mice with the extracts when compared with infected untreated is an indication of the effect of the extracts in preventing anaemic condition as well as reduction in severity of *plasmodium berghei* infection. The extracts may also have some stimulatory effect on the production of red blood cells (erythropoiesis).The erythropoietic stimulating potentials of *M.oleifera* parts is documented by Fahey (2005).

Importantly, at the doses used to inhibit *Plasmodium*, it was observed that no toxic effects of extracts were manifested in the host, because only one mouse in the groups treated with extracts died with exception of two mice in the case of the animals treated with crude extracts from *M.oleifera* seeds. The remaining mice had activity levels similar to those of the normal untreated controls (uninfected control). Although inhibitor extracts may likely reacts with host cysteine proteases, there are several reasons why host cells may be more resistant to the effects of protease inhibitors than single-celled protozoan parasites. Complex animals have a redundancy in protease function that does not exist in protozoan parasites, which are of necessity in more genetically streamlined organisms (Rosenthal, 2011). In addition, parasite proteases may be more accessible to inhibitors than host enzymes, which are found within intracellular compartments.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

6.1 Conclusion

Cysteine protease inhibitors were isolated, purified and characterized from *C.procera* latex and *M.oleifera* seeds, with in *vitro* inhibitory activity. Partially purified CPI from *C.procera* latex and *M.oleifera* seeds showed Temperature and pH characteristics similar to other plant CPI reported. It has also been shown that crude and partially purified extract from both plants has *in-vitro* inhibitory effect on Papain (standard CPI) as well as partially purified Cysteine protease of *Plasmodium berghei*.

Crude and Partially purified extracts from both plants kept the parasitaemia in *P. berghei* infected animals significantly lower than treated mice. The survival period of animals infected with P.berghei but treated with crude and partially purified extracts from both plants was significantly longer compared to untreated mice. The diseases induced anemia caused by the parasite to infected animals was ameliorated with treatment by both plant extracts.

6.2 Recommendation

Inhibitory studies of partially purified extracts from both plants on Human malaria parasite Cysteine protease enzymes and on *in-vitro*, culture parasite is suggested.

Further work on purification, sequencing and molecular ducking is recommended. Synthesis of small peptide molecules containing some of the amino acid implicated in binding to the enzyme. Studies on combination therapy of these inhibitors, especially with other currently used antimalarial agents will be welcome.

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Appendix

Appendix A 1 : Calculation of Inhibtory Protease activity

Protease activity (µmolpNA released) = $A_{410nm} \times d/Ɛ \times t \times v$

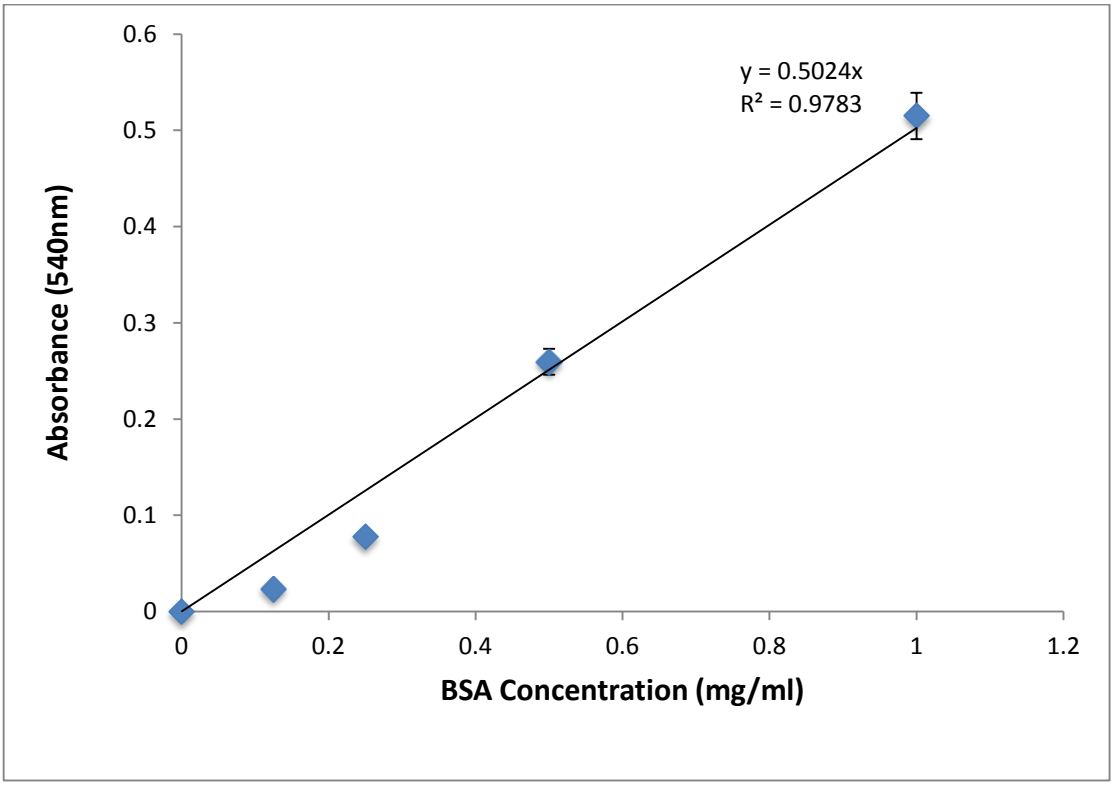
Ɛ=Extintion coefficient of p-nitroaniline (8800 /cm/M), V-vol of sample, d-dilution factor, t-rxn time in min.

Protease Inhibitory activity = $(\mathbf{A_{410nm}c} - \mathbf{A_{410nm}\ t}) \times d / Ɛ \times t \times v$

% Inhibition = $(\mathbf{A_{410nm}c} - \mathbf{A_{410nm}\ t})/\mathbf{A_{410nm}\ c} \times 100$

Where $A_{410nm}c$ and A_{410nm} t are Absorbance at 410nm of the control and the test sample, respectively.

Appendix A 2: Protein Standard curve



Appendix A 3: Median Lethal Dose (LD_{50}) of Cold Acetone Precipitated *Calotropis procera* Latex and *Moringa oleifera* Seeds Extracts

			Death	
Plant sample	Experiment	Dose(mg/kg)	After24hrs	LD_{50}(mg/kg)
Calotropis procera (Latex)	Phase1	10	0/3	490.00
		100	0/3	
		1000	3/3	
	Phase2	200	0/3	
		400	0/3	
		600	2/3	
		800	3/3	
Moringa.oleifera(seeds)	Phase1	10	0/3	690.00
		100	0/3	
		1000	3/3	
	Phase2	200	0/3	
		400	0/3	
		600	0/3	
		800	2/3	

The numerator denotes number of animal which died while the denominator is the number of animals used

LD_{50} was calculated as geometric mean. =√ maximum tolerated dose × minimum toxic dose

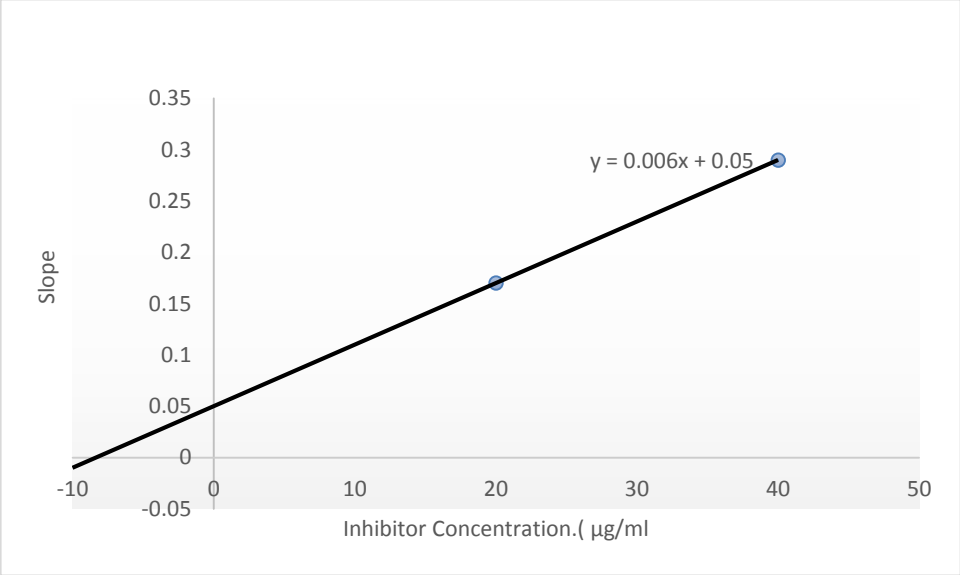
LD_{50} *for Calotropis procera* $=\sqrt{400\times600} = 490$*mg/kg body weigth*

LD_{50} *for Moringa oleifera* $=\sqrt{600\times800} = 690$ *mg/kg body weigth*

Appendix A 4: Ammonium Sulphate Srecipitation of Crude CPI Extracts

Plant	% Ammonium Sulphate $(NH_4)_2SO_4$	Protein Content(mg/ml	Papain Inhibition (%)	Specific inhibitory activity (%activity/mg)
Calotropis procera latex	20	1.45	0.00	0.00
	30	1.15	45.00	39.13
	40	1.30	50.00	38.46
	50	0.90	45.00	50.00
	60	1.20	55.00	45.83
	70	1.60	05.00	3.13
	80	0.80	02.00	2.50
Moringa oleifera seeds	20	1.20	37.00	30.83
	30	1.50	75.00	50.00
	40	1.30	35.00	26.90
	50	0.98	55.00	56.12
	60	1.68	60.00	35.71
	70	1.10	55.00	50.00
	80	0.85	46.00	54.11

Appendix A 5: Secondary plot of *C.procera* inhibitor for the determination of Ki



Appendix A 6: Secondary plot of *M.oleifera* inhibitor for the determination of Ki

