ANTIMALARIAL, IMMUNOMODULATORY AND CONTRACTILE ACTIVITIES OF ALSTONIA BOONEI (APOCYNACEAE)

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ABBREVIATIONS

ABSB	*Alstonia boonei* stem bark
AB-1	Antimalarial active constituent of ABSB
AB-2	Anticomplementary active constituent of ABSB
AP	Alternative pathway
	Alsever's Solution
	Citrate buffered glucose
Aq	Aqueous (water)
CP	Classical pathway
Cq	Chloroquine
DE	Diethylether
EtOH	Ethanol
$EtOA_c$	Ethylacetate
$EtOH_h$	Hydrophylic fraction of EtOH extract
$EtOH_L$	Lypophylic fraction of EtOH extract
HPS	Human pooled Serum
M_1	Methanol insoluble subfraction of $EtOH_h$ extract
M_s	Methanol soluble subfraction of $EtOH_h$ extract
MSP	Mean survival period
PSB	Phosphate buffered saline
PE	Petroleum ether

RaE	Rabbit erythrocytes
ShE	Sheep erythrocytes
ShEA	Sensitized ShE . That is , ShE treated with anti - ShE monoclonal antibody solution.
STZ	Serum treated Zymosan
VSB	Veronal Sodium barbital

ABSTRACT

The major ethnomedical uses of *Alstonia Boonei* (AB) by indigenes of Tropical West African countries are in the treatment of malaria and rheumatoid arthritis. In the pathophysiology of rheumatoid arthritis, the complement and polymorphonuclear neutrophils (PMNs) have been implicated. The main purpose of this study was to isolate the antimalarial and immunomodulatory constituents of the plant using a bioassay guided fractionation technique.

Samples of the stem bark of the plant (Collection number: Lowe 2323; Herbarium number: U.I. H. 13134) were sun-dried, coarsely ground and subjected to soxhlet extraction successively with petroleum ether (PE), diethyl ether (DE), ethylacetate (EtOAc) and ethanol (EtOH). Subsequently, the marc was refluxed in water (AQ). Extracts were dried by evaporation under reduced pressure (PE, DE, EtOAc, and EtOH) or by freeze drying (AQ).

The crude extracts and / the pure anti-malarial compound isolated from the most active extract were assessed against *Plasmodium yoelii nigeriensis (P.y. nigeriensis)* and *P. berghei* ANKA infections in mice and rats, respectively. Activities of the extracts or

isolated compound were investigated in early infection (4-day test) and established infection (Rane's test). Their repository activities were also investigated. Chloroquine and pyrimethamine served as reference anti-malarial drugs. The effects of the crude extracts were also investigated on the immune system *in vitro* using complement system end polymorphonuclear (PMN) neutrophils as immunological parameters. Anticomplementary (classical (CP) and alternative (AP) pathway) activities were assessed using human serum end antibody-sensitized sheep erythrocytes for CP and rabbit erythrocytes for AP. Effects of extracts on phagocytic activities of PMNs were assessed by using PMNs isolated from venous blood of healthy volunteers. Apocynin was used as the reference compound.

In *P.y. nigeriensis* infections, the order of anti-malarial activity of the extracts was DE<EtOAc < AQ < EtOH. The doses of the extract screened ranged from 100 to 800 mg / kg and the mean suppression of parasitaemia in early infections end repository test ranged from 1.23 to 61.4% and 3.3 to 56.7%, respectively. The chemosuppressive effect of chloroquine (5mg/kg) and pyrimethamine (1.5mg/kg) were 85.9% and 87.7% respectively. None of the extracts showed strong activity in established infection. However, mice treated with the highest dose of EtOH extract had prolonged survival over the controls. Activity guided fractionation of EtOH extract using different chromatographic techniques yielded a pure alkaloidal constituent called AB-1, melting

point 208 - 210°C. Doses of 10 - 80 mg/kg of AB-1 produced chemosuppressive effect of 41.3 to 81.9% in early infection and 52.0 to 86.5% in repository test. In established infection, chloroquine (5 mg/kg) and AB-1 (80 mg/kg) produced chemosuppressive effects of 98.0 and 88.5%, respectively on day 8 post infection. The mean survival periods in the control, AB-1-treated and chloroquine-treated mice were 7.4 ± 0.77, 25.7 ± 1.23 and over 30 days, respectively. All doses of AB-1 produced higher chemosuppressive effects in *P. yoelii Inigeriensis* infection than in *P. berghei* ANKA infection.

The order of anticomplementary activity of the extracts was DE = EtOAc > EtOH > AQ. The anticomplementary activity of DE and EtOAc extracts was mediated through the classical pathways. None of the extracts inhibited AP-mediated haemolysis and chemiluminescence, generated by stimulated PMNs. A triterpenoid, called AB-2, isolated from EtOAc extract, showed very strong anticomplementary activity. The concentration producing 50% inhibition of CP-mediated haemolysis was 1.4 mg/ml. The anticomplementary activity increased with increase in temperature and time of preincubation. AB-2 significantly inhibited zymosan-induced mouse footpad swelling.

AQ extracts contracted both guinea pig ileum and rat stomach strip. The contraction was antagonised by methysergide in a competitive manner.

The efficacy of the anti-malarial and anti-inflammatory constituents isolated from *Alstonia boonei* lend strong support for the ethnomedical use of the plant extracts. This is the first report on the anti-complementary activity of *Alstonia boonei* extracts.

PUBLISHED/ACCEPTED PAPERS AND COMMUNICATIONS

RELATING TO THE THESIS

1 J.M. Makinde and O.B. Taiwo (1996). Contractile activity of *Alstonia boonei* stem bark extract on isolated Rat stomach strip and Guinea Pig ileum. *Indian Journal of Pharmacol.* 28, 110 - 112.

2 Taiwo, O.B. Van den Berg, A.J.J., Kroes, B. H., Beukelman, C.J., Horsten, S.F.A.J., Quarles van Ufford, H.C., Van den Worm, E., Makinde, J.M., and Labadie, R.P. (1998). Activity of the stem bark extract of Alstonia boonei De Wild (Apocynaceae) on human complement and polymorphonuclear leukocytes. Indian Journal of Pharmacol. (in press).

DEDICATION

TO:

My mother, brother and sisters

and

My precious and priceless wife, Dr. Omobole Olawumi Taiwo.

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I am very pleased to acknowledge my indebtedness to Dr. Modupe Makinde for supervising this work and for being kind enough to spend part of the WHO grant she received on some aspects of the work. I also thank her for giving me the opportunity to get a University job and for the cordial relationship that developed.

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My wife, Dr. Omobola Olawumi Taiwo, has contributed in no small way to the preparation of this thesis, she single-handedly typed the manuscript. Darling, your

unfailing love and support have been the source of my strength. You always provide the confidence and the atmosphere where peace reigns. Your beauty, inside and outside, has been my pride. Yes, my pride. May the Lord bless you, again and again.

Son, you may not understand what Daddy went through. If there was any time I did not give you total attention, as a result of this program, I ask for your pardon. You have been a peaceful child and mummy and daddy thank God for your live

Finally, I thank the Almighty God for his sustainance through out the entire programme.

CERTIFICATION

This is to certify that this is a record of original research work carried out by Mr. Taiwo, Oludare Bolaninwa of the Department of Pharmacology and Therapeutics, College of Medicine, Unviersity of Ibadan, Nigeria.

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CHAPTER ONE

INTRODUCTION

Plants are a biologically and chemically diverse resource with estimates of 250,000 - 500,000 species occurring on our planet (Cox and Balick, 1994; Borris, 1996). To date, less than 10% of the total plant kingdom have been examined scientifically for their potential in medicine (The Lancet, 1994).

The use of medicinal plants, a major component of Indigenous/traditional medicine, for treating diseases can be traced back to written documents of the early civilizations in China and India as far back as 168 BC (Hamburger and Hostettman, 1991; The Lancet-editorial-1994; Holland, 1994). Although modern medicine has lessened the dependence on medicinal plants as the sole source of drugs, nonetheless, the use of plants is still the almost exclusive source of drugs for over 80% of the world's population which utilise plant-based medicines for primary health care (Hamburger and Hostettmann, 1991; Phillipson and Wright, 1991; Phillipson 1994; Borris, 1996). The greater proportion of the world population that still use extracts from plants to treat wide ranging diseases can be found in developing countries (Cox and Balick, 1994). In industrialised countries, substances derived from higher plants constitute about 25% of

prescribed medicines (Farnsworth and Bingel, 1977; Principe, 1989; The Lancet editorial-1994). Furthermore, the use of plants in the form of phytomedicines is increasingly becoming widespread in some developed countries of Europe like Germany, France, The Netherlands, and Sweden (Bohlin, 1993; King, et al., 1996).

Interest in natural products research particularly that based on medicinal plant has been a cyclical phenomenon over the years. An early scientific approach towards the treasure of traditional medicine in Europe was documented in the late 18th century. In 1785, the British physician William Withering reported that ingestion of dried leaves of the Foxglove (*Digitalis purpurea*) eased dropsy, nowadays known to be caused by heart failure (Cox and Balick, 1994). His original source of information was an old woman known for her great herbal knowledge. Digitalis including its active glycosides, and the pure compounds digoxin and digitoxin isolated from tha plant were later made available for many cardiac patients. This event represents one way by which the medical potential of the plant kingdom together with its diversity of chemical resources can be used for the benefit of mankind. Following the isolation of digitalis compounds, many important substances like morphine, quinine, tubocurarine, pilocarpine, codeine, vinblastine, vincristine (all of which have been known for years) have been

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discovered through scientif.c investigation of folklore claims. Some of them, for example, digitalis cardiac glycosides, are still the drug of choice in their respective areas of therapy (The Lancet, 1994; Cox and Balick, 1994).

Around the 1950s, interest in medicinal plant consumption and research waned after storming advances in synthetic chemistry and molecular biology promised to supply new means for designing drugs in the laboratory. Consequently most pharmaceutical firms abandoned the search for therapeutic compounds in higher plants (Cox and Balick, 1994; Borris, 1996; Turner, 1996). However, since 1970 there has been a resurgence of interest in medicinal plant research and plant-derived products. Ecological awareness and an increased demand for non-classical therapies have been advanced as reasons for the revival of interest (Hamburger and Hostettmann, 1991; Turner, 1996, Borris, 1996). Another major reason is the undisputed clinical efficacy of several plant derived drugs. An exampte is the use of artemisinine, derived from a Chinese medicinal plant, Artemisia annua L(Qinghao) in severe malaria for which chloroquine may not be helpful (UNDP/World Bank/WHO, 1991). Other examples of clinically important plant-derived drugs are taxids (from *Taxus*

The awareness that compounds with less direct therapeutic potential may offer new molecular templates for the design of more effective drugs has also contributed to the renaissance of interest in medicinal plant research (King *et al.*, 1996; Turner, 1996; Borris, 1996).

Bearing in mind the renewed interest in medicinal plant research, it has been reported that the alarmingly rapid rate of species extinction which is currently occurring, due in part to habitat destruction calls for certain urgency to the quest for plant-derived drugs (King *et al.*, 1996, Boris, 1996, Turner, 1996). Moreover since the system of indigenous medicine are based on a vast amount of empincal knowledge about the treatment of diseases, it is recognised that the gradual "extinction" of the traditional medicine practitioners, together with their wealth of knowledge about folk medicine would mean a big loss of valuable and vital ethnomedical information.

The studies carried out in this thesis are in recognition of the resurgence of interest in medicinal plant research. It is beliaved that a concerted effort using the approach of ethnobotany/ethnopharmacognosy and elhnopharmacology may lead to the discovery of yet more novel compounds which may be used directly as therapeutic agents or offer medicinal chemists leads in the design of more

effective therapeutic agents. This prompted investigation of some of the ethnomedical uses of *Alstonia boonei* de wild (Apocynaceae)in this study.

A. boonei de wild (Apocynaceae), the medicinal plant investigated in this thesis, is used in indigenous medicine in the continents of Africa and Asia to treat a number of diseases including malaria, fever, rheumatoid arthritis, dysentery, insomnia, abdominal discomfort, hypertension, and painful micturition (Burkhil, 1935; Perry and Metzger, 1980; Ojewole, 1984; Asuzu and Anaga, 1991). The plant extract has also been given after child birth to help the delivery of the placenta (Dalziel, 1937; Irvine, 1961). The plant is widely grown in West Africa. In Nigeria, it is mainly used in traditional medicine to treat malaria, faver and rheumatic pains (Ojewole, 1984). In Ghana, it is used primarily to treat rheumatoid arthritis (Kweifio-Okai, 1991(a); 1991(b)

The use of the plant to treat malaria by far outweighs other uses and it is not surprising that scientific investigation of the ethnopharmacology of the plant have focussed more on the antimalarial properly than on other biological effects. However the many studies that have so far been carried out have failed to identify a promising antimalarial compound which can either be used directly as a therapeutic agent or as a template for the synthesis of a more effective compound. Furthermore there exists considerable controversies on the efficacy

of extracts of *Alstonia* species or their constituents in malaria (Wright, et al, 1993). Studies *in vitro* using several compounds isolated from the plant have not proven it to be a promising ethnomedicine from which an effective antimalarial drug can be developed. However, the results lend some support to the use of *Alstonia* species in traditional medicine (Phillipson *et al*., 1993). It must be pointed out however that many of the chemical constituents from the plant have not been isolated following activity-guided isolation procedure. Therefore it is probable that the most active antimalarial constituent(s) of the plant has not been isolated (might have been missed). Furthermore, it has been reported that nothing appears to be known about the *in vivo* activities of *Alstonia* species (Phillipson *et al*., 1993). This underscores a need for *in vivo* studies. This is more pertinent if it is assumed that pro-drugs are the active constituents of *Alstonia* species.

In this thesis, two ethnomedical uses of the plant, antimalarial and immunomodulatory (antiinflammatory) were investigated. The choice of these diseases is based on the prevalent use of the plant extract in the two conditions in Africa. Antimalarial activity was investigated in vivo using drug-sensitive and resistant-rodent malaria parasites. Antiinflammatory/immunomodulatory activity was investigated in vivo using zymosan-induced footpad inflammatory model in

resistant-rodent malaria parasites. Antiinflammatory/immunomodulatory activity was investigated in vivo using zymosan-induced footpad inflammatory model in mice, and in vitro on complement system and polymorphonuclear (PMN) leukocytes. The choice of the two immunological parameters is based on their close association with acute inflammatory responses and the implication of complement in the pathogenesis of a variety of rheumatic disorders (Benencia et al, 1994; Morgan 1990; Parnham et al, 1984).

OBJECTIVES OF THE STUDY

The experiments described in this thesis were designed to achieve the following objectives.

1. Investigate the efficacy of *Alstonia boonei* stem bark extracts in rodent malaria using *Plasmodium yoelii nigeriensis* and *Plasmodium berghei* (ANKA).
2. Investigate the modulatory effects of the plant extracts on human complement and neutrophil oxidative burst.

3. Activity-guided isolation of the antimalarial and immunomodulatory (antiinflammatory) compounds in the plant extract.

4. Investigate the efficacy of the isolated compounds in experimentally induced rodent malaria in mice, and experimental rheumatoid arthritis in rat.

5. Investigate the influence of the traditional preparation process on the antimalarial activity of the plant extract. The rationale for this investigation is that most drugs derived from medicinal plants are too expensive for populations which use them (Waller, 1993) as is the case with artheether which is 10 times more expensive than chloroquine. If 80% of the population in developing countries are directly dependent on herbal drugs (Phillipson & Wright, 1991; The Lancet, 1994) and these populations cannot afford the cost of synthetic drugs (especially those derived from herbal drugs) then we feel that the efficacy of the medicinal plant extract and the traditional preparation methods should be investigated with a view to standardizing and optimizing the methods. In this way the poor populations that use the ethnomedicine can derive utmost benefit from it.

CHAPTER TWO

LITERATURE REVIEW

MALARIA

2.1 Introduction

Malaria is an infectious disease caused by parasitic protozoa of the genus *Plasmodium*. The disease is a major cause of high rates of morbidity and mortality in many tropical and sub-tropical regions of the world (WHO, 1989). The discovery in 1897 by Ronald Ross that mosquitoes transmit malaria not only contributed to the understanding of the life cycle of *Plasmodium* but perhaps prematurely motivated people to think that malaria could be easily eradicated. Since that time, eradication of the parasite and mosquito vector via chemical and mechanical means has been only partially successful owing to financial constraints (i.e., confinement of the disease primarily to developing countries) and the appearance of pesticide and drug resistant strains of mosquito and parasite, respectively. Even the control of the infection has not been completely successful owing to several factors including increasing incidence of drug resistance to virtually all existing antimalarial drugs (WHO, 1990, 1991), adverse reactions to some of the available antimalarial drugs

and staggering cost of new drugs (WHO, 1984; 1990). These constraints have necessitated the search for new antimalarial drugs (WHO, 1984; 1990).

In the search for new drugs, apart from molecular modification of existing compounds, intensive screening of plants for bioactive agents has been recognized as a valid approach (Farnsworth and Morris, 1976, Warburton, 1984, Waller, 1993, Bohlin, 1993, The Lancet, 1994, Cox and Balick, 1994).

In this thesis, antimalarial activities of *Alstonia boonei* (Apocyneceae) were investigated using rodent malaria induced by *Plasmodium yoelii nigeriensis* (*P. y. nigeriensis*) and *Plasmodium berghei* (Anka). *P. y. nigeriensis* or *P. berghei* infections in mice, if untreated, are normally always lethal, paralleling to some extent infections with *Plasmodium falciparum* in humans.

The use of an animal model offers a method of administering the extracts or obtaining information that might otherwise be unobtainable in humans for ethical or technical reasons. In addition, rodent plasmodia have played an important role in antimalarial drug development against human plasmodia as they are economical and convenient to handle (Bruce-Chwatt, 1966; Peters, 1982). The discovery and development of some blood schizontocides like chloroquine, primaquine and proguanil were largely based on their activity against *P. Gallinaceum* (Peters and

Howells, 1978). The above advantages nonetheless, caution should be exercised in interpreting the results obtained from the animal model, which may not necessarily apply to *P. falciparum* infections in humans.

2.2 Malaria parasites

Many species of malaria parasites are known. Those that are utilized in research either because of their similarities in life cycle or response to drugs cut across different families including *Eimeridae, Toxoplasmidae, Plasmodiidae, Haemoprofeidae* and *leucocytozidae*.

The only species which occur in man are those belonging to the genus plasmodium (Plasmodiidae). The human parasites and the types of malaria infections associated with them are as follows:

Plasmodium falciparum	Malignant tertian malaria
Plasmodium vivax	Benign tertian malaria
Plasmodium malariae	Quartan malaria
Plasmodium ovale	Milder form of benign tertian malaria

The terms 'tertian' and 'quartan' refer to the periodicity of the bouts of fever. In tertian malaria, fever occurs every 48 hours while in quartan malaria it occurs

every 72 hours. The term 'malignant' refers to the high incidence of severe brain involvement and other complications associated with malaria produced by *P. falciparum* which is the most pathogenic species (Sayers, 1960).

2.2.1 ***Plasmodium falciparum***: It is the most pathogenic of the human plasmodia and is noted for producing cerebral malaria, a life threatening disease common in children. Infection due to *P. falciparum* accounts for more than 90% of malaria cases in Nigerian (Ekanem, Weisfeld, Salako, Nahlem, Ezedinachi, Walker, Breman, Laoye, Hedbrergle, 1990). The parasite is essentially tropical and sub-tropical in its distribution.

2.2.2 ***Plasmodium vivax and Plasmodium ovale:*** *P. vivax* is widely distributed, occuring in tropical, sub-tropical and some parts of the temperate regions. On the other hand, *P. ovale* is essentially tropical. Both produce milder clinical attack than *P. falciparum*. They are responsible for true relapsing malaria due to the presence of the latent exoerythrocytic forms of the parasite in the liver (WHO, 1984).

2.2.3 ***Plasmodium malariae:*** Although it is widely distributed, it is more sub-tropical, and relatively rare. Relapses of the infection to *P. malariae* is attributed to the exoerythrocytic forms remaining in the body for a considerable time (WHO, 1984).

2.3 Animal Plasmodia

Animal *Plasmodia*, especially rodent malaria parasites play an important role in antimalarial drug development against human *plasmodia* (Bruce-chwatt, 1986). The reason is that they are economical and convenient to handle (Peters, 1982). The discovery and development of certain blood schizontocides like chloroquine, primaquine and proguanil were largely based on their activity against *Plasmodium gallinaceum* (Peters and Howells, 1978).

Before the advent of chloroquine-resistant parasites, rodent malaria (*Plasmodium berghei*) provided the basis for the search for new antimalarials (blood schizontocides) in drug screening programmes. Rodent malaria therefore served as a standard model based on drug-sensitive strain of rodent *Plasmodia*. After the emergence of chloroquine-resistant *Plasmodium falciparum*, another model, chloroquine-resistant screening model was developed. At present, there are a battery of drug-resistant models owing to the development of resistance to other antimalarial drugs other than chloroquine. Notwithstanding the widespread occurrence of multidrug resistant *Plasmodium falciparum*, the standard drug-sensitive rodent malaria model still seems to be a highly relevant one in relation to human malaria (Peters and Howells, 1978; Phillipson and Wright, 1991).

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Numerous species and sub-species of rodent *Plasmodia* have been isolated and divided into two main groups, namely, the *berghei* group and the *vinckei* group (Ager, 1984). The *berghei* group is widely used in chemotherapeutic studies. They include *P. berghei* (KBG-173) isolated by Vinckei and Lips (1948), *P. yoelii* 17 x (Landau and Killick-Kendrick, 1966) and *P. yoelii nigeriensis* (N67) (Killick-Kendrick, 1973). Both *P. berghei* and *P. yoelii nigeriensis* are lethal to white mice and young rats (Landau and Boulard, 1978; Killick-Kendrick, 1978; Wyler, 1982). Resistance to both species increases with the age of mice since reticulocytes are fewer in the older animals (Ott, 1968) and trophozoites of both species show a predilection for reticulocytes in the circulation.

The course of rodent malaria has been reported to be influenced by certain factors in addition to age. These include the following:

Sex: Clear sexual differences in mice infected with *Plasmodium berghei* malaria have been reported (Konopka *et al.*, 1966); the females are reported to be more susceptible to infection than the males.

Pregnancy: Pregnant mice are more susceptible to *plasmodium berghei* malaria than non-pregnant mice (Oduola, et al., 1982; Akingbade, 1986).

Diet: Absence of para-amino benzoic acid from diet can lead to unusual low parasitemia (Peters, 1973).

Concomitant infections: This can alter the course of parasite growth (Bafort, 1977; Gothe and Kreiver, 1977).

After inoculation of *Plasmodium berghei* into an uninfected mouse, degree of parasitemia rises steadily until 50% - 80% of the erythrocytes are paresitized. Untreated mouse may die within 5 days or more after infection depending on the strength of inoculum (Raether and Fink, 1979).

2.3.1 *Plasmodium berghei*

Discovered by Vinckei and Lips (1948), *P. berghei* has been found to be an excellent model for primary screening of drugs for eventual use against human plasmodia (Peters, 1975; WHO, 1973). About 200 different strains of *P. berghei* have been isolated. These parasites can be transmitted easily by injection of blood

containing it into laboratory animals such as mice (Bruce-Chwatt, 1980). Maturation of exo-erythrocytic stages of *P. berghei* takes about 50 hours after sporozoite inoculation and can be found in the parenchyma cells of the Liver. The asexual cycle in the blood, from trophozoite to schizonts, takes about 24 hours.

P. berghei has been widely used for experimental work on the parasitology, immunology and chemotherapy of malaria. This rodent *Plasmodium* has been extensively used in the United States in the development of potent antimalarial drugs (Peters, 1965; 1975). This has been possible because the parasite is readily transmitted to mice by syringe - passage. In addition, the intraperitoneal injection in mice has been found to be as good as the intravenous one (Thurston, 1950); penetration of the peritoneal wall into the blood stream occurs within 60 seconds (Black, 1952).

2.3.2 *Plasmodium yoelii (P. yoelii)*

P. yoelii 17 x and *P. yoelii nigeriensis* are subspecies of *P. yoelii* (Killick - Kendrick, 1978). They were formerly thought to be strains of *P. berghei*. *P. yoelii*

nigeriensis has gained prominence as a useful model for primary screening of drugs for eventual use against human plasmodia.

2.4 The Life Cycle of *Plasmodium* Species The life cycle of *Plasmodium* can be divided into four phases, occurring in the host and the mosquito (genus Anopheles). Figure 2.1 shows the division into one sexual and three asexual phases.

Phase 1: Fertilization (sexual stage in the mosquito)

A female Anopheles sucks a blood meal containing both sexual and asexual forms of the malaria parasite. The asexual forms are destroyed in the mosquitos stomach. However, the sexual forms (macro[female] and micro[male] gametocytes) are resistant to the digestive juices of the mosquito and are freed from the erythrocytes to become free macro- and micro-gametes. The microgametocyte produces flagellated microgametes. If contact with a macrogamete occurs, then fertilization results in the production of a zygote. The zygote develops into an invasive ookinete, which plants itself in the stomach wall, migrates through the wall, then differentiates into an oocyst on the outside of the stomach wall.

Phase 2: Sporogony (Asexual Development in the Mosquito)

This phase occurs entirely in the mosquito. The oocyst develops into thousands of (invasive) sporozoites which rupture through the mature cyst and migrate through the body of the mosquito to invade her salivary glands.

Phase 3: Hepatic schizogony (Asexual Development in the Liver)

The subsequent feeding of the mosquito results in the release of sporozoites into the blood stream where they remain for 30 - 60 minutes (Gamham, 1984). Many are phagocylosed, but a varying proportion invade liver cells and become hepatic trophozoites. Exo-erylhrocytic schizogony in the liver produces thousands of invasive merozoites which are then released into the blood. A latent tissue forms (hypnozoites) may persist in the hepatic cells (in *P. vivax* and *P. ovale* infections) causing relapses of malaria with clinical symptoms (WHO, 1986).

Phase 4: Erythrocytic Schizogony (Asexual Development in the Blood)

The exoerythrocytic merozoites (released from the schizonts) which invade the erythrocytes are first visible as tiny rings and then trophozoites which feed on the haemoglobin (protein portion) of the erythrocytes which is ingested by phagocytosis.

MOSQUITO VECTOR

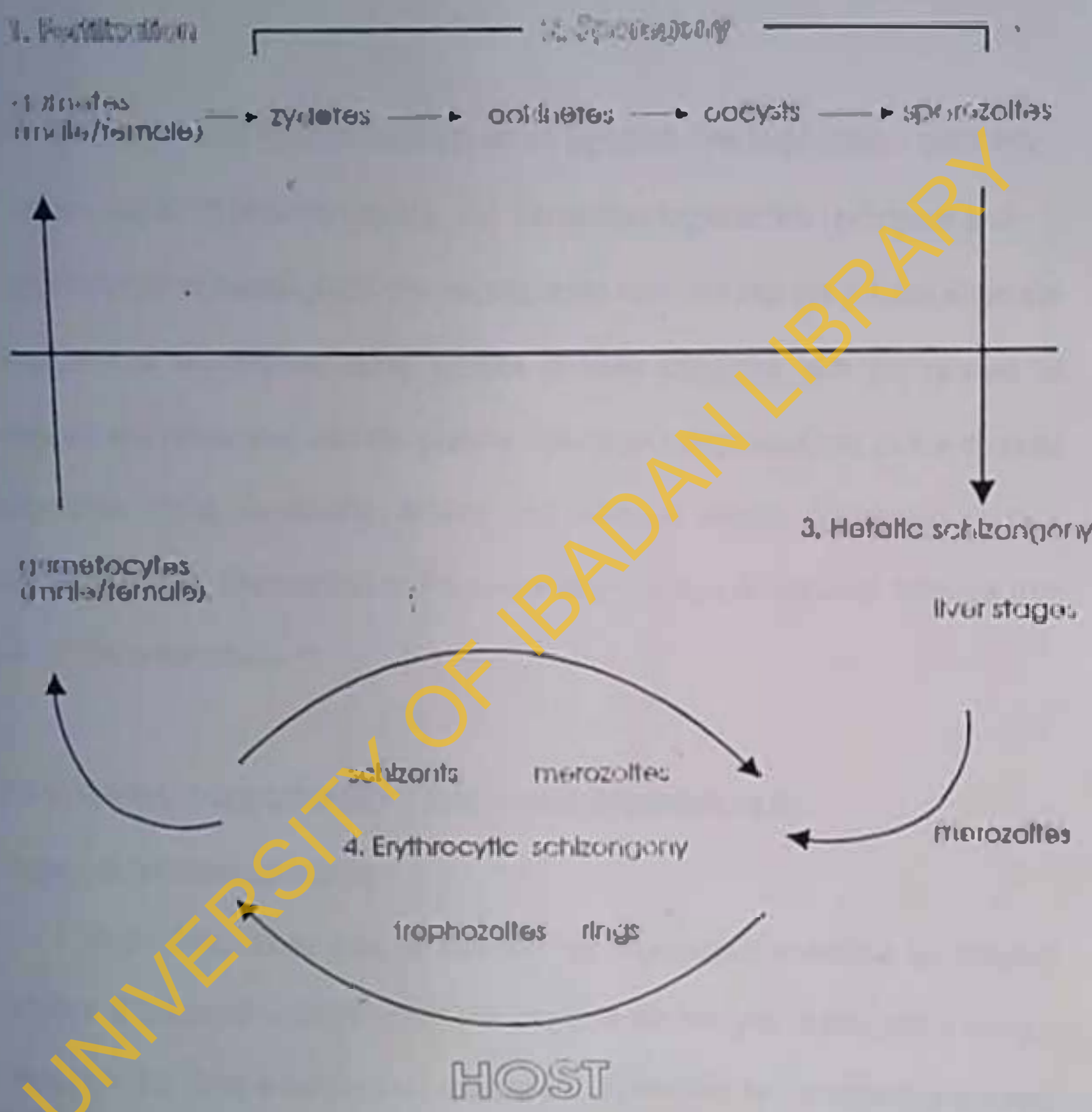

Fig 2. 1: The Life Cycle of *Plasmodium*

The haem portion of the haemoglobin is not digested. The trophozoites grow and divide into 8- 30 new merozoites. The merozoites together with pyrogens and undigested part of haemoglobin are subsequently released into the plasma when the disorganized erythrocytes burst. Attacks of fever coincides with the release of pyrogens and merozoites into the plasma. The cycle is repeated and as the disease progresses, some merozoites develop into male or female gametocytes which circulate, but only develop further if they are taken up by a mosquito to continua that part of the cycle (phase 1).

2.5 MALARIA CHEMOTHERAPY AND CHEMOPROPHYLAXIS:

Types of antimalarial activity

Antimalarial drugs may be classified by their selective actions on different phases of the parasisto's life cycle. The stages of the life cycle in men which may be susceptible to drug action include sporozoites, developing tissue schizonts or latent hypnozoites in the liver, blood schizonts, and gametocytes.

2.5.1 Blood Schizontocides

These are suppressive agents which cause interruption of erythrocytic

schizogony of malaria parasites and subsequently terminate the clinical attack. Blood schizontocides include chloroquine, quinine, halofantrine, artemisinin. Chloroquine is a highly effective blood schizontocide and is the most widely prescribed antimalarial drug (WHO, 1984). The effectiveness and widespread use of the drug is however limited owing to the development and spread of chloroquine-resistant strains of *Plasmodium falciparum* (WHO, 1987). Different mechanisms of action have been described for chloroquine, including DNA intercalation, alkalinization of parasite's lysosomes and formation of a toxic complex with ferriprotoporphyrin IX (FPIX). Although no one mechanism has been generally accepted, it is believed that chloroquine-concentrating mechanism in parasitized cells is important for selective toxicity to malaria parasites.

2.5.2 Tissue Schizontocides

This class of antimalarial drugs are used primarily because they eliminate developing tissue schizonts or latent hypnozoites in the liver. They completely eliminate both the erythrocytic and exo-erythrocytic schizonts, (persisting liver hypnozoites of *Plasmodium vivax* and *Plasmodium ovale*) thus preventing relapses of the disease caused by these parasites and effecting radical cure. Drugs in this group include the 8-aminoquinolines especially primaquine. In falciparum malaria

where there are no hypnozoites, blood schizontocides will achieve radical cure (Rollo, 1970). The mechanism of action of primaquine is poorly understood. It is however thought to act as an oxidant through its quinoline-quinone intermediates. Another agent that can be used for radical cure (in malariae malaria) is quinacrine.

2.5.3 Gametocides/Sporontocidal drugs

Gametocides are suppresive drugs used to prevent infection by mosquitoes by destroying gametocytes in the blood. They include primaquine for *Plasmodium falciparum* and chloroquine for *Plasmodium vivax, Plasmodium malariae* and *Plasmodium ovale*. Sporontocidal drugs are used to render gametocytes non-infective in the mosquitoe but do not destroy the gamelocytes. Examples are pyrimethamine and proguanil.

In malaria chemoprophylaxis, the objective is to prevent infection by suppressing the malaria parasites (sporozoites). The range of drugs available for suppression includes the 4-animoquinollnes (chloroquine), the dihydropteroate-synthase inhibitors (sulphones and sulphonamides), the tetrahydrofolate-dehydrogenase inhibitors (pyrimethamine and proguanil), mefloquine and doxycycline. It has been reported however, that none of these drugs is active against forms of the parasite inoculated by mosquitoes, the sporozoites. Hence

infection is not truly prevented. That is, there is no true causal prophylaxis (WHO, 1988).

2.6 ANTIMALARIAL DRUG DEVELOPMENT

The first chemotherapeutic agent to be used in the treatment of malaria in modern times was quinine which is the chief alkaloid and the active component of the bark of a tree called *Cinchona*. The isolation of quinine and other alkaloids from Cinchona bark was carried out in 1820 (Pinder, 1970) while its synthesis, which was cumbersome and very expensive, was accomplished about 1944.

Prior to the synthesis of quinine, pamaquine was synthesized by the Germans in 1924. Thus the drug became the first synthetic antimalarial drug to be developed. However, due to a number of limitations including some measure of toxicity and inferior activity against *P. falciparum* (Peters, 1980), another drug, mepacrine was synthesized in 1930 shortly before the outbreak of hostilities during the second World War. The drug was effective in the treatment of acute attacks and for suppression of malaria.

Attempts to find more effective antimalarial drugs by the Germans resulted in the development of compounds of the 4-aminoquinolines (Sontochin and Resochin) in 1934, their antimalarial activities were not considered to be superior to Mepacrine

Among the several 4-aminoquinoline derivatives subsequently developed, chloroquine was found in 1944 to have a faster therapeutic action than mepacrine or sontochin and was less toxic (Bruce-chwatt 1986). Amodiaquine, another 4-aminoquinoline was found to be almost as effective as chloroquine.

The extensive research programme at that time also led to the development of the 8-aminoquinolines (pentaquine, isopentaquine and primaquine). Primaquine was better tolerated and gained prominence in the radical cure of infection with the relapsing malarias (Bruce-chwatt, 1986).

In England and the United States, the search for antimalarial drugs culminated in the synthesis of proguanil in 1944 and pyrimethamine in 1951. Although resistance has developed to the two drugs, they are still in use for prophylaxis (WHO, 1990).

Following the release of chloroquine around 1946 and until early 1960s, it remained the drug of choice for the treatment of malaria, and together with proguanil and pyrimethamine played a major role in antimalarial prophylaxis. However, the emergence of *P falciparum* resistant to chloroquine coupled with the outbreak of hostilities in Indonesia increased the search for new antimalarials, particularly

against the resistant parasites (Peters, 1980). The result of the screening exercise was the discovery of the potentials of sulphonamides and the value of combination drugs such as sulphonamides and pyrimethamine, the synergistic action of which not only overcame or delayed resistance to the sulphonamides but were also effective against chloroquine-resistant *P. falciparum* (Bruce-chwatt, 1986). The continued search for antimalarial possessing high activity against chloroquine-resistant *P. falciparum* also led to the development of mefloquine and halofantrine. Although, mefloquine is effective against chloroquine- and quinine-resistant *P. falciparum* (WHO, 1990, 1991), resistant strains have emerged to it (WHO, 1990).

Another promising antimalarial drug is artemisinine, derived from Qinghaosu - a substance extracted from a Chinese plant *Artemisia annua L.* Artemisinine is primarily of use in the treatment of severe malaria (WHO, 1991). Derivatives of artemisinine such as artemether and arteether are in clinical use in China to treat severe and uncomplicated malaria.

2.7 DRUG RESISTANCE

Drug resistance in malaria has been defined as the ability of a parasite strain to survive and/or to multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within the limits of the subject (WHO, 1965, 1973).

A major problem inhibiting the control of malaria is the increasing incidence of drug resistance especially that due to multi-drug resistant - *Plasmodium falciparum* (WHO, 1990). The extent of spread, the degree of resistance and the mechanism(s) of resistance (where it is known) vary from one drug to another.

Chloroquine

The geographical distribution of chloroquine-resistant *P. falciparum* is very wide. It extends from its original foci in South and Central America to most countries in Asia and recently to many African countries (WHO, 1984, 1986). From its focal point in Kenya (East Africa) where it was first discovered on the continent (Kean, 1979) it has spread within a short time to other countries within and outside the region (WHO, 1986; Oduola, 1992). It has been pointed out that the rapidity of spread of the parasite in Africa may not be unconnected with uncontrolled

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transmission, owing to lack of vector control measures and wide occurrence of drug pressure (WHO, 1986).

Quinine

Quinine is a drug of choice for cases of severe and complicated malaria (WHO, 1984; UNDP/WORLD BANK/WHO, 1991). It is also used for uncomplicated malaria. Resistance to this drug occurred many years before the advent of synthetic antimalarial (WHO, 1984), and has spread at an alarming rate especially in South-East Asia (UNDP/WORLD BANK/WHO, 1991). Earlier reports from that land (Jaroonvesama *et al.*, 1974; Migasena *et al.*, 1980; Chongsuphajaisiddhi *et al.*, 1981) had shown widespread occurrence of quinine-resistance *P. falciparum*. In Africa, resistance to the drug has been reported in Tanzania, Burundi, Kenya, Zaire (WHO, 1987).

Although less widespread than chloroquine resistance, quinine resistance is nevertheless of great concern because of its great utility in severe and complicated malaria.

Sulphadoxine/Pyrimethamine combination

Sulphadoxine/pyrimethamine combination was the first drug developed against chloroquine-resistant *P. falciparum* (WHO, 1984). The combined drug exerts a more rapid and potent action than either of the drug in combination

Resistance to the potentiating combination observed shortly after its introduction is now well documented in South-East Asia, South America and in Africa (Reacher *et al.*, 1980; Pinichpongse *et al.*, 1982; Eichenlaub *et al.*, 1983.

Mefloquine and Halofantrine

Mefloquine and halofantrine are among the most recent antimalarial drugs developed against chloroquine-resistant *P. falciparum*. Cases of mefloquine resistance in *in vivo and in vitro* have been reported in Thailand (Boudreau *et al.*, 1982) and in Africa (Bygbjerg *et al.*, 1983; Burchard, 1983; Oduola *et al.*, 1987; 1992). Report on hatofantrine resistance is scanty. However, recent report (Ringwald *et al.*, 1990) demonstrating reduced *in vitro* susceptibility to the drug in some West African countries where this drug had not been introduced has been taken to imply the presence of innate resistance to the drug as in mefloquine (Oduola *et al.*, 1987).

Artemisinin: It is a new antimalarial drug which is used primarily in treating severe malaria (UNDP/WORLD BANK/WHO, 1991). It is yet to be licensed for use in many countries. In Nigeria (West Africa) where this drug is still undergoing clinical trials, transient resistance *in vitro* has been observed (Oduola et al., 1992).

2.8 ANTIMALARIAL MEDICINAL PLANTS

Many medicinal plants belonging to different families are utilized in traditional medicine for treating fevers and malaria. Scientific investigation of the antimalarial activities of these plants using *in vivo* and *in vitro* methods have not only provided experimental support for the traditional use of some of the medicinal plants, but it has also led to the isolation of antimalarial principles in them. The following are some medicinal plants which are used either alone or in combination with other plants to treat fever and malaria.

***Azadirachta indica* Juss (Meliaceae):** It is an evergreen tree which is about 80 feet tall. It is native to India, but it has naturalized in West Africa. In Nigeria, the chief use of the plant is in the treatment of fever and malaria. However, there are conflicting reports regarding its antimalarial activity. For example, whereas Tella (1976) reported that high concentration of the leaf extract was not effective in *Plasmodium berghei* malaria, Ekanem (1978), Obih and Makinde (1985) reported antimalarial activity against *Plasmodium falciparum* and *Plasmodium berghei*, respectively. However, antimalarial effect against *Plasmodium berghei* malaria was demonstrated in early infection but not in established infection. High concentration of the leaf extract has been reported to cause liver damage (Basak, 1968).

***Picralima nitida* (Apocynaceae)**

It is a deciduous tree reaching 25m in height - it is widely but sparsely distributed in Ghana and Nigeria (Irvine, 1961). Extractives of the stem bark, the seed, the root and the fruit have been reported to be used in the treatment of fever, malaria, and jaundice (Dalziel, 1937). The water suspension of the plant was found to have activity in *P. yoelii nigeriensis*-induced early infection, and repository test but not active in established infection (Taiwo, 1989). The main alkaloids from *Picralima nitida* include akuammine, pseudoakuammidine, akuammiline and akuamicine (Raymond-Hamet 1951). Akuammine, has been reported to be inactive in malaria both in pharmacological and clinical trials. It however has a local anaesthetic action almost equal to that of cocaine (Reymond-Hamet, 1951). Akuammiline has sympatholytic, hypotensive and local anaesthetic actions (Raymond-Hamet, 1944).

Morinda lucida (Rubiaceae): The stem bark, roots and leaves are bitter and astringent. The leaves are widely used in the treatment of fever and malaria in Nigeria (Dalziel, 1937; Oliver, 1960). *Morinda lucida* is also used in combination therapy with other medicinal plants such as *Alstonia boonei*, *Cassia podocarpa*, *Cimbopogon citratus* for treating malaria and fever (Gbile, 1986). Odetola and Bassir (1980) reported that the extractive of the leaf suppressed *Plasmodium gallinaceum* in chicks but not *Plasmodium berghei* in mice. Obih and Makinde (1985) however reported antimalarial activity against *Plasmodium berghei* in mice. Laoye (1988), using *in vivo* and *in vitro* methods confirmed the antimalarial effect of *Morinda lucida* in *Plasmodium berghei* malaria and also reported activity against

Plasmodium falciparum. Some active principles isolated from *Morinda lucida* include oruwal, oruwalol, oruwacin (Adesogan, 1973; 1979).

Khaya species (Meliaceae): All *khaya* species found in West Africa are reported to be used as antimalarial agents (Adesina, 1988). The species include *Khaya senegalensis*, *Khaya ivorensis*, *Khaya grandifoliola*. All of them are used either alone or in combination with other antimalarial plants. Awe and Makinde (1991) compared the antimalarial activity of 3 khaya species against *Plasmodium berghei* infection in mice and reported that *Khaya ivorensis* produced the highest chemo-suppression of parasitemia while *Khaya senegalensis* produced the least chemosuppressive effect in early infection. Surprisingly, they reported that *Khaya grandifoliola* which produced an intermediate schizontocidal effect in early infection was the only active species in established infection. The extractives from the stem bark of these plants are used in indigenous medicine. The stem bark is either soaked in water or boiled for a few hours. Alternatively, alcohol is used as the medium. Most active compounds from *Khaya* species are steroids and triterpenes. The triterpenes are very bitter and the antimalarial activity of the species are linked to the bitter principles.

Spathodea campanulata: The water or alcoholic decoction of the leaves and stem bark of the plant is used in indigenous medicine for the treatment of malaria. Makinde, Adesogan, Amusan, 1987; Makinde, Awe, Agbedahunsi,1988; Makinde, Amusan, Adesogan, 1990) reported the antimalarial effects of the crude extracts of the leaves and stem bark of the plant and also of the chromatographic fractions of the stem bark extract. The crude extracts and the chromatographic fractions were more active in early infection than in established infection.

The foregoing plants are widely used in Nigeria either alone or as a decoction. Other plants used locally and which have been investigated scientifically using *in vivo* methods in mice or rats and reported to be active include *Solanum enanthum* (Makinde, Obih and Jimoh, 1987) and *Cymbopogon citratus* (Obih and Makinde, 1986).

To date, the most important antimalarial plants are *Cinchona* and *Artemisia annual* L. (Qinghao), furnishing quinine and artemisinine respectively. Other promising antimalarial plants undergoing investigation include *Dichroea febrifuga* Saxifragacea) and *Triclisia subcordata*. The former is a Chinese plant with a strong reputation for the treatment of malaria (Anon. 1975, 1985). Although considered toxic for use as an antimalarial drug it has been used clinically for the treatment of

Plasmodium ovale and *Plasmodium vivax* malaria (Phillipson and Wright, 1991). *Triclisia subcordata* on the other hand is a Togolese plant widely used to treat malaria. An alkaloid from this plant has been reported to possess activity against drug-resistant forms of *Plasmodium falciparum* (Richardson *et al.*, 1991).

Some other antimalarial plants, and the countries in which they are used (in parentheses) include *Brucea javanica* (China, Thailand); *Eurycoma longifolia* (Malaysia); *Simarcuba amara*; *Picramnia antidesma* (Central America) and *Celastrus paniculatus* (Thailand). Extracts of these plants have been investigated for antimalarial effect on drug-resistant forms of *Plasmodium falciparum*, and the concentrations of the extracts that inhibited 50% of asexual parasite growth to schizont stage were reported to be in order of ug/ml (Phillipson and Wright, 1991).

2.9 ANTIMALARIAL COMPOUNDS FROM MEDICINAL PLANTS

A range of chemical compounds belonging to different groups including alkaloids, sesquiterpenes, terpenoids, quinones and phenolics have been isolated from antimalarial plants used traditionally at different foci around the world. The following are some of the important compounds and their activity.

Quassinoids

Quassinoids are the bitter principles of the plant family Simaroubacea (Polonsky, 1973). They are terpenoids biosynthetically obtained from triterpenoids. Quassinoids have been isolated from some medicinal plants that have traditional reputations for antimalarial or other antiprotozoal activities. Such medicinal plants include *Brucea javanica* (South East, Asia, China, Thailand), *Eurycoma longifolia* (Malaysia), *Ailanthus altissima* (India), *Simarouba amara* (Central America) and *Picramnia antidesma* (Central America).

Following the observation that extracts of *simaroubaceae* are active against avian malarias several species including those mentioned above were investigated for antimalarial activity and were reported to be active against *Plasmodium falciparum* (K1, multi-drug resistant strain) (Phillipson *et al*., 1993).

Several reports relating to the antimalarial activity of quassinoids *in vitro* have appeared in the literature (Trager and Polonsky, 1981; Guru, et al., 1983; Fandeur, Mercereau and Polonsky, 1985; O. Neill *et al*., 1986; Bray et al., 1987). All of the reports indicated that many of the quassinoids tested showed superior activity to chloroquine and quinine under the same test conditions. There is paucity of information on the antimalarial activity *in vivo* of quassinoids owing to limited *in vivo*

studies (O'Neill *et al*., 1987). However, the few studies carried out indicate a level of activity comparable in some cases to quinine in *P. berghei* infected mice (Bray *et al*., 1987).

The mechanism of antimalarial action of quassinoids has been reported to be related to disruption of normal ribosome function and irreversible inhibition of protein synthesis in eukaryotic cells (Liao, *et al*., 1976; Fresno *et al*., 1978; Beran *et al*., 1980; Hall *et al*., 1983; Patel *et al*., 1989; Kirby *et al*., 1989).

In addition to antimalarial effects, activity against other protozoal and neoplasm have been reported for quassinoids. For example activity of quassinoids against *Leishmania donovani* promastigotes and amoebic dysentery has been reported (Robert-Gero *et al*., 1985; Keene *et al*., 1986). Further, several quassinoids have also been reported to possess cytotoxic activity against KB cells (human epidermoid cancer of the mouth). However, it has been reported that cytotoxicity does not necessarily parallel antiplasmodial activity (Anderson *et al*., 1991; 1992).

Sesquiterpene

Interest in antimalarial medicinal plant research has been stimulated by reports of the clinical use of the endoperoxide sesquiterpene lactone, artemisinine,

which has been identified as the active principle of the Chinese traditional antimalarial herb *Artemisia annua*. *Artemisia annua* has been used for centuries in China for treating fever and malaria (Phillipson and Wright, 1991). Artemisinine has been reported to be effective in the treatment of cerebral malaria caused by *P. falciparum* resistant to chloroquine (WHO, 1990) and its action is superior to that of quinine or chloroquine (O'Neill *et al.*, 1985). The drug localizes in parasite membrane and may have an oxidant mode of action due to the presence of the trioxane structure (Vennerstrom and Eaton, 1988).

Owing to the lipophilic nature of artemisinine there are problems associated with its administration. To circumvent the problem and improve on its pharmacokinetics, a series of derivatives have been synthesized, and the most active include artemether, arteether and sodium artesunate. All these derivatives, in various dosage forms including oral, suppository and injectable are used clinically in China (WHO, 1991).

Another novel sesquiterpene which has been reported to have antimalarial activity is parthenin, an active principle isolated from *Parthenium hysterophorus*. Parthenin has been reported to be active *in vitro* against e multidrug resistant strain of *P. falciparum* (KI) (Phillipson and Wright, 1991).

Alkaloids

Quinine, from Cinchona is the first alkaloid in nature. It has been in use for many years as an antimalarial agent. Many important antimalarial drugs such as chloroquine and mefloquine have been obtained using the quinine molecule as a template (Phillipson and Wright, 1991). In recent times, many alkaloids with wide distribution in plant families including Annoaceae (Berberine alkaloids), Menispermaceae (Bisbenzylisoquinoline and Berberine alkaloids), Papaveraceae (Berberine alkaloids), Simaroubaceae (β - carbonine alkaloids) are now known to be in wide use in traditional medicine, including for the treatment of malaria (Vennerstram and Kleyman, 1988; Phillipson *et al.*, 1993). Most of the alkaloids have been tested for antimalarial activity using *in vitro* and/or *in vivo* systems, and many of them have been reported to have activity against sensitive and multidrug resistant *P. falciparum* comparable to that of chloroquine and other standard antimalarial drugs.

Bisbenzylisoquinoline alkaloids: Many bisbenzylisoquine alkaloids have been isolated from the plant family menispermaceae. They include phaeanthine, pycnamine, aromoline (from *Triclisia patens* - one of the most active medicinal plant used in Sierra Leone for the treatment of fevers and malaria (Partridge *et al.*, 1988). The IC50 value of the 3 alkaloids against *Plasmodium falciparum* (Kl) ranged from

0.15-1.43 ug/ml under the same test conditions (Tackie *et al.*, 1974; Dwuma-Badu *et al.*, 1975).

Other bisbenzylisoquinoline alkaloids include liliacorine, liliacorinine and nortiliacorinine A. They have been isolated from *Tiliacora triandra* (Menispermaceae) which is used as an antimalarial in Thailand. Their IC50 values against *P. falciparum* ranged between 0.56 and 3.5 ug/ml (Parvanand *et al.*, 1989).

The *in vitro* activities of bisbenzylisoquinoline alkaloids and the crude extracts from which the alkaloids have been isolated have been reported to lend support for the traditional reputations of the species of plants in Sierra Leone and Thailand as remedies for the treatment of malaria (Phillipson *et al.*, 1993).

Berberine and Related Alkaloids: Berberine and related alkaloids (Protoberberine alkaloids) have been isolated from *Enantia chlorantha*, and are widely used in the treatment of Protozoal diseases including malaria, amoebiasis and leishmaniasis (Vennerstrom and Klayman, 1988). Three closely related alkaloids, berberine, palmatine, and jatrororrhizine have been reported to have activity against two chloroquine-resistant strains, (D-6 and W-2) of *P. falciparum* (Partridge *et al.*, 1990). The IC50 ranged between 0.14 and 0.36 ug/ml for berberine, 0.16-0.28 ug/ml for palmatine and 0.42-1.6 ug/ml for jatrorrhizine.

Cryptolepine: Crytolepine is an alkaloid isolated from *Cryptolepis sanguinolenta*. It has been reported to be highly active against *P. falciparum* (KI). Its in vitro activity is comparable to that of chloroquine base under the same test conditions (Noamesi *et al.*, 1991). The alkaloid however lacked in vivo activity against *P. berghei*, no reduction in parasitaemia occurred. The alkaloid has anti-inflammatory activity as it reduced carageenin-induced oedema in rat paw (Bamgbose and Noamesi, 1981). The root bark from which cryptolepine is isolated is used to treat malarial patients. The anti-inflammatory property has been proposed to be contributory to its effect in malaria.

2.10 THE COMPLEMENT SYSTEM AND POLYMORPHONUCLEAR LEUKOCYTES (PMNs): GENERAL FEATURES AND ROLES IN INFLAMMATION

Rheumatoid arthritis is an inflammatory disease in which cure has remained elusive (Brune, 1989; Paulus *et al.*, 1992). Although the inhibitors of phospholipid-derived mediators of inflammation, mainly the cyclo-oxygenase inhibitors, are effective, they are limited by their often deleterious side effects, and dietary management has provided only transient benefits (Kremer *et al.*, 1985). As such, other unexplored area of arthritis management including immunomodulation based

on demonstrated antibodies to joint components (Hayner *et al*., 1986; Morgan, 1990) are receiving attention.

The immune system is an important system involved in the maintenance of homeostasis by defending the body against microbial infections. It is composed of cellular and humoral elements which act in concert to build an immune response and to regulate it. One of the many processes in which immune responses are involved is inflammation. Inflammation is a reaction resulting from and accompanying an immune response to usually a foreign particle called antigen. It may be acute, involving the complement and polymorphonuclear neutrophils (PMNs) or chronic, involving T-lymphocytes, lymphokines and macrophages in addition to the involvement of complement and PMNs (Hood *et al*., 1984). In a rheumatoid joint, immune complexes [(antigen (Ag) - antibody(Ab)] often including rheumatoid factors (RF) activate complement via the classical and alternative pathways. Complement activation leads to the release of complement chemotactic factors (C3a, C5a) which attract phagocytic cells into the joint. The phagocytes are activated by complement products (e.g. C3b) and while phagocytosing the immune complex, they release lysosomal enzymes, a variety of proinflammatory

molecules including reactive oxygen metabolites (ROM) which initiate tissue injury in such diseases as rheumatoid arthritis (Fantone and Ward, 1985).

The demonstration of complement consumption in biological fluids and of complement deposition in the affected tissues in human disease and animal models, together with the ameliorating effects of decomplementation on the model diseases, strongly implicates complement in the pathogenesis of a variety of rheumatic disorders (Morgan, 1990). Although the initiating factors and copathogens may differ among the diverse diseases, the possibilities remain that measures aimed at specifically inhibiting complement activation might be of therapeutic value in many of the conditions (Morgan, 1990; Fantone and Ward, 1985).

In the following sections, some aspects of the biological effects of complement and PMNs activation are highlighted.

2.10.1 THE COMPLEMENT SYSTEM

Complement is an umbrella name for a complex series of proteins which forms one of the enzyme systems present in the plasma. The system is composed of at least 25 proteins of which 12 components C_1-C_9, factors B, D and P are directly involved in the pathways constituting the system, while the remainder functions as essential regulators (Morgan, 1990). Activation of the system initiates a sequence of biochemical reactions, each component activating the next in a cascade fashion. The cascade mechanism allows rapid and considerable amplification to occur in the system thus leading to the formation of large quantities of active products. The consequences of complement activation include opsonization of antigenic particles (including microorganisms), activation of leucocytes and lysis of target cells (Frank, 1992; Liszewski and Atkinson, 1993).

Four functional units can be distinguished, two activation pathways, an amplification loop, and a terminal activation route (Kierx, 1985). The two activation pathways are known as the classical and alternative pathways (CP and AP, respectively). Activation of complement via either of these pathways results in the proteolytic cleavage of complement factors resulting in small split products, and the generation of high molecular complexes of complement components.

The complexes may eventually lead to the lysis of target cells, whereas the small fragments play a role in many immunoregulatory processes (Fig. 2.2; Roitt *et al.*, 1989).

2.10.1.1 Classical Pathway Activation

The classical complement pathway (CP) can be triggered by the immunoglobulins IgG1, IgG3 and IgM, but also by other substances as certain micro-organisms (Ziccardi, 1984; Liszewski and Atkinson, 1993) or the lipid A part of bacterial lipopolysaccharides (Cooper and Morrision, 1978). CP activation is initiated by the direct binding of the C1q subunit of C1 to the target, and leads to a conformational change in the whole C1 complex which results in the activation of subcomponents C1r and C1s. C1 activation is dependent on the presence of Ca^{2+} (Johnson, 1977), since the C1q, r end s complex is held together by a calcium ion. Activated C1s splits factor C4 into a small fragment C4a, and a large fragment C4b, which binds to the target. In the presence of Mg^{2+}, C2 binds to the membrane-bound C4b and in close proximity of activated C1, C2 is converted into C2a by splitting off the fragment C2b. The C4b2a complex is known as the CP-dependent C3-convertase. C2a in this complex cleaves C3 into C3a and C3b. The latter binds to

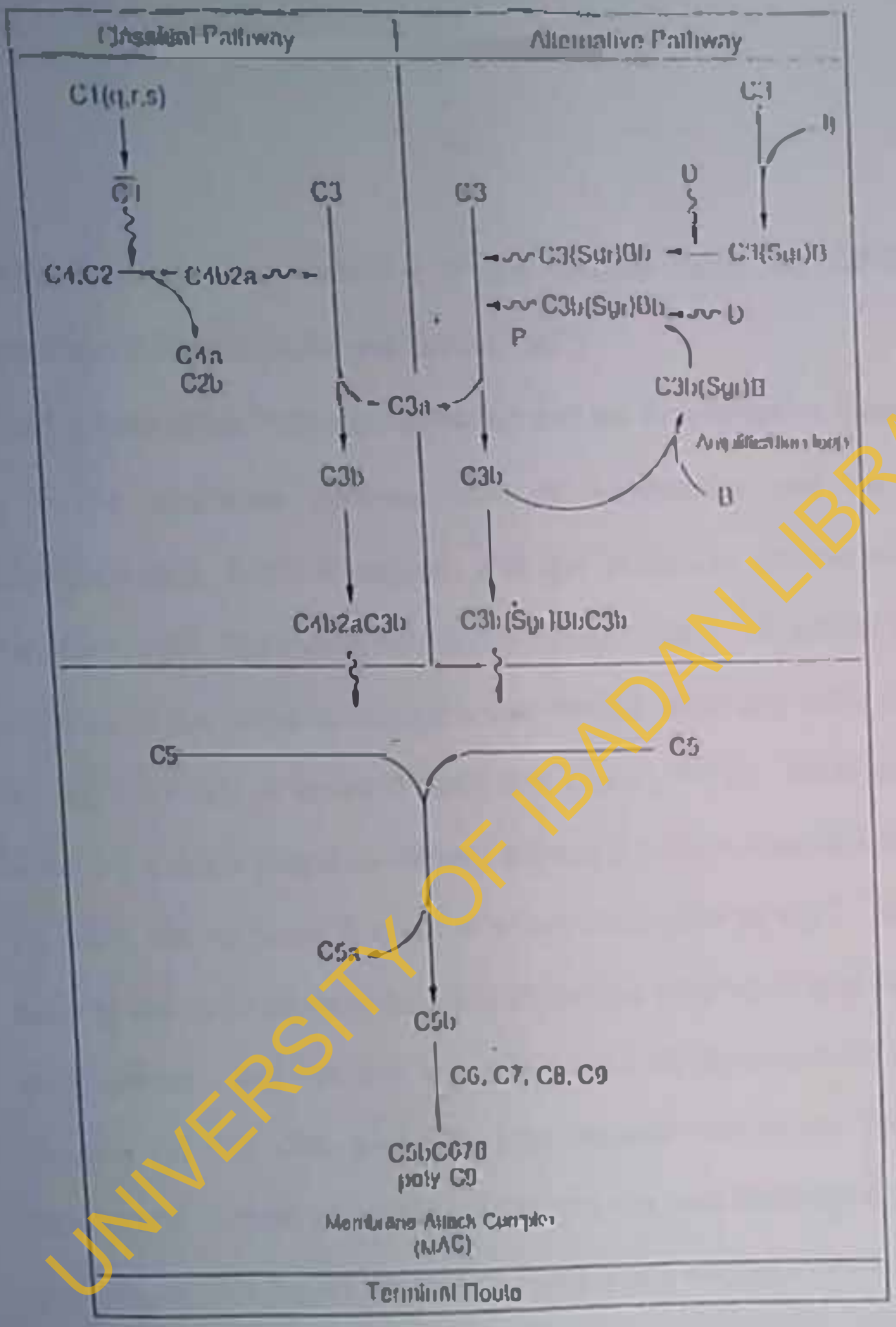

Fig. 2.2 The Complement System or Cascade

C4b2a resulting in an activator of the terminal route, the CP-dependent C5 convertase (C4b2a3b) (Law and Levine, 1977).

2.10.1.2 Alternative Pathway Activation and the Amplification Loop

The alternative pathway (AP) of complement can be activated by polysaccharides, bacteria, viruses, and IgA antibodies (Heideman et al., 1988; Pangburn, 1983; Muller-Eberhard and Schreiber, 1980). AP activity is based on the presence of low levels of spontaneously formed, extremely labile C3 intermediate [+(C=O) - C3-(S)-] in serum (Fearon and Austen, 1977). When this intermediate binds to a suitable (sugar-containing) surface, it forms a covalent ester which carries a binding site for factor B which is in turn dependent on Mg^{2+}. After cleavage by factor D, the small fragment Ba splits off and the initial enzyme of the AP, C3(sugar) Bb is formed. This complex is known as the AP-dependent C3 convertase, and converts C3 into C3a and C3b. C3b behaves functionally like the labile C3 intermediate; it binds to sugar moieties and has also an acceptor site for factor B- giving rise to C3b(sugar) Bb. Factor P(properdin) acts as a stabilizer of C3b(sugar) Bb, thus promoting AP-dependent complement activation. Like C3(sugar) Bb, C3b(sugar) Bb is also a C3 convertase, which gives rise to a continuous generation of new C3b molecules (the amplification loop). Eventually, C3b(sugar) BbC3b [end

C3 (sugar) BpC3B] complexes are formed which constitute the AP-dependent C5 convertase (Roitt *et al*., 1989; Medicus *et al*., 1976).

2.10.1.3 The Terminal Route

The terminal route of complement is initiated by the activation of C5 by C5 convertases formed via either the CP(C4b2a3b) or the AP [C3b(sugar) Bb C3b] routes. During activation, C5 is cleaved into the smaller fragment C5a and C5b. The latter interacts with C6 to form the meta-stable C5bC6 complex, which may reversibly bind to surfaces, but may also be released into watery solutions. After binding of C7, a short-lived C5bC67 is produced which is either inactivated by soluble inhibitors in serum (vitronectin or clusterin) or inserts into cell membranes. After the binding of C8, the C5bC678 complex is formed which mediates the polymerization of C9 (Klerx, 1985). C5bc678 and the polymerized product of C9 can cause cell death through membrane damage (lysis). The complex of C5 through C9 is known as the membrane attack complex (MAC) (Heideman *et al*., 1988).

2.10.2 Biological Activities Associated with Complement Activation

Several biological activities have been attributed to complement split products. These small peptides (about 12KDa) liberated during complement

activation mediate the triggering of several cell-dependent immune functions (Morgan *et al.*, 1983; Sundsmo, 1983). Fragments C3a and C5a have anaphylactic properties (Muller-Eberhard, 1975; Minta and Movat, 1979). They cause degranulation of mast cells, enhance vascular permeability and induce smooth muscle contraction, although the latter effects may also be partly due to histamine release from mast cells. Moreover, C5a is a major chemotactic factor and priming agent for neutrophils, promoting their migration from blood vessels into tissues. Split product C3b (and to a lesser extent C4b) are opsonizing agents (Liszewski and Atkinson, 1993). The coating of microorganisms with C3b belongs to the most important function of complement. Neutrophyl, monocytes and macrophages have receptors for C3b and therefore, covering (Pathogenic) micro-organisms with C3b facilitates their recognition and uptake by these phagocytes.

Complement has both negative and positive roles. Complement helps maintain immune complex in solution, preventing their deposition in the tissues, and also solubilizes precipitated complexes (Morgan, 1990). However, it is one of the major initiation pathways in the process of inflammation, and complement factors are intimately involved in rheumatoid inflammation (Parnham *et al.*, 1984). Activation of complement by immune complexes in the tissues will exacerbate inflammation and

tissue damage. Complement is harmful under several circumstances including the following:

- if activated systemically on a large scale; e.g. in Gram-negative septicaemia.
- if activated by tissue necrosis, e.g. during myocardial infarction.
- if activated by an autoimmune response to host tissues. For instance, in auto-immune disorders involving auto antigen-antibody complexes, complement activity can cause tissue damage. Some examples of auto-immune diseases, in which complement activation has adverse effects include gout, rashes and inflammatory events associated with rheumatoid arthritis and systemic lupus erythematosus.

2.10.3 POLYMORPHONUCLEAR LEUKOCYTES

The role of complement in the defence of the human body towards infections has been discussed. Other components of the immune system which play a key role in the nonspecific host defence are polymorphonuclear leukocytes (PMNs) and monocytes. The two cells are specialized in phagocytosis and PMNs in particular provide the primary cellular defence against bacteria in humans and are an important component of the acute inflammatory response (Fantone and Ward, 1985,

Simons *et al.*, 1990). In addition, PMNs are involved in the extracellular killing of virus - infected and tumour cells (Simons *et al.*, 1990).

The phagocytic process comprises of the binding of usually opsonized particles, the formation of a phagosome by endocytosis and the inactivation of the ingested material after fusion of phagosomes with lysosomes (Cline, and Territo, 1980). The latter inactivation is mediated by proteolytic enzymes, and reactive oxygen species (ROS). Formation of ROS is characterized by a cellular increase in the consumption of oxygen. This phenomenon is called respiratory or oxidative oxygen burst (Barbior et al., 1973). In the respiratory burst, free molecular oxygen (O_2) is enzymatically reduced to superoxide anions ($O_2^{\cdot}$) by a membrane-bound NADPH-dependent oxido-reductase (NADPH-oxidase). The superoxide anion which is regarded as a low toxic metabolite is further converted into other ROS (hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$), hypohalites (OCL^{-}, OI^{-}) with much more pronounced bactericidal and cytotoxic activities (Barbior, 1984; Bellavite, 1988).

The activity of ROS are both beneficial and harmful. Whereas the microbicidal effects and toxicity towards virusinfected cells or tumour cells are beneficial, their cytotoxicity may cause harmful effects. For example, in a number of autoimmune diseases, such as rheumatoid arthritis, ROS are believed to be responsible for the lesions in the joint tissue (Halliwell *et al.*, 1988).

2.11 *ALSTONIA* SPECIES: Use in Traditional Medicine, Botany, Chemistry and Ethnopharmacology

2.11.1 Introduction:

Alstonia is the generic name of a group of trees belonging to a family called Apocynaceae. They show a wide ranging properties which are suitable for industrial and medicinal exploitation. This review focus on the medicinal properties. There are about 50 species of *Alstonia*, widely distributed in the continents of Africa, Asia and America (Perry and Metzger, 1980; Maberly, 1990). Those species that have been investigated for chemical constituents and biological activities include *A. scholaris*, *A angustifolia*, *A. boonei*, *A coriacaea*, *A. constricta*, *A. congensis*, *A. macrophylla*, *A. muelleriana*, *A. venenata*, *A. undulata*, *A. yunnanansis*. The most widely distributed species is *A. scholaris* occurring throughout Asia from India to South China, Indonesia, the Philippines and the Solomon Islands (Perry and Metzger, 1980). Others are distributed throughout Africa, Central America, China, South East Asia and the Pacific (Maberly, 1990). In West Africa, the prevalent species are *A. boonei* De-Wild and *A congensis* (Ojewole, 1984; Oliver-Bever, 1986; Keay (1989).

Alstonia species have been extensively investigated for their chemical constituents and biological activities (Goodson, 1932; Faparusi and Bassir, 1972;

Ojewole, 1984; Kweifio-Okai, 1991; Awe and Opeke, 1990; Vasanth *et al.*, 1990; Wright *et al.*, 1993; Makinde and Taiwo, 1996) Virtually all the publications on the chemical constituents of the species are in agreement, but reports on the biological activity especially those concerning antimalarial effects have been controversial.

2.11.2 Chemistry

Extensive phytochemical studies have been carried out on *Alstonia* species. This is reflected in the vast amount of publications since 1934 when a monograph in the British Pharmaceutical codex defined *Alstonia* as the bark of either *Alstonia scholaris* R. Br (Apocynaceae) from trees growing in India, Burma, the Phillipine Islands, or of *A. constricta* F. Muell from Australia (Anon, 1934). It seems much of the phytochemical work has not been borne out of desire to isolate compounds which may be of therapeutic interest but, it has been out of desire to draw a relationship between plant chemistry and phylogeny. Little wonder then that many of the isolated compounds lack the biological (medicinal) properties associated with the particular species from which they have been isolated.

The chemical constituents reported can be divided into several structure classes including alkaloids, terpenes and steroids. Over 90% of the isolated chemical constituents of the genus are alkaloids (Goodson, 1932; Hamilton, *et al*., 1962 Ray and Chatterjee, 1968; Faparusi and Bassir, 1972; Kucera *et al*., 1972, Ojewole, 1984; Kweifio-Okai, 1991; Wright *et al*., 1993).

The major alkaloid is echitamine (Goodson, 1932; Kucera *et al*., 1972; Faparusi and Bassir, 1972; Wright *et al*., 1993). Like echitamine, many of the alkaloids are of the indole types (Hamilton *et al*., 1962; Wright *et al*., 1993). Others have dihydroindole moieties similar in structure to the indole types. However, corialstonine and corialstonidine from the new caledonian species, *A. coriacea* have quinoline moities similar in structure to the alkaloids of the quinine type (Wright *et al*., 1993). Antiplasmodial activity of the alkaloids have been investigated in vitro using both drug sensitive and resistant strains of *P. falciparum*. In one such screen, IC50 values of 42.6 ± 34 uM, 5.71 ± 0.033 and 5.4 ± 0.11 uM were reported for echitamine, corialstonine and corialstonidine respectively (Wright *et al*., 1993). Chloroquine diphosphate and quinine hydrochloride had IC50 values of 0.44 and

0.56 uM, respectively in the test system. The report concluded that echitamine had no significant antiplasmodial activity *in vitro*.

Over 130 alkaloids have been isolated from the genus *Alstonia*, and only a small number has been assessed for antimalarial activity with a preponderance of in vitro studies (Wright *et al.*, 1993; Phillipson and Wright, 1993). The only recent publication on the in vivo antimalarial activity of echitamine was by Vasanth *et al.* (1990). The authors reported an ED50 of 1.6 mg/kg against *P. berghei* in mice.

2.11.3 Ethnomedical Use/Ethnopharmacology

Alstonia species are used in traditional medicine to treat a variety of diseases including malaria, fever, heart diseases, hypertension, painful micturition, insomnia, chronic diarrhoea, and rheumatic pains (Dalziel, 1937; Burkhill, 1935; Irvine, 1961; Faparusi and Bassir, 1972; Perry and Metzger, 1980; Ojewole, 1984; Oliver-Bever, 1986; Abbiw, 1990; Asuzu and Anaga, 1991; Kweifio-Okei (1991 (a) and (b) Wright *et al.*, 1993). The most often cited disease for which extracts of the species are indicated (in traditional medicine) are fever and malaria. Consequently, most ethnopharmacological investigation of the species have focussed on the antimalarial property. However, the result has been disappointing as no candidate compound

which can be used directly in therapeutics or as templates for the synthesis of effective compound has been found.

The species that have received the most attention are *A. boonei*, *A. scholaris*, *A. congensis*, *A. constricta*, *A. macrophylla*, and *A. coriacea*. Antimalarial screen of alkaloids from the barks of four of the species, namely, *A. scholaris*, *A. constricta*, *A macrophylla and A. congensis* have been done on avian malaria, *Plasmodium inconstants*. The total alkaloids from the first two species showed activity at oral doses of 125 mg/kg and 500 mg/kg respectively, for 6 days. The total alkaloids from the two remaining species did not show activity (Wright *et al.*, 1993 citing Goodson et al., 1930). More recently, extracts of *A. scholaris* were reported to be active against *P. berghei* malaria (Ghandi and Vinayak, 1990; Awe and Opeka, 1990). However, Awe and Opeka (1990) differentiated between two tests, early suppressive test (4-day test) and Rane test (established infection). The extract was found to have significant suppressive activity in the former but no activity in the latter.

The many ethnopharmacological studies carried out on *Alstonia* species seem not conclusive enough. This is reflected in the conclusion of Wright *et al.*, (1993) that the overall position of *Alstonia* and its antimalarial effects are still not clear, and the reported effectiveness of the species in traditional medicine could be due to

other effects in humans including antipyretic, antiinflammatory and immunomodulatory actions.

2.12 *Alstonia boonei* (Apocynacea)

Alstonia boonei (Ahun in Yoruba) is a large perennial tree widely distributed in tropical Africa (Ojewole, 1984). It is distinguished from *A. congolensis* Engl which is also prevalent in tropical Africa, by a glabrous inflorescence and shorter corolla tuble (Wright et al 1993). Different parts of *A. boonei* are widely used in traditional medicine to treat various diseases such as malaria, fever, painful micturition, insomnia, chronic diarrhoea, rheumatic pains (Dalziel, 1937; Ojewole, 1986; Oliver-Bever, 1986; Abbiw, 1990; Wright *et al.*, 1993).

There is a paucity of information on the antimalarial activities of *A. boonei*. The aqueous extract of the plant was reported to have strong schizontocidal activity in early infection (4-day test) and also strong repository activity, but ineffective in estasblished infection (Rane's test) (Makinde, Obih and Salako, 1987; Jimoh 1985, Awe, 1991). The epository activity was found to be more then the schizontocidal action in early infection (Awe, 1991).

The chemical constituents that have been isolated from *A. boonei* include echitamine (common to most species of *Alstonia*), and echitamidine (Goodson, 1932; Kucera *et al.* 1972; Burkill, 1985).

In addition, a lactone, and triterpines, amyrin and lupeol and ursolic acid as well as steroids, sitosterol and sapogenin have been reported (Faparusi and Bassir, 1972). *A. boonei* is either boiled in water and taken in form of aqueous teas or soaked in alcohol. The possibility therefore exists that the alkaloid constituents from them may be responsible for antimalarial activity. Echilamine, the major alkaloid in *A. boonei* has been extensively investigated for antimalarial activity. So far, the reports have been controversial. Whereas Marquis and Ojewole (1976) and Wright *et al.* (1993) reported ineffectiveness of echitamine *in vivo* against *P. berghei* and *in vitro* against *P. falciparum* respectively, Vasanth *et al.* (1990) reported that echitamine was effective against *P. berghei* in mice. Ursolic acid, isolated from *Spathodea campanulata* has been reported to be active against *P. berghei* malaria in mice (Amusan, 1990). The antimalarial activity of ursolic acid isolated from *A. boonei* has not been reported.

In addition to the widespread use of extracts of *Alstonia* to treat malaria and fever, they also have a reputation of use in the treatment of joint swellings and rheumatoid arthritis. In West Africa (Nigeria and Ghana), the species used for inflammatory disease mentioned above is *Alstonia boonei*. There is paucity of information on the ethnopharmacology of the plant in arthritis. Some of the recent studies on the indigenous use of the plant to treat rheumatoid arthritis were carried out by Kweilio Okai (1991 (i) and (ii)). He reported that a decoction of the root bark

of *A. boonei* and *Rauwolfia vomitora* and *Elaeis guineensis* nuts suppressed the early and late phases of carrageenin oedema in rats. The decoction was also effective in adjuvant athritis in rats.

CHAPTER THREE

GENERAL MATERIALS AND METHODS

ANTIMALARIAL STUDY

3.1 Animals: Male Swiss albino mice (18-20g) and male Wister rats (150-200g) were used. The mice were bred in the Animal house of the Department of Pharmacology and Therapeutics, College of Medicine, University of Ibadan. They were maintained at room temperature and kept in groups of five in plastic cages (48cm x 12cm x 12cm) with wood shavings for their beddings and fed standard mouse cubes and water *ad libitum*. The rats were obtained from the Central Animal Unit, Department of Laboratory Animal Science, University of Utrecht, the Netherlands.

3.2 Parasite: *Plasmodium yoelii nigeriensis* (*P. y. nigeriensis*) and chloroquine-resistant *Plasmodium berghei* strain (*P. berghei*)ANKA were used. *P. y. nigeriensis* was obtained from Nigerian Institute of Medical Research (NIMR),

Lagos. The *P. berghei* used was obtained from Rijks Instituut voor Volksgezondheid en Milieuhgyiene (R.I.V.M), Bilthoven, the Netherlands. The parasites were maintained by serial passage of blood from mouse to mouse or from rat to rat.

3.3 Syringes, needles and microscope: Sterile disposable plastic synnges and needles (10G and 25 G) were used. The Leitz laborlux 12 research light microscope (Leitz Wetzlar, Germany) and 100x oil immersion objective and 6-8 X eye pieces were used. Immersion oil (olympus) and a graticule (25 squares) were used to facilitate counting and improve accuracy.

3.4 Slides: Clean grease free microscope glass slides (7.5cm x 2.5cm) were used. Commercially pre-cleaned slides were soaked in water with a detergent for a few days. They were removed and pieced in several changes of clean water. Each slide was wiped dry and polished with a dry, clean lint free cloth. Used slides were first soaked in a detergent solution for 24 hours. The slides were then cleaned with a pledget of cotton wool until all traces of blood film and oil have been removed. Thereafter, the slides were transferred to a fresh

solution of detergent and later to running water or several changes of clean water, before drying with clean cotton wool.

3.5 Oesophageal Cannula: A syringe carrying an oesophageal cannula was used for the oral administration of drug preparations to the animals.

3.6 Giemsa Stain: The Giemsa stain was prepared from Giemsa powder (Difco laboratories) using the method of Bruce-chwatt (1980).

Giemsa powder	3.8g
Methanol	250ml
Glycerol	250ml

Preparation: Thirty solid glass beads were put into a dark bottle. The measured amount of methanol was poured into the bottle followed by addition of the measured stain powder. The bottle was lightly stoppered. The stain powder was allowed to sink slowly through the methanol until it settled to the bottom. The bottle was shaken with a circular motion for 2-3 minutes. Then, the measured amount of glycerol was added and the mixture was shaken 2-3 minutes at half-hourly intervals six times. The bottle was left unused for 4 days, being shaken 3 times each day until the stain was thoroughly mixed

The prepared stock solution was filtered to remove the undissolved powder and was kept in a dark bottle with a close fitting polythene stopper at 4°C.

3.7 Normal Saline: 0.9% Nacl solution w/v was prepared by dissolving 9g of Nacl in 1 litre of distilled water. The solution was sterilized by autoclaving and kept in a refrigerator.

3.8 Buffer Solution: A phosphate buffer solulion (pH 7.2) was used. A concentrated solution was prepared by dissolving 3.0g of disodium hydrogen Phosphate (Na_2 HPO_4) anhydrous and 2.1g of Potassium dihydrogen phosphate (KH_2 PO_4) in 25ml of distilled water. The pH was adjusted up to 7.2 by adding small quantities of a 2%Na_2 HPO_4 solution or down to 7.2 by adding small quantities of a 2% solution of KH_2 PO_4. The solution was stored in a dark bottle at 4°C and usually allowed to reach room temperature before use. A working solution was made by diluting 1ml of the concentrate to 20ml of distilled water.

3.9 Drugs:

1. Chloroquine diphosphate (May & Baker Nig. Plc.)
2. Pyrimethamine (Ciba Nig. Plc.)
3. Extracts of *Alstonia boonei* stem bark (ABSB)
   - Petroleum ether extract
   - Diethyl ether extract
   - Ethyl acetate extract
   - Ethanolic extract
   - Aqueous extract
4. Isolated antimalarial active constituent (AB-1) of *A. boonei* stem bark.

3.10 Drug Solutions:

All drugs including extracts, fractions, subfractions and the pure antimalarial compound were dissolved in 1% "Tween 80". In preparing chloroquine solution, the ratio of base to salt was taken into consideration. For example, the salt of chloroquine used was the diphosphate

Molecular weight of chloroquine diphosphate = 515.9g

Molecular weight of the 2 phosphate groups (2 (H_3PO_4) = 2 (3+30.97+64) = 195.94g

Molecular weight of chloroquine (the active substance) = 515.9 - 195.94

= 319.96g

Therefore, 319.96g of chloroquine base is contained in 515.9g chloroquine diphosphate.

Ratio of base to salt = 319.96 : 515.9

= 1 : 1.6124

= 1 : 1.6

Thus 1g of chloroquine base is contained in 1.61g of the diphosphate. This was taken into account when weighing chloroquine diphosphate. For example, to weigh 20mg of chloroquine base, 20 x 1.61 = 32.2mg of chloroquine diphosphate was weighed and dissolved in 5% 'Tween 80' in distilled water. The solution was made up to 20ml to give a final solution of 1mg/ml of chloroquine base.

3.11 Administration of Drug/Extract: Drugs or extracts were administered to mice/rats orally (Per Os) or subcutaneously (Sc). In Oral administration, drugs or extracts were given in a volume of 0.4ml per 20g mouse or 0.6ml per 200g rat with the aid of an oral cannula. For subcutaneous administration drugs or

extracts were given in volumes of 0.1ml per 20g mouse and 0.3ml per 200g rat. A short needle (25G) was used for rats and a smaller needle for mice.

3.12 Preparation of Blood Films and Staining Technique:

A small drop of blood was obtained from the caudal vein of animal (mouse/rat) by carefully cutting the tip of the tail with a pair of fine dissection scissors. It was placed on a clean grease free slide and smeared along the full length of the slide with the edge of another slide (placed at an angle of about 40 degrees to the surface of the first slide) to make a thin film. The film was allowed to dry, and was fixed by dabbing gently with a pledget of cotton wool dampened with methanol, or by dipping it in a conlainer of methanol for a few seconds. After drying, the slides were placed back to back in a staining trough. A 3% Giemsa solution in pH 7.2 buffered distilled water in sufficient quantity to fill the number of troughs being used was prepared. The stain solution was poured gently into the trough until the slides were totally covered. The film was allowed to stain for 30-45 minutes. Clean water was poured into the trough to float off the iridescent scum on the surface of the stain. The remaining stain was gently poured off and the slides were rinsed in clean

water for a few seconds. After pouring off the water, the slides were removed one by one and placed in a slide rack to drain and dry.

3.13 Evaluation of Parasitaemia:

Thin blood films were made from infected animals as described above. A parasite count was made under oil immersion with the objective lens of the microscope using a tally counter. In each field, the total number of red blood cells was first counted and then the number of parasitized red blood cells was also counted. The degree of parasitaemia expressed as percentage parasitaemia for each animal was calulated as follows:

$$\% \text{ Parasitaemia} = \frac{\text{Number of parasitized red blood cells}}{\text{Total number of red blood cells}} \times 100$$

Ten fields were counted on each slide and the mean percentage parasitaemia was calculated for each animal.

3.14 Preparation of Inoculum:

Thin blood films of infected animals were made and stained between 3-5 days after the day of inoculation. Animal(s) with a parasitaemia of between

35-40% were chosen as donors. The range of parasitaemia indicated that infection was established and the parasites were viable intraperitoneally.

An haemocytometer was used to determine the number of red blood cells per millimeter of the blood of the donor animal. Knowledge of the percentage parasitaemia and the number of red blood cells per unit volume enabled the calculation of the number of parasitized red blood cells in any volume and the number of red blood cells in the volume of interest. Blood was obtained from the donor animal by cardiac puncture using a sterilized syringe cotaining about 10.i.u of heparin. The blood was suitably diluted with sterile normal saline so that the final inoculum of 0.2ml for each mouse would contain the required number of parasitized red blood cells which is recommended to be 10^7 (Peters, 1975; Knight and Peters, 1980).

During each experiment, a single donor animal was used to infect all the animals in order to minimize variability in ensuing parasitaemia of the test animals.

Example of calculation of inoculum size:

Percentage parasitaemia in donor mouse = 40.0%

80 small squares of haemocytometer had 450 red blood cells (RBCs).

Volume of 1 small square of haemocytometer = $\frac{1}{4000}$ mm^3

Volume of 80 small squares = $\frac{80}{4000}$ mm^3

Dilution factor = 200

$\frac{80}{4000}$ mm^3 diluted blood contains 450 RBCs

$1mm^3$ diluted blood contains $\frac{450 \times 4000}{80}$ x RBCs

$1mm^3$ undiluted blood contains $\frac{450 \times 4000}{80}$ x 200 RBCs

1ml undiluted blood contains $\frac{450 \times 4000}{80} \times 200 \times 10^3$ RBCs

$= 4.5 \times 10^9$ RBCs

Since percentage parasitaemia = 40.0%, then

1 ml undiluted blood contains $\frac{40}{100} \times 4.5 \times 10^9$ parasitized RBCs (pRBCs)

If 10^7 pRBCs are required in 0.2 ml of blood, then 1 ml of blood is

required to contain 5×10^7 pRBCs.

Dilution factor $= \frac{0.4 \times 4.5 \times 10^9}{5 \times 10^7} = 36$

Thus the amount of blood collected from the donor animal should be diluted 36 times with normal saline so that 0.2ml of the resultant suspension will contain 10^7 pRBCs, which is the standard inoculum for the infection of a single animal.

3.15 Infection of Animals:

Infection was initiated in each mouse or rat by intraperitoneal (I.P) injection of 0.2ml suspension of the inoculum prepared as described above into each mouse/rat.

3.16 Evaluation of the Blood Schizontocidal Activity of Extract on Early Infection (4-day Test)

The aim of this experiment was to determine if the extracts of the plant had a suppressive effect in an early infection. The method used was described by Knight and Peters (1980). Animals were inoculated with an inoculum of 0.2ml containing 10^7 *Plasmodium yoelii nigeriensis* or *P. berghei* Anka. The infected animals were randomly allocated into several groups of 5 animals each. The number of groups depended on the doses of the extract prepared. However, mice were usually allocated into 2 control groups, one group (positive control) received chloroquine (a reference drug) while the second group (negative control) received 1% "Tween 80" - the vehicle. The day infection was initiated was regarded as day 0 (D_0) and subsequent days D_1, D_2 and so on. Different dosage of drug/extract/vehicle were administered orally or subcutaneously within one hour of inoculation of parasite for 4 days (day 0, 1, 2, 3). Drugs were administered once or twice daily. On day 4, a thin blood

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film of each animal was made and the percentage parasitaemia calculated. Animals were monitored for 30 days post infection, and survival period was calculated.

The average percentage suppression of parasitaemia by each dose of drug was determined using the formula below.

$$\text{Average percentage suppression} = \frac{\text{Av. percentage parasitaemia in untreated controls} - \text{Av. percentage parasitaemia in treated group}}{\text{Av. percentage parasitaemia in untreated controls}}$$

Source (Knights and Peters, 1980).

A suppression of parasitaemia greater than 50% is regarded as indicating drug activity.

3.17 Evaluation of the Blood Schizontocidal Activity of Extract in an Established Infection (Rane Test):

An established disease is less sensitive to treatment then a disease in early stages of development. In order to investigate the actions of the extracts in Rane's test, treatment was withheld until 72 hr post infection. The method of

Ryley and Peters (1970) was used. Animal distribution and dosage of inoculum were as described above. Administration of drug/extract/vehicle started after 72h (day 3) of initiation of patent infection and continued daily for 4 days (day 6). Drugs were administered once or twice daily. Thin blood films were prepared from each animal immediately before drug administration on days 3 - 8. Thereafter it was prepared weekly. Parasitaemia was enumerated from each thin smear to monitor responses of infections to treatment. Efficacy and effects of route of administration of drug/extract were assessed by comparing survival times of infected treated and infected non-treated animals. Death occurring in infected treated animals before day 6 or 7 when infected, untreated controls started to die was attributed to drug toxicity.

A survival time on the treated animals greater than twice that of the control animals indicated drug activity. Animals surviving for 30 days post inoculation with negative parasitaemia were regarded cured of infection.

3.18 Evaluation of the Repository activity of the Extract:

The method used was similar to that described by Peters (1965). Animals were divided into several groups of 5 animals each. The number of groups depended on the number of doses of extract prepared. Pyrimethamine

(1.5mg/kg) served as a reference drug. Pyrimethamine (1.5mg/kg), vehicle and various doses of the extract in volumes of 0.4ml per 20g mouse were administered to animals orally or subcutaneously once or twice daily for 3, consecutive days (day 0, 1, 2). On day 3, animals were inoculated with 0.2ml of inoculum containing $x10^7$ parasitized red blood cells. Thin blood films were made from the animals 72h after inoculation and the average percentage suppression of parasitaemia determined. Animals were monitored until their death.

3.19 IMMUNOMODULATORY TEST.

Haemolytic Assays for Human Complement Activity.

Buffers Required.

Stock of 5 times concentrated Veronal Saline Buffer (5xVSB). Nacl (41.5g) and veronal (Sodium Barbital) (5.1g) were dissolved in 800ml of distilled water. The pH was adjusted to 7.35 and the volume made up to 1 litre.

Stock Ca/Mg Solution. $Mgcl_2.6H_2O$ (10.17g) and $Cacl_2.2H_2O$ (2.21g) were dissolved in 100ml of distilled water.

Stock of ethyleneglycol-bis (2-aminoethyl) tetraacetic acid (EGTA) Solution. EGTA (7.6g) were dissolved in 200ml distilled water. The pH was adjusted to 7.35; $Mg504.7H_20$ (3.075g) was added and the solution made up to 1 litre with distilled water.

***VSB**.** VSB stock solution (200ml) was mixed with Ca/Mg stock solution (1ml). The solution was made up to 1 litre and the pH adjusted to 7.35.

EGTA-VSB. VSB stock solution (200ml) was mixed with EGTA-stock solution (40ml). The solution was made up to 100ml and the pH adjusted to 7.35.

Saline. Necl (9g) was dissolved in 1 litre distilled water.

Alsever's Solution. Glucose (2.05g), hydrated sodium citrate (0.8g) and Nacl (0.42g) were dissolved in distilled water (50ml). The pH was adjusted to 6.1 using a 10% sodium citrate solution. The solution was made up to 90ml and served out in 15ml-portions. The solution was sterilized at 121°c for 15 minutes

3.20 Reagents.

Sheep erythrocytes in Alsever's solution for CP activity

Rabbit erythrocytes in Alsever's solution for AP activity

Amboceptor: Anti-sheep erythrocyte antibodies

Human pooled serum (HPS)

Heat inactivated human pooled serum (iHPS)

3.21 Materials:

Microtitre plates

Plastic tubes (12ml)

Plastic tubes (50ml)

3.22 Chemiluminescence Assay

3.22.1 Buffers and reagents for the measurement of the oxidative burst of polymorphonuclear neutrophils.

3.22.1.1 Phosphate Buffered Saline (PBS) (44.9g/L of distilled water) was added to a solution of K_2HPO_4 (33.7g/L of distilled water) until pH = 7.4. 10ml of the resulting solution was mixed with 100ml of Nacl (87.5g/L of distilled water). The final solution was made up to 1 litre with distilled water.

3.22.1.2 PBS/Heparin 100ml of PBS was mixed with 200 uL of a 5000 IE/ml heparin solution

3.22.1.3 Percoll Solution 10ml of (87.5 g/L) Nacl was mixed with 55ml of Percoll and 35ml of deionized water. The solution was stored at 4 ^{o}C until use.

3.22.1.4 Lysis Buffer 8.3g NH_4Cl and 1g $KHCO_3$ and 37.2mg Na_2 EDTA were dissolved in 1 litre of distilled water.

3.22.1.5 Gelatin 10g of gelatin were dissolved in 100ml of boiling distilled water on a heated magnetic stirrer. The solution of gelatin was dispensed in 1ml aliquots and stored at 37 ^{o}C.

3.22.1.6 HBSS-gel Hanks Buffured Salt Solution (HBSS) was prepared by dissolving 9.75g of Hanks Buffer Salt (Gibco) and 350mg of $NaHCO_3$ in 1 litre of distilled water. HBSS-gel was prepared by mixing 100ml of HBSS with 1ml of gelatin solution. The buffer was made fresh daily. Gel prevents PMN from aggregating.

3.22.1.7 Turk Solution 5mg cristal violet was dissolved in 1ml acetic acid. The solution was made up to 1 litre with distilled water.

3.22.1.8 Serum Treated Zymosan A mixture of 62.5mg of zymosan (sigma)

and 100ml of HBSS was incubated for 1 hour in a boiling water bath. The solution was cooled to room temperature and centrifuged for 10 minutes at 2500rpm. The pellet was resuspended in 10ml HBSS and 30ml of human pooled serum added. The mixture was shaken continuosly during a 30 minute incubation at 37 °C. Following centrifugation (10 minutes at 2500rpm, 1250xg) the pellet was suspended in 37.5 ml of HBSS. The serum treated zymosan was stored at -20 °C in 2.6ml aliquots.

3.22.1.9 Luminol Stock Solution 13.3g of luminol (5 amino 2, 3 dihydro 1, 4 phtalazinedione was dissolved in 5ml DMSO. The solution was made up to 30ml with HBSS. It was dispensed in 400 uL aliquots and stored at -20 °C.

3.23 Statistical analysis Data were expressed as mean ± standard error of the mean (mean±s e m), and analysed using Student's t-test, Dunnet t-test or Duncan's multiple range Test. Level of significance was set at $P \leq 0.05$

CHAPTER FOUR

4.1 EXTRACTION AND CHROMATOGRAPHY

4.1.1 Plant Material: Stem barks of *Alstonia boonei* were collected in February / March 1991, 1992, 1993, from the Department of Botany, University of Ibadan. The collection and Herbarium numbers were Lowe 2323 and U.I.H. 13134, respectively. The plant material was cut into small pieces and oven dried (35°C).

4.1.2 Extraction Procedure:

4.1.2.1 Soxhlet extract: Coarsely ground plant material was extracted in a Soxhlet apparatus with petroleum ether (PE, 10hr), Diethyl ether (DE, 10hr), Ethyl acetate (EtOAC, 10hr) and Ethanol (EtOH, 10hr). The marc was dried and refluxed in water (AQ, 3hr). The soxhlet fractions were concentrated under reduced pressure, and subsequently lyophilized along with aqueous extract.

4.1.2.2 Cold Extract: In order to rule out any possibility that application of heat as in soxhlet extraction effects the activity of the extracts, the extraction

was carried out in the cold so that the activity of soxhlet and cold extracts could be compared

Coarsely ground plant material was steeped in petroleum ether for 3 nights followed by filtration. The residue which was dried at room temperature was weighed and soaked in diethyl ether for 3 nights and filtered. The marc was dried, weighed and steeped in ethylacetate for 3 nights and filtered. The marc from ethylacetate was dried, weighed and steeped in ethanol for 3 nights followed by filtration. Finally, the marc from alcohol extraction was dried, weighed and steeped in distilled water for 3 nights followed by filtration. The extracts of petroleum ether, diethyl ether, ethyl acetate and alcohol were first concentrated in vacuo. They were later lyophilized together with aqueous extract. The extracts of petroleum ether, diethyl ether, ethylacetate, ethanol and aqueous extract were referred to as cPE, cDE, cEtOAc, cEtOH and cAQ, respectively.

4.1.3 Fractionation of EtOH extract The EtOH extract was dissolved in dichloromethane (CH_2Cl_2) and subjected to liquid - liquid extraction with distilled water. The mixture was shaken for about 15 minutes, after which two

layers developed, namely, the lipophilic layer and the hydrophilic layer. Both layers were separated in round bottom flasks, and each was evaporated under reduced pressure and lyophilized. The concentrate from the lipophilic and hydrophilic layers were referred to as $EtOH_L$ and $EtOH_h$, respectively. $EtOH_h$ was partitioned into MeOH soluble (Ms) and MeOH insoluble (Mi) subfractions. The Ms subfraction later crystallized out on cooling (refrigeration) to give white crystals. The crystals, called *Alstonia boonei* -1 (AB-1), were separated from the mixture by washing with MeOH in a buckner funnel under low pressure and thereafter dried. The filtrate (Msf) and the MeOH insoluble subfraction (Mi) were concentrated under reduced pressure and a stream of nitrogen followed by freeze drying.

4.1.4 Extract prepared by prolonged boiling

This test was carried out in order to find out how the traditional method of preparing the decoction of *A. boonei* using water can be optimized. Whole, ground *A. boonei* stem bark was divided into 7 equal parts of 1g each. One part was steeped in distilled water for 3 days. Each of the remaining 6 parts was refluxed in distilled water for varying times (2-64 hrs). The starting volume of

distilled water in all 6 parts was the same (2L). At each reflux, a constant starting volume was maintained. All extracts were concentrated in vacuo and lyophilized

4.1.5 Determination of the Purity of the Antimalarial Compound (AB-1)

In pharmaceutical preparations, natural products from higher plants are used either as pure compounds or as extracts (Phillipson, 1994). In traditional medicine, one plant or several are combined in one prescription whereas in modern medicine a single active ingredient, (usually synthesized) is prescribed (Phillipson, 1994). Thus in traditional medicine, unlike in modern medicine, the activity and clinical efficacy of prescriptions cannot be attributed to a single active ingredient (Phillipson, 1994). When plants are extracted and fractionated in order to obtain a single active ingredient, the purity of the constituents isolated can be checked by TLC, HPLC and spectroscopy. In this section, TLC and HPLC methods are used as described below.

4.1.6 HPLC analysis.

Apparatus: HPLC analysis was performed on a Kratos liquid chromatograph equipped with a Kratos spectroflow 400 solvent delivery system type 5140 solvent programmer (Kipp & Zn) and Rheodyne model 7125 injector. The injector was connected to a HP 1040A diode-array detector (Hewlett-Packard)

with HP series 9000 workstation (Purity determination) or Kratos spectroflow 757 absorbance detector with CRIB chromatopac data processor (shimadzu, quantitative analysis). A chromspher Si 200x3mm I.D. column (Chrompack Cat No. 28277) was used. Mixtures of EtOAc/MeoH = 70:30 served as eluent (flow rate 0.5ml/min).

Methods: Solutions of 100,ug/ml of AB-1 were prepared and aliquots of 20,uL were injected into the HPLC. The mobile phase consisted of EtOAc/MeoH = 70:30. The spectrum was scanned from 220 to 320nm.

4.1.7 Thin Layer Chromatography (TLC) of AB-1

TLC is a chromatographic technique which effects the physical separation of two or more components in a mixture on a plane surface. Separation is achieved by the differences in adsorption of components of the mixture on the stationary phase (solid) and the solubility in the mobile phase. It can be used to determine a suitable solvent or solvent system for separating components of, for example a plant extract. It can also be used to determine the purity of an organic material.

TLC was performed on pre-coated plates (silica gel $60F_{254}$; Merck, Cat. No 5729) with a thickness of 0.25mm. Volumes of 5ul of the solution of the compound (4mg/ml) in MeoH were applied to the TLC plates in bands.

The plates were developed with EtOAc/MeoH = 70:30 in a saturated chamber. Spots were sprayed with vanillin - H_2SO_4 (Vanillin 1% in ethanol and H_2SO_4, 5% in EtOH), fast blue (1mg/100ml of H_2O) and Draggendorf's reagents A & B. UV detection was at 254 and 366nm.

4.2 RESULTS AND DISCUSSION

The yield of the extracts ranged from 0.3% to 3.5% for soxhlet extracts and 0.26 to 3.1% for cold extracts (Table 4.1). The percent yield relates to the polarity of extracting solvent, the more polar the solvent, the higher the yield. This observation applies to both solvent and cold extracts. For each solvent extract, the yield of soxhlet extract was higher than those of cold extracts. This observation suggests that application of heat may elute more extractives from the plant material.

Table 4.1 Yield of Soxhlet and Cold extracts of *A. boonei* stem bark

Extract	% yield
Soxhlet	
DE extract	0.30
EtOAc extract	0.44
EtOH extract	2.90
AQ extract	3.60
Cold	
cDE extract	0.26
cEtOAc extract	0.35
cEtOH extract	2.19
cAQ extract	3.10

c = Cold.

Liquid-liquid partitioning of EtOH extract into lipophilic fraction ($EtOH_L$) and hydrophilic fraction ($EtOH_h$) led to simple purification of EtOH extract. The yield of $EtOH_h$ was approximately $2^1/_2$ times higher than that of $EtOH_L$(Table 4.2). Part of $EtOH_L$ was lost during lyophilisation, and may account for the smaller weight of $EtOH_L$. In addition, the loss during lyophilisation suggests that $EtOH_L$ may be volatile. As the antimalarial activity of $EtOH_L$ was much lower than that of $EtOH_h$, the yield of the former is not of interest. $EtOH_h$ was incompletely soluble in methanol. This partial solubility enabled the separation of $EtOH_h$ into methanol soluble subfraction (Ms) and methanol insoluble subfraction (M_I). The yield of Ms was approximately 6 times higher than M_I (Table 4.2). Ms accounted for the bulk weight of $EtOH_h$ and exhibited higher activity than the parent extract ($EtOH_h$). The yield of antimelariel constituent (AB-1) crystallizing from Ms was approximately 21% of the starting material (Table 4.2).

4.2.1 Extract prepared by prolonged boiling

The yield of extract prepared by steeping *A. boonei* stem bark in water (at room temperature) was lower compared to those obtained by refluxing the stem bark in water for between 2-64 hr (Table 4.3). There was no difference in the yield of refluxed extracts (4-64 hr), however, the yield of *A. boonei* refluxed in water for 2 hr was slighlty

lower compared to those refluxed for between 4-64 hr (Table 4.3). It would appear that boiling of *A. boonei* stem bark in water for more than 2 hrs may not significantly increase the total amount of extractives eluted from the plant.

Table 4.2 Yield of fractions from EtOH extract and isolated active antimalarial constituent AB-1

Extract	% Yield
Lipophilic fraction of EtOH extract ($EtOH_L$)	28.5
Hydrophilic fraction of EtOH extract ($EtOH_h$)	70.1
Methanol Soluble fraction of $EtOH_h$ extract (M_{sl})	80.9
Methanol insoluble fraction of $EtOH_h$ extract (M_i)	14.2
AB-1 (crystallizing from M_s)	21.4

Table 4.3 Yield of extracts of *A. boonei* stem bark obtained by varying the time of boiling

Extract	% Yield
cAQw	7.1
rAQw (2hr)	11.2
rAQw(4hr)	13.5
rAQw (8hr)	13.6
rAQw (16hr)	14.1
rAQw (32hr)	14.0
rAQw (64hr)	13.9

c = water extract obtained by steeping *A. boonei* stem bark in water (at room temperature) for 2 nights.

r = water extract obtained by refluxing *A. boonei* stem bark in water for different time intervals indicated

w = whole plant material (*A. boonei* stem bark). The water extract in this extraction is different from the AQ

extract earlier described in which the marc remaining after extracting with diethyl ether, ethyl acetate and ethanol

was later refluxed in water

4.2.2 Purity of (AB-1)

The TLC chromatogram of AB-1 in EtOAc : MeOH (70:30) showed a single spot with a retardation factor (Rf) of 0.56 (Fig. 4.1).

$$Rf = \frac{\text{Distance moved from origin by a solute}}{\text{Distance moved from the origin by solvent front}}$$

AB-1 neither produced quenching in UV-254nm nor fluoresced in UV-365nm. Treatment of the chromatogram with Dragendorff reagent gave a red-brown glowing spot which was not stable, disappearing within 3 min. The positive test of AB-1 with Dragendorff reagent indicates that AB-1 is an alkaloid. AB-1 melted between 280 - 210°c indicating that AB-1 is pure. HPLC analysis of AB-1 further showed that it is very pure (Fig. 4.2). The constituent giving the sharp peak was eluted at retention time of 2.07 min. The mobile phase was EtOAc/MeOH (70:30).

FIGURE 4.1

Fig. 4.1 Chromatogram of AB-1 on tlc plate
Eluent: EtOAc: MeOH = 70:30
Detection = Dragendorff reagent

FIG 4.2

Fig. 4.2. HPLC of AB-1
Eluent = EtOAc: MeOH (70:30)
λ = from 220 - 320nm
The eluent was injected first into the HPLC before injecting the sample

FIG 4.2

Fig. 4.2. HPLC of AB-1
Eluent = EtOAc: MeOH (70:30)
λ = from 220 - 320nm
The eluent was injected first into the HPLC before injecting the sample

CHAPTER FIVE

ACUTE TOXICITY STUDY

5.1 Introduction

The aim of acute toxicity test is to define the potential risk associated with the administration of a drug. In this work, **AB-1**, the isolated antimalarial constituent from *A boonei* stem bark was used. The study was designed to determine the medial lethal dose and to establish the dose-response relationship of the lethal action of AB-1 using oral and subcutaneous routes. The data from this study may provide preliminary and valuable insight into the pharmacological responses which may be associated with accidental poisoning with AB-1. Knowledge of LD50 may also be used as a guide for selecting screening doses of a drug for efficacy test. This is one reason why the acute toxicity test was carried out in this work.

5.2 Materials and Methods

5.2.1 Oral LD50 Determination The Up-and-Down method of Bruce (1985) was used. Thirty-two Swiss Albino mice divided into 8 equal groups were given single doses of

AB-1 orally. The control group of 4 mice received normal saline. All mice were observed over a period of 14 days.

5.2.2 Subcutaneous (Sc) LD50 determination

The same method as in oral LD50 determination was used. Fifty-six Swiss Albino mice were divided into 14 equal groups. Single doses of AB-1 were administered subcutaneously. The control group received equivalent volume of normal saline. All mice were observed over a period of 14 days.

5.3 RESULTS AND DISCUSSION

Lethality and behavioural changes

Death did not occur in mice treated with an oral dosage of AB-1 ranging from 10mg/kg to 1,280mg/kg (Table 5.1). No observable symptoms were also seen for one week after dosing the mice. This shows that the oral LD50 of AB-1 must be greater than 1,280mg/kg. When AB-1 was administered subculaneously, the LD50 was approximately 750mg/kg. Percent mortality at different doses are shown in Table 5.2. Death of mice occurred 45 min after the injection of AB-1. Mice treated with AB-1 also

showed toxic signs including crawling gait and shaking of heads at a dosage of 550mg/kg and above.

The absence of lethality at 1,280mg/kg oral dose of AB-1 and the very high subcutaneous LD50 suggest that AB-1 is better tolerated orally than subcutaneously. It is possible that the rate of detoxification and excretion of AB-1 was more rapid when administered orally than when given subcutaneously. It could also be that the bioavailability of AB-1 was reduced orally then subcutaneously. Other possible explanation are inactivation of AB-1 by enzymes in gastrointestinal tract (GIT) or liver or both.

Table 5.1: LD50 (oral) of AB-1 in male albino mice.

Group of mice*	Dose of AB-1 (mg/kg)	Mortality	Mortality (%)	LD 50 (mg/kg)
1	10	0/6	0	
2	20	0/6	0	
3	40	0/6	0	
4	80	0/6	0	
5	160	0/6	0	
6	320	0/6	0	
7	640	0/6	0	
8	1280	0/6	0	
9 (control normal saline)	0.2ml	0/6	0	

* n=6

The oral LD50 of AB-1 must be greater than 1,280 mg/kg.

Table 5.2 : LD50 (subcutaneous) of AB-1 in male albino mice.

Group of mice*	Dose of AB-1 (mg/kg)	Mortality	Mortality (%) (mg/kg)	LD 50
1	300	0	0	
2	350	0	0	
3	400	0	0	
4	450	0	0	
5	500	0	0	
6	550[a]	0	0	
7	600	0	0	
8	650	0	0	
9	700	1/6	16.7	
10	750	3/6	50	~750
11	800	5/6	83.3	
12	900	6/6	100	
13	1000	6/6	100	
14 (normal saline)	0.2ml	0/6	0	

* n=6
[a] toxic signs from 550 mg/kg

CHAPTER SIX

Antimalarial study of *A. boonei* stem bark extracts, fractions and isolated antimalarial constituent (AB-1)

6.1 Introduction

It has been mentioned in Chapter 1 that *A. boonei* (AB) is widely used to treat fever and malaria in traditional medicine. The culture of using the plant as a remedy for malaria may continue for a long time. Studies on the antimalarial activities of the plant have been restricted largely to *in vitro* models; results from such studies lend support to the traditional use of *Alstonia* species (Phillipson *et al.*, 1993). However, nothing appears to be known of the *in vivo* activity or mode of action of these plants (Phillipson *et al.*, 1993, Wright *et al.*, 1993).

Considering the prevalent use of AB to treat malaria and the paucity of information, particularly on the *in vivo* antimalarial activities, there is a need for further studies on the plant extracts to put to rest the question whether AB or its chemical constituents have antimalarial properties for which they are used in traditional medicine to treat malaria. A systematic bioassay guided fractionation will be required to resolve the question.

In this chapter, the activities of crude extracts, fractions and the isolated antimalarial chemical constituent (AB-1) were assessed in chloroquine sensitive *P. y. nigeriensis* and chloroquine resistant *P. berghei* ANKA infections in mice.

6.2 Experimental procedure

6.2.1 Evaluation of the blood schizontocidal activity of soxhlet extracts of *A. boonei* stem bark in early infection (4-day test).

The method of Knight and Peters (1980) described in 3.16 was used. Mice infected on D_0 were allocated into several groups of 5 mice eech, and doses of 100, 200, 400 and 800mg/kg of each of DE, EtOAc, EtOH and AO extracts of the plant were administered orally either once or twice daily. One group of mice received orally a dose of 5mg/kg chloroquine (the reference drug) while another group received orally 0.4ml of the vehicle (5% Tween 80). Administration of the extract / chloroquine/ vehicle was repeated on D_1, D_2 and D_3. A thin blood film of each mouse was made on D_4 and the percentage parasitaemia calculated using the formula in section 3.16.

6.2.2 Evaluation of the blood schizontocidal activity of soxhlet and cold exracts of *A. boonei* stem bark in early infection

The aim of this experiment was to find out whether the blood schizontocidal action of the soxhlet extracts used in the previous test was affected by heat. In this experiment, a single high dose (400mg/kg) of each of the soxhlet or cold extracts was administered orally to groups of 5 mice as shown in Table 6.0. The extracts, chloroquine or 5% 'Tween 80' were administered on D_0, D_1, D_2 and D_3. A thin blood film of each mouse was made on D_4 and the percentage parasilaemia of each mouse calculated using the formula in section 3.16.

6.2.3 Evaluation of blood schizontocidal activity of EtOH and AQ extracts of *A. boonei* stem bark in an established infection.

The method of Ryley and Peters (1970) described in 3.17 was used. Seventy mice infected as decribed previously were allocated in fives into 10 groups. Doses of 100, 200, 400 and 800mg/kg of either EtOH or AQ extracts were administered orally 72hr post infection (D_3). In some cases, EtOH was administered twice daily to groups of mice. Chloroquine (5mg/kg) and 0.4ml of 5% 'Tween 80' were administered to positive (chloroquine treated) and negative control (Tween 80-treated) groups respectively.

Table 6.0

Group of mice	Extract or drug	Dose (mg/kg)
1	DE extract	400
2	cDE extract	400
3	EtOAc extract	400
4	cEtOAc extract	400
5	EtOH extract	400
6	cEtOH extract	400
7	AQ	400
8	cAQ	400
9	Chloroquine	5
10	5% 'Tween 80'	0.4ml

c = cold.

Drug administration continued daily up to D_6. Thin blood films were made daily for 5 days (D_3-D_7) and average percentage parasitaemia plotted against time in days. The number of deaths in each group was recorded daily for 30 days to give the mean survival period (MSP) for each extract, chloroquine and 5% 'Tween 80' and their respective doses.

6.2.4 Evaluation of the repository activity of EtOH extract of *A. boonei* stem bark

The method used has been described in 3.18. Fifty mice were allocated in fives into 10 groups. One group served as the positive control and received 1.5mg/kg of pyrimethamine. Another group, a negative control, received 5% Tween 80. The remaining 8 groups received 100, 200, 400 or 800mg/kg of EtOH extract either once or twice daily. Drugs were administered orally for 3 consecutive days (D_0, D_1, D_2). On D_3, all the mice were challenged with $1x10^7$ pRBCs. Thin blood smears were made from each mouse 72hr post infection (D_6) and the average percentage parasitaemia determined. Number of death in each group was recorded and the MSP determined.

6.2.5 Evaluation of blood schizontocidal activity of $EtOH_L$ and $EtOH_h$ in an established infection.

Twenty five mice were allocated in fives into 5 groups. Mice were infected with *P. y. nigeriensis* as previously described. A dose of 400mg/kg of EtOH, $EtOH_h$ and $EtOH_L$ (see section 4.1.3) was administered orally 72hr post infection (D_3). The activities of $EtOH_h$ and $EtOH_L$ were compared with the parent extract (EtOH). Chloroquine (5mg/kg) and 5% 'Tween 80' served as positive and negative controls, respectively. Drug administration continued up to D_6. Assessment of drug activity was as described in 6.2.3.

6.2.6 Evaluation of the repository activity of $EtOH_L$ and $EtOH_h$

The procedure was essentially similar to that described in section 6.5. Twenty mice were allocated into four groups. One group received pyrimethamine (1.5mg/kg). The remaining groups received 400mg/kg of either $EtOH_L$, $EtOH_h$ or EtOH. Drugs were administered orally for 3 consecutive days (D_0, D_1, D_2). On day 3, all mice were challenged with $1x10^7$ pRBCs. The remainder of the procedure was identical to that described in 6.2.4.

6.2.7 Evaluation of blood schizontocidal activity of methanol soluble (M_s) and methanol insoluble (M_i) subfractions of $EtOH_h$ in an established infection.

Twenty mice infected as previously described were allocated in fives into 4 groups. One group received 100mg/kg of M_s while another group received 100mg/kg of M_i. The remaining 2 groups received either chloroquine (5mg/kg) or 5% 'Tween 80'. Drug administration continued up to D_6. Drug activity was assessed as described in 6.2.3.

6.2.8 Evaluation of blood schizontocidal activity of (AB-1) in *P. y. nigeriensis* established infection.

Thirty five mice infected as previously described were allocated in fives into 7 groups. Doses of 5, 10, 20, 40 and 80mg/kg of AB-1 were administered orally to 5 groups of mice. The remaining 2 groups received either 5mg/kg chloroquine or 0.4ml of 5% Tween 80. Drugs were administered between D_3 and D_4 postinfection. Assessment of drug activity was as described in section 6.2.3.

6.2.9 Evaluation of blood schizontocidal activity of AB-1 in *P. berghei* ANKA established infection.

Thirty rats were used. Infection of rats was as previously described. Animals were allocated in fives into 6 groups. Doses of 10, 20, 40 and 80mg/kg of AB-1 were administered orally to 4 groups of rats. The remaining 2 groups received either 5mg/kg chloroquine or 5% 'Tween 80'. Drugs were administered for 4 days starting from D3, post infection. Assessment of drug activity was as described in section 6.2.3.

6.2.10 Evaluation of the blood schizontocidal activity of AB-1 in early infection (4-day test).

The procedure has been described in 6.2.1. Thirty mice were used. Doses of 10, 20, 40 and 80mg/kg of AB-1 were administered orally to *P. y. nigeriensis* infected mice. Chloroquine (5mg/kg) and 5% 'Tween 80' were administered to positive and negative controls, respectively.

6.2.11 Evaluation of the repository activity of AB-1 in *P. y. nigeriensis* infection.

The procedure was identical to that described in 6.2.4. Thirty mice were used. Doses of 10, 20, 40 and 80mg/kg of AB-1 were administered orally or subcutaneously to *P. y.*

nigeriensis infected mice. Pyrimethamine (1.5mg/kg) and 5% 'Tween 80' served as controls.

6.2.12 Evaluation of the effect of prolonged boiling on the blood schizontocidal activity of water extract of whole stem bark of *A. boonei*

Extracts of *A. boonei* stem bark obtained by refluxing the crushed stem bark in water for varying time intervals were assessed for antimalarial activity in *P. berghei* ANKA infection as described in 6.2.4. Thirty five rats were used. 400mg/kg of each of the refluxed extracts was administered orally to rats which were later challenged with $1x10^7$ rRBCs of *P. berghei* ANKA. Thin blood smears were made and percent suppression of parasitaemia was calculated.

6.3 RESULTS

6.3.1 Course of infection of *P. y. nigeriensis* and *P. berghei* ANKA

Infection induced by intraperitoneal inoculation of $1x10^7$ red blood cells parasitized with *P. y. nigeriensis* or *P. berghei* ANKA was viable in mice. Infected, non-treated mice were usually patent on D3, 72hr post-inoculation (post-infection) with parasitaemia ranging from 15-16%.

Parasitaemia increased rapidly initially, and then slowly until between D6 and D8 (post-infection) when infected, non-treated controls began to die. That is, the mean survival period (MSP) ranged from 6-8 days. Parasitaemia before death of animals was usually above 50%. The course of infection of *P. y. nigeriensis* in mice and *P. berghei* ANKA in rats was similar. However, death in *P. berghei* infected rats usually occurred between D8 and D10.

6.3.2 Blood schizontocidal actions of extracts of *A. boonei* stem bark in early infections (4-day test).

The degree of parasitaemia in mice treated with doses of diethylether extract (DE) of *A. boonei* ranging from 100-800mg/kg did not differ significantly from that of the control ($P \geq 0.05$) (Table 6.1). When DE was administerd twice daily, there was little or no difference in the chemosuppression produced compared with single daily administration (Fig. 6.1).

Mice treated with EtOAc (100-400mg/kg) also experienced a high level of parasitaemia that was not significantly different from the control (Table 6.2). It was only

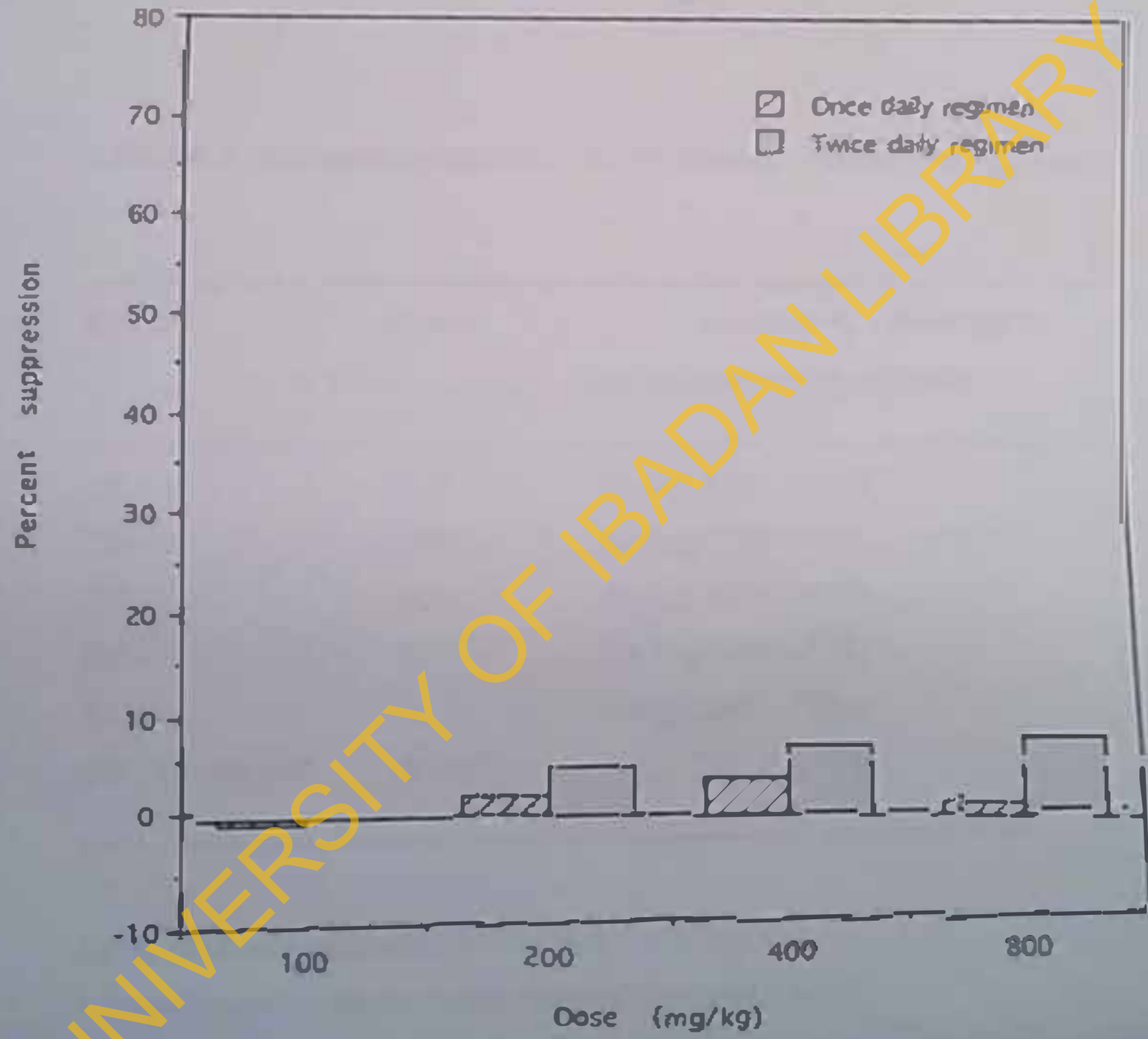

Fig. 6.1. Blood schizontocidal actions of De administered once or twice daily in early infection (4 - day test).

Table 6.1: Schizontocidal action of DE in early infection (4 - day test).

Extract	Dose (mg/kg)	Average % parasitaemia	Average % suppression
DE	100	16.4 ± 1.35	-0.60
DE	200	16.0 ± 1.39	1.84
DE	400	15.2 ± 2.10	6.75
DE	800	16.1 ± 1.60	1.23
CQ	5	2.3 ± 0.41*	85.9
5% 'Tween 80'	0.4ml	16.3 ± 1.66	-

Drug was administered once daily for 4 days.

* Significant compared with control (Dunnet t-test).

Table 6.2: Schizontocidal action of EtOAc in early infection (4 - day test).

Extract	Dose (mg/kg)	Average % parasitaemia	Average % suppression
EtOAc	100	16.0 ± 1.56	1.64
EtOAc	200	15.7 ± 0.98	3.7
EtOAc	400	15.8 ± 0.56	3.1
EtOAc	800	13.4 ± 1.20*	17.6
CQ	5	2.3 ± 0.41*	85.9
5% 'Tween 80'	0.4ml	16.3 ± 1.66	-

Drug was administered once daily for 4 days.

* Significant compared with control (Dunnet t-test).

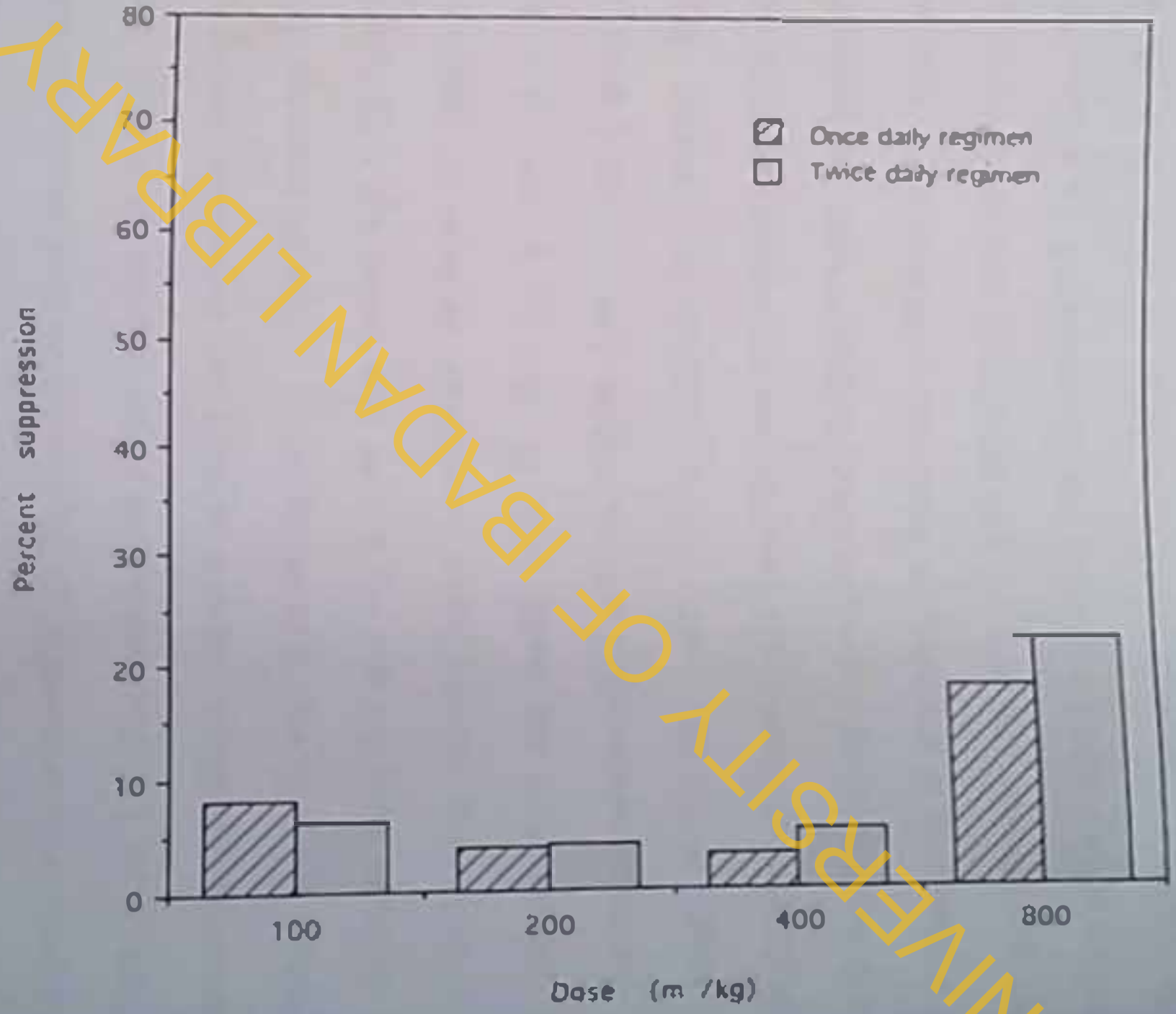

Fig. 6.2 Blood schizontocidal actions of EtOAc administered once or twice daily in early infection (4-day test)

at the highest dosage of 800mg/kg that the degree of parasitaemia was statistically significant compared with the control ($P \leq 0.05$); the suppression of parasitaemia at this dose was 17.8% (Table 6.2). Chloroquine gave a chemosuppression of 85.9%. Twice daily administration of EtOAc did not produce an appreciable increase in chemosuppression over once daily regimen (Fig. 6.2). The schizontocidal action of EtOH is shown in Table 6.3 and Fig. 6.3. All mice treated with EtOH with the exception of the lowest dosage group experienced decrease of parasitaemia significantly different from the control ($P \leq 0.05$). The extract produced a dose dependent chemosuppressive effect with the highest dose (800mg/kg) giving the maximum effect of 61.4%, while 5mg/kg Chloroquine produced 85.9% chemosuppressive effect (Table 6.3). When EtOH was administered twice daily, there was a small increase in chemosuppressive effect produced by each dose tested (Fig. 6.3). At the highest dose of 800mg/kg, the chemosuppression produced by twice daily regimen was approximately 72% compared with that of single administration which was 57%. The result of the schizontocidal action of aqueous extract (AQ) is shown in Table 6.4 and Fig. 6.4. The chemosuppression produced by the active doses (400 and 800mg/kg) of AQ were 16.6 and 27%, respectively. CQ gave chemosuppressive effect

of 85.9%. When AQ was administered twice daily, the active doses gave chemosuppressive effects that were significantly higher than those produced by single daily regimen (Fig. 6.4).

Comparison of chemosuppressive effect of EtOH and AQ shows that for each dose of extract above 100mg/kg, the chemosuppression produced by EtOH was more than twice that of AQ (Table 6.5).

The result of the test comparing the schizontocidal actions of soxhlet and cold extracts of *A. boonei* stem bark is shown in Fig. 6.5. The dose of all extracts tested was 400mg/kg. The chemosuppressive actions of the cold (c) and soxhlet extracts were comparable and for the most part similar (Fig. 6.5). The chemosuppression produced by cold and soxhlet extracts of DE and EtOAc were below 10% and were not significantly different from each other. Also, the suppression of parasitaemia produced by soxhlet extracts of EtOH and AQ was not significantly different from that produced by the cold extract of either EtOH or AQ (Fig. 6.5).

Table 6.3: Schizontocidal action of EtOH extract in early infection (4 - day test).

Extract	Dose (mg/kg)	Average % parasitaemia	Average % suppression
EtOH	100	16.0 ± 1.44	3.1
EtOH	200	13.4 ± 1.67*	17.2
EtOH	400	9.8 ± 0.98*	39.9
EtOH	800	6.3 ± 1.0*	61.4
CQ	5	2.3 ± 0.41*	85.9
5% 'Tween 80'	0.4ml	16.3 ± 1.66	-

Drug was administered once daily for 4 days.
* Significant compared with control (Dunnet t-test).

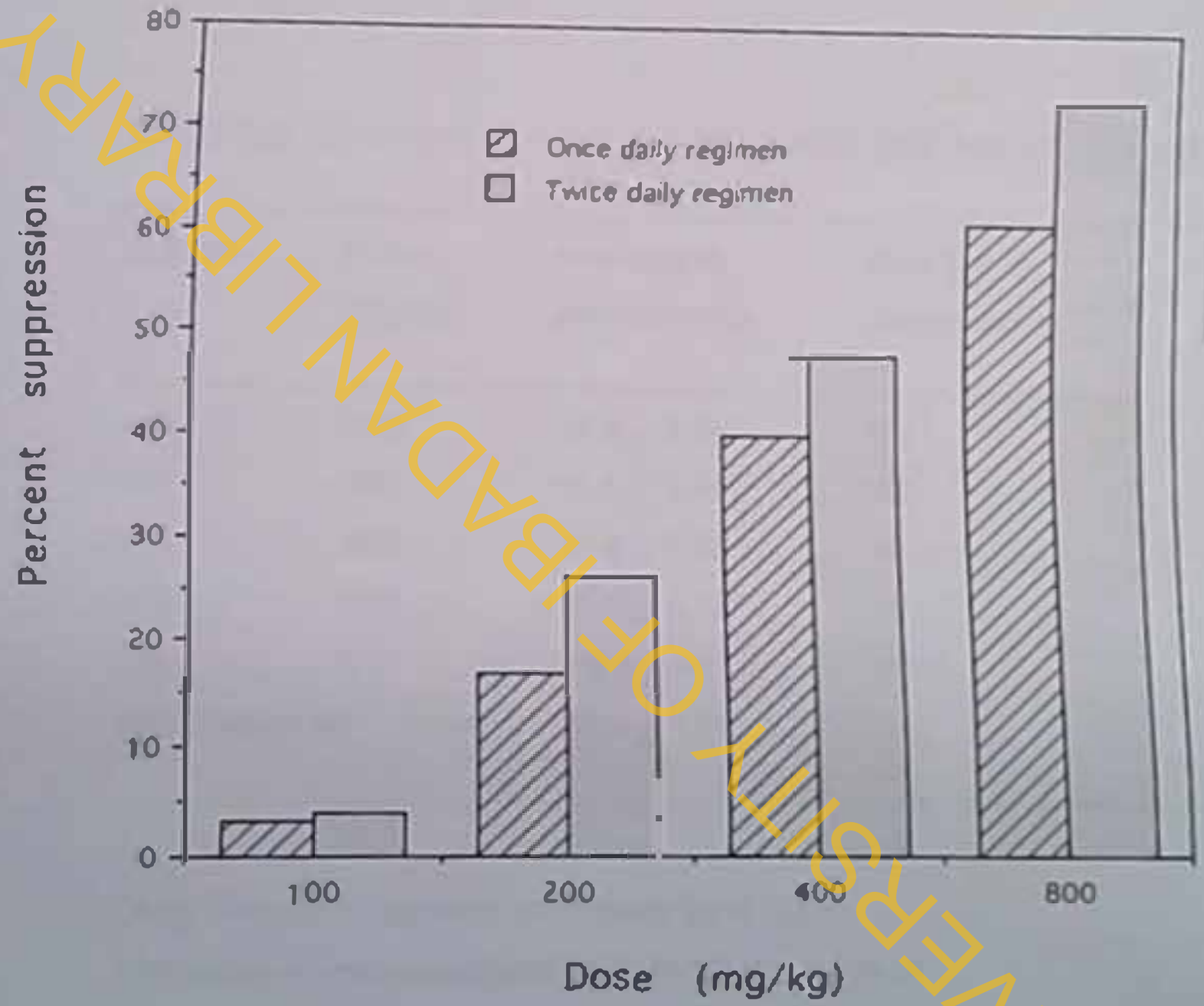

Fig. 6.3 Blood schizontocidal activity of EtOH administered once or twice daily in early infection (4-day test).

Table 6.4: Schizontocidal action of AQ in early infection (4 - day test).

Extract	Dose (mg/kg)	Average % parasitaemia	Average % suppression
AQ	100	15.9 ± 1.54	2.5
AQ	200	14.9 ± 1.46	8.9
AQ	400	13.6 ± 0.09*	16.6
AQ	800	11.7 ± 2.12*	27.0
CQ	5	2.3 ± 0.41*	85.9
5% 'Tween 80'	0.4ml	16.3 ± 1.66	-

Drug was administered once daily for 4 days.

* Significant compared with control (Dunnet t-test).

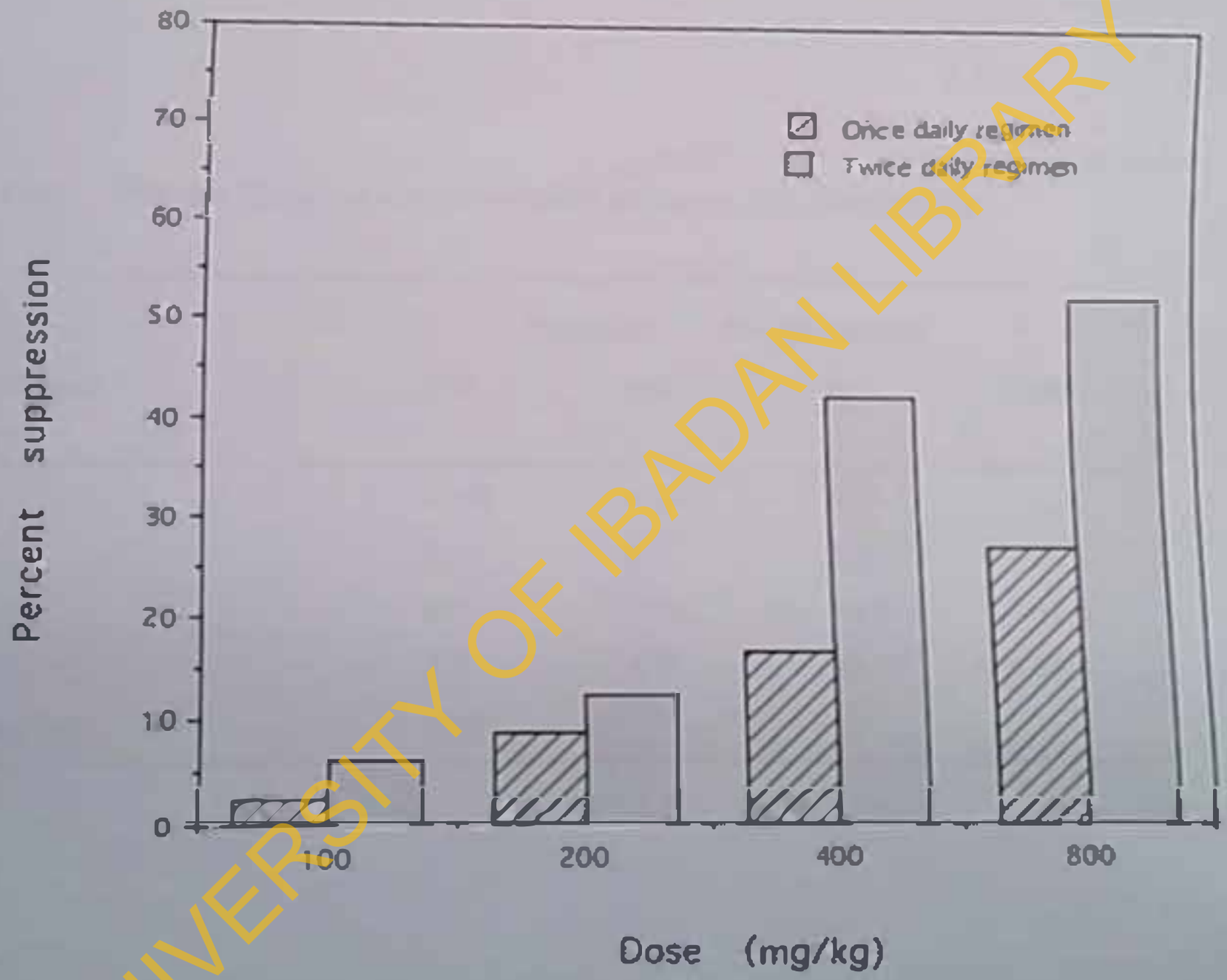

Fig. 6.4. Blood schizontocidal actions of AQ administered once or twice daily in early infection (4 - day test).

Table 6.5: Percent Suppression of soxhlet extracts and chloroquine.

Extract/Doses (mg/kg)	Percent Suppression			
	100	200	400	800
DE	-0.60	1.84	6.75	1.23
EtOAC	7.9	3.7	3.1	17.8
EtOH	3.1	17.2	39.9	61.4
AQ	2.5	8.9	16.6	27.0
CQ (5mg/kg)	85.9			

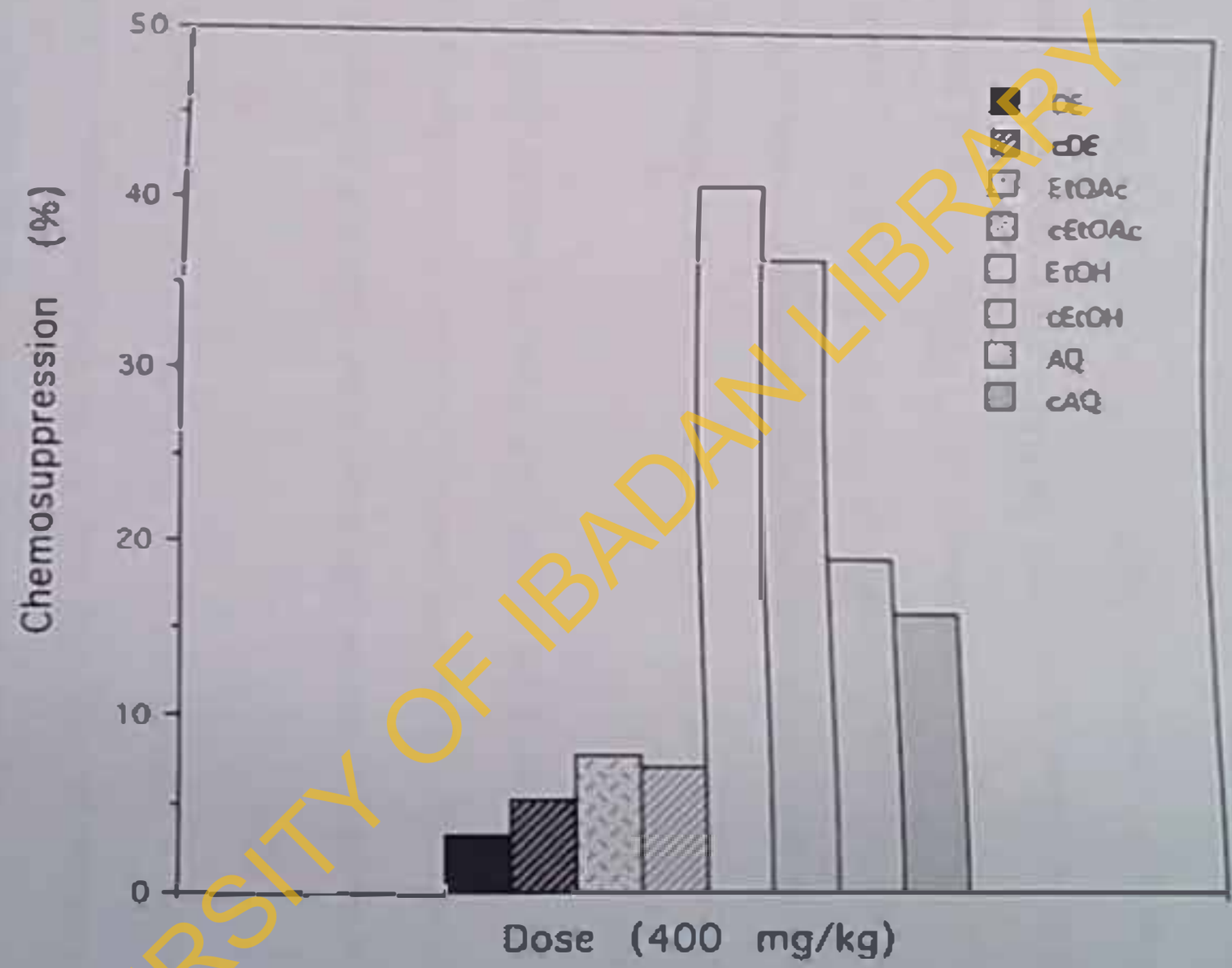

Fig. 6.5. Chemosuppression of soxhlet and cold extracts of A. boonei stembark in P. y. nigeriensis malaria (4- day test).

6.3.3 Effect of EtOH on an established infection (Rane's test)

Control mice experienced a gradual increase in parasitaemia from D3 to D7 (Fig 6.6). One mouse died on D5. Mice that received chloroquine (5mg/kg) experienced a marked reduction in parasitaemia daily (Fig. 6.6). By D8, the parasitaemia in these mice had dropped from 15.1 ± 1.4% to 0.9 ±0.32. The mean survival period (MSP) of control mice was 7.3 ± 0.37 days.

Mice that were infected and treated with lower doses (100 and 200mg/kg) of EtOH either once or twice daily, did not experience a reduction in parasitaemia that was significantly different from that of the control mice (Figs. 6.6 and 6.7). Although mice treated with 400 and

800mg/kg of EtOH extract, either once or twice daily, did not experience a decrease in parasitaemia, the parasitaemias in these mice were nevertheless significantly different from the control and the two low-dosed groups (Figs. 6.6 and 6.7). Similarly, the MSPs of the high-dosed groups were significantly different ($P < 0.05$) from those of the low-dosed groups (Table 6.6). In either applications, the MSPs of the group treated with 800mg/kg of EtOH extract were significantly higher ($P \leq 0.05$) than the MSPs of mice treated with lower doses of the extract (Table 6.6). In addition, the MSP of the group

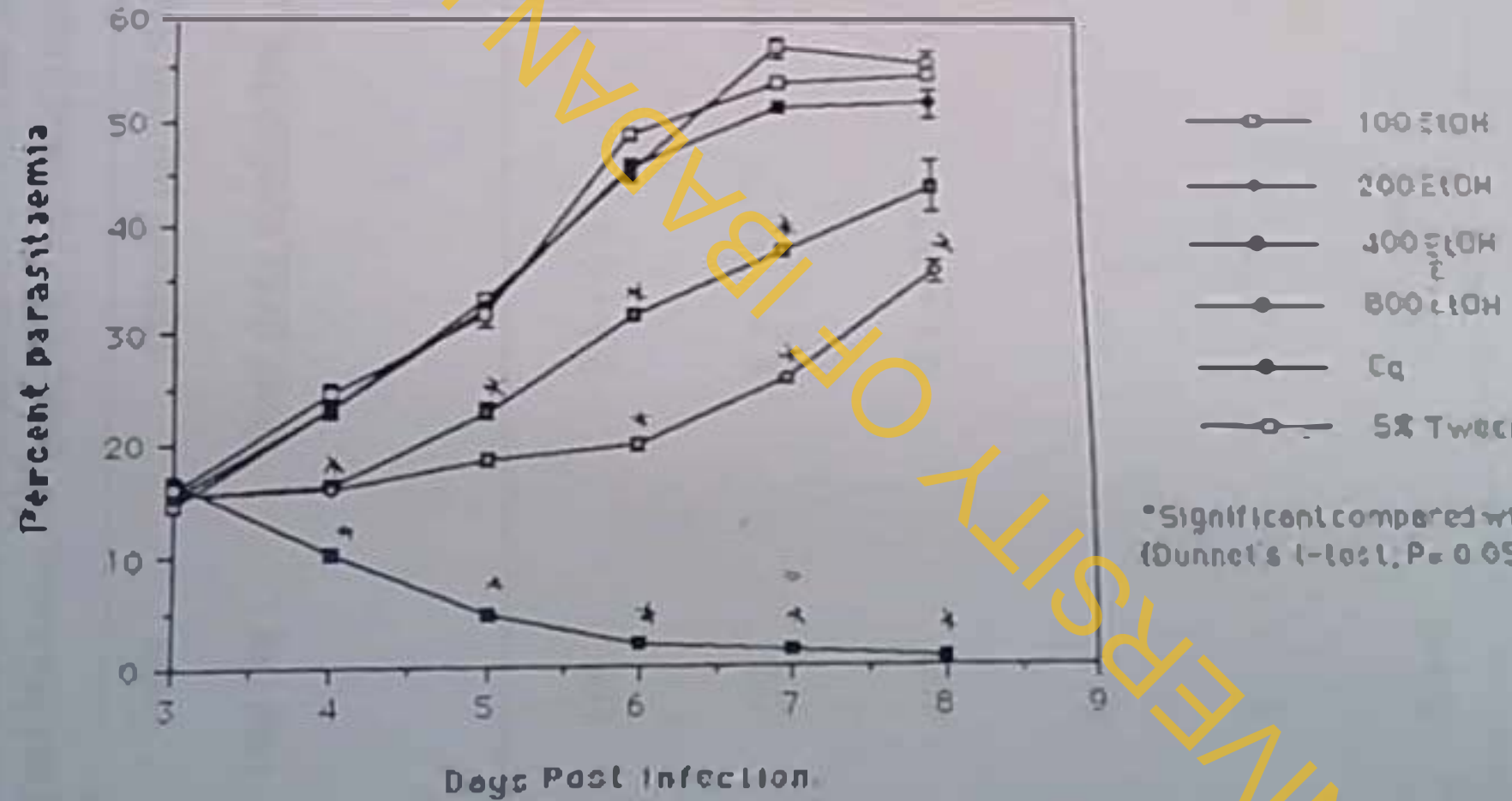

Fig. 6.6. Effect of soxhlet extract of EtOH on P. y. nigeriensis parasitaemia in Rane's test. Extracts were administered orally and once daily. Vertical lines indicate sem

Table 6..6 Mean survival period of infected mice trected once and twice daily with EtOH extract, CQ and 5% Tween 80' (once daily) in Rane's test.

Drug / extract application	Dose (mg /kg)	Average percent Suppression	
		Once daily application	Twice daily
EtOH	100	7.4 ± 0.4^{a}	7.3 ± 0.42^{a}
EtOH	200	8.2 ± 0.3^{abc}	7.9 ± 36^{ab}
EtOH	400	$9.4 \pm 0.5^{c*}$	$11.2 \pm 0.71^{bc*}$
EtOH	800	$10.6 \pm 0.4^{d*}$	$17.2 \pm 0.51^{c*}$
CQ	5	$30 \pm 0.0^{f*}$	
5% Tween 80'	0.4ml	7.3 ± 0.37^{a}	

Significant compared with control ($P \leq 0.05$) using Dunnet t - test.
Means followed by the same letters in each column or roll are not significantly different at ($P \leq 0.05$) following Duncan's Multiple Range Test

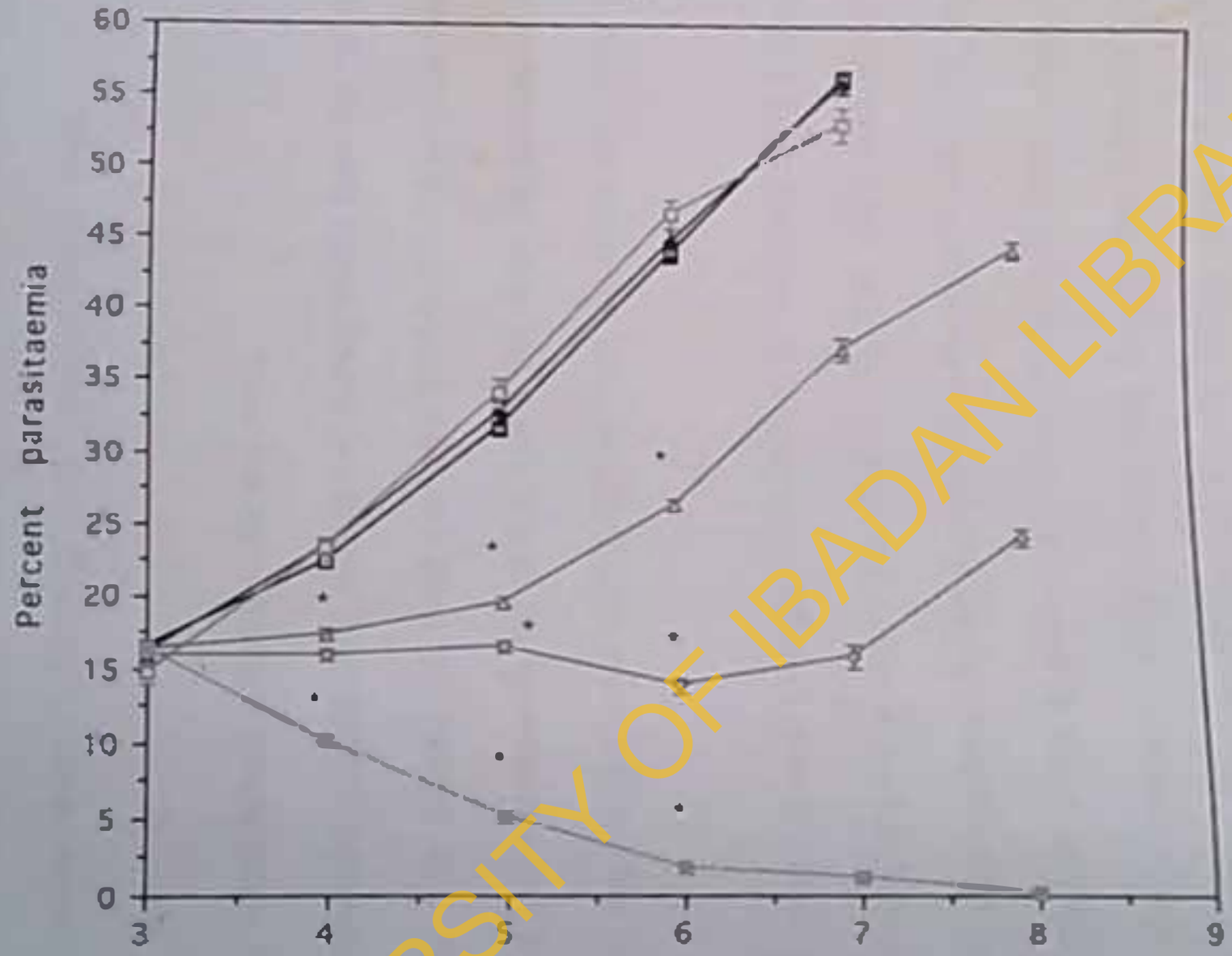

Fig. 6.7. Effect of soxhlet extracts of EtOH on P. y. nigeriensis parasitaemia in Rane's Test. Extracts were administered orally and twice daily. Vertical lines indicate s.e.m.

treated twice daily with 800mg/kg of EtOH twice daily was more than twice that of the control (Table 6.6). Mice treated with CQ (5mg/kg) lived for more than 30 days

6.3.4 Effect of AQ on an established infection (Rane's test).

Control mice experienced a rapid daily increase in parasitaemia from D3 to D7, when death occurred (Fig. 6.8). In contrast, mice treated with chloroquine experienced a drastic daily reduction in parasitaemia up to D6 when the drop in parasitaemia became gradual. By D8, the parasitaemia had dropped from approximately 15% to 0.9%.

Mice treated with lower doses (100 and 200mg/kg) of AQ exhibited a daily increase in parasitaemia comparable to that of the control (Fig. 6.8). Mice treated with 400 and 800 mg/kg of AQ also experienced an increase in parasiteamia with time, but the increase was rather gradual unlike in control and mice treated with lower doses of AQ extract in which the daily increase in parasitaemia was draslic. The MSPs of the control mice and the group that received 800mg/kg of AQ extract were 7.6 and 9.9 days, respectively (Table 6.7). The group treated with CQ extract lived beyond the 30 day observation period. The MSP of the group treated with 800mg/kg was significanlly greater ($P \leq 0.05$)than the control, but not up to twice that of the control (Table 6.7).

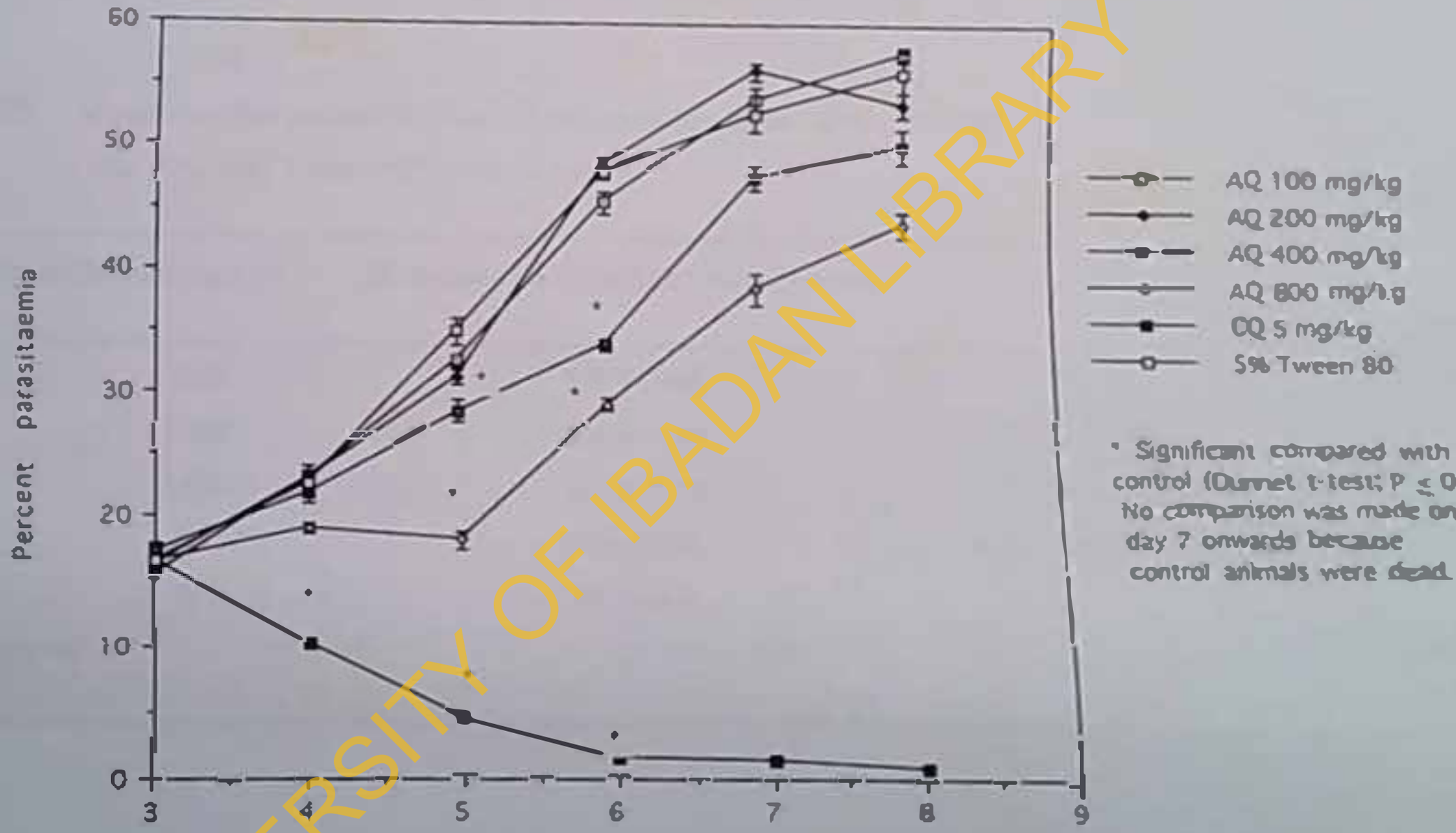

Fig. 6.8 Effect of AQ on P. y. nigeriensis parasitaemia in Rane's test. Extracts were administered orally and once daily. Vertical lines indicate sem.

Table 6.7: Mean survival period of infected mice treated once daily with AQ, CQ and 5% 'Tween 80' in Rane test.

Drug/extract	Dose (mg/kg)	Mean survival period (days)± sem
AQ	100	7.5 ± 0.49
AQ	200	7.4 ± 0.55
AQ	400	8.7 ± 0.39
AQ	800	9.9± 0.72
CQ	5	30 ± 0.0
5% 'Tween 80'	0.4 ml	7.6 ± 0.51

6.3.5 Repository action of EtOH

The results are shown in Fig. 6.9 and Tables 6.8 and 6.9. EtOH was administered orally, once and twice daily to mice. Percent parasitaemia in the control was approximately 15%. In contrast, percent parasitaemia in the group treated with pyrimethamine (1.5 mg/kg) was 2.1% with a chemosuppression of 86.7% (Table 6.8). EtOH at the doses used did not prevent infection. The percent parasitaemia in the lowest-dose group was comparable to that of the control. Higher doses, especially 400 and 800mg/kg of EtOH produced marked reduction in parasitaemia (Fig. 6.9) with the highest dose producing a chemosuppresion of approximately 47 and 53% when given once or twice daily, respectively.

The percent parasitaemia exhibited when doses of 400 and 800mg/kg were administered once daily were approximately 9% and 7-8%, respectively. When the same doses were administered twice daily, the percent parasitaemias slightly dropped to approximately 8 and 7%. The Perent parasitaemia exhibited by 400 and 800mg/kg in the two regimens were significantly different from that of the control. In addition they were dose-related (Fig. 6.9).

The MSPs of the mice treated with lower doses of EtOH (100 and 200mg/kg) either once or twice daily were not significantly different from that of the control which

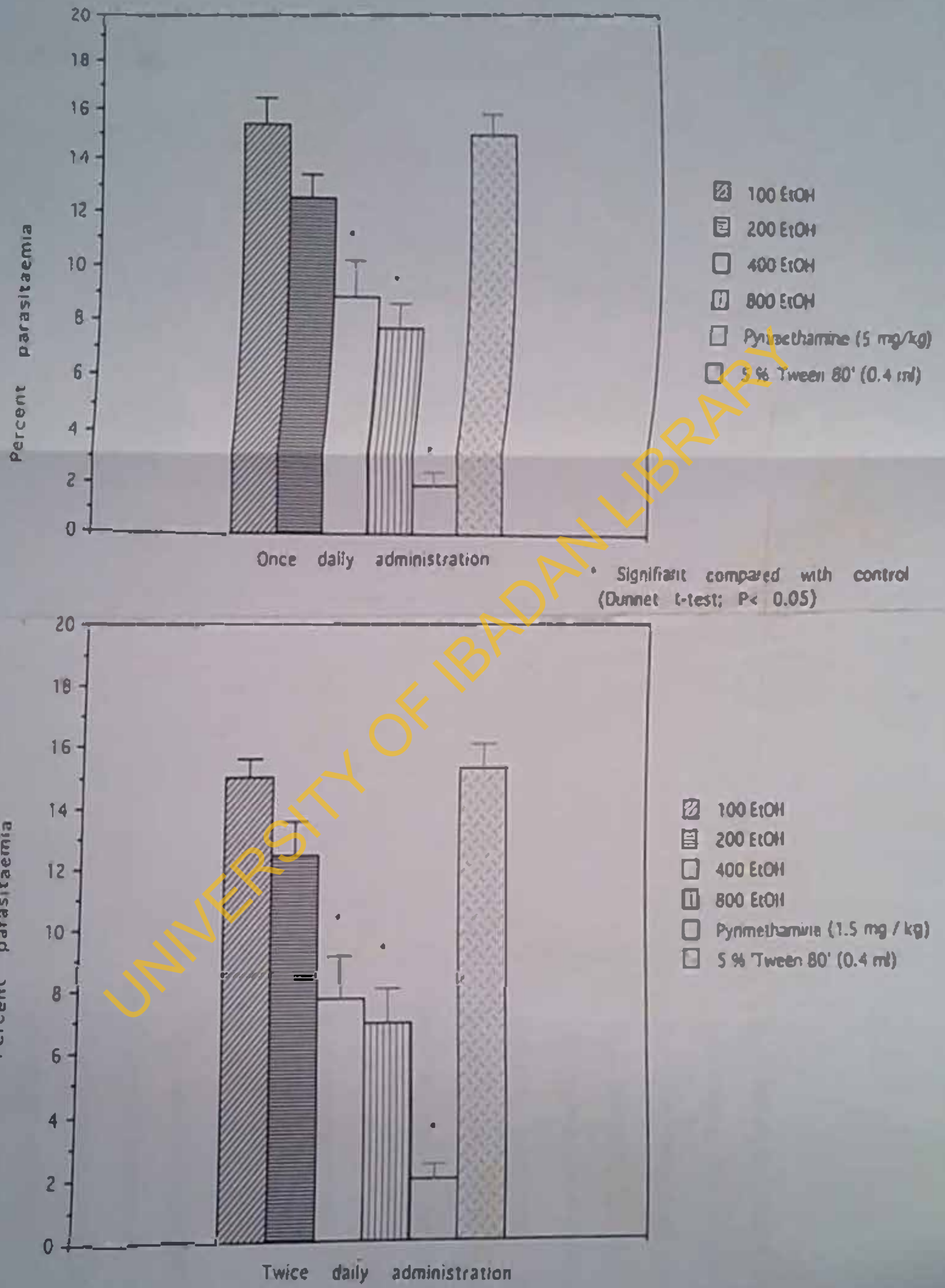

Fig. 6.9. Repository activity of EtOH on P. y. nigeriensis parasitaemia. EtOH was administered orally (Top) once daily and (bottom) twice daily. Pyrimethamine was administered orally and once daily. Vertical lines indicate s.e.m.

was 7.9 days. In contrast, the MSPs of mice treated with higher doses (400 and 800 mg/kg) were significantly higher than that of the control ($P \leq 0.05$; Table 6.9). In addition, the MSP of the group treated with 800 mg/kg, twice daily, was more than twice that of the control. Mice treated with pyrimethamine lived longer than 30 days (the observation period).

6.3.6 Blood schizontocidal actions of EtOH subfractions ($EtOH_L$ and $EtOH_H$) in Rane's test.

$EtOH_L$ (lypophilic portion) and $EtOH_H$ (hydrophilic portion) were the sub-fractions of EtOH obtained by liquid-liquid extraction. As shown in Fig. 6.10, the control mice and the mice treated with $EtOH_L$ experienced a daily increase in parasitaemia. All the animals in the control group died by D7. 4/5 of the mice treated with $EtOH_L$ died by D7. The remainder did not die until D16. This mouse experienced a somewhat erratic change in percent parasitaemia. The percent parasitaemia increased to 32% on D5. By D7, the parasitaemia had dropped to 17%. It started to rise again by D8 and the increase was gradual up to D16 when death occurred (not shown in the graph).

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Table 68 Schizontocidal action of EtOH extract administered once or twice daily in reponsitory test.

Drug / extract	Dose (mg/kg)	Average percent Suppression	
		Once daily application	Twice daily
application			
EtOH	100	3.3	0.3
EtOH	200	16.7	18
EtOH	400	40.0	48
EtOH	800	46.7	53.3
Pyrimethamine	1.5	86.7	-
5% Tween 80'	0.4ml	-	-

Table 6.9: Mean survival period of mice in which residual activity of EtOH extract was investigated

Drug/extract	Dose (mg/kg)	Mean survival period (days) ± sem	
		Once daily application	Twice daily application
EtOH	100	7.3 ± 0.53a	7.9 ± 0.51a
EtOH	200	7.0 ± 0.46a	7.3 ± 0.37ab
EtOH	400	10.3 ± 0.41b	11.98 ± 0.77b*
EtOH	800	12.3 ± 0.61b*	18.8 ± 0.83c*
Pyrimethamine	5	30 ± 0.00d*	
5 % 'Tween 80'	0.4ml	7.9 ± 0.45a	

Pyrimethamine served as a reference drug

* Significant compared with control ($p \leq 0.05$) Dunnet t-test

Means followed by the same letters in each column or row are not significantly different at ($P \leq 0.05$) following Duncan's Multiple Range Test

erratic parasitaemia expressed by this particular mouse may be due to sequestration of parasite in tissues.

Mice treated with EtOH and $EtOH_h$ experienced daily increases in parasitaemia which were significantly lower ($P \leq 0.05$) compared to the control and $EtOH_L$-treated mice. It should be pointed out that mice treated with $EtOH_h$ experienced a fall in parasitaemia on D5 before their percent parasitaemia gradually increased on D6 up to D8 (Fig. 6.10). Mice treated with CQ experienced a steady decrease in parasitaemia up to D8 when the experiment was terminated.

6.3.7 Repository action of EtOH, $EtOh_L$ and $EtOH_h$

The degree of parasitaemia in the negative control group was approximately 16%. Pyrimethamine gave a chemosuppression of 88% while EtOH, $EtOh_L$ and $EtOH_h$ produced chemosuppressive effects of 46, 2 and 63%, respectively (Fig. 6.11). The repository activity of $EtOH_h$ was 1.5 times higher than that of the parent extract (EtOH) whereas the repository activity of $EtOh_L$ was 23 times lower than that of the parent extract.

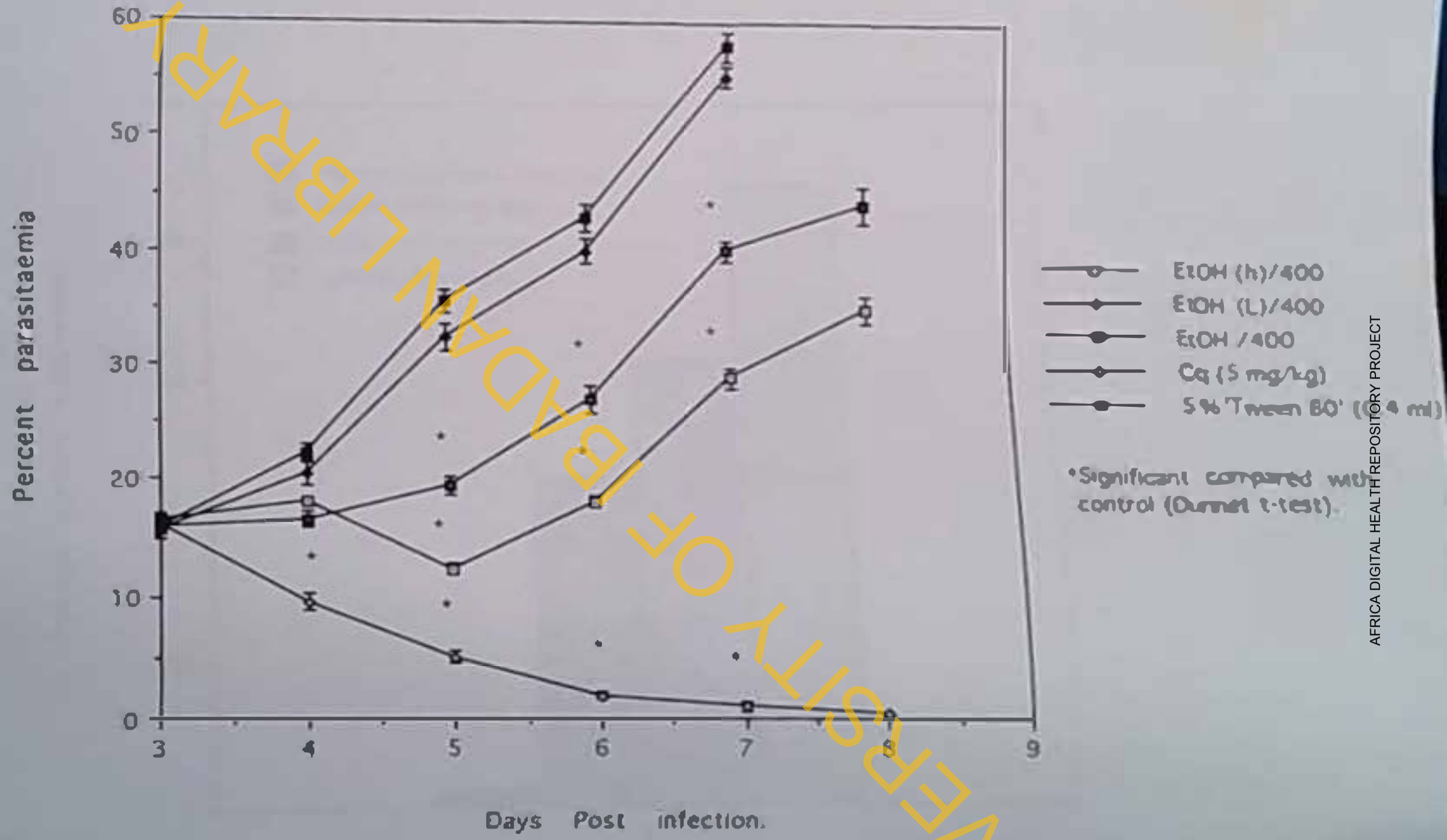

Fig. 6.10. Effect of EtOH(h), EtOH(L), EtOH, chloroquine on *P. y. nigeriensis* parasitaemia in Rane's Test. Extracts/drugs were administered orally and once daily. Vertical lines indicate s.e.m.

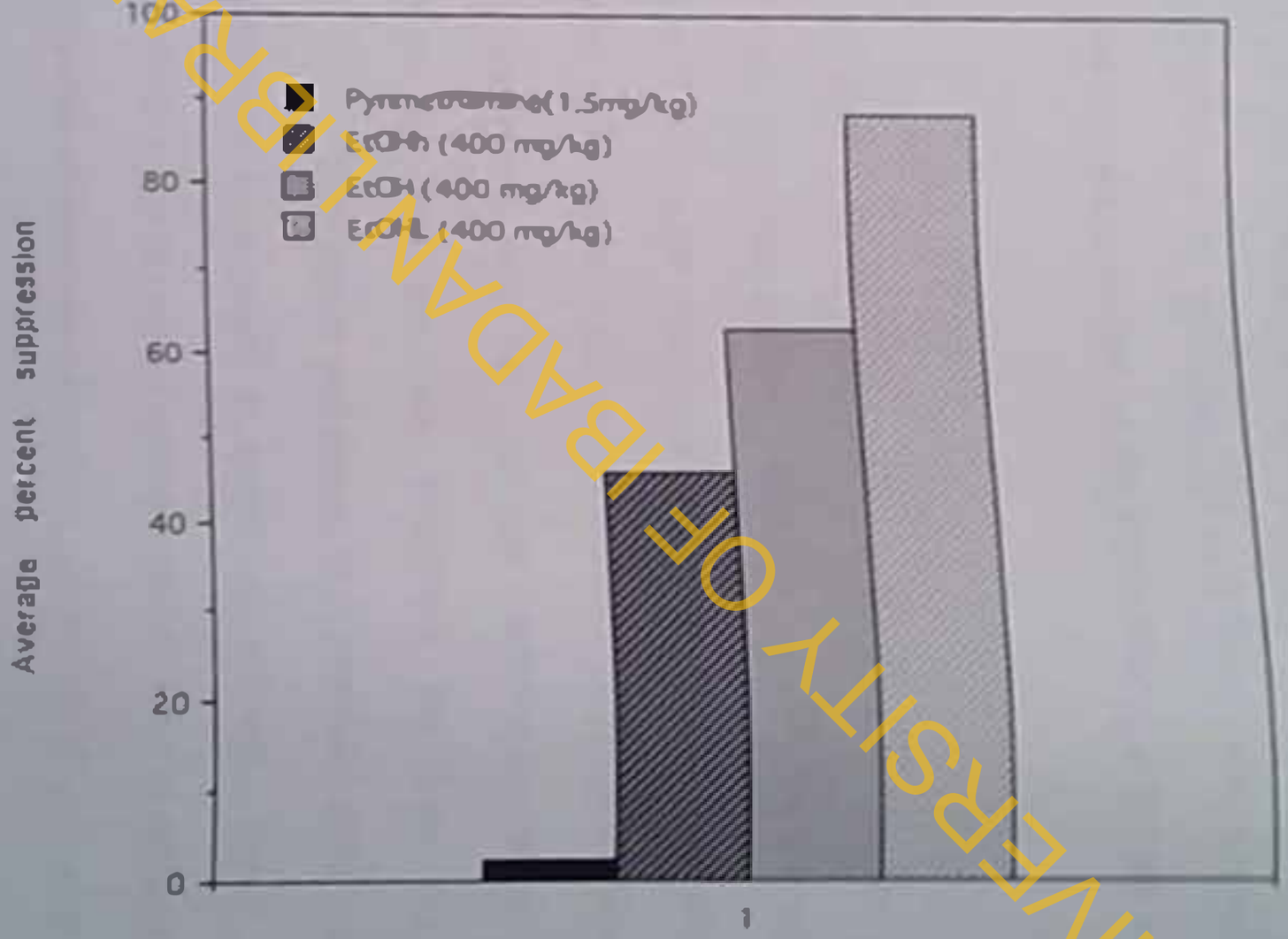

Fig. 6.11. Repository activity of EtOH, EtOH, EtOH and Pyrimethamine against P. y. nigeriensis. Extracts were administered orally.

6.3.8 Blood schizontocidal actions of M_s, M_i and AB-1 in Rane test.

The result is shown in Fig. 6.12. The methanol insoluble fraction (M_i) of $EtOH_h$ was not active against *P. y. nigeriensis*. The percent parasitaemia increased daily as in the control. In contrast M_s, the methanol soluble fraction of $EtOH_h$ showed a moderate schizontocidal action. Although the degree of parasitaemia increased daily, the increase was less rapid compared to M_i-treated and control mice.

Unlike M_i, AB-1 the crystallized compound from Ms showed strong schizontocidal action. Mice treated with AB-1 like those treated with chloroquine (5mg/kg) experienced drastic reduction in parasitaemia. By day 8, the parasitaemia in chloroquine- and AB-1-treated mice had dropped to approximately 6% and 1%, respectively. Death in all M_i-treated and control mice occurred on D7. Mice treated with Ms, AB-1 and chloroquine lived up to 15 days post infection when the mice were euthanized.

6.3.9 Activity of AB-1 in *P. y. nigeriensis* and *P. berghei* ANKA infection

The results are shown in Fig. 6.13 and Table 6.10. All control mice infected with *P. y. nigeriensis* experienced a daily increase in parasitaemia until D7 when death occurred in 3 of 5 animals. The remainder died on D8. The mean percent parasitaemia on D7 was 54%. In contrast, mice treated with chloroquine (5mg/kg) experienced a

drastic decrease in parasitaemia each day. The mean percent parasitaemia on D7 in these mice was approximately 1% corresponding to a chemosuppression of 98.1% (Fig. 6.13 and Table 6.10A).

Mice treated with AB-1 (5mg/kg) experieced a daily increase in parasitaemia which was somewhat less rapid compared with the control group (Fig. 6.13). In contrast, mice treated with 20-80mg/kg of AB-1 experienced a decrease in parasitaemia until D6 when it started to rise. Parasitaemia in 80mg/kg group fell again on D8. The percent parasitaemia and suppression in the highest dosed group on D8 were 6.2% and 88.5%, respectively (Table 6.10A). The chemosuppression of AB-1 was dose-dependent and the mean survival period was also dose dependent with values ranging from 10.9 days in the least dosed group to 19.4 in the highest dosed group. The MSP of the groups administered with 40 and 80mg/kg of AB-1 was more than twice that of the control.

On day 15, the blood film from mice dosed with 5mg/kg of CQ was parasite negative. This remained so until day 30 when the experiment was terminated. All mice treated with 10-20mg/kg of AB-1 ware dead by day 15. Three of the mice treated with 40mg/kg were dead by day 15; the remainder died on day 17. In the group treated with

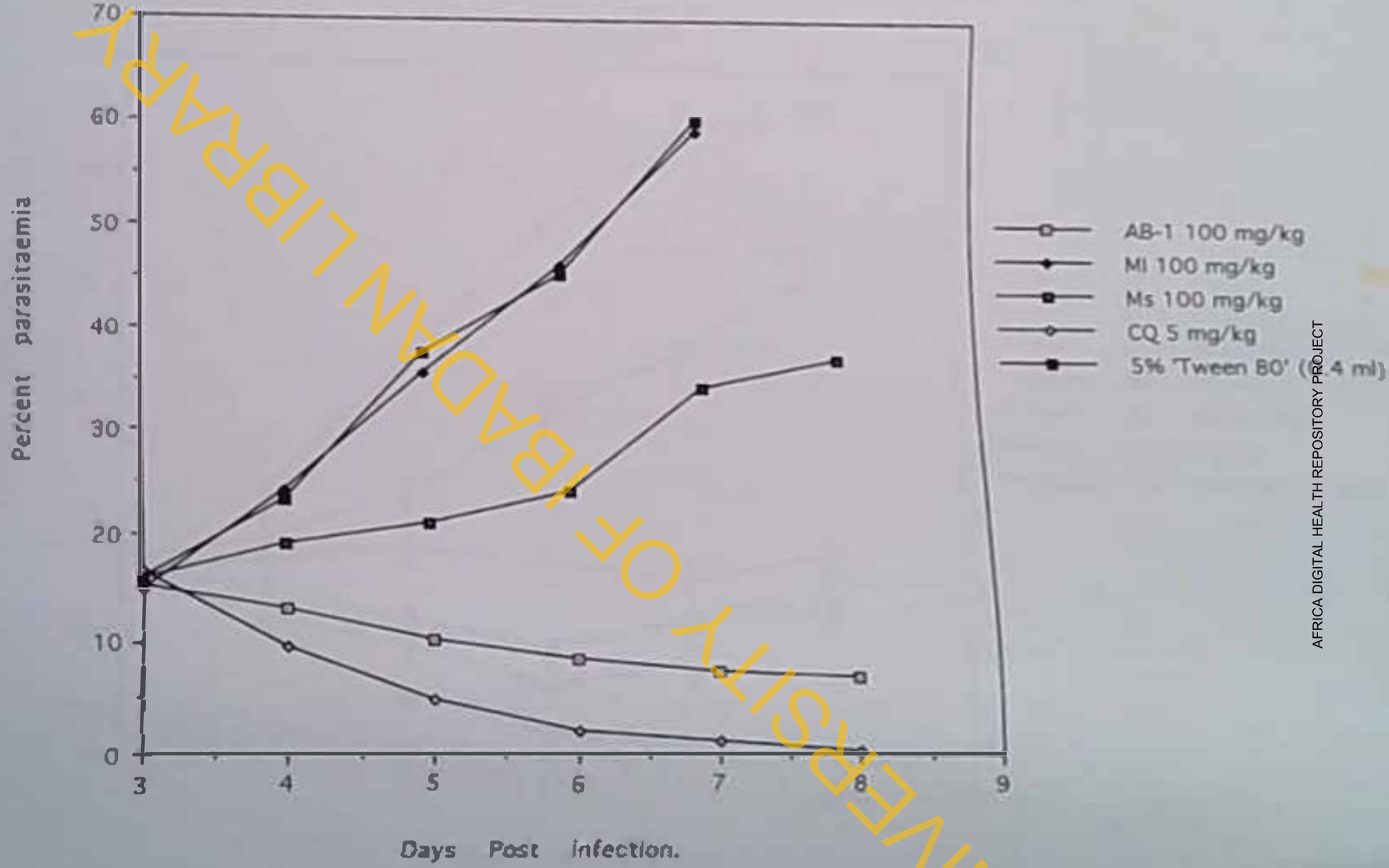

Fig. 6.12 Effect of Ms, MI, AB-1 on P. y. nigeriensis parasitaemia in Rane's test. Extract or drug were administered orally and once daily. Vertical lines indicate sem.

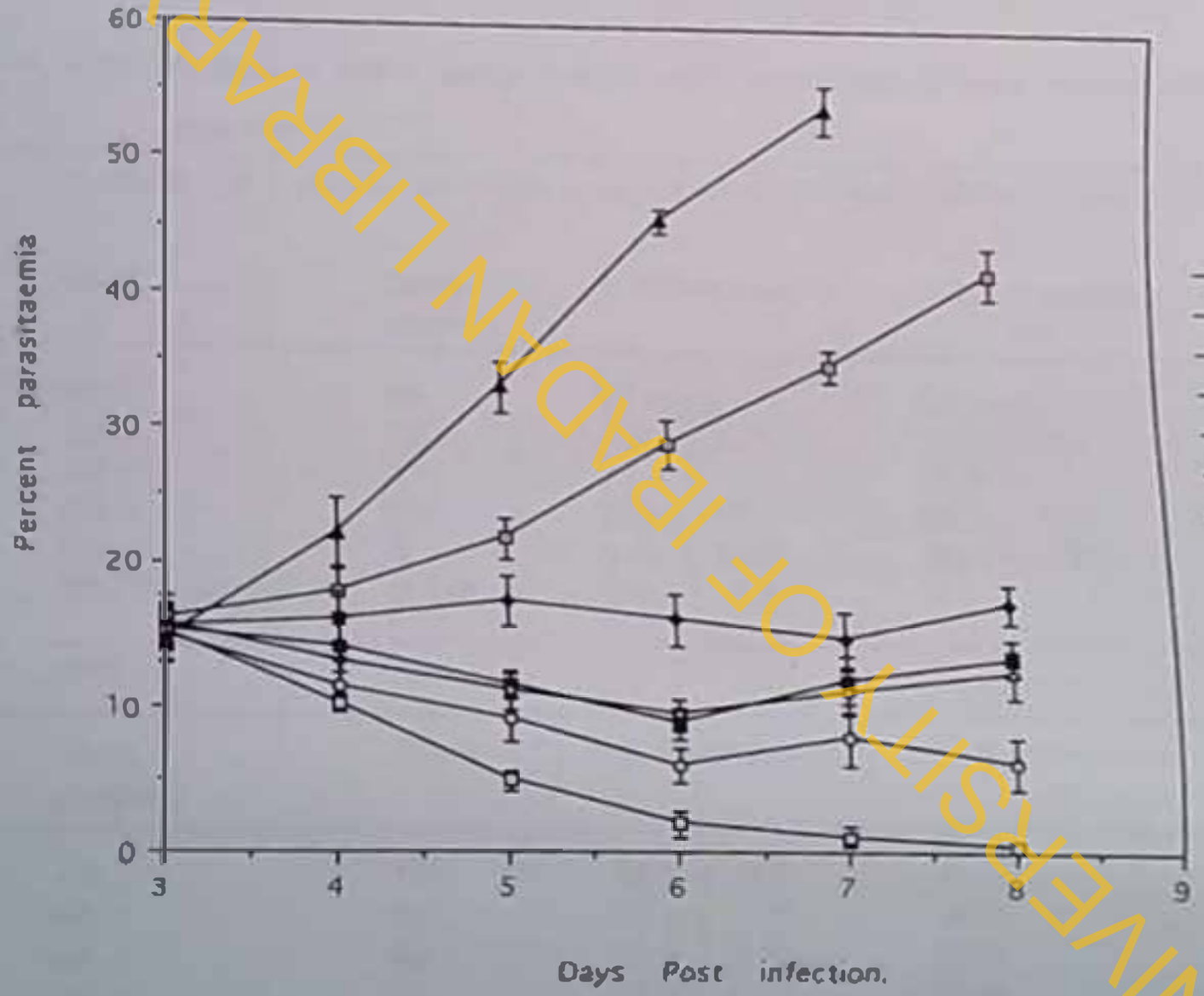

Fig. 6.13 Effect of AB-1 on P. y. nigeriensis parasitaemia in Rane's test. AB-1 was administered orally and once daily. Vertical lines indicate sem

Table 6.10. Activity of AB-1 administered orally once daily to male mice infected with *P. y. nigeriensis* (Table 6.10A) and to male rats infected with *P. berghei* ANKA (Table 6.10B) in Rane's test.

A.	Drug	Dose (mg/kg)	% Parasitaemis	% Suppression D8	Mean Survival Period (days)
	AB-1	10	17.0 ± 2.9*	68.5 ± 2.1	10.9 + 0.42
	AB-1	20	13.1 ± 2.2*	75.7 ± 1.9	10.0 + 0.72
	AB-1	40	11.1 ± 1.7*	79.0 ± 1.1	14.8 + 0.92
	AB-1	80	6.2 ± 1.8*	68.5 ± 2.9	19.4 + 1.2
	CO	5	1.0 ± 0.9*	98.1 ± 0.8	> 30 days
	5% 'Tween 80'	0.4ml	54.0 ± 1.8	—	7.4 + 0.77

n=3

B	Drug (mg/kg)	Dose	% Parasitaemia	% Suppression D8	Mean Survival Period (days)
	AB-1	10	35.5 ± 3.5	43.0	9.6
	AB-1	20	33.6 ± 2.9*	46.0	10.1
	AB-1	40	29.2 ± 3.1*	53.0	12.7
	AB-1	80	18.9 ± 1.9*	69.5	15.0
	CO	5	6.3 ± 1.5*	89.9	22.2
	5% 'Tween 80'	0.4ml	62.2 ± 2.3	—	10.5

* Significant compared to control at $P \leq 0.05$ (Dunnett t-test)

80mg/kg, one died on day 15, 3 on day 19 and the last one died on day 25. AB-1 was also active on *P. berghei* ANKA but less so compared to its activity on *P .y. nigeriensis*. The parasitaemia and the chemosuppresive action of 5mg/kg of CQ on day 8 were 17.9±1.5 and 71.2%, respectively (Table 6.10^{B}). The parasitaemia rose to 52.4% by day 15. One animal died by day 17, 2 by day 22, one on day 24 and the remainder died on day 26. Doses of AB-1 ranging from 10-80mg/kg produced chemosuppressive effect of 43 to 69.5%. The MSP ranged from 9.6 to 15.0 days. All the mice dosed with 10 - 40mg/kg of AB-1 died before day 15. In mice treated with 80mg/kg, one died by day 13, two by day 15 (one before blood film was taken) and the remaining 2 by day 16. The parasitaemias of the mice alive on day 15 and 16 were 71.5 and 69.6%, respectively (data not shown).

6.3.10 **Blood schizontocidal actions of AB-1 in early infection (4-day-test)**

In a '4-day test', chloroquine gave a suppression of 88.67 percent (Table 6.11). AB-1-treated mice gave chemosuppressive effects ranging from 41.3% (lowest dose = 10mg/kg) to 81.9% (highest dose = 80mg/kg). At dosages of 20 and 40mg/kg, the

Table 6.11: Blood schizontocidal actions of AB-1 in *P. y. nigeriensis* early infection.

Drug	Dose (mg/kg)	% Suppressioion
AB-1	10	41.3
AB-1	20	60.1
AB-1	40	71.2
AB-1	80	81.9
cq	5	88.6

chemosuppressive effects were 60 and 71.2%. These show a dose dependent effect (Table 6.11). At the highest dose of 80mg/kg, the chemosuppressive effect of AB-1 is comparable to that of 5mg/kg chloroquine.

6.3.11 Repository action of AB-1

In the repository test, doses of 10, 20, 40 and 80mg/kg produced chemosuppressive effects of 52, 62.4, 79.9 and 86.5 percent, respectively (Table 6.12). Pyrimethamine (1.5mg/kg), produced a suppression of 89.4%. The actions of AB-1 in repository test were also dose dependent. Further, a dosage of 80mg/kg produced schizontocidal effects that were similar to that of pyrimethamine (1.5mg/kg). In addition, doses of AB-1 used in this study, as well as the dose of pyrimethamine did not prevent infection 72 hours after inoculation of mice with $1x10^7$ pRBCs.

When AB-1 was administered orally and subcutaneously in a repository test, the chemosuppressive effects produced via both routes of administration were similar and comparable (Fig. 6.14).

Table 6.12: Repository action of AB-1 in *P.y. nigeriensis* induced malaria

Drug	Dose (mg/kg)	% Suppression
AB-1	10	52
AB-1	20	62.4
AB-1	40	79.9
AB-1	80	86.5
Pyrimethamine	5	89.4

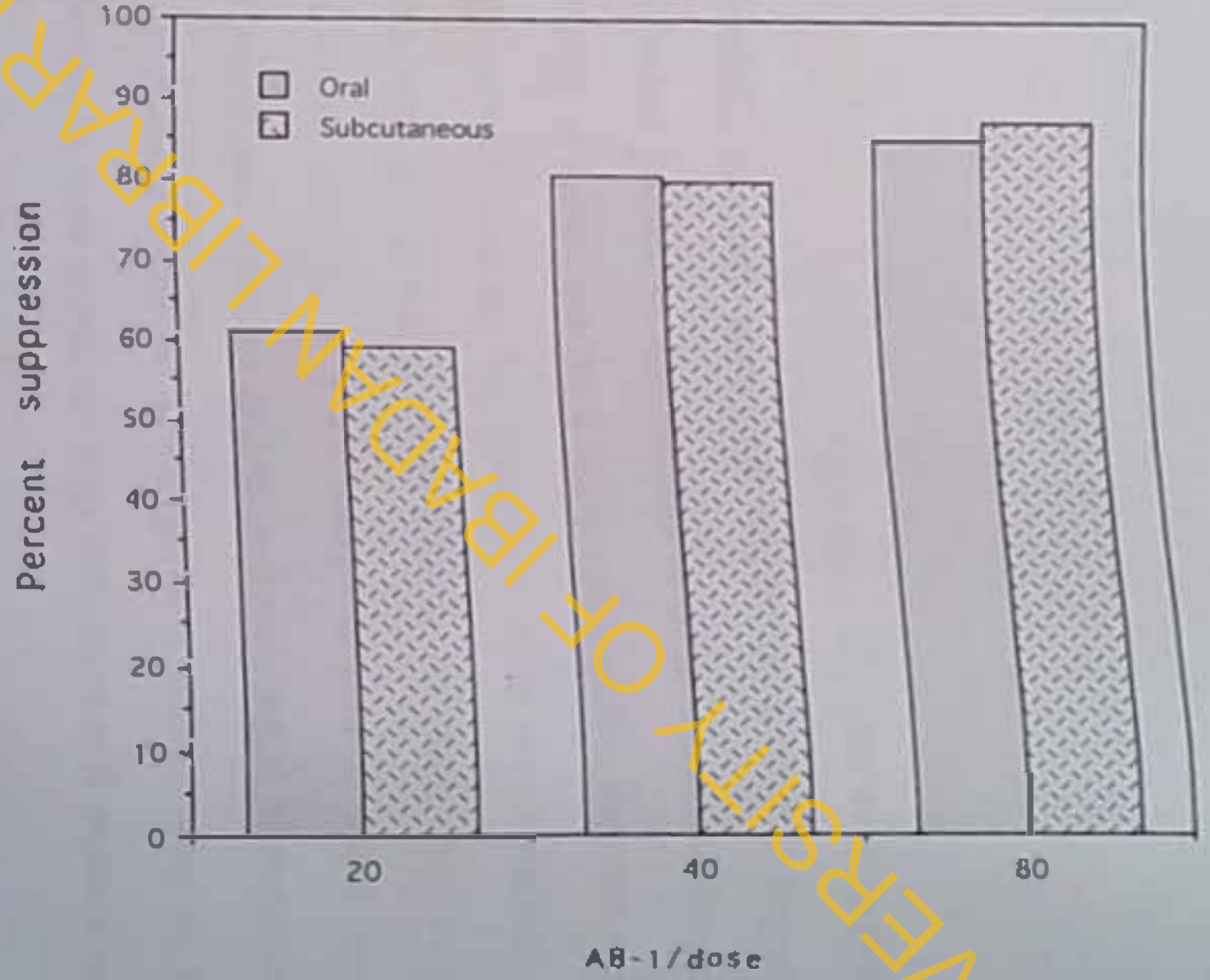

Fig. 6.14. Repository actions of AB-1 administered orally and subcutaneously.

6.3.12 Effects of prolonged boiling on the antimalarial activity of *A. boonei* stem bark extract.

Whole plant material (pulverized) was either steeped in water or refluxed in water continuosly for times ranging from 2-64 hours. As shown in Fig. 6.15, the chemosuppression produced by the whole plant material steeped in water (cAQw) was approximately 33%; those of extracts refluxed for 2 to 16 hours ranged from approximately 32 to 33%. These values were similar to that of cold whole extract indicating that prolonged boiling for up to 16 hours may not affect the antimalarial activity of *A. boonei* stem bark extract. When plant material was refluxed for 32 and 64 hours, the percent suppression dropped to approximately 19, and 16, respectively. Thus prolonged boiling for periods ranging from 32-64 hours resulted in a significant decrease in the activity of the plant extract (Fig. 6.15).

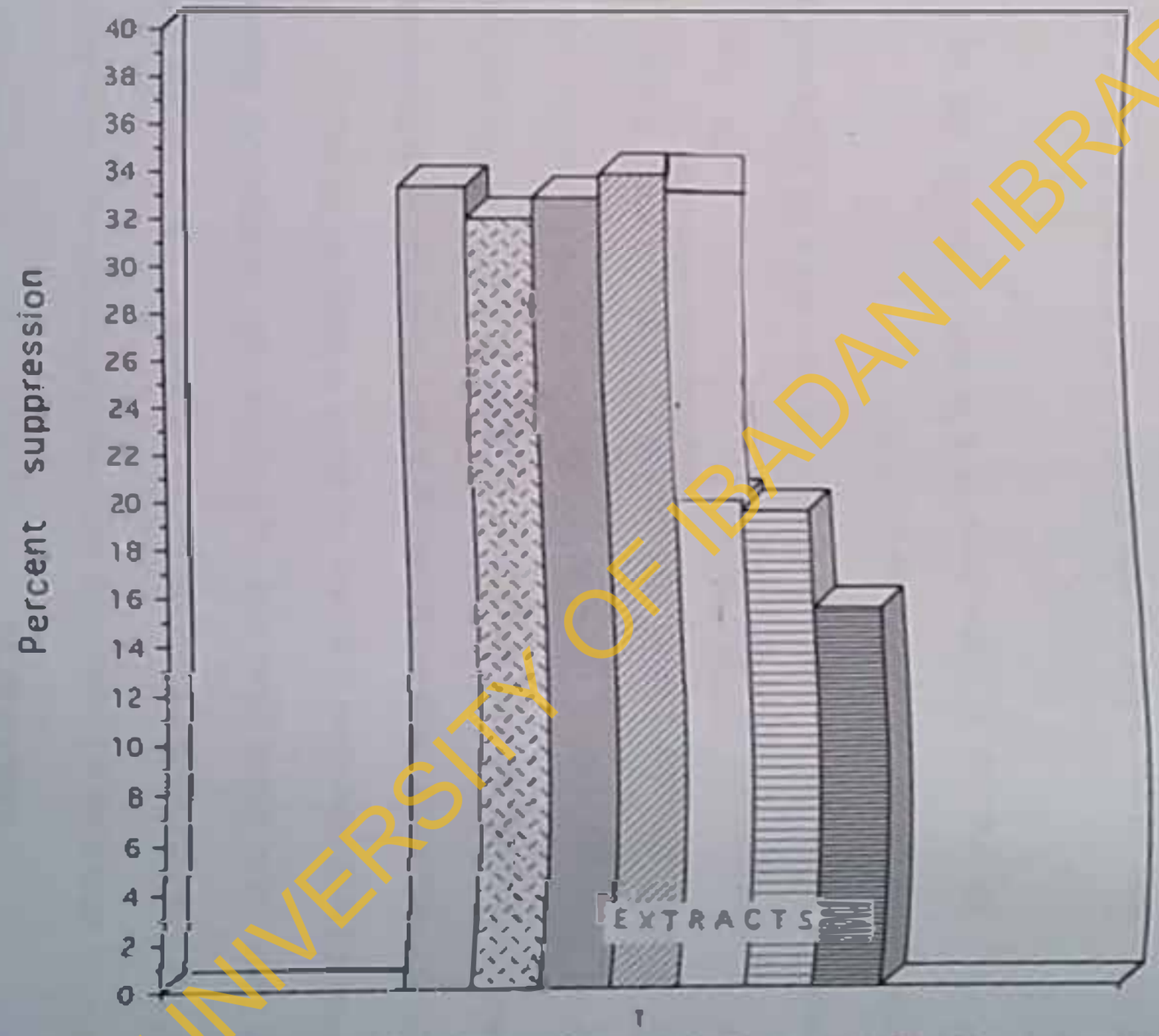

Fig.6.15 Effects of prolonged boiling on the antimalarial activity of Alstonia boonei stem bark. Repository activity was investigated. Whole plant material was steeped in distilled water (AQ) or refluxed in distilled water for various hours (2-64 hr).

5.4

DISCUSSION

In this work, the blood schizontocidal activities of active antimalarial constituent of *Alstonia boonei* stem bark were assessed in *P. y. nigeriensis* and *P. berghei* ANKA infections in male swiss albino mice and male Wistar rats, respectively. Bioassay guided fractionation was performed to isolate the active antimalarial constituent.

In all tests in which the blood schizontocidal actions of extracts were assessed in early infections (4-day test), an inoculum of 0.2mls containing $1x10^7$ pRBCs produced a parasitaemia of approximately 16% in infected, non-treated controls and a parasitaemia of approximately 2% in chloroquine (5 mg/kg)-treated group. The suppression of parasitaemia in this latter group was 85.9%. This level of chemosuppresion is adequate for the purpose of the present study as it indicates the parasite sensitivity to the drug (blood film was parasite negative on D15). So the use of chloroquine as a reference drug in this work is justifiable.

In early infection, all the doses of diethylether extract (DE) tested did not show blood schizontocidal activity. The chemosuppression of parasitaemia at the highest dose of 800 mg/kg was only 1.23%. This is extremely low compared with that of chloroquine (85.9%) at a dose of 5 mg/kg. Even when DE was given twice daily, there

was little or no increase in the chemosuppressive effect indicating that DE was not active in the test.

The activity of EtOAc extract compared with that of DE extract. The percent parasitaemias in mice dosed with 100-400 mg/kg of EtOAc extract did not differ significantly from the control indicating that the extract at the doses indicated, lacked blood schizontocidal activity. At a higher dosage of 800 mg/kg, there was a significant reduction in parasitaemia ($P \leq 0.05$) compared with the control. The chemosuppresive effect of 17.8 associated with this dosage is probably due to the increase in the amount of antimalarial active constituents in the 800 mg/kg compared with lower (100 - 400 mg/kg) dosages.

The blood schizontocidal action of aq. extract was higher than those of DE extract and EtOAc extract. At the highest dose of 800mg/kg, the chemosuppressive effect was 27%. The effect of AQ extract was dose related. When AQ extract was administered twice daily, a substantial and significant increase in the chemosuppression produced by higher doses (400 and 800mg/kg) occurred. This is a departure from the trend noticed with DE and EtOAc extracts.

Result of activity of EtOH extract shows that the extract was effective in suppressing *P. y. nigeriensis* infection in mice. The extract produced a dose-dependent

chemosuppressive effect in the dose range of 100-800mg/kg; the highest dose producing a chemosuppression of 57-61.4% (once daily regimen) and 72% (twice daily regimen) compared to 5mg/kg of chloroquine which produced 85.9%. This observation indicates that ethanolic extract of *A. boonei* stem bark had some blood schizontocidal effect in early infection initiated by *P. y. nigeriensis* in mice.

At the highest dose of 800mg/kg, the activity of EtOH extract was more than twice that of AQ extract, approximately 3 times that of EtOAc extract and about 50 times that of DE extract. This strongly suggests that the most active antimalarial constituent(s) of *A. boonei* is present in the EtOH extract. Further, it suggests that the antimalarial active constituents are of intermediate polarity.

When EtOH was administered twice daily, there was a significant increase in chemosuppressive effect of each dose (with the exception of 100mg/kg). This again is a departure from the trend noticed with DE and EtOAc where twice daily administration of extracts produced little or no increase in chemosuppression. This observation indicates that twice daily administration of DE, EtOAc may not have an important therapeutic advantage over once daily regimen. Moreover, the small increase in chemosuppression that occurred when the dose was increased, as a consequence of twice daily administration may not compensate for any likely adverse effect which the

extract may show. In contrast, twice daily administration of EtOH and AQ extracts may be beneficial.

Considering the results of the above tests, it can be concluded that DE extract did not contain antimalarial active constituent; EtOAc contained trace amounts of antimalarial active constituent whereas EtOH and AQ contained a large proportion of the antimalarial active constituents present in the stem bark of *A. boonei*. It is possible that EtOH and AQ contained the same active constituents. If so, then the compound(s) are more in EtOH then in AQ. Thus, ethanol would appear to be the most appropriate solvent for preparing a decoction of *A. boonei* stem bark.

The actions of the stem bark extracts of *A. boonei* reported in this work is at variance with that reported by Awe (1991). In that study, crushed stem bark of the plant was extracted with distilled water in a soxhlet apparatus. The resulting aq. extract was concentrated using a rotary evaporator and tested against *P. berghei berghei* in mice. The highest dose of 200mg/kg produced a chemosuppression of 58.2% while the lowest dose of 25mg/kg produced a chemosuppression of 18.5%.

In the present study, the aq. extract (AQ) was obtained by refluxing the marc remaining after extracting the crushed *A. boonei* stem bark with diethylether, ethylacetate and ethanol (in that order) in a soxhlet apparatus. At 200 mg/kg, the AQ extract prepared by this latter method gave a chemosuppressive effect of only 8.9%.

This shows that the AQ extract used by Awe (1991) was more potent than that used in the present work. Several factors may be responsible for the discrepancy in the two results.

First is the different methods of preparing the AQ extracts. Ethanolic extract was more active than AQ extract in the present work. Thus the use of ethanol before water in the extraction procedure described in this thesis may have removed some active constituents which would be present in the AQ extract used by Awe (1991) as no ethanol was used in that work. Second, the rodent *Plasmodia* used in both studies were different. The *P. berghei berghei* used by Awe (1991) would appear to be more sensitive to the AQ extract than *P. y. nigeriensis* is.

Another remote source of discrepancy is seasonal variation. Plants growing in the wild are subject to certain influences including climatic factors, seasonal variation and disease (Phillipson, 1994). This implies that the composition of secondary metabolite may vary according to season (Waller, 1993). This phenomenom has been suggested to affect the composition of certain chemical constituents of *Morinda lucida* (Adesogan, 1979) and to affect the antimalarial activity of crude extracts of *M. lucida* (Awe, 1991). Samples were collected during different seasons (Makinde, Awe and Salako, 1993). Samples of *M. lucida* stem bark used in the present study were collected in February while those used by Awe (1991) were collected in August.

In its indigenous use, *A. boonei* stem bark is either soaked in alcohol or boiled in water and taken in the form of aqueous tea. This method of preparation of the extract ensures that most of the compounds present in the solution of the tea are polar in nature. That is, the active antimalarial constituents in the tea are polar or of intermediate polarity. In the present study, it has been demonstrated that the ethanolic extract (EtOH) and the aqueous extract (AQ) showed considerably higher blood schizontocidal action than diethylether (DE) and ethylacetate extracts (EtOAc). The two active extracts invariably contain polar or semipolar constituents. Thus there is good correlation between the extracts found active in this study and the extract used for treating malaria in traditional medicine. Hence, this present study lends some support to the solvent used in the extraction of antimalarial sap of *A. boonei* stem bark.

As mentioned earlier, *A. boonei* stem bark is either soaked in alcohol or boiled in water and drunk. In this study, the antimalarial activities of cold extracts obtained by soaking *A. boonei* stem bark in different organic solvents were compared with those extracts obtained by the use of soxhlet apparatus. The result shows that the chemosuppressive effects of cold extracts were not significantly different ($P > 0.05$) from those of soxhlet extracts. However, exposure of plant material to heat for 3 hours and above significantly reduced the chemosuppressive effect.

in the 4-day test, only EtOH and AQ extracts showed strong blood schizontocidal action. So they were tested for activity in established infection (Rene's test). Both extracts were not curative in the test as there was a daily increase in parasitaemia in the control. However, the increase was less rapid in the case of mice treated with 400 and 800mg/kg dose of EtOH and AQ extracts. The low multiplication of parasite in the mice treated with higher doses of both EtOH and AQ extracts probably accounted for their prolonged survival over the controls. Further, the observation that the MSP of mice treated twice daily (with 800 mg/kg of EtOH) was more than twice that of the control is indicative of blood schizontocidal action of EtOH extracts according to Rogers (1983).

The results of the Rene's test shows that extracts of *A. boonei* were not effective in established infection. This observation agrees with the findings of Jimoh (1985) and Rene (1991). However, the extract used in the present study produced some benefits in terms of prolonging the survival period of treated mice over the control. The longer MSP in the mice treated with extract as opposed to the shorter MSP in the control mice may be attributed to additional properties of the extract which have been reported. These properties include antipyretic and analgesic effects (Wrights *et al.* 1993). The blood effect may however be absent or minimal since according to Asuzu and Anaga (1991), up to 200mg/kg of the aqueous extract of the stem bark of the plant did not

show analgesic effect when used alone, but potentiated morphine-induced analgesia in mice.

Results of the repository test shows that the most active extract (EtOH extract) in the 4-day and Rane's test, like pyrimethamine (1.5 mg/kg) did not prevent parasitaemie 72 hours postinfection in mice, but significantly reduced parasite multiplication rate. Mice treated with 800mg/kg had prolonged survival period over the control. When the dosage was administered twice daily, the MSP was more than twice that of the control. This may be taken as an evidence of drug activity.

It is not known whether *A. boonei* stem bark extract is used for prophylactic purposes. The activity observed for EtOH extract in the repository test would suggest that some benefits may be derived from the plant extract. Thus the plant may be regarded as having a great potential for residual antimalarial activity.

Out of all the extracts of *A. boonei* stem bark tested for blood schizontocidal activities against *P. y. nigeriensis* in the above tests, EtOH extract has shown the most promising activity. So the ethanolic extract was considered for bioassay guided fractionation in order to isolate the active antimalarial constituent in the extract.

Liquid-liquid extraction using dichloromethane ($CH_2=CH_2$) and water yielded lipophilic and hydrophyllic extracts referred to as $EtOH_L$ and $EtOH_H$ respectively. The

two subfractions were tested for blood schizontocidal activity using Rane's test and repository test.

In the Rane's test, the actions of $EtOH_L$ and $EtOH_H$ were similar to the parent extract (EtOH), in that mice treated with the extracts generally experienced an increase in parasitaemia until death. However, the observation that mice treated with $EtOH_H$ experienced less rapid increase in parasitaemia compared to mice treated with $EtOH_L$ is important and indicate that $EtOH_H$ contained more active antimalarial constituent(s) than $EtOH_L$. This inference has been strengthened by the result of the repository test (Fig. 3.11) in which the order of activity of the extracts would be $EtOH_H > EtOH >> EtOH_L$. This further strengthened the suspicions that the active antimalarial principle is of intermediate/ high polarity.

The antimalarial action of $EtOH_H$ is 1.4 times lower than that of pyrimethamine in repository test. Although the subfraction was far less effective than chloroquine in Rane's test, the prolonged survival of mice treated with the extract in the latter test coupled with the promising activity in repository test led to further fractionation of $EtOH_H$ into methanol soluble (Ms) and methanol insoluble (M_i) parts.

In the Rane's test, the activity of Ms was approximately 7 times higher than that of M_i. The insignificant action of M_i compared to the moderate activity of Ms may mean that M_i contained trace amounts of antimalarial constituents whereas Ms contained

higher amounts. This inference is based on the assumption that M_l and Ms contained the same antimalarial principes. If not, then it woud mean that the antimalarial constituent(s) of Ms was more potent than that of M_l. In the same test, AB-1, the constituent crystalizing from Ms had a blood schizontocidal action comparable to that of chloroquine. By D7, the degrees of parasitaemia in AB-1- and choroquine-treated mice were 7 and 1.5%, respeclively (Fig. 6.3).

Data on the activity of AB-1 in *P. y. nigeriensis* and *P. berghei* ANKA showed that the efficacy of AB-1 was greater against the former than against the latter. Eighty (80) mg/kg of AB-1 produced a chemosuppression of 88.5 and a MSP of 19.4 days in *P. y. nigeriensis* infection whereas the same dose produced a lower chemosuppressive effect of 69.5 and a MSP of 15 days in *P. berghei* ANKA infection. This result must be taken with circumspection because of differences in the strains of animals used.

When AB-1 was tested against *P. y. nigeriensis* in a 4-day and repository tests, the actions of AB-1 in both tests were similar. Thus it would appear that AB-1 was equally effective in both tests. Makinde *et al.* (1987) and Awe (1991) have reported that the ether extract of *A. boonei* showed greater activity in the repository test than in the 4-day test. The same conclusion can not be drawn from the present study.

In the study comparing the effectiveness of oral or subcutaneous AB-1 administration of the antimalarial compound via the subcutaneous route did not seem to increase the effectiveness of the compound. So, it would appear that AB-1 did not undergo considerable first-pass effect. So, it seems reasonable to consider oral route suitable for administering AB-1 for the reason of convenience.

On the whole, the activity of AB-1 reported in this thesis is promising. It is low compared to the activtiy of echitamine against *P. berghei* reported by Vasanth *et al* (1980) In that study, echitamine was found to be effective by subcutaneous route at 16mg/kg against *P. berghei* in mice. In the present study, AB-1 at 80mg/kg did not cure the infection induced by *P. y. nigeriensis* and *P. berghei* ANKA. However, it substantially suppressed the infections. The observation that up to 1,200mg/kg oral dose of AB-1 was not lethal in mice shows that AB-1 may be safe in mice. The ratio of 1,200mg/kg (causing no mortality) to 80mg/kg causing 85% suppression of parasitaemia in Rane's test is 1:15. This implies that higher doses (more than 80mg/kg) could still be tolerated.

It may not be valid to compare the activity of echitamine against *P. berghei* with that of AB-1 against *P. y. nigeriensis* or *P. berghei* ANKA. The reason is that *P. y. nigeriensis* is known to have intrinsic resistance to chloroquine while *P. berghei* ANKA

resistant to chloroquine at a low level. The *P. berghei* used by Vasanth *et al.* (1990) was chloroquine sensitive.

In view of the activity of AB-1 in the present studies, this chemical constituent may have a great potential in the treatment of chloroquine resistant *P. falciparum* malaria in humans. This is because *P. y. nigeriensis in vivo* model used in this study has always been used as a model for chloroquine-resistant *P. falciparum* malaria (W. Peters, Personal Communication). Mechanistic, spectroscopic, pharmacokinetic and clinical studies on AB-1 may shed more light on the utility of the compound in human malaria.

CHAPTER SEVEN

IMMUNOMODULATORY AND ANTIINFLAMMATORY STUDY OF *ALSTONIA BOONEI*

7.1 INTRODUCTION

Rheumatoid arthritis is one of the many diseases for which *A. boonei* is indicated in traditional medicine. The water-soluble principle of the stem bark is used as an external application for rheumatoid pains (Ojewole, 1986) and the stem bark latex is smeared on 'Calabar swellings' caused by filarial worms in Nigeria (Ojewole, 1986). A decoction of the root bark of *A. boonei*, rool bark of *Rauvolfia vomitona* and *Elais guineensis* (nut without pericarp) is used for the management of rhematoid arthritis in Ghana (Kwelfio-okai, 1991a; 1991b; 1995. The composition of *A. boonei* in the mixture is approximately 95% (Kwelfio-okei, 1991(a). *A. boonei* is responsible for the antirheumatoid effect of the decoction (Dalziel, 1937). *R. vomitona* provides sedative cover (Sofowora, 1982) while *E. guineensis* reduced the toxicity of *R. vomitona* (Kwelfio-okai, 1991a.

Rheumatoid arthritis is an inflammatory disease that affects millions of people the world over. Despite intensive research into the disease and the development of many new antiarthritic drugs over the years, cures of the condition remain elusive (Brune, 1989). Although the use of phospholipid-derived mediators of inflammation (mainly the cyclooxygenase) play a major role in alleviating the condition, they are limited by their often deleterious side effects (Kweifio-okai, 1991b). Immunomodulation has been reported (Hayner *et al*., 1986; Mollenhauer and Brune, 1988) to be a factor in the pathogenesis of the disesase. Regrettably, this area of arthritis management has remained largely unexplored (Kweifio-okai, 1991b).

In this chapter, the activities of *A. boonei* on complement system and polymorphonuclear (PMNs) leukocyles were investigated *in vitro* and *in vivo* (anti-inflammatory test). The rationale being that, if activation of these components of immune system, by immune complexes are responsible in part for rheumatoid arthritis, then inhibitors of either the complement activation or PMNs may be beneficial in rheumatoid arthritis.

7.2 MATERIALS AND METHODS

7.2.1 Bio-assays

Haemolytic Assay for Human complement Activity (Procedure)

Five times-concentrated veronal saline buffer, pH 7.35 (VSB-5x), prepared according to Mayer (1961), served as stock solution for the preparation of (i)VSB^{2+} containing 0.5 mMg^{2+} and 0.15 mM Ca^{2+}, (ii) EGTA-VB, containing 2.5 mM Mg^{2+} and 8 mM ethyteneglycol-bis(B-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA, Aldrich).

Sheep and rabbit blood diluted 1:2 in citrate-buffered glucose (Alsever's solution) served as sources of sheep erythrocytes (ShE) and rabbit erythrocytes (RaE) respectively. To elute possibly adsorbed serum proteins (Freedman and Massey, 1979), the erythrocytes were washed three times with isotonic sodium chloride (0.154M) before use.

ShE were suspended in VSB^{2+} to a concentration of $3x10^8$ cells/ml and were sensitized by incubation with an equal volume of 1:800 diluted anti-ShE monoclonal antibody solution (haemolytic amboceptor, RIVM, Bilthoven) at room temperature for 10 minutes (sensitized cells are further referred to as ShEA). Subsequently, the suspension was centrifuged at 1,500 x g for 10 min,

he ShEA were washed once with VSB^{2+} and resuspended in VSB^{2+} to a final concentration of 1.5×10^8 cells/ml. RaE were resuspended in EGTA-VB to a final concentration of 1.5×10^8 cells/ml.

Human pooled serum (HPS) from healthy volunteers was used as a source of complement. Classical (CP) and alternative pathway (AP) activities were determined by a modified version of the microassay described by Klerx et al (1983). The test was performed in U-well microtitre plates (no. 650102 Greiner, Germany). Logarithmic dilutions of extract/fractions/isolated compound were prepared in VSB^{2+} (CP) or EGTA-VB (AP). To each well containing 50 ul (CP) or 100 ul (AP) of these dilutions, 50 ul of HPS dilution in VSB^{2+} (CP) or 25 ul dilution of HPS (10^{-03}) in EGTA-VB (AP) were added after which the microtitre plates were preincubated at 37 °C for 30 minutes. Subsequently, 50 ul of ShEA (CP) or 25 ul of RaE (AP) were added. The plates were incubated at 37 °C for 60 minutes (CP) or 30 minutes (AP). After incubation, the plates were centrifuged at 1,500 x g for 5 minutes to precipitate react cells and cell ghosts. To determine the degree of haemolysis, 50 ul of supernatants were mixed with 200 ul of deml.water in 96 wells flat-bottom microtitre plates (no. 655102, Greiner). Absorbance at 405 nm was measured

using an automatic ELISA reader (SLT instruments, model SF-plus). Controls in these assays consisted of similarly treated incubates without sample, but with buffer (VSB^{2+} or EGTA-VB; 0% inhibition), incubates in which HPS was replaced by dilutions of heat-inactivated (56 °C for 30 minutes) serum (0% haemolysis), and incubates of erythrocytes and demineralized water (100% haemolysis). See Fig. 7.0 for outline of plate.

7.2.2 Chemiluminescence Assay (Procedure)

Experiments were performed in Hank's balanced salt solution buffered at pH 7.35 with $NaHCO_3$ (HBSS, Gibco, Paisly, Scotland). Before use, 0.1% (w/v) of gelatin was added to the buffer to avoid cell aggregation (HBSS-gel).

Polymorphonuclear leukocytes (PMNs) were isolated from venous blood of healthy volunteers as described by Verbrugh et al. (1978). Zymosan was opsonized by incubation with diluted HPS for 30 minutes at 37 °C (serum-treated zymosan, STZ). In 2 ml flatbottom vials (Sterlin Ltd, Middlesex, UK), 50 ul of a suspension of PMNs (1×10^7 cells/ml HBSS-gel), 100 ul of a luminol solution (30 uM in HBSS-gel), and a metal spin bar were added to 500 ul of logarithmic dilutions of isolated compound in HBSS-gel. Subsequently, the

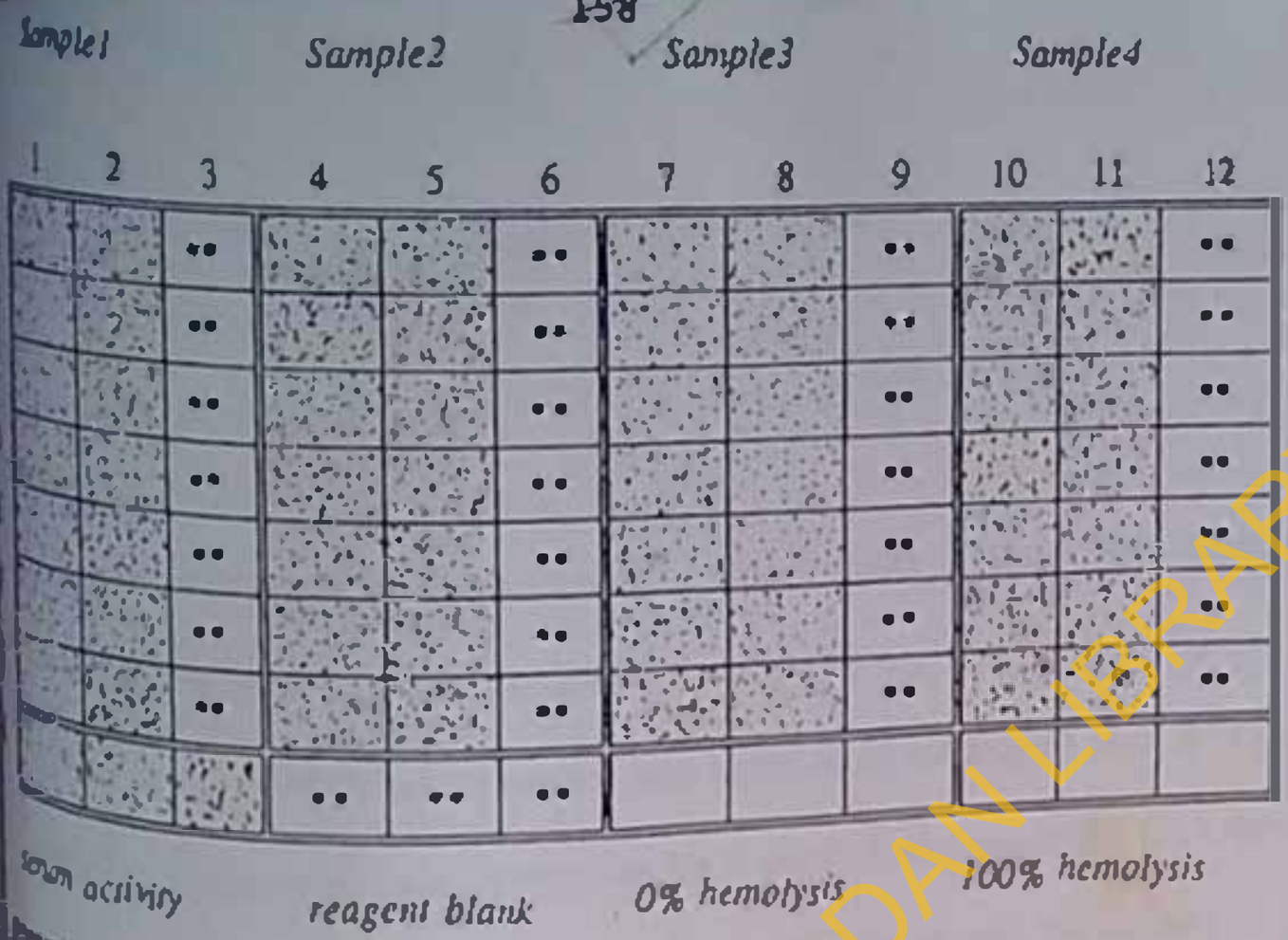

Fig. 7.0. Outline of plate and dosing procedure

vials were placed in a Packard Piclite Model 6500 Luminometer (Packard United Technologies, Downers Grave IL, USA) to equilibrate at 37 °C under gentle stirring. Chemiluminescence was induced by adding 50 ul of STZ (0.6 mg/ml) and monitored every 2 minutes for 5 secs. The peak levels (PL), which were reached after 8 to 10 minutes, were used to calculate the activity of the sample in comparison with a control (identical incubates without the test compound or mixture). Modulatory effects on the chemiluminescence are expressed as $PL_{sample}/PL_{control} \times 100\%$. Tests were performed in duplicate

7.2.3 Plant Material: The stem bark of the plant was collected in August 1995 from the Department of Botany, University of Ibaden, Ibadan, Nigeria. The collection and herbarium numbers were lowe 2323 and U.I.H. 13134 respectively. The plant material was cut into small pieces, oven dried (35°C) and transported to the Netherlands by air

7.2.4 Analytical Method

Tlc: Pre-coated plates (0.25mm) silica gel 60 F_{254} (Merck)

Ptlc: Pre-coated plates (0.5mm); silica gel 60 F_{245} (Merck).

Preparative Column Chromatography: MiniPrep LC apparatus (Jobin Yvon, France); Column dimension: 1.4cm i.d x 40cm; Column pressure: 8 bar; Silica gel 60 F_{245} (Merck); Sephadex LH-20 (Pharmacia) Column Chromatography.

7.2.5 HPLC (straight phase)

Solvent system: Ethyl acetate: Methanol (80:15 V/V); cyclohex: MeOH: isopropOH= 24:8:8; Methanol.

7.2.6 EXTRACTION

Coarsely ground plant material was extracted successively in a Soxhlet apparatus with Petroleum ether (PE,12hr), Diethyl ether (DE, 14hr), Ethyl acetate (EtOAc, 14hr) and Ethanol (EtOH, 18hr). The marc was dried and refluxed in water (H2O, 2hr). The extraction was monitored by TLC. The extracts were concentrated under reduced pressure, and subsequently lyophilized along with aqueous extract. The percentage yield of each extract was determined, and their activities were evaluated in the bioassay described in 7.2.1.

7.2.7 Purification of the most active extract.

DE and EtOAc extracts showed the most inhibitory effect in the assay described 7.2.1 Each of the extract was partitioned in methanol into methanol soluble part (Ms) and methanol insoluble part . That is, for DE, the methanol soluble and methanol insoluble parts were DE_{MS} and DE_{MI} respectively. The methanol soluble and methanol insoluble parts of EtOAc are likewise referred to as $EtOAc_{MS}$ and $EtOAc_{MI}$ respectively. The four extracts were assessed for activity according to the bioasay previously described in 7.2.1 and 7.2.2.

7.2.8 Column chromatography of $EtOAc_{MS}$-Using Miniprep (LC) method

$EtOAc_{MS}$ and DE_{MS} showed strong anticomplementary activities. On account of a higher yield, $EtOAc_{MS}$ was selected for further fractionation steps. It was fractionated by column chromatography on silica gel using a miniprep LC apparatus described in 7.2.4 The column dimension and pressure were 1.4cm i.d x 40cm and 8 bar, respectively. Eluting solvents were cyclohex : MeOH : IsoprOH = 24 :8 : 8 end the solvent pressure was 2 bar. Fractions of 3ml were collected and concentrated under N_2 to a small volume. A total of 53 fractions were collected. Classicel (CP) pathway activities of the fractions were determined on volume basis (Fig. 7.1). The fractions were combined

ding to their activity (Fig 7.1 and TLC Pattern into 5 pooled fractions labelled A-E
Table 7.3). The pooled fractions were concentrated under N_2 and lyophilized. The
lyophilized fractions were tested on the assay for activity on CP-mediated haemolysis
shown in Table 7.3.

7.2.9 Column chromatography of Pooled fraction C (from Table 7.3)

23mg of Pooled fraction C (Table 7.3) was dissolved in 1ml of MeOH and
chromatographed over Sephadex LH-20 (Pharmacia, column dimensions: 1.4 cm i.d x 40cm)
with MeOH as eluting solvent (flow : 0.35ml/min). A total of 29 Fractions of 5ml were
collected (Fig 7.2) and tested on volume basis in the classical complement assay
previously described. The fractions were combined according to their activity and TLC
patterns into 4 pooled fractions labelled A-D (Table 7.4). The pooled fractions were
concentrated under N_2 and lyophilized. The lyophilized, pooled fractions were tested for
activity (Table 7.4) in the complement assay described previously.

7.2.10 Preparative TLC of pooled fraction B (Table 7.4).

PTLC (silica gel F_{254}, 0.5mm) of pooled fraction B (from Table 7.4) was done
using EtOAc : MeOH = 85 : 15 as eluting solvent. The chromatogram was observed

der UV, sprayed with Van-H_2SO_4 and fast blue solutions. Based on the patttern of the
romatogram and reactions with the reagents, sections of it called Ptlc1, Ptlc2, Ptlc3
d PtlcA (Table 7.5) were scraped. Each of the scraped silica was eluted using dry
ethanol solutions. The methanol solutions of each were concentrated under reduced
ressure, and lyophilized. They were assessed for activity, using the test described in
ection 7.2.1.

7.2.11 Thin layer chromatography of the fractions obtained from Ptlc

Thin layer chromatography (tlc) of each Ptlc fraction was done using EtOAc :
MeOH = 85 : 15 and $CHCl_3$: MeOH = 80 : 20 as solvent systems. 1, 2 and 5mg of
each Ptlc fraction was dissolved in MeOH and developed with the solvent system of
ither EtOAc : MeOH (85 : 15) or $CHCl_3$: MeOH (80 : 20). The chromatograms were
bserved under UV 254 and 365nm and then treated with different reagents. PTLC 3
as found pure and subsequently named AB-2.

7.2.12 Determination of purity of AB-2

The same HPLC method previously described for AB-1 in 4.1.6 was used. The
obile phase however, unlike in

AB-1 was EtOAc: MeOH = 90:10. The spectrum was similarly scanned from 220 to 320nm.

2.13 Analysis of the Anticomplement Effect of AB-2 *in vitro*:

In this section, effects of preincubation, Ca^{2+}/Mg^{2+} chelation, and kinetics of AB-2 were studied. Accurately weighed amounts of the compound were dissolved in a few microlitres of DMSO and further diluted with VSB^{2+}. Studies on the inhibition of haemolytic complement activity were performed as described previously using the next variations.

2.14 Ca^{2+}/Mg^{2+} chelation: To exclude a Ca^{2+}- or Mg^{2+}-chelating effect, additional Ca^{2+} and Mg^{2+} ions (0.15, 0.3, 0.6 or 1.2 mM Ca^{2+} and 0.5, 1, 2 or 4 mM Mg^{2+}) were added to the VSB^{2+} buffer. The standard procedure was followed to determine complement inhibition.

2.15 Kinetics: Activation of complement components was studied by incubating diluted HPS ($10^{-1.8}$) with AB-2 at 37 °C or 0°C for different time periods (0, 15, 30 and 60 minutes, or 30 minutes, respectively).

7.2.16 Acute Toxicity Study.

An Up-and- Down procedure for acute toxicity testing described in 5.2.1 was used. Thirly six Balb/c mice divided into 8 equal groups were used. Single doses of AB-2 were given orally. The control group received 2% DMSO. All mice were observed over a period of 7 days.

7.2.17 IN VIVO ANTI-INFLAMMATORY ACTIVITY OF AB-2

Anti-inflammatory activity of AB-2 was tested in a zymosan-induced footpad inflammation model.

7.2.18 Animals

Male BALB/c mice were obtained from and maintained at the central facility of the Universiteit Utrecht for laboratory animals (Gemeenscheppelijk Dieren laboratorium) under the following conditions; temperature 22 °C, relative humidity 60%, lighting 12 h/day, and ventilation 15 cycles/h. Animals were fed standard rodent chow (Hope Farms; Cat. no. 1110) and water ad libitum.

7.2.19 Zymosan-induced Inflammation

Effects of AB-2 on zymosan-induced inflammation were studied with 20 male BALB/c mice of 16 weeks old. The animals were allocated into 4 groups of 5 animals each. Inflammation was induced in all the animals by injecting 300 ug of zymosan (suspended in 25 ul of sterllized saline) in the left hind footpad. AB-2 or apocynin (the reference drug) dissolved in saline was administered intra-peritoneally (I.p.) 1 hour before zymosen injection. The mice from the control group received the vehicle (saline). Footpad swelling was measured before and 4 hrs after induction of inflammation with an antomated plethysmometer.

7.2.20 Statistical Analysis

Statistical Analysis was performed using student's - t - test or Duncan's Multiple range test and level of significance was determined at $P < 0.05$. Data are reported as mean ± sem.

RESULTS

7.1 Anti-complementary activity

Activity of soxhlet extracts and cold extracts of *A. boonei* stem bark extract on complement-mediated haemolysis.

The yields of the extracts, DE, EtOAc, EtOH and AQ were 0.25%, 0.39%, 3.4% and 3.9%, respectively. The activities of the extracts were expressed as the extract concentration (ug/ml) causing a 50% inhibition (IC50) of heemolysis. The lower the IC50, the higher the inhibitory activity. The soxhlet (boiled) extracts and cold extracts of the plant showed similar inhibitory effect on the CP assay, but little or no activity on the AP assay (Table 7.1). The IC50 of DE, cDE, EtOAc and cEtOAc extracts were 1.7 ± 0.19 ± 0.13, 2.2 ± 0.30 and 2.3 ± 0.22, respectively. These IC50s were similar and about 12 and 15 times lower than those of EtOH (IC50 = 26.0 ± 0.62) and AQ extracts (IC50 = 32.5 ± 0.79) respectively. Each of the extract displayed dose-dependent inhibitory effects in the CP assay.

DE and EtOAc which showed strong anticomplementary activity towards CP-mediated haemolysis were partitioned into methanol soluble (DE_{ms} and $EtOAc_{ms}$) and methanol insoluble (DE_{mi} and $EtOAc_{mi}$) parts (Table 7.2). Whereas DE_{ms} and $EtOAc_{ms}$

showed stronger inhibition of complement than the parent (DE and EtOAc) fractions, DE_{CMS} and $EtOA_{CMS}$ produced little inhibition of complement activity (Tables 7.1 and 7.2).

DE_{CMS} and $EtOA_{CMS}$ were the most active subfractions of *A. boonei* stem bark. The IC_{50}s of DE_{CMS} and $EtOA_{CMS}$ were 1.2 and 1.7 ug/ml, respectively (Table 7.2). These values were not significantly different. On account of greater yield of $EtOA_{CMS}$ over DE_{CMS}, the former was fractionated by preparative column chromatography on silica gel H (Merck, cat. no. 7736) using a miniprep LC apparatus with cyclohexane : methanol : isopropyl alcohol = 24:8:8 as eluting solvent. Fractions of 10ml were collected and concentrated under N_2.

In Fig. 7.1, where extinctions (absorption 405nm) were plotted against fraction nos., low extinction relates to low percentage of lysis of the sheep erythrocytes, and hence high activity of the fraction. Fraction numbers 10-14, particularly fractions 11 and 12 showed the lowest extinctions (Fig. 7.1).

The pooled fractions (fraction numbers 10-14) referred to as pooled fraction C, showed the highest inhibitory activity ($IC50 = 2.5 \pm 0.9$; Table 7.3). The inhibitory activity of pooled fraction E was also high ($IC50 = 4.8 \pm 2.0$). However, the extinction (on a volume basis) corresponding to the pooled fraction numbers 33-53 was also high (Fig. 7.1). The high extinction (low inhibitory activity) of the pooled fractions 33-53

Table 7.1: Anticomplementary activity of crude soxhlet (boiled) and cold extracts of *Alstonia boonei* stem bark

Extract	IC50 (ug/ml)	
	CP	AP
Soxhlet		
DE	1.7 ± 0.19^{a}	184 ± 1.13^{d}
EtO Ac	2.2 ± 0.30^{a}	200 ± 1.60^{d}
EtOH	26.0 ± 0.62^{b}	208 ± 2.46^{cd}
Aq	32.6 ± 0.79^{a}	206 ± 3.90^{cd}
Cold		
DE	1.9 ± 0.13^{a}	208 ± 1.90^{cd}
EtO Ac	2.3 ± 0.22^{a}	223 ± 1.50^{b}
EtOH	24.8 ± 1.0^{b}	214 ± 2.0^{c}
Aq	37.4 ± 0.9^{c}	301 ± 2.5^{a}

CP = Classical pathway
AP = Alternative pathway
Cold
Data are mean ± s.e.m., n=4

Means followed by different letters in each column are significantly different at $P \leq 0.05$
Duncan's Multiple Range Test

Table 7.2 : Anticomplementary activity* of DE_{MS}, DE_{MI}, $EtOA_{CMS}$ and $EtOA_{CMI}$

Extract	% Yield	IC50 (ug/ml)	
		CP	AP
soxhlet			
DE_{MS}	0.19	1.2 ± 0.3[c]	201 ±1.42[c]
DE_{MI}	0.6	46.9 ± 1.1[a]	199 ± 1.76[c]
$EtOA_{CMS}$	0.29	1.7 ± 0.9[c]	250 ± 2.92[a]
$EtOA_{CMI}$	0.9	34.6 ± 0.8[b]	225 ± 1.87[b]

* Means followed by the same letters in each column are not significantly different at P≤ 0.05 following Duncan's Multiple Range Test.

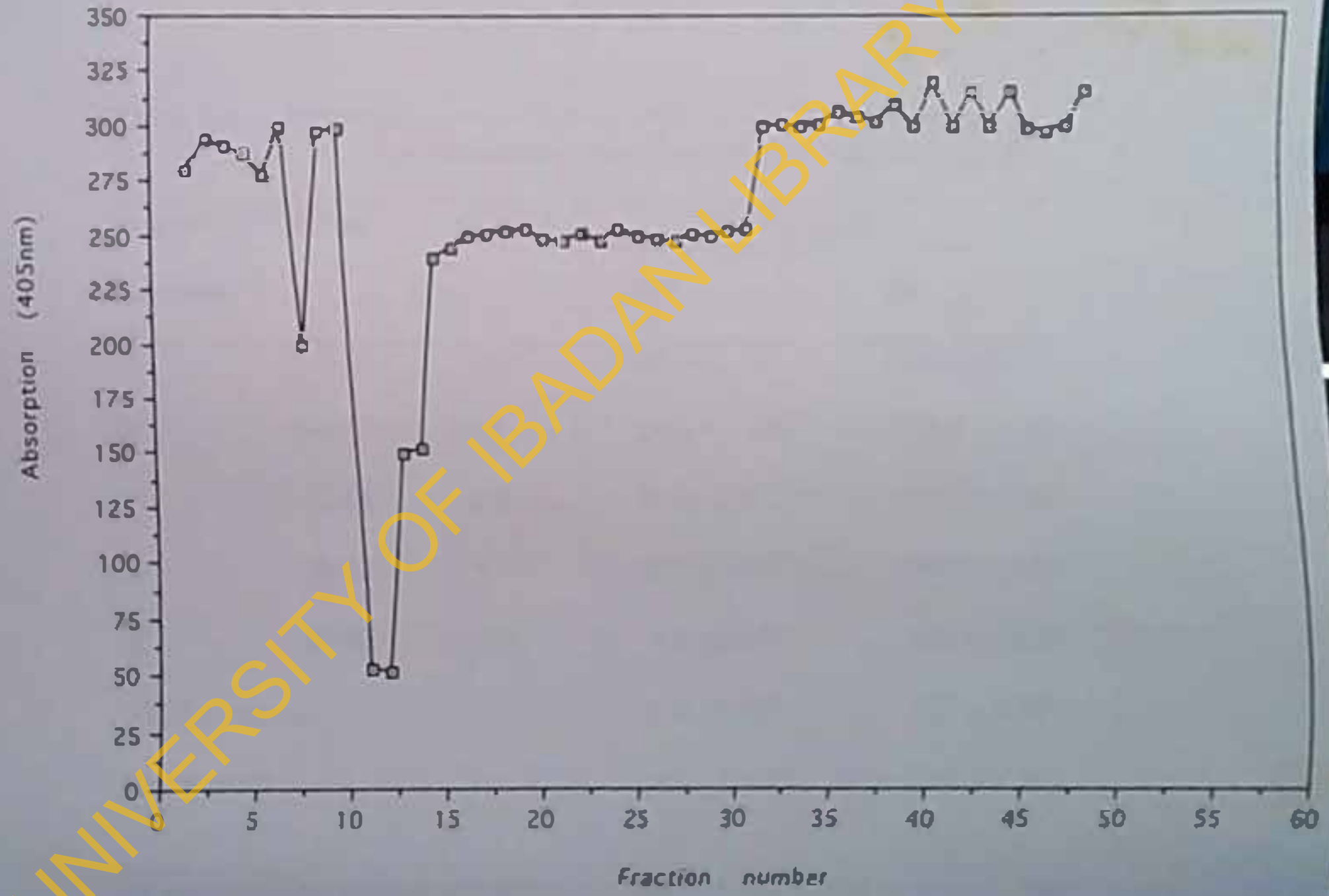

Fig. 7.1 Anticomplementary activity of muruprep fractions. Fractions were tested on volume basis and diluted 100-fold. Solvent system for Muruprep [illegible]

Table 7.3: Anticomplementary activity of pooled miniprep fractions
Solvent system = cyclohex : MeOH : isoprOH = 24:8:8

Pooled fractions	Tubes	% Yield	IC 50 (ug/ml)	
			CP*	AP*
A	1-7	0.3	28.1 ± 2.70^{a}	240 ±3.00^{a}
B	8-9	0.2	30.4 ± 1.90^{a}	190.0 ± 3.10^{b}
C	10-14	2.8	2.5 ± 0.9^{cd}	170.8 ±3.10^{c}
D	15-32	3.1	9.7 ± 1.80^{b}	154.2 ± 4.90^{d}
E	33-53	1.6	4.8 ± 2.00^{c}	192.1 ± 4.87^{b}
EtOAc extract			3.2 ±0.30^{c}	200 ± 3.70^{b}
EtOAcms**			1.7 ± 0.90^{d}	240 ± 1.9^{a}

Pooled fractions were concentrated, lyophilized and tested on weight basis.
** Values represent the mean ± sem (n=3). Means followed by the same letters in each column are not significantly different at P = 0.05 following Duncan's Multiple Range Test.*
*** EtOAcms was chromatographed*

then tested on volume basis could be due to non-homogenous solution of the fractions.

The activity profile of the fractions obtained via Sephadex LH-20 chromatography was shown in Fig. 7.2. Low extinctions (high inhibitory activity) were shown by fraction numbers 8-13 (pooled fraction B) and 26-27 (pooled fraction D) corresponding to IC50 values of 2.1 ± 0.86 and 7.9 ± 1.1 ug/ml, respectively (Table 7.4). In contrast the extinction of fractions 1-7 (pooled fraction A) and 14-25 (pooled fraction C) were quite high corresponding to IC50 values of 42.1 ± 3.0 and 19.6 ±1.4 ug/ml, respectively (Table 7.4). The IC50 values of pooled fraction D was significantly lower than that of pooled fraction E. So pooled fraction B was selected for further chromatographic work. Preparative thin layer chromatography (Ptlc) of pooled fraction B using ethyl acetate : methanol = 85 : 15 as a carrier (mobile phase) yielded a pure compound which gave a quenching at UV 254nm, reacted with van-H_2SO_4 (red colour) and iodine vapour. The compound was referred to as AB-2. The M. P. of AB-2 was 217° with Rf of 0.75. The IC50 value of AB-2 was 1.4 ± 0.94 ug/ml (Table 7.5). This value was significantly lower ($p \leq 0.05$) compared with the IC50 of Ptlc2 which was 9.5 ±1.2 (Table 7.5).

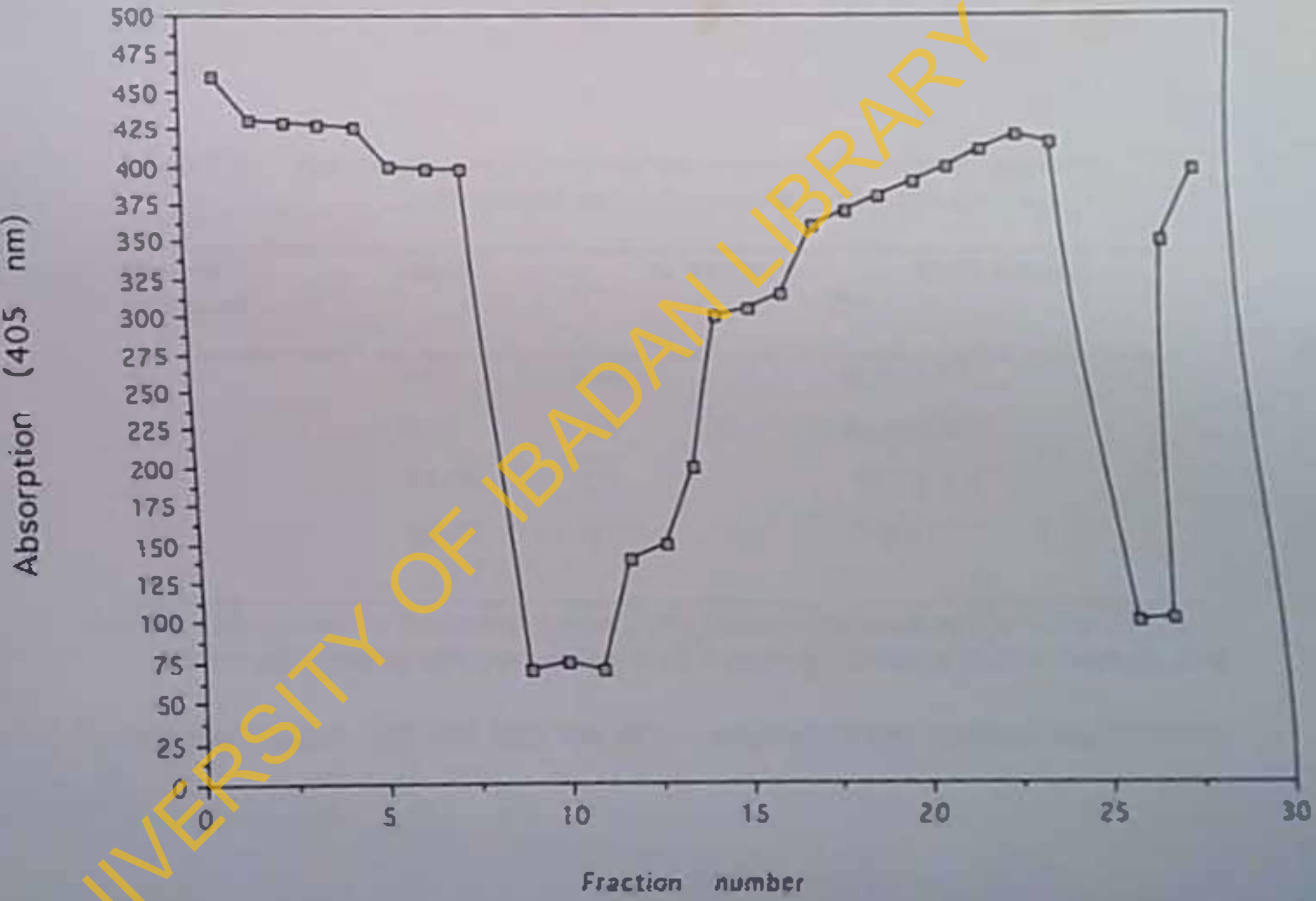

Fig. 7.2 Anticomplementary activity of LH-20 Column chromatography fractions. Fractions were tested on volume basis. Fractions were diluted 100-fold (Eluting solvent = MeOH. Pooled fraction C (Table 7.3) was chromatographed

Table 7.4: Anticomplementary activity* of pooled fractions from Sephadex LH-20 column chromatography. Eluting solvent : MeOH

Pooled fractions	Tubes	% Yield	IC 50 (ug/ml) CP
A	1-7	0.43	42.1±3.0[a]
B	8-13	1.8	2.1 ± 0.86[d]
C	14-25	2.1	19.6 ± 1.4[b]
D	26-27	2.91	7.9 ± 1.1[c]

*Values represent the mean ± sem (n=3). Means followed by the same letters are not significantly different at P ≤ 0.05 following Duncan's Multiple Range Test

Pooled fraction C (Table 7.3) was chromatographed and tested on weight basis.

Table 7.5 Anticomplementary activity of AB-2 obtained by Ptlc.
Solvent system : EtOAC : MeOH = 85: 15.

Scraped part of Ptlc	Yield (mg)	Yield (%)	IC 50 ug / ml CP*
Ptlc 1	7.1	1.01	40.6 ± 2.9[a]
Ptlc 2	2.4	0.34	9.5 ± 1.2[c]
Ptlc 3 (AB-2)	21.8	3.11	1.9 ± 0.94[d]
Ptlc 4	41.2	5.89	26.1 ± 1.9[b]

AB-2 scraped from the third layer of Ptlc plate was the most active and also the only pure fraction

Pooled fraction (Table 7.4) was chromatographed

Means followed by the same letter (s) are not significantly different at P = 0.05 following Duncan's Multiple Range Test

13.2 Anticomplementary analysis of AB-2

The mechanism of action of anticomplementary effect of AB-2 was investigated in the CP-mediated haemolysis using different preincubation conditions as shown in Fig 7.3. The anticomplementary activities of AB-2 were affected by changes in pre-incubation conditions; increases in temperature and time of pre-incubation are proportionate to increases in anticomplementary effect.

When the concentrations of Ca^{2+} and / or Mg^{2+} ions were increased up to 8 times in VSB^{2+} there was no significant alteration in complement inhibition under standard conditions (data not shown).

13.3 Activity of soxhlet extracts of *A. boonei* stem bark on luminol-enhanced chemiluminescence generated by stimulating PMNs with opsonized zymosan.

None of the extracts inhibited luminol-dependent chemiluminescence (CLIZ) generated by zymosan stimulated PMN (Table 7.6). The IC50 of each extract was greater than 250 ug/ml. In contrast, apocynin, the reference compound used in this assay showed an IC50 of 1.7 ± 0.94 ug/ml.

13.4 Antiinflammatory activity of AB-2

There was a significant swelling of the foot pad of mice 4 hours after administration of zymosan (Table 7.7). AB-2 significantly reduced the foot pad swelling.

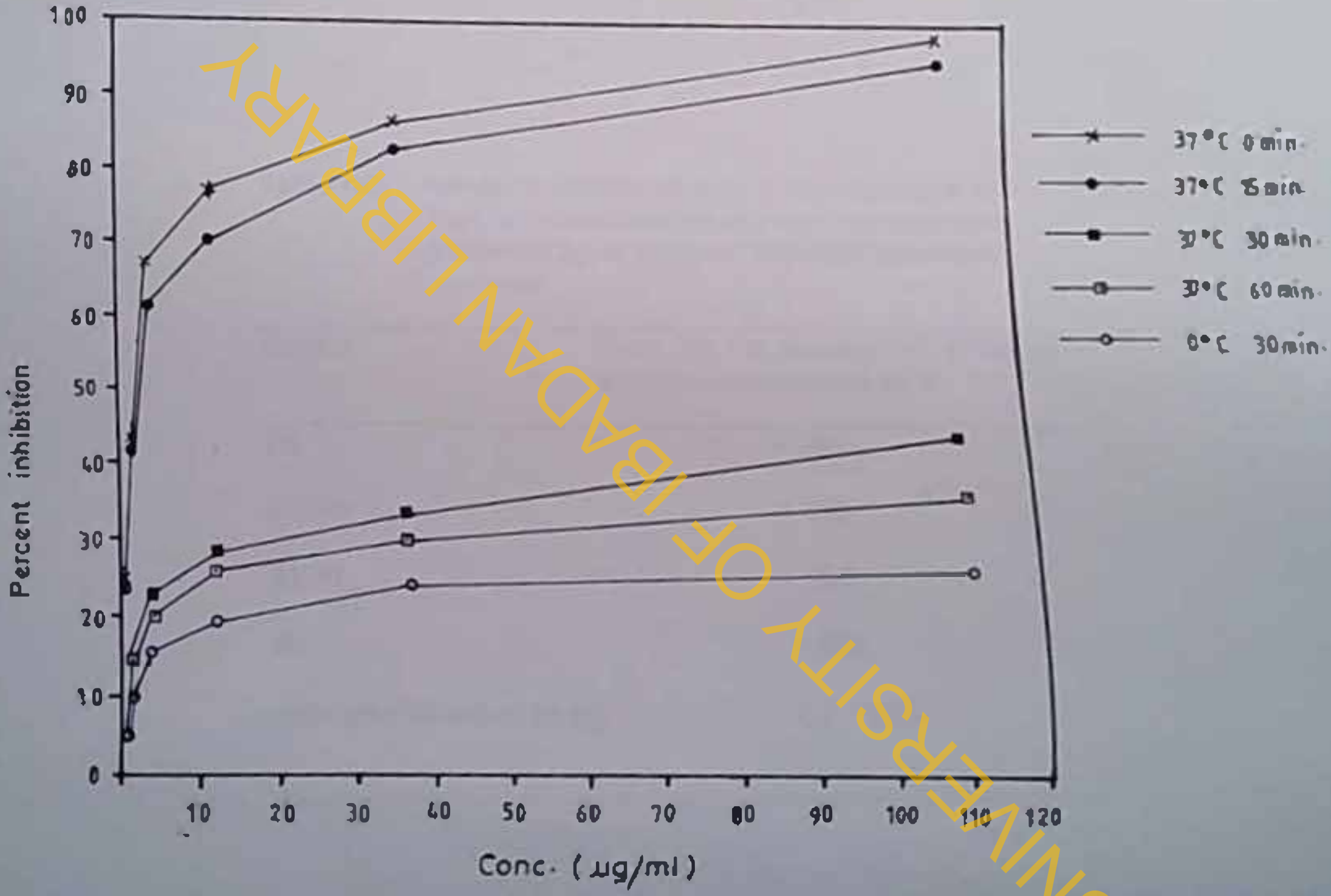

Fig. 7.3 : Effect of preincubation conditions on the inhibitory activity of AB-2 on classical pathway activation.

Table 7.6: Activity of soxhlet exracts of *Alstonia boonei* stem bark on luminol-enhanced chemiluminescence generated by stimulating PMNs with opsonized zymosan.

Extract	Conc. (ug /ml) causing 50% inhibition of chemiluminescence IC 50
DE	> 250
EtOAc	> 250
EtOH	> 250
Aq	> 250
Apocynin (standard drug)	1.7 ± 0.94

Table 7.7: Effect of AB-2 on zymosan-induced mouse footpad swelling.

Group	Treatment	Average Increase in paw diameter (mm) Mean ± sem	% Inhibition
i	Control (saline)	2.2 ± 0.19	-
ii	A B-2 (5 mg / kg)	1.5 ± 0.14	31.80
iii	AB-2 (10 mg / kg)	0.93 ± 0.21*	57.70
iv	AB-2 (20 mg / kg)	0.58 ± 0.15*	73.64
v	Apocynin (10 mg / kg)	0.8 ± 0.16*	63.64

Number of mice per group = 5
* Significantly different ($P \leq 0.05$) from control using Student t-test

The increase in foot pad swelling was significantly higher in control mice than in all mice given AB-2 indicating that AB-2 was active. The effect of AB-2 was dose dependent with the lowest (5 mg/kg) and the highest dose (20 mg/kg) causing 31.8 and 73.6% inhibition of foot pad swelling, respectively. At the highest dose of AB-2, the percent inhibition of mice foot pad swelling was greater than that of apocynin (10 mg/kg) - (Table 7.7).

13.5 Lethality of AB-2

The oral LD50 of AB-2 was approximately 1000 mg/Kg. Percent mortality at different dosages are shown in Table 7.8. Death of mice occured between 2 and 3 hours after oral administration of AB-2.

13.6 Determination of purity of AB-2 by HPLC with diode-array detection.

In order to investigate the purity of AB-2, solutions of 100 ug/ml of AB-2 were prepared and subjected to HPLC analysis. The mobile phase consisted of diethylether : methanol = 80 : 20. Scanning the spectrum from 220 - 320nm, chromatograms were recorded as shown in Fig. 7.4. The UV spectrum obtained for the peak was superimposed and the plot normalised. The peak was shown to be chromatographically pure by the exact coincidence of the corresponding UV spectrum.

Table 7.8: LD50 (oral) of AB-2 in male BALB/C mice

Group of mice	Dose of AB-2 (mg/kg)	Mortality	% Mortality	LD50
1	100	0/6	0	
2	200	0/6	0	
3	400	0/6	0	
4	800	1/6	16.6	
5	1,000	3/6	50	~ 1,000
6	1,200	6/6	100	
7	1,400	6/6	100	
8 (2% DMSO)	0.6ml	0/6	0	

Oral LD50 of AB-2 is 1,000mg/kg

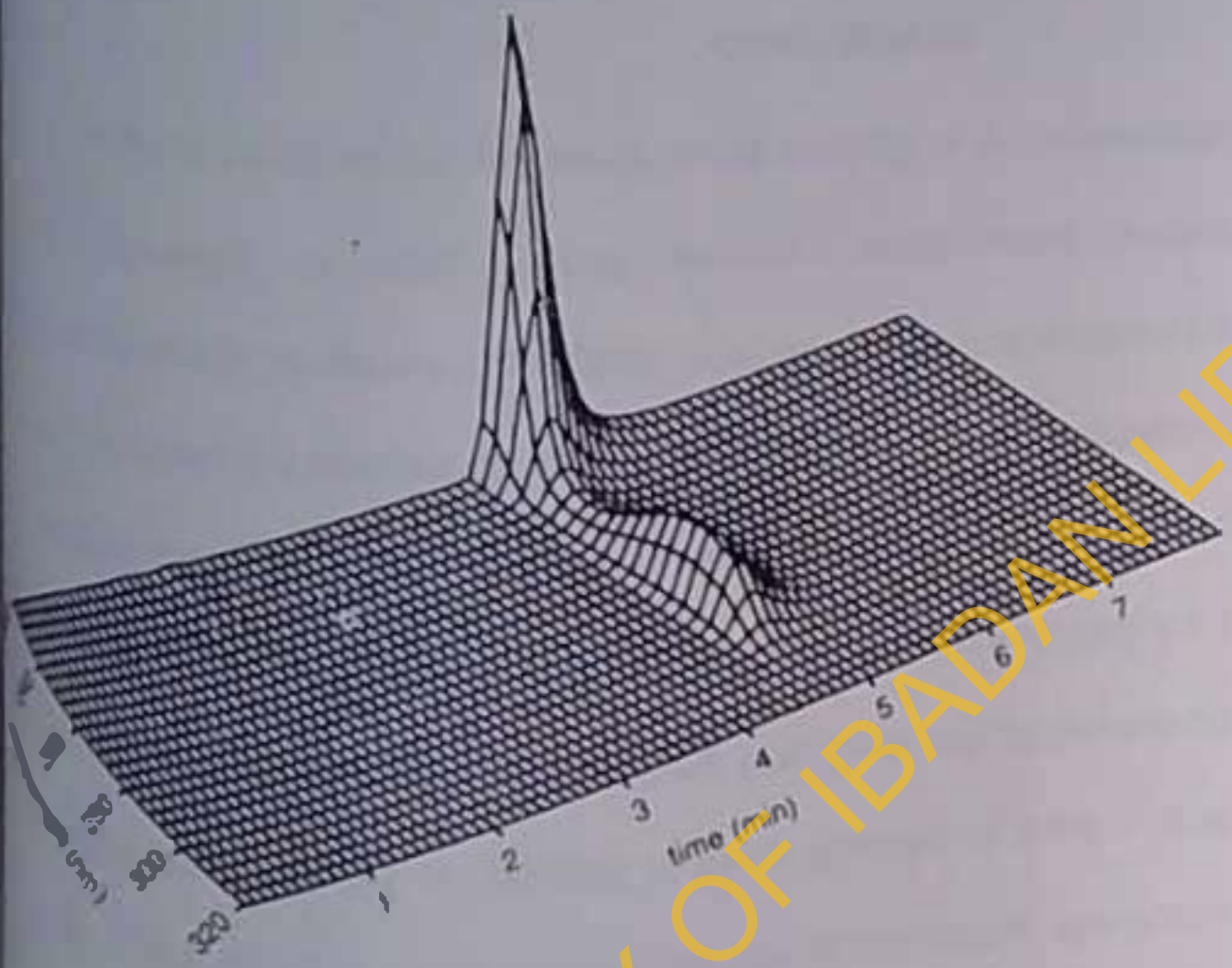

Fig 7.4 Chromatogram of AB-2 recorded as 3-D plots of absorbance as a function of wavelength and relention time UV spectra was computer-normalised and the plots superimposed

DISCUSSION

In the present study, the modulatory activity of *A. boonei* stem bark extracts has been assessed *in vitro* using human complement haemolytic assay and chemiluminescence assay. The immunological models selected for the screening of these modulatory activities all deal with inflammatory and degenerative diseases. The complement system is an important source of pro-inflammatory mediators, especially in autoimmune disorders where the cascade is generally triggerred by antigen-antibody complexes (Morgan, 1990). Phagocytes can give rise to severe tissue lesions by the extracellular production of reactive oxygen species (Fantone and Ward, 1985). The results of the present study show that the complement system was inhibited by the extracts of *A. boonei* stem bark. The observation that the diethylether (DE) and the ethylacetate (EtOAc) extracts exhibited the strongest complement inhibition indicates that the anticomplementary constituents in the stem bark of the plant are most likely non polar, of low or intermediate polarity. Since inhibitory action of the extracts was directed towards the classical pathway (CP) alone, it is most likely that only a few components of the complement cascade are involved.

In contrast to the effect found on complement activation, none of the extracts affected the phagocytic activity of PMN leukocytes. So it would appear that *A. boonei* stem bark extracts do not affect the functionality of PMN. It has been reported that the

nfiltration of PMNs into injured tissues is induced by complement chemotactic factors Susen, 1977). The inhibition of the complement by the extracts may therefore explain he lack of effect of the extracts on PMNs functionality.

The use of silica gel 60 F_{245} in classical column chromatography (intermediate ion) and Sephadex LH-20 in column chromatography under low pressure was suitable or activity-guided fractionation of AB-2. Each of the above method yielded fractions hat were more active than the original sample chromatographed. The use of reparative thin layer chromatography (Ptlc) proved effective in the isolation of one of he most active anticomplementary constituents of *A. boonei* stem bark. A particular roblem with the use of ptlc is poor separation of components of the mixture hromatographed. The problem was circumvented by the use of a preparative plate that s between the analytical grade, and the 1mm preparative grade that is, the 0.5mm hick.

Inhibition of Classical pathway of the human complement may be accomplished by the interaction of test fraction/substance with CP components or by chelating ivalent cations, Ca^{2+} and Mg^{2+} (Kosasi, 'tHart, Van Dijk and Labadie, 1989). To xamine possible interaction with complement components, AB-2 was investigated in he CP-mediated haemolysis using different preincubation conditions as shown in Fig. 3. The inhibitory effect of AB-2 was decreased when preincubation was performed at

gic indicating that the interference may occur at the enzymatic level. Also increase in
he length of preincubation led to enhancement of anticomplementary activity. These
ndings suggest that the mode of action of AB-2 involves complement activation and
hereby depletion or complement components rather than a direct functional
nactivation.

Results of the anticomplementary activity of AB-2 stimulated the evaluation of
AB-2 for *in vivo* anti-inflammatory test. Graded doses of AB-2 were tested for their
ivity towards acute inflammatory process. In the zymosan-induced inflammation,
otpad swelling was measured 4 hours after induction, hence it represents an acute
ammatory response. The exudate in this type of inflammation consists mainly of
eutrophils (Schalkwijk *et al.* 1985). The anti-inflammatory activity of drugs towards
ymosan-induced inflammation may be due to interference with complement or PMN
unctions (Roitt, Brostoff and Male, 1989). AB-2 has shown high inhibition of CP-
duced haemolytic assay, but has little or no effect on the chemiluminescence
erated by zymosan-stimulated PMNs. Therefore it is reasonable to attribute the
ibition of zymosan-induced footpad swelling in mice used in the present study to
erference with complement function.

Considering the very high oral LD50 of AB-2; about 50 times the effective dose inhibiting the footpad swelling, then the compound probably has a wide margin of safety in mice.

Since activated complement is involved in inflammatory responses by increasing capillary permeability, degranulating mast cells or promoting neutrophil activation and chemotaxis, the inhibition of complement activation reported in this thesis may in part explain the anti-inflammatory (antirheumatoid) effects claimed for *Alstonia boonei* stem bark extracts preparations in traditonal systems of medicine.

CHAPTER EIGHT

CONTRACTILE ACTIVITY OF AQUEOUS EXTRACT OF *ALSTONIA BOONEI* STEM BARK.

8.1 INTRODUCTION

A boonei stem bark extracts have been used in traditional medicine to treat diseases affecting gastro-intestinal tract (GIT). For example, it is used to treat dysentery (Ojewole, 1984; Wright *et al.*, 1993).

One of the chemical constituents of the plant, echitamine, has been subjected to a battery of pharmacological tests based on the indigenous use of the crude extracts of related species to treat many diseases. Some of the actions of echitamine include lowering of arterial blood pressure in normotensive anaesthetized animals (Ojewole, 1984) and a fall in blood pressure in hypertensive cats (Kucera *et al.*, 1973; Marquis, 1975). It has also been reported to induce negative chronotropic and inotropic effects in isolated atrial muscles strips, relaxation of isolated vascular and extra-vascular smooth muscles, inhibition of electrically-provoked and agonist-induced contractions or relaxations of isolated smooth muscle preparations, paralysis of electrically-evoked skeletal muscle twitches, and induction of diuresis (Ojewole, 1984).

Echitamine and a few alkaloids of *Alstonia* species have been assessed for *in vitro* activity against *Giardia intestinalis*, the organism responsible for giardiasis. All the alkaloids were inactive against *G. intestinalis* at the concentrations tested (Wright *et al.* 1993).

The aqueous extract of *Alstonia boonei* and echitamine isolated from it have also been investigated for activity on isolated smooth muscle preparations. Whereas the aqueous extract of the plant neither contracted nor relaxed isolated guinea pig ileum (Ajaiyeoba and Anaga, 1991), echitamine isolated from it relaxed the guinea pig isolated ileum in a concentrated-dependent manner (Ojewole, 1984). Further, the alkaloid blocked or abolished the agonist (acetylcholine-, histamine-, nicoline-, 5-hydroxytryptamine-, potassium-, or barium-) induced contractions of guinea pig isolated ileum in a dose-related manner (Ojewole, 1984).

The two reports above on the relaxant activity of echitamine and the lack of activity of the aqueous extract of *A. boonei* on guinea pig isolated ileum are not in agreement. In the present study, the action of lyophilized aqueous extract of *A. boonei* stem bark was investigated in rat stomach strip and guinea pig isolated ileum.

12

MATERIALS AND METHODS

121 Plant material and extract preparation

The stem bark of the plant was collected in March from the Department of botany, University of Ibadan, Nigeria. Its identity (collection number : Lowe 2323, herbarium number : U.I.H. 13134) was confirmed by Dr. Joyce Lowe of the same University. The stem bark of the plant was air dried and powdered. The powdered extract was steeped in water for 3 nights to allow for sufficient extraction. The solution was filtered and the filtrate was concentrated into a dark brown dried form using a lyophylizer (Lab Conco-Lyph lock 6). The yield was 8.3%. It was studied immediately or after storage in the Deep freezer.

122 Preparation of rat stomach strip

Rats of either sex (200-300 g) were stunned and bled. The stomach strip was prepared as described by Vane (1957). Briefly, the abdomen was cut open and lower part of the stomach removed and cleaned. A longitudinal strip was made from the stomach and suspended in a 20 ml organ bath containing Tyrode solution (composition, mmol/L; NaCl, 136.0; KCl, 2.7; $CaCl_2$, 1.8; $MgCl_2$, 1.0; NaH_2PO_4, 0.3; glucose, 5.5 and NaH_2CO_3, 12.0) maintained at 37°C and gassed with air. The preparation was allowed to equilibrate under a resting tension of 1 g

for 60 min during which the Tyrode solution was changed at 20 min intervals. Isotonic contractions (Magnification x 7) were recorded on a smoked paper through a frontal writing lever.

3.2.3 Preparation of guinea pig ileum

Adult guinea pig of either sex (300-350 g) were stunned and bled. Segments of the ileum (4 cm long) were removed 10 cm from the caecum and treated as in the case of stomach strip.

3.2.4 Pharmacological test

Two series of experiments were carried out.

1. Concentration-response curves were constructed to different agonists acetylcholine (Ach) and 5-hydroxytryptamine (5-HT) followed by the extract. The contractions were expressed as percentage of the maximal response induced by the standard agonist (5-HT).

2. Antagonism to the extract (rat stomach strip): Concentration- response curves were constructed to the extract in the absence and then in the presence of two antagonists, viz., atropine sulphate (BDH) and methysergide (Sandoz).

In each case of the extract-antagonist pair, the stomach strip was incubated with each antagonist for 30 min before constructing concentration-response curves to the extract.

In all the above experiments five animals each were used. The pH of the extract, before adding to the organ bath, and when in the bath was measured. Contact time of extract or standard agonist was maintained at 5 min intervals.

12.5 Statistics

Statistical analysis was performed using Student's t-test and a P value less than 0.05 was considered significant. Data are presented as mean ± sem.

RESULTS

13.1 CONTRACTILE ACTIVITY OF AQ EXTRACT OF A. BOONEI

The pH of the extract before adding to the Organ bath, and in the organ bath, remained at 7.0

Contractile effect of the extract

The extract had a contractile effect on rat stomach strip and guinea pig ileum. The contractile response was concentration-dependent. The onset of action was about 1min and the duration of action ranged between 1 and 2 min. The contractile effect was more on rat stomach strip than on guinea pig ileum. The concentration of extract required to develop 50% of the maximal response on the rat stomach strip and the guinea pig ileum (EC50) were 2.7 ± 0.1 mg/ml and 4.4 ± 0.2 mg/ml, respectively. These values were significantly different ($P \leq 0.05$).

The maximal response by extract on guinea pig ileum was 62% (n =5) of that induced by 5-HT on the same tissue.

8.1.2 Antagonism to extract

Atropine (up to 1mg/ml) did not have any effect on the contractions induced by the extract (Fig. 8.1). In contrast, methysergide (1 and 10 ug/ml) inhibited the induced contractions (Fig. 8.2). Concentration-response curves in the presence of methysergide were progressively shifted to the right, maximum response was achieved by a higher concentration of extract (Fig. 8.3).

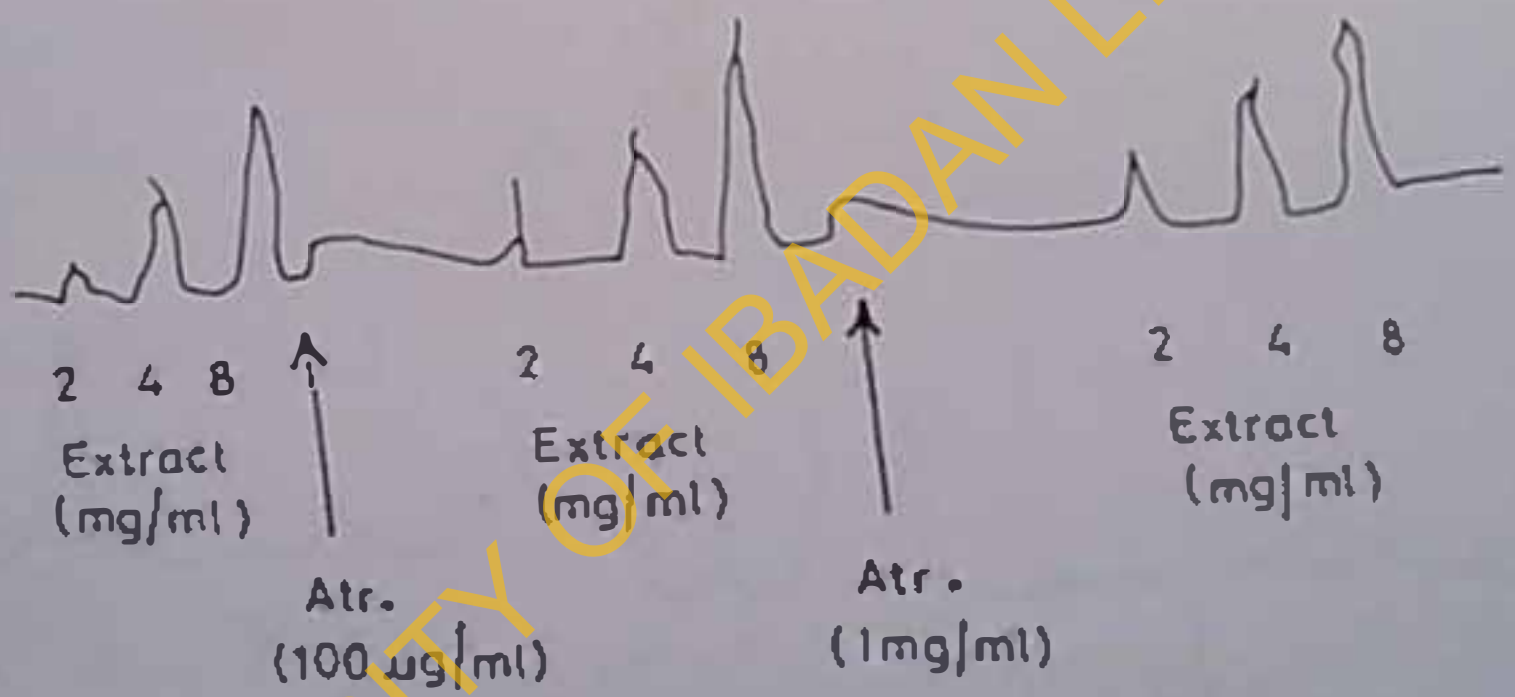

Fig. 8·1: Responses of stomach strip to aqueous extract of Alstonia boonei stem bark alone and after incubation with Atropine (Atr.)

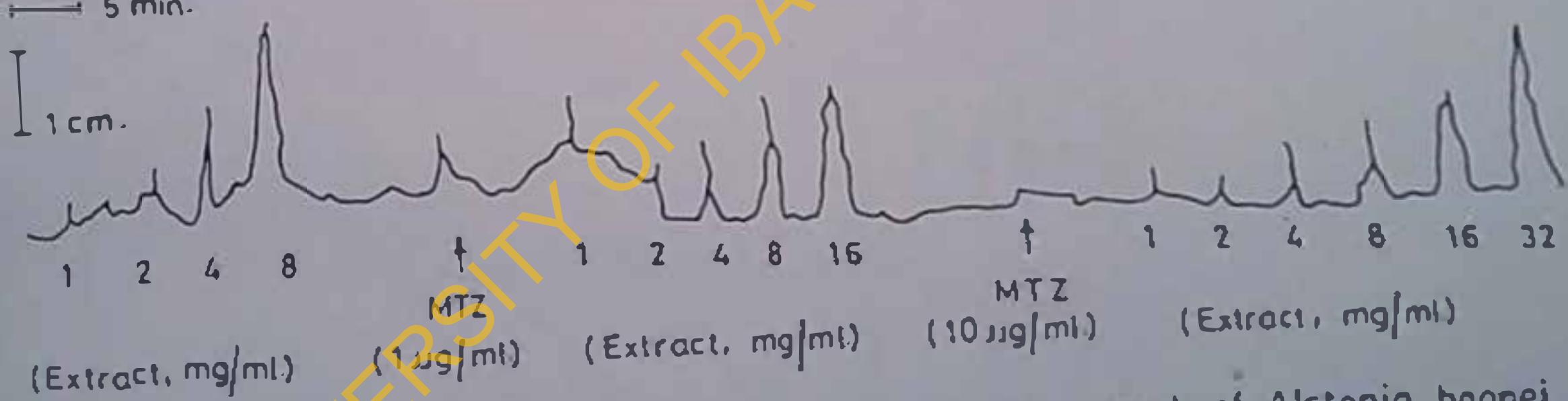

Fig. 8·2.: Responses of stomach strip to aqueous extract of Alstonia boonei stem bark alone and after incubation with methysergide (MTZ)

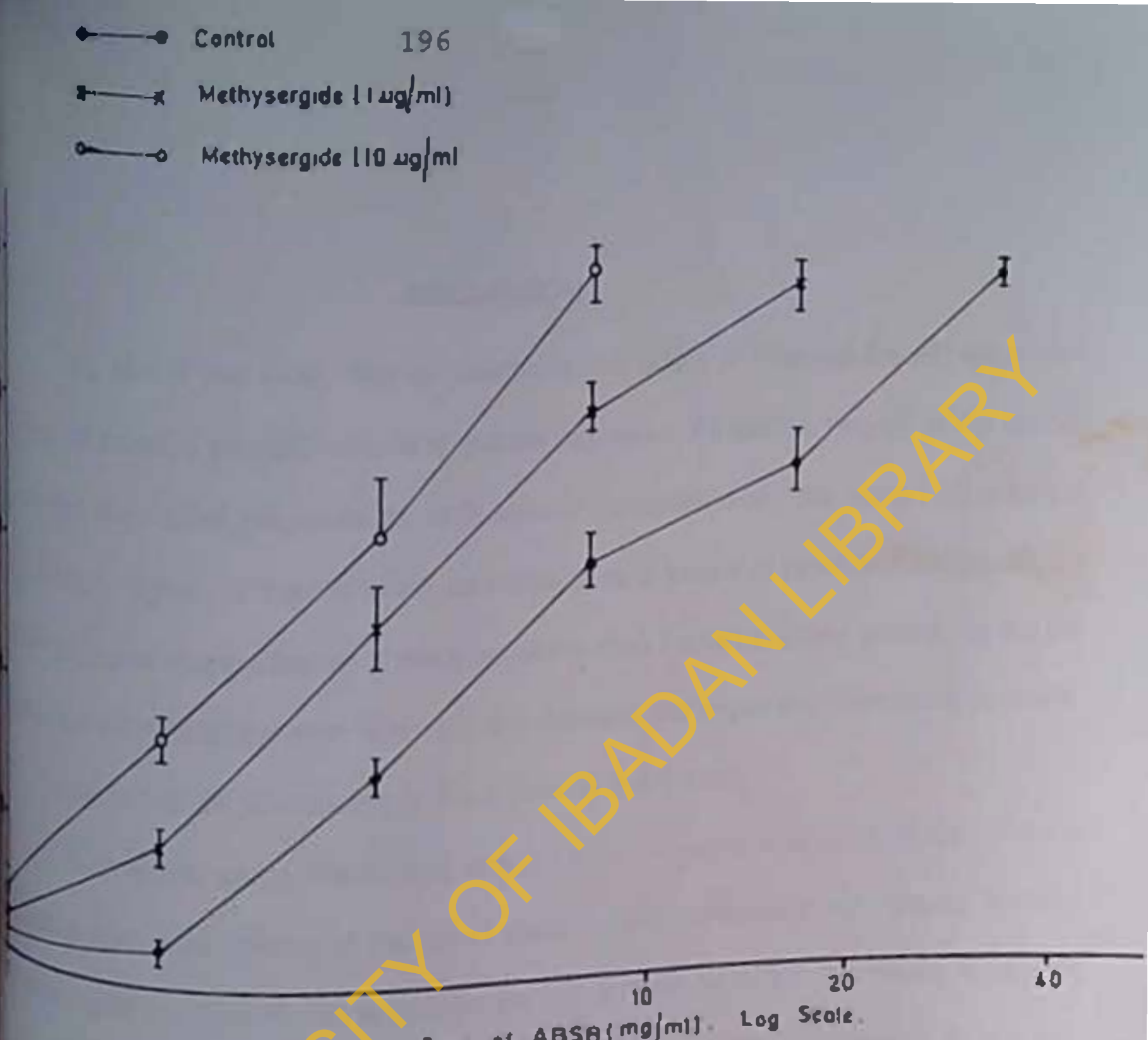

Fig. 8·3: Alstonia boonei stem bark - stimulated concentration — effect curve in the absence (● control) and in the presense of methysergide, 1 ug/ml (x) and 10 ug/ml (•). mean values are shown; vertical lines indicate sem ; n=5. Tissue = rat stomach strip.

DISCUSSION

The aim of this study was to determine the effect of *Alstonia boonei* stem bark extract on isolated stomach strip and guinea pig ileum. As low as 1mg/ml of the extract produced contractile response on both smooth muscles. That the extract produced a significantly higher contractile effect on stomach strip than that produced on guinea pig ileum suggests that the former is more sensitive than the latter to the extract. As the pH of the extract before and after adding to the tissue in the organ bath remained constant, it is not likely that pH contributed to the activity of the extract.

In an earlier study, Asuzu and Anaga (1991) reported that up to 40mg/ml of the aqueous stem bark extract of the same plant neither contracted nor relaxed isolated guinea pig ileum. Their report is at variance with that presented in this thesis. Seasonal and ecological variation could probably be the cause of the discrepancy in the two studies.

The contractile activity of the aqueous extract of *A. boonei* reported in this thesis is also at variance with the relaxant effect of echitamine reported by Ojewole (1984). It is probable that *A. boonei* may contain another active principle that may have a relaxant effect on isolated guinea pig ileum.

The lack of effect of atropine on the tonic contractions induced by the extract may rule out the involvement of acetylcholine in the contraction. On the other hand, the blockade of the induced contraction by methysergide may suggest the following. First, the extract may contain 5-HT like substances. Second, the extract may cause the release of 5-HT from the tissue. If the second possibility is considered, then it may explain the relatively delayed onset of action of the extract.

The nature of antagonism between methysergide and the extract may be competitive as there is a progressive shifting of the dose-response curve to the right with maximum response could be maintained with higher doses of extract.

CHAPTER NINE

9.0 GENERAL DISCUSSION, SUMMARY, CONCLUSION AND SUGGESTIONS FOR FURTHER STUDIES.

The main use of *Alstonia* species in Africa, Latin America and Asia is to treat malaria. In West Africa, *A. boonei* is the prevalent species and is mainly used to treat malaria and inflammatory diseases particularly rheumatoid arthritis. These two ethnomedical uses of the plant have been investigated in this thesis.

In the antimalarial studies, the diethylether and ethylacetate extracts showed little or no schizontocidal action whereas the ethanol and water extracts showed some blood schizontocidal effect. This finding agrees with the traditional preparatory methods in which the plant material is soaked in alcohol or boiled in water. In all the tests, the ethanol extract consistently showed higher blood schizontocidal action than the water extract. This implies that the ethanol extract was more potent and efficacious than the water extract. Hence, the use of alcohol in the traditional preparation of the plant extract is suggested.

The use of aqueous extract usually requires boiling the plant material. It has been demonstrated in the present study that application of heat (boiling of plant material in water) for 32hr or more decreases the effectiveness of the water extract

Although this finding may be considered academic for the simple reason that the plant material is not subjected to heat continuously for that length of time, the observation nonetheless indicates that the constituents responsible for antimalarial effect of *Alstonia boonei* stem bark are probably heat labile.

The chemosuppression produced by boiling plant sample for between 2 and 16 hours is not significantly different from that of the extract prepared by steeping the plant material in water (without boiling). Thus the extract may be prepared cold in water. This method however necessitates steeping the plant sample in water for a few days before consuming it. The issue of quick intervention of the disease (malaria) process may then become an important consideration.

The blood schizontocidal effect of ethanolic and aqueous extracts was observed only in the early infaction (4-day test) and repository test whereas it was absent in established infections (Rane's test). This suggests that the extracts are not curative in established *P. y. nigeriensis* infection. The reduction in the rate of parasite multiplication which occurred in early infection end repository tests may be the therapeutic benefit derivable from the use of the plant to treat malaria in traditional medicine.

The method of isolation of AB-1, the antimalarial constituent isolated from *A. boonei* stem bark in this study is simple. The most active fraction or subfraction from

ethanol extract at each fractionation step had higher blood schizontocidal effect than the parent extract. The effectiveness of AB-1 in early and established infections as well as the strong blood schizontocidal action in repository test removes any doubt about the antimalarial property of *Alstonia boonei* in rodent malaria initiated by *P. y. nigeriensis* and *P. berghei* ANKA. Although caution has always been exercised in extrapolating animal data to humans, nevertheless, the strong activity of AB-1 in the present study coupled with the reported moderate to high activity of certain constituents from the family of *Alstonia* against *P. falciparum* in vitro strongly suggests that the plant has a great potential as a candidate plant for the development of new antimalarial drugs. Furthermore, the fact that *Alstonia* species have been in use for centuries as a remedy for malaria means that some benefit is derived from it.

The second ethnopharmacological property of *Alstonia boonei* stem bark investigated in this thesis is its use to treat rheumatoid arthritis and rheumatic pains. These conditions have inflammatory component and the immune system is known to be deranged. The complement system and polymorphonuclear leukocytes are components of immune system which have been implicated in the pathogenesis of rheumatoid arthritis. The activation of the complement cascade via the classical pathway (CP) and the alternative pathway (AP) and zymosan-induced luminol dependent

emiluminescence (CLIZ) by PMNs have been used as immunological parameters in
f is study.

Theoretically, the inhibition of CP-activity could be beneficial in some
inflammatory processes in which immune reactions are deranged, by preventing the
formation of biologically active complement split products (Vogt 1985). It has been
demonstrated in the present study that diethyl ether and ethylacetate extracts of A.
boonei stem bark strongly inhibited the CP-activity whereas the AP-activity was little
affected. In the activity guided fractionation of ethylacetate extract, the use of a
combination of miniprep column chromatography and Sephadex LH-20 systems proved
suitable for purifying the extracts and for the isolation of the active constituent, AB-2.

AB-2 showed a strong anticomplementary activity on the CP assay and also
inhibited zymosan-induced footpad swelling in mice. Thus the *in vivo* anti-inflammatory
activity of
AB-2 may be mediated via the classical pathway of human complement. This
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On the whole, studies carried out in this thesis lend support for the ethnomedical uses of *A. boonei* stem bark to treat malaria and rheumatoid arthritis. These two diseases are somehow affected by the complement system. It has been reported that complement factors are intimately involved in rheumatoid inflammation (Pamham *et al.*, 1984). In the two pathways of complement activation, the CP initiated by the immune complexes is reported to be of greatest importance in rheumatoid arthritis (Morgan, 1990). The effect of complement in malaria has also been reported. *Plasmodium*, the protozoan responsible for malaria is an obligatory intracellular parasite, residing in erythrocytes. It has been reported that infected erythrocytes activate the CP via the AP in the presence of antibody (Morgan, 1990). CP activation leads to stimulation of phagocytosis, thus clearing infected cells (Morgan, 1990). The overall contribution of this phenomenon to parasite destruction is not known.

In this thesis, the compounds, AB-1 and AB-2, isolated from *A. boonei* stem bark extracts have been shown to have antimalaria (AB-1) and immunomodulatory and antiarthritic (AB-2) effects. These are the 2 major ethnomedical uses of the plant extracts. These findings agree, in part, with the suggestion by Wright *et al.* (1993) that the effectiveness of *Alstonia* species in traditional medicine may be due to such effects as antipyretic, anti-inflammatory and immunomodulatory actions.

SUMMARY

The blood schizontocidal activities of crude extracts of *Alstonia boonei* stem bark were assessed against *P. yoelii nigeriensis* (in mice) and *P. berghei* ANKA (in rats). Activities of the extracts and the active constituent (AB-1) isolated from the ethanolic extract were investigated in early infection (4 - day test) and established infection (Rane's test). Their repository activities were also investigated. Chloroquine and pyrimethamine served as reference antimalarial drugs.

The effects of the crude extracts were also investigated on the immune system in vitro using complement system and polymorphonuclear leukocytes (PMNS) as immunological parameters. Anticomplementary (classical (CP) and alternative (AP) pathway activities were assessed using human serum and antibody - sensitized sheep erythrocytes or rabbit erythrocytes. Effects of extracts on phagocytic activities of PMNS was assessed by using PMNS isolated from venors blood of healthy volunteers. Isoniazid served as a reference compound.

A. boonei stem bark extracts had repository activity and a high blood schizontocidal action in early infection but no effect in established infection induced by

P. y nigeriensis. The antiplasmodial activity was concentrated in the ethanolic and the water extract.

The antiplasmodial constituent (AB-1) of the plant isolated via activity guided fractionation was very active in early and established infections as well as having a strong repository action.

A high oral dose of 1,200 mg/kg of AB-1 did not cause any mortality in mice in acute toxicity study. This shows that the constituent is well tolerated when administered orally.

The extracts of *A. boonei* stem bark had an anticomplementary activity mediated via the CP. However, this activity unlike the antimalarial effect was concentrated in the less polar extracts of diethylether and ethylacetate. The extracts appered not to be active on the AP of the complement assay and on the chemiluminescence assay using OZ-stimulated PMNs.

The anticomplementary constituent (AB-2) isolated from ethylacetate extract of *A. boonei* stem bark in this study inhibited zymosan induced footpad swelling in mice. This finding lends support for the use of the plant extract to treat rheumatoid arthritis in traditional medicine. This is the first time that *A. boonei* stem bark extract or its constituents will be shown to have anticomplementary activity.

The inhibition of complement activation by AB-2 increased with an increase in the time of preincubation. This finding suggests that the mode of anticomplementary action of AB-2 involves complement activation and hence depletion of complement component.

The water extract of *A. boonei* stem bark had a contractile effect on isolated guinea pig ileum and rat stomach strip. The contraction induced by the extract was antagonised by methysergide but not by atropine. The antegonism by methysergide appered to be competitive.

The antimalarial constituent of *A. boonei* stem bark (AB-1) is probably heat labile as continuous application of heat for 32h and above reduced the antimalarial activity of aqueous extract of *A. boneei* stem bark.

CONCLUSION

Results obtained in this study showed that ethanolic and aqueous extracts of *Alstonia boonei* stem bark have some blood schizontocidal activity in *P. yoelii nigeriensis* - induced infection in mice. AB-1, the antimalarial constituent isolated from the stem bark of the plant showed stronger blood schizontocidal effect than the parent, crude ethanolic extract. The potency of AB-1 is lower than that of chloroquine in both *P. yoelii nigeriensis* and *P. berghei* ANKA infections.

Diethyl ether and ethylacetate extracts of the stem bark of *Alstonia boonei* had strong anticomplementary activity mediated via the classical pathway. The anticompleentary activity increased with an increase in temperature end time of preincubation. AB-2 significantly inhibited zymosan - induced mouse footpad swelling.

The anticomplementary activity of AB-2 mey be the in vitro correlate of the in vivo anti-inflammatory activity.

Suggestion for further studies

1 Ethanolic extract and the pure antimalarial constituent (AB-1) isolated from it should be further assessed for antimalarial activity using human plasmodia. The use of in vitro model is strongly recommended

2 AB-2, the anticomplementary and antiinflammatory constituent isolated from ethylacetate extract should further be investigated for antiinflammatory activity using carragennan as a phlogistic agent.

3 Structure elucidation of AB-1 and AB-2 should be undertaken

4 A detailed study of the contractile activity of aqueous extract of *Alstonia boonei* sterm bark should be carried out. Since the contractile effect of aqueous extract of the plant was antagonised by methysergide, other drugs such as tetrodotoxin, morphine, Ca^{2+} channel blockers - D - 600 could be used to further elucidate the action of the extract

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