Establishment of *Plasmodium falciparum* culture for the first time in Bangladesh and *in vitro* antimalarial activity assessment of methanolic extract of *Clerodendrum infortunatum* Linn.

A Dissertation submitted to the Department of Pharmacy, East West University, in the partial fulfillment of the requirements for the degree of Master of Pharmacy.

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THIS THESIS PAPER
IS DEDICATED
TO MY BELOVED PARENTS

APPROVAL CERTIFICATE

This is to certify that the dissertation entitled "Establishment of *Plasmodium falciparum* culture for the first time in Bangladesh and *in vitro* antimalarial activity assessment of methanolic extract of *Clerodendrum infortunatum* Linn." was carried out by Jannatul Ferdous (ID: 2015-01-79-014), in the partial fulfillment of the requirement for the degree of Master of Pharmacy for the session Spring-2016 of East West University, Dhaka, Bangladesh. This work was carried out under our supervision and the style and content of the dissertation have been approved and recommended for the award of Master degree

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I, Jannatul Ferdous (ID: 2015-01-79-014), hereby declare that this dissertation, entitled "Establishment of *Plasmodium falciparum* culture for the first time in Bangladesh and *in vitro* antimalarial activity assessment of methanolic extract of *Clerodendrum infortunatum* Linn. " submitted to the Department of Pharmacy, East West University, in the partial fulfillment of the requirement for the degree of Master of Pharmacy, is a genuine & authentic research work carried out by me under the guidance and supervision of Dr. Sufia Islam, Professor, Department of Pharmacy, East West University, Dhaka. The contents of this dissertation, in full or in parts, have not been submitted to any other Institute or University for the award of any Degree or Diploma of Fellowship.

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ABSTRACT

Malaria is still the most destructive and dangerous parasitic infection in many tropical and subtropical countries. The burden of this disease is getting worse, mainly due to the increasing resistance of *Plasmodium falciparum* against the widely available antimalarial drugs. There is an urgent need for new, more affordable and accessible antimalarial agents possessing original modes of action. Developing countries, where malaria is endemic, depend strongly on traditional medicine as a source for inexpensive treatment of this disease. Consequently, it is important that antimalarial medicinal plants are investigated, in order to establish their efficacy and to determine their potential roles of new antimalarial drugs. Thus the objectives of the present study are to establish *Plasmodium falciparum* culture for the first time in Bangladesh and to assess *In-vitro* antimalarial activity of methanolic extract of *Clerodendrum infortunatum* Linn. In this study, we established culture of *P. falciparum* 3D7 strain (MRA-102 and MRA-1240) for the first time in Bangladesh. Strains were cultured in human RBC at 5% hematocrit in complete medium composed of RPMI 1640 medium supplemented with 25 ml HEPES buffer (pH 7.4) and 10% AB human serum. Culture jars were incubated at 37°C under a gaseous mixture of 5% CO_2, 5% O_2, and 90% N_2. Parasites were synchronized by using 5% sorbitol. Then the antimalarial properties of crude extract *Clerodendrum infortunatum* Linn. Was evaluated. The whole parts of the plant contain different medicinally active substances which were variously used in Ayurveda, Unani and Homeopathy system of medicines. Its root, leaf and stem extract are used in microbial infection as well as traditionally used against malaria and fever, mainly from Asia regions including Bangladesh. The air-dried powdered plant parts (roots, leaves,) or whole plant was extracted with solvents (Methanol). Schizontocidal activity was measured using a standard *In vitro* assay, with 3D7 *Plasmodium falciparum* wild type *(MRA-1240) and* chloroquine resistance *(MRA-102)* strain. The methanolic extract of *Clerodendrum infortunatum* Linn. (0.35, 0.116, 0.038, 0.012, 0.004, 1.43×10^{-3}, 4.79×10^{-4}, 1.59×10^{-4} and 5.3×10^{-5} µg/ml) showed antimalarial activity against 3D7 (Chloroquine resistance) strain of *P. falciparum.* In addition, other concentrations (0.2, 0.04, 0.008, 1.6×10^{-3}, 3.2×10^{-4} and 6.4×10^{-5} µg/ml) of extract of *Clerodendrum infortunatum* Linn. showed antimalarial activity against 3D7 (chloroquine resistance) strain of *P. falciparum.* The methanolic extract of *Clerodendrum infortunatum Linn.* (0.35, 0.116, 0.038, 0.012, 0.004, 1.43×10^{-3}, 4.79×10^{-4}, 1.59×10^{-4} and 5.3×10^{-5} µg/ml) showed antimalarial activity against 3D7 (Wild type) strain of *P. falciparum*. We conclude that methanolic extract of *Clerodendrum infortunatum* Linn. showed antimalarial activity. Further study is needed to find out the efficacy and safety of the *Clerodendrum infortunatum* Linn. extract.

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Chapter One
INTRODUCTION

1. BACKGROUND

Malaria has been a major disease of human race for thousands of years. It is referred to in numerous biblical passages and in the writings of Hippocrates. Although drugs are available for cure, malaria is still considered by many to be the most important infectious disease of humans. There are approximately 200 million to 500 million new cases each year in the world, and the disease is the direct cause of 1 million to 2.5 million deaths per year. *(Baron et al. 1997)*

It has long been understood that mosquito numbers depend on climate and that meteorological variables are of use in predicting the onset and severity of malaria epidemics. Decades later, our understanding of how factors such as temperature, humidity and rainfall influence mosquito population dynamics has improved considerably, along with the sophistication with which weather variables can be used to predict malaria transmission rates. *(Hay et al. 1998).*

Bangladesh has a history of endemic malaria transmission in 13 of 64 districts. Up to 70 000 laboratory-confirmed and 900 000 clinical cases, with more than 500 deaths per year, were reported in the late 1990s. Unreported cases might have been as high as 250 000 each year. A cross-sectional survey in 2007 reported a crude prevalence of 4% in the 13 malaria-endemic districts. More than 90% of cases were *Plasmodium falciparum*. The highest prevalence (>10%) was identified in three districts of the Chittagong Hill Tracts in southeastern Bangladesh. The Global Fund to fight AIDS, Tuberculosis and Malaria (Global Fund) approved funds in 20064 (Round 6) and 2009 (Round 9) to support the Bangladesh National Malaria Control Program (NMCP). The NMCP was implemented by the Bangladesh Ministry of Health with BRAC (a national non-governmental development organization). The goals were to: (1) reduce malaria morbidity and mortality; (2) provide community-based services that increased access to diagnosis and treatment with artemisinin-based combination therapies in hard-toreach regions; (3) provide longlasting insecticidal nets (LLINs) to 100% of households in the three malariaendemic districts with the highest malaria burden and 80% coverage in the other ten malaria-endemic districts; (4) strengthen the malaria epidemiological surveillance system; (5) strengthen partnerships in malaria control; and (6) provide periodic (every 3 years) treatment of non-LLIN with suitable insecticides. (*Haque et al. 2014*)

Plasmodium falciparum causes the majority of malaria morbidity and mortality worldwide, with an estimated 781,000 deaths and 225 million cases of malaria annually. Malaria disease occurs during the asexual blood stage of infection, when the merozoite form of the parasites invades host red blood cells (RBCs) and establishes an intracellular trophozoite. The intraerythrocytic parasite grows and divides over approximately 48 h into a schizont form containing up to 30 daughter merozoites that are released for subsequent invasion and replication. Currently there is no vaccine against malaria and with the emergence of resistance to the artemisinin class of antimalarial drugs, new approaches to tackling disease are desperately needed. Since the merozoite form of Plasmodium spp. is extracellular and is exposed to antibodies and immune cells in the blood stream, merozoite antigens have long been regarded as attractive targets for vaccine development. There is also growing interest in the identification and development of novel drug therapies *(Boyle et al. 2013).*

1.1. HISTORY OF MALARIA.

Malaria occupies a unique place in the annals of history. Over millennia, its victims have included Neolithic dwellers, early Chinese and Greeks, princes and paupers. In the 20th century alone, malaria claimed between 150 million and 300 million lives, accounting for 2 to 5 percent of all deaths *(Carter and Mendis, et al. 2002).* Although its chief sufferers today are the poor of sub-Saharan Africa, Asia, the Amazon basin, and other tropical regions, 40 percent of the world's population still lives in areas where malaria is transmitted. Ancient writings and artifacts testify to malaria's long reign. Clay tablets with cuneiform script from Mesopotamia mention deadly periodic fevers suggestive of malaria. Malaria antigen was recently detected in Egyptian remains dating from 3200 and 1304 BC *(Miller et al., 1994).* Indian writings of the Vedic period (1500 to 800 BC) called malaria the "king of diseases." In 270 BC, the Chinese medical canon known as the Nei Chin linked tertian (every third day) and quartan (every fourth day) fevers with enlargement of the spleen (a common finding in malaria), and blamed malaria's headaches, chills, and fevers on three demons—one carrying a hammer, another a pail of water, and the third a stove *(Bruce-Chwatt, et al. 1988).* The Greek poet Homer (circa 750 BC) mentions malaria in The Iliad, as does Aristophanes (445-385 BC) in The Wasps, and Aristotle (384-322 BC), Plato (428-347 BC), and Sophocles (496-406 BC).

Like Homer, Hippocrates (450-370 BC) linked the appearance of Sirius the dog star (in late summer and autumn) with malarial fever and misery (Sherman, et al. 1998).

Malaria's probable arrival in Rome in the first century AD was a turning point in European history. From the African rain forest, the disease most likely traveled down the Nile to the Mediterranean, then spread east to the Fertile Crescent, and north to Greece. Greek traders and colonists brought it to Italy. From there, Roman soldiers and merchants would ultimately carry it as far north as England and Denmark *(*Karlen, et al. 1995). For the next 2,000 years, wherever Europe harbored crowded settlements and standing water, malaria flourished, rendering people seasonally ill, and chronically weak and apathetic. Many historians speculate that falciparum malaria (the deadliest form of malaria species in humans) contributed to the fall of Rome. The malaria epidemic of 79 AD devastated the fertile, marshy croplands surrounding the city, causing local farmers to abandon their fields and villages. As late as the 19th century, travelers to these same areas remarked on the feebleness of the population, their squalid life and miserable agriculture (Cartwright, et al. 1991). The Roman Campagna would remain sparsely settled until finally cleared of malaria in the late 1930s.

In India and China, population growth drove people into semitropical southern zones that favored malaria. India's oldest settled region was the relatively dry Indus valley to the north. Migrants to the hot, wet Ganges valley to the south were disproportionately plagued by malaria, and other mosquito- and water-borne diseases. Millions of peasants who left the Yellow River for hot and humid rice paddies bordering the Yangtse encountered similar hazards. Due to the unequal burden of disease, for centuries, the development of China's south lagged behind its north. Although some scientists speculate that *vivax* malaria may have accompanied the earliest New World immigrants who arrived via the Bering Strait, there are no records of malaria in the Americas before European explorers, conquistadores, and colonists carried Plasmodium malariae, and P. *vivax* as microscopic cargo (Sherman, et al. 1998). Falciparum malaria was subsequently imported to the New World by African slaves initially protected by age-old genetic defenses (sickle cell anemia, and G6PD deficiency) plus partial immunity gained through lifelong exposure. Their descendants, as well as Native Americans and settlers of European ancestry, were more vulnerable, however. Deforestation and "wet" agriculture such as rice farming facilitated breeding of Anopheles mosquitoes. By 1750, both *vivax* and *falciparum* malaria were common from the tropics of Latin America to the

Mississippi valley to New England. Malaria, both epidemic and endemic, continued to plague the United States until the early 20th century. It struck presidents from Washington to Lincoln, weakened Civil War soldiers by the hundreds of thousands (in 1862, Washington, D.C., and its surroundings were so malarious that General McClellan's Army en route to Yorktown was stopped in its tracks), traveled to California with the Gold Rush, and claimed Native American lives across the continent. Until the Tennessee Valley Authority brought hydroelectric power and modernization to the rural South in the 1930s, malaria drained the physical and economic health of the entire region. Just as the United States was eradicating its last indigenous pockets of infection, malaria reclaimed Americans' attention during World War II. During the early days of the Pacific campaign, more soldiers fell to malaria than to enemy forces. The United States' premier public health agency—the Centers for Disease Control and Prevention—was founded because of malaria. By the time of the Vietnam War, the American military discovered that drugresistant malaria was already widespread in Southeast Asia, a harbinger of the worldwide hazard it was destined to become. But nowhere—past or present—has malaria exacted a greater toll than on Africa. A powerful defensive pathogen, it was a leading obstacle to Africa's colonization. Portuguese traders who entered the African coastal plain in the late 1400s and early 1500s were the first foreigners to confront the killing fever. For the next 3 centuries, whenever European powers tried to establish outposts on the continent, they were repelled time and again by malaria, yellow fever, and other tropical scourges. By the 18th century, the dark specter of disease earned West and central Africa the famous epitaph, "the White Man's Grave." Even stronger testimony to malaria's ancient hold on Africa is the selective survival of hemoglobin S—the cause of the inherited hemoglobin disorder sickle cell anemia. Since individuals who inherit two copies of the hemoglobin S gene (one from each parent) are unlikely to survive and reproduce, the disease should be exceedingly rare. However, in those people who have inherited only one sickle cell gene (such individuals are sickle cell "carriers"—they suffer few if any complications of sickle cell disease), needle-shaped clumps of hemoglobin S within red blood cells confer strong protection against malaria (Bayoumi, et al. 1987). Thus, the sickle cell gene is perpetuated in malarious regions by one set of individuals who reap its benefits while another set pays the price. In some parts of Africa, up to 20 percent of the population carry a single copy of the abnormal gene (Marsh, et al. 2002).

In recent years, by virtue of climate, ecology, and poverty, sub-Saharan Africa has been home to 80 to 90 percent of the world's malaria cases and deaths, although some predict that resurgent malaria in southern Asia is already altering that proportion.

1.2. SYMPTOMS OF MALARIA

Malaria is an acute febrile illness. In a non-immune individual, symptoms appear 7 days or more (usually 10–15 days) after the infective mosquito bite. The first symptoms – fever, headache, chills and vomiting – may be mild and difficult to recognize as malaria. If not treated within 24 hours, P. falciparum malaria can progress to severe illness, often leading to death.

Children with severe malaria frequently develop one or more of the following symptoms: severe anaemia, respiratory distress in relation to metabolic acidosis, or cerebral malaria. In adults, multi-organ involvement is also frequent. In malaria endemic areas, people may develop partial immunity, allowing asymptomatic infections to occur. *(Malaria, Fact sheet 2016)*

1.3. TRANSMISSION OF MALARIA

In most cases, malaria is transmitted through the bites of female Anopheles mosquitoes. There are more than 400 different species of Anopheles mosquito; around 30 are malaria vectors of major importance. All of the important vector species bite between dusk and dawn. The intensity of transmission depends on factors related to the parasite, the vector, the human host, and the environment.

Anopheles mosquitoes lay their eggs in water, which hatch into larvae, eventually emerging as adult mosquitoes. The female mosquitoes seek a blood meal to nurture their eggs. Each species of Anopheles mosquito has its own preferred aquatic habitat; for example, some prefer small, shallow collections of fresh water, such as puddles and hoof prints, which are abundant during the rainy season in tropical countries.

Transmission is more intense in places where the mosquito lifespan is longer (so that the parasite has time to complete its development inside the mosquito) and where it prefers to

bite humans rather than other animals. The long lifespan and strong human-biting habit of the African vector species is the main reason why nearly 90% of the world's malaria cases are in Africa.

Transmission also depends on climatic conditions that may affect the number and survival of mosquitoes, such as rainfall patterns, temperature and humidity. In many places, transmission is seasonal, with the peak during and just after the rainy season. Malaria epidemics can occur when climate and other conditions suddenly favour transmission in areas where people have little or no immunity to malaria. They can also occur when people with low immunity move into areas with intense malaria transmission, for instance to find work, or as refugees.

Human immunity is another important factor, especially among adults in areas of moderate or intense transmission conditions. Partial immunity is developed over years of exposure, and while it never provides complete protection, it does reduce the risk that malaria infection will cause severe disease. For this reason, most malaria deaths in Africa occur in young children, whereas in areas with less transmission and low immunity, all age groups are at risk. *(Malaria, Fact sheet 2016)*

1.4. PREVENTION OF MALARIA

Vector control is the main way to prevent and reduce malaria transmission. If coverage of vector control interventions within a specific area is high enough, then a measure of protection will be conferred across the community.

WHO recommends protection for all people at risk of malaria with effective malaria vector control. Two forms of vector control – insecticide-treated mosquito nets and indoor residual spraying – are effective in a wide range of circumstances. *(Malaria, Fact sheet 2016)*

1.5. DIAGNOSIS AND TREATMENT OF MALARIA

Early diagnosis and treatment of malaria reduces disease and prevents deaths. It also contributes to reducing malaria transmission. The best available treatment, particularly for *P. falciparum* malaria, is artemisinin-based combination therapy (ACT).

WHO recommends that all cases of suspected malaria be confirmed using parasite-based diagnostic testing (either microscopy or rapid diagnostic test) before administering treatment. Results of parasitological confirmation can be available in 30 minutes or less. Treatment, solely on the basis of symptoms should only be considered when a parasitological diagnosis is not possible. More detailed recommendations are available in the "WHO Guidelines for the treatment of malaria", third edition, published in April 2015. *(Malaria, Fact sheet 2016)*

1.5.1 WHO response about malaria

The WHO Global Technical Strategy for Malaria 2016-2030 – adopted by the World Health Assembly in May 2015 – provides a technical framework for all malaria-endemic countries. It is intended to guide and support regional and country programmes as they work towards malaria control and elimination.

The Strategy sets ambitious but achievable global targets, including:

- ✓ Reducing malaria case incidence by at least 90% by 2030.
- ✓ Reducing malaria mortality rates by at least 90% by 2030.
- ✓ Eliminating malaria in at least 35 countries by 2030.
- ✓ Preventing a resurgence of malaria in all countries that are malaria-free.

This Strategy was the result of an extensive consultative process that spanned 2 years and involved the participation of more than 400 technical experts from 70 Member States. It is based on 3 key pillars:

1. ensuring universal access to malaria prevention, diagnosis and treatment;
2. accelerating efforts towards elimination and attainment of malaria-free status; and

3. transforming malaria surveillance into a core intervention.

The WHO Global Malaria Programme (GMP) coordinates WHO's global efforts to control and eliminate malaria by:

- setting, communicating and promoting the adoption of evidence-based norms, standards, policies, technical strategies, and guidelines;
- keeping independent score of global progress;
- developing approaches for capacity building, systems strengthening, and surveillance; and
- identifying threats to malaria control and elimination as well as new areas for action.

GMP is supported and advised by the Malaria Policy Advisory Committee (MPAC), a group of 15 global malaria experts appointed following an open nomination process. The MPAC, which meets twice yearly, provides independent advice to WHO to develop policy recommendations for the control and elimination of malaria. The mandate of MPAC is to provide strategic advice and technical input, and extends to all aspects of malaria control and elimination, as part of a transparent, responsive and credible policy setting process. *(Malaria, Fact sheet 2016)*

1.6. CLINICAL MANIFESTATIONS OF MALARIA

The most characteristic symptom of malaria is fever. Other common symptoms include chills, headache, myalgias, nausea, and vomiting. Diarrhea, abdominal pain, and cough are occasionally seen. As the disease progresses, some patients may develop the classic malaria paroxysm with bouts of illness alternating with symptom-free periods. The malaria paroxysm comprises three successive stages. The first is a 15-to-60 minute cold stage characterized by shivering and a feeling of cold. Next comes the 2-to-6 hour hot stage, in which there is fever, sometimes reaching 41°C, flushed, dry skin, and often headache, nausea, and vomiting. Finally, there is the 2-to-4 hour sweating stage during which the fever drops rapidly and the patient sweats. In all types of malaria the periodic febrile response is caused by rupture of mature schizonts. In *P vivax* and *P ovale* malaria,

a brood of schizonts matures every 48 hr, so the periodicity of fever is tertian ("tertian malaria"), whereas in *P malariae* disease, fever occurs every 72 hours ("quartan malaria"). The fever in falciparum malaria may occur every 48 hr, but is usually irregular, showing no distinct periodicity. These classic fever patterns are usually not seen early in the course of malaria, and therefore the absence of periodic, synchronized fevers does not rule out a diagnosis of malaria. *(Baron et al. 1997)*

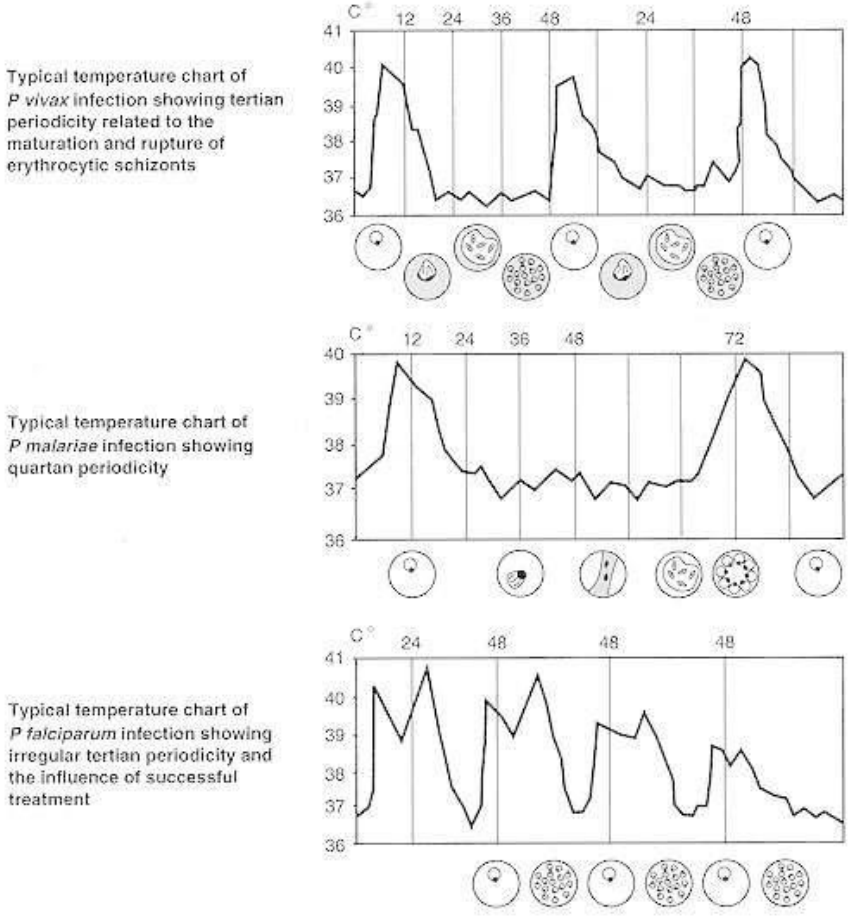

Figure 1: Typical temperature charts of malarial infections.

Physical findings in malaria are nonspecific and offer little aid in diagnosis. In many cases there may be no positive findings other than fever. Splenomegaly is common but may not be apparent early in disease. Hepatomegaly, jaundice, hypotension and abdominal tenderness may also be seen. Malaria does not cause lymphadenopathy and is not associated with a rash.

A variety of laboratory abnormalities may be seen in a case of uncomplicated malaria. These include normochromic, normocytic anemia, thrombocytopenia, leukocytosis or

leukopenia, hypoglycemia, hyponatremia, elevated liver and renal function tests, proteinuria, and laboratory evidence of disseminated intravascular coagulation (although clinically important bleeding is rare). Eosinophilia is not seen. Patients with complicated malaria occasionally show evidence of massive intravascular hemolysis with hemoglobinemia and hemoglobinuria.

If the diagnosis of malaria is missed or delayed, especially with *P falciparum* infection, potentially fatal complicated malaria may develop. The most frequent and serious complications of malaria are cerebral malaria and severe anemia. Cerebral malaria is defined as any abnormality of mental status in a person with malaria and has a case fatality rate of 15 to 50 percent. Other complications include: hyperparasitemia (more than 3 to 5 percent of the erythrocytes parasitized); severe hypoglycemia; lactic acidosis; prolonged hyperthermia; shock; pulmonary, cardiac, hepatic, or renal dysfunction; seizures; spontaneous bleeding; or high-output diarrhea or vomiting. These manifestations are associated with poor prognosis. Persons at increased risk of severe disease from malaria include older persons, children, pregnant women, nonimmune persons and those with underlying chronic illness. Other complications of malaria infection include gram-negative sepsis, aspiration pneumonia and splenic rupture.

1.7. MANIFESTATIONS AND IMPACT OF MALARIAL DISEASE AROUND THE WORLD

The meeting began with a series of presentations and discussions on clinical and epidemiological aspects of malarial disease as experienced in different endemic regions of the world.

1.7.1. The general picture

Malaria occurs throughout the tropical world, where it remains one of the most prevalent of infectious diseases. *(SturchIer, et al. 1989)* The aetiologic agents of malaria are recognized as four distinct species of Plasmodium: P. falciparum, P. vivax, P. malariae and P. ovale. The distribution of these species and the spectrum of clinical conditions that arise from infection are highly diverse among individual infections and in their general

characteristics in different endemic regions. Wherever it occurs, malaria ranks high among the main causes of mortality and/or morbidity due to infectious agents, and the mortality can be attributed virtually entirely to a single species, P. falciparum.

The picture of malarial disease in the South Asian region was described as represented in parts of India and Sri Lanka. Transmission of malaria, which includes both P. vivax and P. falciparum, is focal but usually stable where it occurs. Annual incidence rates (number of cases per person per year) are relatively low but may rise above lOO%, ie. exceed one case per person per year, in some localities. The commonest presentation for both species is an acutely febrile illness which, in the case of P. vivux, gives rise to distinct paroxysms (chills, rigor, fever) at 48 h intervals. Associated symptoms are headache, anorexia and nausea, myalgia, arthralgia and general prostration. Adults and children are typically affected to similar degrees, although individuals differ widely in the nature and intensity of their symptoms. Parasitaemia without symptoms can occur, but usually proceeds to symptomatic infection. Severe and complicated disease, in the sense discussed below, and as defined by WHO criteria, *(Anon, et al.1990)* is relatively rare in any age group in Sri Lanka. This picture is not, however, a constant or uniform feature; devastating outbreaks with high mortality rates have occurred in the past. Moreover, in both India and Sri Lanka, severe disease tends to be concentrated in certain localities, as in North Central Province around Anuradapura in Sri Lanka and in Orissa State in India, where the incidence of cerebral malaria is relatively high in young adults. Chloroquine remains the drug of choice for most of the region, although Rl (recurrence of parasitaemia after initial clearance of detectable parasites and symptoms following drug treatment) levels of resistance in P. falciparum infections are now widespread in Sri Lanka.

These general aspects of malaria, typical of India and Sri Lanka, appear to distinguish the conditions in this region from those in much of Southeast Asia, where cerebral and other forms of severe and complicated malaria (which also occur typically in adults) are more frequent. High levels of both chloroquine resistance and multidrug resistance of P. falciparum are also widespread in Southeast Asia, contributing significantly to the large amount of unmanageable severe disease in these regions. *(Mendis et al. 1995)*

1.7.2. The impact of malarial disease

The overall burden of malarial disease on human populations is hard to assess and varies qualitatively and quantitatively in different regions of the world. Outside Africa and New Guinea, the burden of disease is spread through most age groups. Malaria is a major cause of morbidity, and to a variable extent mortality, in adults in several non-African regions of the world. In Sri Lanka, it is estimated that a single episode of malaria incapacitates an adult from work for an average of two weeks, while up to one-quarter of hospital admissions in endemic regions in Sri Lanka are currently due to malaria. In Southeast Asia, the risk of severe malaria has risen dramatically in recent decades due to the spread of multidrug resistance in *P. fulcipurum* malaria. *(Mendis et al. 1995)*

1.8. ANTIMALARIAL DRUG RESISTANCE IN BANGLADESH

Malaria is endemic in 13 of 64 districts in Bangladesh, where approximately 8% of the population live in risk areas. *(Reid et al. 2012)* The ecoepidemiological settings of the country's 152.4 million population where malaria occurs include rural, forest, forest fringe and foothills, these endemic districts, bordering India and Myanmar, are covered with hilly forest and difficult to access. The Plasmodium species *Plasmodium falciparum, P. vivax, P. malariae* and *P. ovale* exist in the region. *(Haque et al. 2009, Rahman et al. 2010, Fuehrer et al. 2010)* Malaria became epidemic in Bangladesh during the 1990s, *(Haque et al. 2010)*probably as a result of the banning of dichlorodiphenyltrichloroethane (DDT) in 1985, the lack of continued malaria control efforts and the development of resistance to chloroquine (CQ), which until then was the most widely used antimalarial drug. *(Haque et al. 2010)* In 1996, the Bangladesh National Malaria Control Program reported approximately 137 000 malaria cases. *(Bangladesh. Database site of NMCP 2013)* New interventions, including rapid diagnostic tests (RDT), the distribution of long-lasting insecticidal nets (LLIN) and artemisinin-based combination therapy (ACT, Coartem) were introduced in 2007 with the support of the Global Fund to Fight AIDS, Tuberculosis and Malaria. By 2012, malaria cases had been reduced to 29 522 reported cases with 11 reported deaths. *(Bangladesh. Database site of NMCP 2013)* The proportion of P. falciparum cases increased to approximately 94% of the total in 2012 from around 54% in 1996, in spite of the annual prevalence of P. falciparum infection decreasing from 4.87 to 1.95 cases per 1000 population over the same period.

The apparent change in species dynamics in Bangladesh might be attributable to improved diagnosis as a result of increased use of RDTs. *(Bangladesh. Database site of NMCP 2013)* The recent surge in antimalarial drug resistance is an obstacle to the treatment, control and ultimately elimination of malaria from Bangladesh. Resistance to CQ in P. falciparum malaria was first reported from the north of the country in 1970, *(Alam et al. 2008)* and next observed in 1975 in the north east, where Bangladesh borders the Indian state of Tripura. *(Rosenberg et al. 1976)* Resistance to CQ subsequently increased from 10% in 1979 to 45% in 1987, and 57% in 1992. *(Rosenberg et al. 1976)* Although no systematic drug resistance monitoring system was in place in Bangladesh, various research organizations initiated clinical trials. A malaria case definition and structured treatment regimen was introduced in 1994, *(Alam et al. 2008)* when malaria was classified as 'uncomplicated', 'treatment failure' (i.e. failure to clear detectable parasites from the blood) *(White et al. 2009)* and 'severe' *(WHO. 2000)* In 2004.

1.9. CLASSIFICATION OF *Plasmodium.*

Only four species of the protozoan genus *Plasmodium* usually infect humans: *P falciparum, P vivax, P malariae,* and *P ovale*. *P falciparum* and *P vivax* account for the vast majority of cases. *P falciparum* causes the most severe disease. *(Baron et al. 1997)*

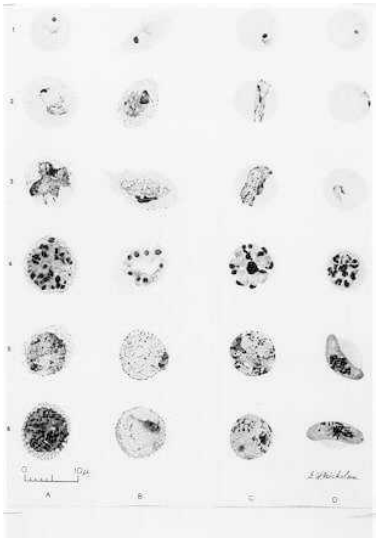

Figure 2: Blood stages of Plasmodium.

1.10. STRUCTURE AND LIFE CYCLE OF MALARIA PARASITE

Like many protozoa, plasmodia pass through a number of stages in the course of their two-host life cycle. The stage infective for humans is the uninucleate, lancet-shaped sporozoite (approximately 1 × 7 μm). Sporozoites are produced by sexual reproduction in the midgut of vector anopheline mosquitoes and migrate to the salivary gland. When an infected *Anopheles* mosquito bites a human, she may inject sporozoites along with saliva into small blood vessels. Sporozoites are thought to enter liver parenchymal cells within 30 minutes of inoculation. In the liver cell, the parasite develops into a spherical, multinucleate liver-stage schizont which contains 2,000 to 40,000 uninucleate merozoites. This process of enormous amplification is called exoerythrocytic schizogony. This exoerythrocytic or liver phase of the disease usually takes between 5 and 21 days, depending on the species of *plasmodium*. However, in *P vivax* and *P ovale* infections, maturation of liver-stage schizonts may be delayed for as long as 1 to 2 years. These quiescent liver-phase parasites are called hypnozoites.

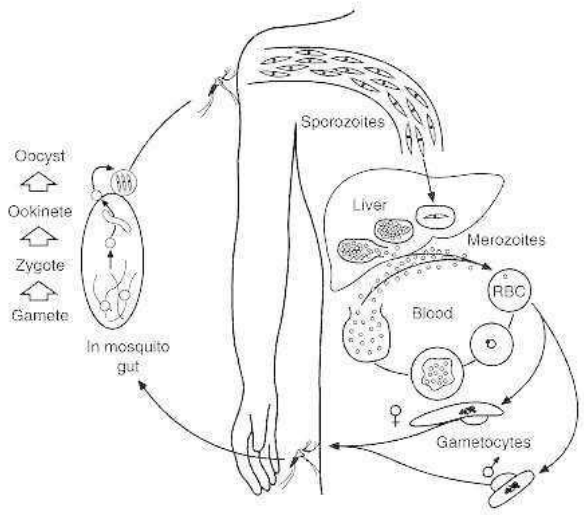

Figure 3: Life cycle of malaria parasite

Regardless of the time required for development, the mature schizonts eventually rupture, releasing thousands of uninucleate merozoites into the bloodstream. Each merozoite can infect a red blood cell. Within the red cell, the merozoite develops to form either an erythrocytic-stage (blood-stage) schizont (by the process of erythrocytic schizogony) or a

spherical or banana-shaped, uninucleate gametocyte. The mature erythrocytic-stage schizont contains 8 to 36 merozoites, each 5 to 10 μm long, which are released into the blood when the schizont ruptures. These merozoites proceed to infect another generation of erythrocytes. The time required for erythrocytic schizogony-which determines the interval between the release of successive generations of merozoites-varies with the species of *plasmodium* and is responsible for the classic periodicity of fever in malaria.

The gametocyte, which is the sexual stage of the *plasmodium*, is infectious for mosquitoes that ingest it while feeding. Within the mosquito, gametocytes develop into female and male gametes (macrogametes and microgametes, respectively), which undergo fertilization and then develop over 2 to 3 weeks into sporozoites that can infect humans. The delay between infection of a mosquito and maturation of sporozoites means that female mosquitoes mustlive a minimum of 2 to 3 weeks to be able to transmit malaria. This fact is important in malaria control efforts. *(Baron et al. 1997)*

1.11. PATHOGENESIS OF MALARIA

Clinical illness is caused by the erythrocytic stage of the parasite. No disease is associated with sporozoites, the developing liver stage of the parasite, the merozoites released from the liver, or gametocytes.

The first symptoms and signs of malaria are associated with the rupture of erythrocytes when erythrocytic-stage schizonts mature. This release of parasite material presumably triggers a host immune response. The cytokines, reactive oxygen intermediates, and other cellular products released during the immune response play a prominent role in pathogenesis, and are probably responsible for the fever, chills, sweats, weakness, and other systemic symptoms associated with malaria. In the case of falciparum malaria (the form that causes most deaths), infected erythrocytes adhere to the endothelium of capillaries and postcapillary venules, leading to obstruction of the microcirculation and local tissue anoxia. In the brain this causes cerebral malaria; in the kidneys it may cause acute tubular necrosis and renal failure; and in the intestines it can cause ischemia and ulceration, leading to gastrointestinal bleeding and to bacteremia secondary to the entry of intestinal bacteria into the systemic circulation. The severity of malaria-associated anemia tends to be related to the degree of parasitemia. The pathogenesis of this anemia appears

to be multifactorial. Hemolysis or phagocytosis of parasitized erythrocytes and ineffective erythropoiesis are the most important factors, and phagocytosis of uninfected erythrocytes and an autoimmune hemolytic anemia have also been implicated. Massive intravascular hemolysis leading to hemoglobinuria and renal failure is referred to as blackwater fever. It was described more frequently in the past than currently. Hemolysis may also occur after the use of certain antimalarials (especially primaquine) in patients with glucose 6-phosphate dehydrogenase deficiency. *(Baron et al. 1997)*

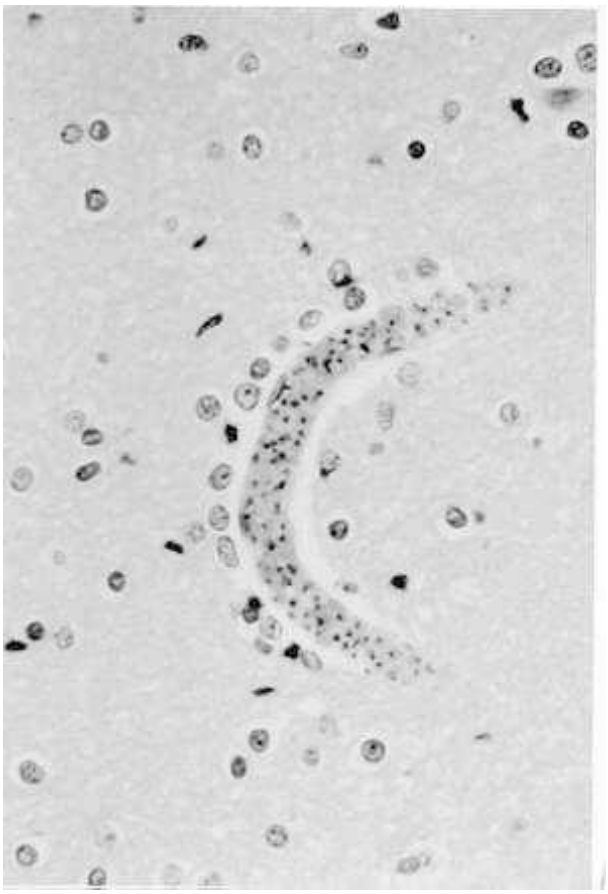

Figure 4: Light micrograph of a cerebral capillary blocked with parasitized erythrocytes

1.12. CULTURE SYSTEMS OF *Plasmodium falciparum*

Plate cultures are prepared with a 5% hematocrit and about 1% parasitemia. The lower the initial parasitemia is, the greater the increase in numbers of parasites that will occur

during in vitro growth is. Trager *(Trager et al. 1994)* obtained 20- to 50-fold increase in parasite numbers with a starting parasitemia of 0.1%. Parasitemia of cultures can be increased to about 20% by changing medium in cultures every 8 h *(Jensen et al. 1978).* Monitoring of parasitemia is accomplished by preparing blood films, staining with Giemsa stain following methanol fixation, and counting infected red blood cells microscopically. While the simplest system for cultivation of parasites uses petri or Linbro plates in a candle jar, this system is laborintensive, requiring constant attention and daily changes of medium in order to maintain parasite growth. In this static system infected erythrocytes settle out to form a layer, producing microenvironments high in lactic acid in the region of the proliferating parasites. This may lead to conditions unfavorable for schizont development and penetration of merozoites into uninfected erythrocytes. Lactic acid production taxes the buffering capacity of the medium and leads to a drop in pH, which is detrimental to the growth of Plasmodium. Optimal parasite yields occurred with an extracellular pH of 7.2 to 7.45 and a lactate concentration below 12 mM *(Jensen et al. 1983)*; higher lactate concentrations were postulated to cause negative feedback of glycolysis.

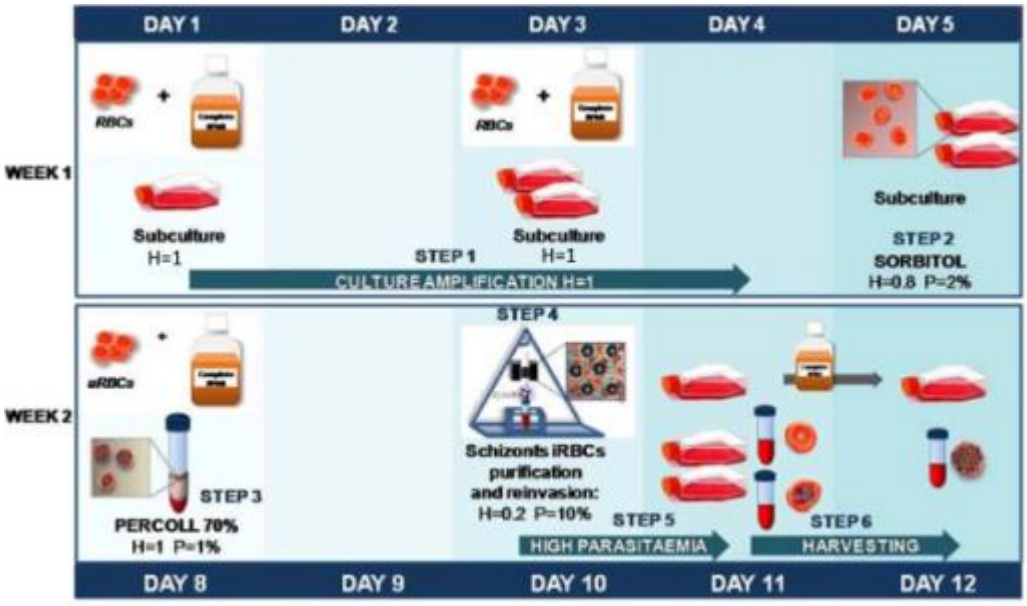

Figure 5: Summary of the complete protocol. Plasmodium falciparum culture is maintained.

at 1% haematocrit during the first week to scale up the P. falciparum culture. Plasmodium falciparum culture is synchronized with 5% wt/v sorbitol and then adjusted to H = 0.8% and P = 2%. Percoll is carried out after three days to purify the schizonts and P. falciparum culture is adjusted to H = 1% and P = 1%. Forty-eight hours later, P. falciparum culture enriched with mature forms is passed through the vario mac magnetic column to isolate the schizonts-enriched iRBCs. iRBCs obtained are adjusted to H = 0.2% and P = 10% and after 18 hours P. falciparum ring-enriched culture at approximately 40% parasitaemia is harvested. If a trophozoite or schizont-enriched population is required, media has to be changed and P. falciparum culture will be harvested next day.

Glucose diffusion into the cell layer also becomes limiting *(Jensen et al. 1978)*. Several devices to semiautomate cultivation of plasmodia have been described previously *(Jensen et al. 1988)*. These devices allow for continuous flow of medium through growth vessels, with a controlled gas phase of 2 to 5% CO_2, 3 to 18% O_2, and the remainder being N_2. Semiautomated devices reduce the amount of time spent on maintenance of stock cultures of the parasite. Although the malarial parasite is found in red blood cells, it is microaerophilic in its oxygen preference. Taylor-Robinson *(Taylor-Robinson et al. 1998)* used a commercial system designed for growth of Campylobacter spp. to generate the low O_2 and high CO_2 levels favored by P. falciparum. Anaerobic jars and gas-generating envelopes were employed to grow parasites in tissue culture flasks or microtiter plates in an atmosphere of 6% O_2–8% CO_2–86% N_2 *(Taylor-Robinson et al. 1998)*. Suspension cultures of parasites have also been tried. Zolg et al. *(Zolg et al. 1982)* reported improved yields with shaking of cultures, but these claims have been disputed *(Jensen et al. 1988)*. Fragility of the infected red blood cells is an important factor in agitated cultures, but this has been countered to some extent by the use of methylcellulose in agitated cultures *(Zolg et al. 1982)*. Mons et al. 1988 have used a culture flask with a stirring bar for cultivation of the rodent parasite P. berghei. Induction of synchrony in vitro. In its human host, P. falciparum exhibits a synchrony of about 48-h duration. Blood sampled at any one time from an infected host will reveal a parasite population at the same stage in its developmental cycle, i.e., mostly ring stages or mostly schizonts, etc. This synchrony is in part a response to the circadian rhythmicity of the host's body. Synchrony can be artificially imposed in vitro upon developing malaria parasites by one of several methods. Most popular is the use of sorbitol or mannitol treatment of infected erythrocytes.

Infected cells are treated with 5% sorbitol, which causes lysis of erythrocytes containing late stages and preferentially selects for red blood cells with early ring stages. The effect is not osmotic, but has to do with the permeability of infected cells and the sensitivity of the parasites to sorbitol (Trager et al. 1994). Treatment can be repeated at 34 h to further select for young stages and improve on the synchrony. Other techniques involve the separation of late-stage parasites, as by sedimentation in Plasmagel or gelatin. Most strains of P. falciparum produce knobs over the surface of the host erythrocyte when the parasite reaches the late trophozoite to schizont stage. Such red cells do not form rouleaux in the Plasmagel, as do uninfected red cells or those containing ring-stage organisms. As a result, the cells with late-stage parasites remain in suspension while the uninfected ones and those with rings settle out. If the late-stage parasites are then mixed with fresh red cells and put under culture conditions, merozoites formed by them invade new cells. If one allows invasion to go on for 3 h and then treats the cells with sorbitol to kill all late-stage parasites, one gets a population of rings just 0 to 3 h old. Such a tightly synchronized culture will remain quite synchronous through about three cycles. Shifting cultures of *P. falciparum* from 37 to 28°C has been used to delay the asexual cycle for 12 to 16 h (Rojas et al. 1993).

1.13. PLANT PROFILE OF *Clerodendrum infortunatum* Linn.

Figure 6: Plants and leaf of *Clerodendrum infortunatum* Linn.

a) **Botanical description:** An undershrub or shrub, often gregarious, forming dense thicket, branches obscurely tetragonal, channeled, covered with densely yellowish-brown appressed tomentose. Leaves simple, decussate-opposite, 10-25 x 7.5-20 cm, ovate, acute acuminate, rounded or cordate at the base, more or less covered with rough hairs with small round glands on the lower surface, margin denticulate or serrate, rarely entire, petioles 2.5-10.0 cm long, densely pubescent. Flowers white, tinted with red to purplish red, pleasantly scented, inflorescence terminal, trichotomous, pyramid-shaped panicle, 20 cm long, peduncles 1-6 cm long, red or purplish-red, panicle branches ended by 3 flowers. Fruit a drupe, globular, 8 mm across, at first green, become bluish-black when mature, shining, enclosed by the enlarged fleshy red calyx. *(Motaleb et al. 2013)*

b) **Flowering and fruiting:** January to July.

c) **Ecological adaptation:** It normally grows in the fallow lands, along roadsides, village thickets, slope or bank of ponds, sometimes on cultivated field margin and also along railway tracts.

d) **Distribution:** India, Myanmar, Thailand, China, Indonesia, Sri Lanka and Philippines. In Bangladesh, it occurs throughout the country, often abundant as an undergrowth of Sal forests and village thickets.

e) **Propagation and management:** Propagated through seeds and old rootstocks.
f) **Chemical constituents:** The plant contains saponin, flavonoids, alkaloids, a new glycoside, clerodendroside, lupeol, benzoic acid derivaties and β-sitosterol. The plant also contains clerosterol, clerodolone, clerodone. Leaves contain protein, free reducing sugar, a bitter principle, clerodin, a sterol, oleic, stearic and lignoceric acids, tannin, glucuronide and gallic acid. Roots contain lupeol and β-sitosterol, the antifungul flavonoids, cabruvin and quercetin. The seeds contain fatty oil, in which the major fatty acids are palmitic, oleic and linoleic acids. Clerodin and hentriacontane have been isolated from flowers. *(Motaleb et al. 2013)*

g) **Medicinal uses:** The medicinal use of the species collected through FGD and one to one discussions are shown below,

Table 1: Medicinal uses of *Clerodendrum infortunatum* Linn.

Disease name	Parts use	Preparation process	User group
Contiguous disease/itching	Whole plant and root	Bath with plant and root boiled water.	Khumi and Marma
Abdominal pain of children	Leaf	Leaf juice is taken thrice a day for 3-5 days.	Tripura
Lice in hair	Leaf	Hair washes with leaf boiled water.	Khumi

The species is also used in the treatment of anaemia, cluster headache, diabetes, diarrhoea, fever, food poisoning, gallstone, gastric ulcer, general weakness, gout, headache, impotence, indigestion, itching, jaundice, malaria, obstructive labor, osteoarthritis, paralysis, piles, rheumatism (gout), scabies, stomachache, tuberculosis and vaginitis *(Uddin, 2006)*; chest pain and cough *(Kirtikar et al., 1935)*; insect bites, skin diseases, scorpion sting and tumor *(Ghani, 2003)*; asthma, cough, malarial fever and snake bite *(Chopra et al., 1956)*; skin diseases *(Moldenke and Moldenke, 1983)*; ascaris and liver pain *(Partha and Hossain, 2007)*; diabetes and sores *(Roy et al., 2008)*; abdominal pain, dental caries, pain, roundworms with indigestion and vomiting *(Rahman et al., 2007)*; fever and healing cut injury *(Uddin et al., 2006)* and asthma, chest complaint, cough, impotency, jaundice, malaria, scabies, skin diseases and tumors *(Yusuf et al., 2009, Motaleb et al. 2013).*

Plant extract of *Clerodendrum infortunatum* Linn. has been traditionally used in the treatment of malaria *(Chopra et al., 1956)*. However no *in vitro* study has been done to assess the antimalarial activity of the plant extract. Therefor this present study has been carried out to assay *In-vitro* antimalarial activity of methanolic extract of *Clerodendrum infortunatum* Linn.

Chapter Two
OBJECTIVES

2. OBJECTIVE OF THE STUDY

2.1. This study has two major objectives:

1) To establish *Plasmodium falciparum* culture for the first time in Bangladesh

2) To asses *In-vitro* antimalarial activity of methanolic extract of *Clerodendrum infortunatum* Linn.

Chapter Three

MATERIAL AND METHODS

3.1. Plant Material and Extraction

- Theleaves of *Clerodendrumin fortunatum* Linn were collected from the Jahangirnagar University area, Savar, Bangladesh in January 2016.
- About 400 g of dried and powdered material have to be soaked in 1000 mL methanol at 25 ± 2 °C for 72 h in a beaker and mixture needs to be stirred every 18 h using a sterile glass rod.
- Filtrate was obtained 3 times with the help of no. 102 filter paper and sterilized cotton filter.
- The solvent was removed by rotary evaporator and 11.56 g extract was obtained.
- This crude extract was used for further investigations of anti-malarial effect.

3.2. Biological materials

The *P. falciparum* 3D7 strain (MRA-102 and MRA-1240) was cultured in human RBC at 5% hematocrit in complete medium composed of RPMI 1640 medium supplemented with 25 mMHEPES buffer (pH 7.4) and 10% AB human serum . Petri dishes were incubated at 37°C under a gaseous mixture of 5% CO2, 5% O2, and 90% N2.Parasites were synchronized by using 5% sorbitol.

3.3 Culture of *Plasmodium falciparum* blood stages

3.3.1 Materials

- RPMI medium for washing
- Complete medium for the culture of *P. falciparum* (RPMI medium supplemented with 10% serum)
- Uninfected erythrocytes (blood group O)
- Sodium chloride
- Freshly prepared Giemsa stain
- 25 cm2 sterile plugged sealed tissue culture flasks
- Sterile propipettes

- Sterile graduated pipettes
- Disposable microscope slides

3.4 Preparation of complete medium for malaria culture

3.4.1 Materials

- Sterile graduated pipettes
- 100mL volumetric flask
- 1L volumetric flask
- Cups and spatula
- Disposable 0.22μM sterilisation filter unit and filter unit receiver
- Sterile vials
- RPMI 1640, powdered medium, stored at 4°C
- Sodium bicarbonate, NaHCO3, FW 84.01, stored at RT
- HEPES, C8H18N2O4S, FW 238.1, stored at RT
- Hypoxanthine, C5H4N4O, FW 136.11, stored at RT
- L-glutamine, C5H10N2O3, FW 146.1, stored at RT
- Glucose, C6H12O6, FW 180.16, stored at RT
- Orotic acid, C5H4N2O4, FW156.1 (optional), stored at RT
- Gentamicin (optional), C21H43N5O7, FW 477.596, stored at -20°C WWARN
- Sterile human serum stored at -20 °C or Albumax stored at + 4 °C, protected from extended exposure to light
- Hydrochloric acid 1N
- Sodium hydroxide 1N
- Water for cell culture or double-distilled water

3.5 Synchronisation of *Plasmodium falciparum*

3.5.1 Materials

- RPMI medium for washing
- Percoll
- PBS
- D-sorbitol

- Water for cell culture or orbidistillated water
- 25 cm2 sterile plugged seal tissue culture flasks
- Sterile propipettes
- Sterile graduated pipettes
- Disposable microscope slides
- 100 mL volumetric flask
- Cups and spatula
- Disposable sterilisation filter unit and filter unit receiver
- Sterile vials.

3.6 Preparation of Predosed Plates

3.6.1 Materials

- Sterile graduated pipettes
- Sterile vials
- 12 channel multipipette
- Clear, cell culture-treated 96-well microtiter plates
- 1mL serological pipettes
- Sterile micropipette tips
- Volumetric flask
- Ethyl alcohol
- MethanolK
- Sterile pure water
- Standardized antimalarials pre-weighed in vials:
    - Dihydroartemisinin
    - Piperaquine
    - Lumefantrine
    - Desethylamodiaquine
    - Chloroquine
    - Mefloquine
    - Quinine
    - Pyronaridine
    - Doxycycline
    - Atovaquon

3.7 Estimation of *Plasmodium falciparum* drug susceptibility *ex vivo* by HRP2 ELISA

3.7.1 Materials

- 96-well microculture plates e.g. Costar 3599 or Falcon 3070 pre-coated with antimalarial drugs. See WWARN procedure INV03: preparation of fresh and dried pre-dosed plates.
- Complete RPMI 1640 Medium (10.43g RPMI 1640 powder + 6 g HEPES + 25 mg gentamycin + plus 0.5% w/v of Albumax I + distilled water to 1L). Add NAHCO3 (2.8 ml of 7.5% NAHCO3 per 100ml medium before use).RBCs (blood group 0 or same as patient).
- Sterile disposable phlebotomy tool
- Sterile trays
- PBS pH 7.4
- Tween® 20 (e.g. Sigma-Aldrich, P1379)
- BSA (e.g. Sigma-Aldrich, A9647)
- TMB
- 96-well, high protein-binding capacity, ELISA plates
- Sulphuric acid
- Antibody 1: MPFM-55A (e.g. Immunology Consultants Laboratories, Inc, Newberg, OR, USA or equivalent)
- Antibody 2: MPFG-55P (e.g. Immunology Consultants Laboratories, Inc, Newberg, OR, USA or equivalent

3.8 Culture of Plasmodium falciparum blood stages

3.8.1 Equipment

1. Cryogenic equipment at 4° C
2. Cryogenic equipment at –20° C
3. Cryogenic equipment at –196° C
4. Laminar flow hood

5. Incubator with a reliable source of CO2 or incubation chamber with gas mixture or candle jar
6. Microscope with a 100x oil immersion objective
7. Centrifuge
8. Water-bath or heater block
9. Shaker

Figure 7: Equipment 1-6

Figure 8: Equipment 7-9

3.9 Preparation of complete medium for malaria culture

3.9.1 Equipment

1. Analytical balance
2. Magnetic stir plate
3. Laminar flow hood
4. Vacuum trapk
5. Cryogenic equipment at + 4 °C
6. Cryogenic equipment at – 20 °C
7. Water-bath or heater block
8. pH meter
9. Osmometer

Figure 9: Equipment 1, 8

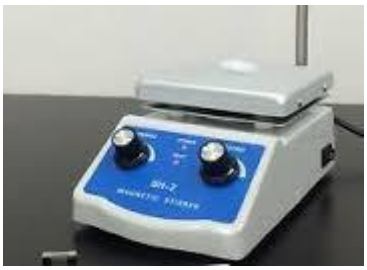

Figure 10: Equipment 2, 9

3.10 Synchronisation of *Plasmodium falciparum*

3.10.1 Equipment

1. Cryogenic equipment at 4° C
2. Laminar Flow hood
3. Vacuum trap
4. Incubator with a reliable source of CO2 or candle jar
5. Microscope with a 100x oil immersion objective
6. Centrifuge
7. Water-bath or heater block
8. Shaker
9. Densitometer.

Figure 11: Equipment 9

3.11 Preparation of Predosed Plates

3.11.1 Equipment

- Laminar flow hood
- Cryogenic equipment at 4 °C

- Cryogenic equipment at 20 oC

3.12 Estimation of *Plasmodium falciparum* drug susceptibility *ex vivo* by HRP2 ELISA

3.12.1 Equipment

1. Incubator
2. Candle jar or incubation chamber with gas mixture
3. Freezer (-20°C or below)
4. ELISA
5. plate reader (capable of reading at 450nM)

Figure 12: 5, 6, 7

6. Multichannel pipette (20-200 µL)
7. One set of adjustable pipettes (20-200 µL; 100-1000 µL)

3.13 Culture of Plasmodium falciparum blood stages

3.13.1 Procedure

3.13.1.1 Preparation of red blood cells

Red blood cells are used in dilutions of culture to lower parasitemia and maintain hematocrit.

I. Warm RPMI medium to 37° C in water-bath or heater block.

II. Centrifuge red blood cells at 500 g for 5 minutes.

III. Add 2:1 v/v RPMI medium to erythrocytes pellet to return to the original volume.

IV. Stir 3–5 minutes at room temperature on a shaker.

V. Centrifuge at 500 g for 5 minutes.

VI. Remove supernatant.

VII. Wash the pellet of packed red cells two more times as above.

3.13.1.2 Preparation of the sample

3.13.1.2.1 Clones

Clones are stored in glycerolyte at –196° C in liquid nitrogen.

I. Prepare a sterile NaCl solution at 3.5%. Conserve at 4° C.

II. Warm RPMI medium to 37° C in water-bath or heater block.

III. Thaw the frozen sample at room temperature in 1–2 minutes.

IV. Transfer the contents to a new sterile tube.

V. Add NaCl solution drop by drop, very slowly, inverting gently each time and waiting for a minute or two. This slow timing is crucial to avoid lysis of the red cells and will take 5–10 minutes.

VI. Agitate gently 3–5 minutes at room temperature on a shaker.

VII. Centrifuge at 500 g for 5 minutes.

VIII. Remove supernatant.

IX. Wash the pellet two more times with RPMI medium 1:9 v/v.

3.13.1.2.2 Isolates

There is a possibility for short term culture of patient isolates before running an *in vitro* test but this may select clones. Isolates must be washed three times in RPMI and the white cells removed. An isolate is successfully adapted in continuous culture if there is a more than five-fold increase in growth per cycle.

3.13.1.3 Culture and culture condition

I. Warm complete medium to 37° C in water-bath or heater block.

II. In a flask of 25 cm2, add 8 mL of complete medium.

III. Add packed uninfected erythrocytes to packed infected erythrocytes as to obtain a total volume of 500 µL.

IV. Add this volume to the medium to obtain a 5% hematocrit.

V. Stir gently.

VI. Maintain the culture of *P. falciparum* in candle jar or in incubator in the following conditions:

- temperature: 37° C
- gas mixture: 5% CO2, 5–10% O2 and 85–90% N2
- humidity: > 90%

3.13.1.4 Long-term culture

a) Medium Renewal

The culture medium has to be changed every 24 hours in a sterile environment.

I. With a sterile graduated pipette, withdraw the medium above sedimented cells. Note the amount removed volume.

II. Add the same volume of fresh medium to the flask.

b) Control of parasitemia

At every medium renewal, parasitemia must be assessed.

I. Make thin blood films.

II. Stain with Wright's stain or Giemsa method.

III. By microscopic examination:

- determine the parasitemia
- assure that there is no contamination

IV. If the parasitemia exceeds 5%:

- centrifuge at 500 g for 5 minutes
- discard the supernatant
- dilute the pellet with uninfected red blood cells to obtain a 0.5–1%parasitemia and with complete medium to 5% hematocrit.

3.13.1.5 Short term culture for *in vitro* testing

The first stage in the *in vitro* drug sensitivity assays is incubation of the culture. In each method, the culture must be diluted to the value specified according to the corresponding procedure for preparing the plate for the test.

I. Centrifuge at 500 g for 5 minutes.
II. Discard the supernatant.
III. Dilute the pellet with uninfected red blood cells to obtain the required parasitemia and with complete medium to 1.5% hematocrit.

3.13.1.6 Synchronisation

To measure drug sensitivity, clones must be synchronised in the ring stage.

3.13.1.7 Quality control

QC records must be kept and approved. Preparation and sterility of red blood cells and NaCl solution must be assessed by:

- preparation date
- powder weights
- reagent batch numbers

3.14 Preparation of complete medium for malaria culture

3.14.1 Procedures

a. 5% sodium bicarbonate solution I. Weigh 5g sodium bicarbonate II. Add 100mL of cell culture water in volumetric flask III. Stir on magnetic stirring plate until dissolved IV. Store at room temperature V. Use within 24 hours of preparation.

b. RPMI washing medium Used to wash samples and red blood cells. I. Weigh 10.43g of powdered RPMI medium and add to 1L volumetric flask II. Add 5.95g HEPES (25mM final concentration) III. Add 42mL 5% sodium bicarbonate (as prepared in 6.1) (25mM final concentration) IV. Fill to 1L with cell culture water V. Stir the solution until dissolved VI. Using a calibrated meter, check medium pH. If necessary, adjust to pH7.0 ± 0.3 with either hydrochloric acid (1N) or sodium hydroxide (1N). The medium should be orange, not red/purple or yellow, in colour due to the phenol red dye VII. Using a calibrated meter, check that the medium osmolarity = 292 ± 5% mOsm. If necessary, adjust with sterile water VIII. In a sterile environment, use a 0.22μM filter to sterilise the medium IX. Aseptically dispense medium into sterile bottles X. Store at – 20 °C for a

maximum 6 months XI. After first use, store at + 4 °C for one week WWARN procedure: Preparation of complete medium for malaria cultures v1.2 Page 6/7

c. Complete medium Used to culture *P. falciparum* isolates or clones. I. Weigh 10.43g of powdered RPMI 1640 medium (RPMI 1640 with Lglutamine) into a 1L volumetric flask II. Add 5.95g HEPES (25mM final concentration) III. Add 42mL 5% sodium bicarbonate (as prepared in 6.1) (25mM final concentration) IV. Add: a. 50mg hypoxanthine

Note: Hypoxanthine should **not** be added if culturing *P.falciparum*for 3 H-hypoxanthine uptake inhibition assays (INV07) b. 430mg L-glutamine (0.3 g/L) c. 1g glucose d. 0.25mg orotic acid (0.25 mg/L; optional) e. 10mg gentamycin or 32mg neomycin (10 mg/L; optional) V. Adjust volume to 1L with cell culture water VI. Stir until dissolved VII. Using a calibrated meter, check medium pH. If necessary, adjust to pH7.0 ± 0.3 with either hydrochloric acid (1N) or sodium hydroxide (1N). The medium should be orange, not red/purple or yellow, in colour due to phenol red dye VIII. Using a calibrated meter, check osmolarity = 292 ± 5% mOsm. If necessary, adjust with sterile water IX. In an aseptic environment, sterilize the medium using a 0.22µM filter X. Add sterile human serum to a final concentration of 10% or AlbuMAX™ I (lipid-enriched bovine serum albumin) to a final concentration of 0.5%. XI. Aseptically dispense medium into sterile bottles XII. Store at – 20 °C for a maximum 6 months XIII. After first use, store at 4 °C for one week XIV. Protect bottles from extended exposure to light

d. Quality control (QC) Record: I. Preparation date II. Recorded powder weights III. Reagents' batch numbers IV. pH QC records should be approved by a competent person and stored in a safe location.

3.15 Synchronisation of *Plasmodium falciparum*

3.15.1 Procedure

In a sterile environment:

First stage: schizont forms selection

Parasitemia of the culture must be assessed by making a coloured thin blood film. If at 5% parasitemia, schizont forms represent the majority, it can be separated from parasites in other stages.

a) Percoll solution preparation

I. Add 8 mL of 10X PBS in 17.5 ml of RPMI 1640.
II. Add 72 mL of Percoll.
III. Stir gently to obtain a homogeneous solution.
IV. Measure the density of the mix and adjust it at 1.085:
    - by adding RPMI 1640 if the density > 1.085
    - by adding Percoll if the density < 1.085.

NOTE: Percoll increases density.

If Percoll is used for adjustment, add PBS to maintain a 9 v/v Percoll/PBS ratio.

V. In a sterile environment, filter sterilise medium with 0.22 μM filter.

VI. Dispatch 4 mL of solution in 5 mL tubes.

VII. Store at 4° C. The solution is good for 3 months.

b) Schizont forms sedimentation

I. Warm a tube of Percoll solution to 37° C in water-bath or heater block.

II. Centrifuge the culture at 500 g for 5 minutes.

III. Discard supernatant and stir the cell pellet.

IV. With a sterile pipette, collect the cell pellet.

WWARN Procedure: Synchronisation of *P. falciparum* v1.1 Page 6/7

V. Gently lay down the collected blood at the surface of Percoll solution.

VI. Centrifuge the tube at 1300 g for 15 minutes.

VII. With a sterile graduated pipette, withdraw the ring created by schizonts forms in the superior phase of solution.

VIII. Add 1:9 v/v RPMI medium to cell supernatant.

IX. Stir 3–5 minutes at room temperature on a shaker.

X. Centrifuge at 500 g for 5 minutes.

XI. Remove supernatant.

XII. Wash packed red blood cells two more times.

XIII. In a flask of 25 cm2, add 8 mL of complete medium.

XIV. Add washed infected cell pellet.

XV. Complete to 400 μL with uninfected erythrocytes to obtain a 5% hematocrit.

XVI. Stir gently.

Second stage: culture

Maintain the culture of *P. falciparum* in candle jar or in incubator for 6 hours in conditions determined in procedure culture of *P. falciparum*.

NOTE: An alternative method to enrich a culture in schizonts is to use a magnetic separation kit.1

Third stage: ring forms selection

At this stage, parasitemia must be assessed by making a coloured thin blood film.

- If ring % > 95%, the drug sensibility of the culture can be assessed directly.
- If ring % < 95%, proceed with the next step as ring forms are not sufficient.

6.3.1 Sorbitol solution preparation

I. Weigh 5 g of D-sorbitol.

II. Dissolve sorbitol with cell culture water in a 100 mL volumetric flask.

Ribaut C *et al.*, Concentration and purification by magnetic separation of the erythrocytic stages of

all human Plasmodium species. *Malaria Journal* 2008 Mar 5; 7:45. Abstract available from: http://www.ncbi.nlm.nih.gov/pubmed/18321384 (Accessed 29 November 2010).

III. In a sterile environment, filter sterilised medium with 0.22 µM filter.

IV. Dispatch 3 mL of solution in 5 mL tubes.

V. Store at 4° C. The solution is good for 3 months.

6.3.2 Ring forms selection

I. Warm a tube of sorbitol solution to 37° C in water-bath or heater block.

II. Centrifuge the culture at 500 g for 5 minutes.

III. Discard supernatant and stir the cell pellet.

IV. Add 5:1 v/v sorbitol to cell pellet.

V. Stir 5 minutes at room temperature on a shaker.

VI. Wash one more time with RPMI medium.

VII. In a flask of 25 cm2, add 8 mL of complete medium.

VIII. Add washed infected cell pellet.

IX. Complete to 400 µL with uninfected erythrocytes to obtain a 5% hematocrit.

X. Stir gently.

XI. Maintain the culture of *P. falciparum* in candle jar or in an incubator for 2 (3) hours.

Quality control

Quality control records must be kept and approved by a competent person.

Preparation and sterility of solution must be assessed by:

- Preparation date
- Powder weights
- Osmolarity
- Density

3.16 Preparation of Predosed Plates

3.16.1 Procedure

Preparation of stock solution

Each test compound is packaged as a powder in a sterile vial. Vials are stored at +4 °C. Dissolve drug powder in the volume of solvent according to required drug range to obtain stock solution concentration. Appendix A notes appropriate solvent and storage instructions the for the stock solution

NOTE: Stock solutions have a shelf life of up to 1 month when stored at +4 gc. They can be successfully stored as aliquots at -8o gc with increased shelf-lives.

Preparation of working solution

Prepare working solutions by further diluting the stock solution in the appropriate solvent. The final dilution should be matched against the highest concentration of drug in the in vitro test plate.

Preparation of plates

a) Dry plates

I. A batch of plates can be prepared, dried and stored for later use.
II. Prepare the required range of reference drug concentrations by diluting stepwise 1 in 1.5, or 1 in 1.25 (depending on the compound under test) 21 times

III. When the dilutions are finished, use a 12 channel multipipette to dispense one row at a time with either 25ul (200ul final volume culture) or 12.5HL (100HL final volume of culture) of drug dilution into each well of a sterile 96-well microplate
IV. The last row should not contain any drug dilution as it will be used as the control
V. The test plates must be thoroughly air-dried before use (for several hours to overnight) in a sterile environment.
VI. Protect pre-dosed plate with a film or a cover.
VII. Store plates at +4 ac until ready for use.

b) Fresh plates

I. Plates can be prepared fresh and used within the day.
II. In tubes, prepare a twofold serial dilution of the drug final working solution. The dilutions are made in complete medium for malaria culture and mixed thoroughly by aspiration
III. use a 12 channel multipipette to dispense, one row at a time, 2sul of drug dilution into each well of a sterile 96-well microplate.
IV. The last row does not contain any drug dilution as it will be used as the control.
V. Protect plates with a film or a cover.
VI. Store plates at +4 c and use them within the day.

c) Quality control

Each batch should be validated against reference clone 3D7 to assure that drug quality was maintained over the period of use.

Validation criteria should be assessed by each laboratory as they vary depending on the in vitro drug susceptibility testing method used.

QC records should be retained and approved. Uniformity of plate batches must be assessed using:

- Clones
- Drug susceptibility values.

3.17 Estimation of *Plasmodium falciparum* drug susceptibility *ex vivo* by HRP2 ELISA

3.17.1 Procedure

Sample collection:

- Samples from patients with *P. falciparum* mono-infections and parasite densities of **0.002 % or greater** (approx. 100 parasites per µL or more). Samples with parasite densities of 1% or more may be used but should be diluted before culture with uninfected RBCs to obtain a density of approximately 0.2% to limit the inoculum effect (i.e. the influence of high parasite densities on test outcome).

- After thorough disinfection of the skin, collect a minimum 1 mL of blood by venipuncture using a sterile disposable phlebotomy tool and a heparinized container.

- Prepare thick and thin blood films, thoroughly dry the slides, fix thin films with methanol, and stain with Giemsa (3%, 20 minutes), microscopically examine the slides (oil, 1000x magnification), assess and record parasite density, and the proportion of rings and trophozoites.

Sample preparation and culture:

- The HRP2 drug sensitivity field test uses 72 hours of incubation at 37°C.

- For assays requiring one complete 96 well plate, prepare 25 mL of parasitized blood diluted in culture medium (cell-medium mixture, CMM). Dispense **24.06 mL** of complete RPMI 1640 into a sterile, disposable tube (note: medium filled tubes may be prepared in advance and stored at 4°C for several days). Add **0.94 mL** of parasitized blood per tube to obtain a CMM with approximately 1.5% hematocrit (assuming a 40% hematocrit in the parasitized blood sample).

- Add 200 µL of the resulting CMM to each well of the pre-dosed plates using a multichannel pipette (start with row A and proceed to higher drug concentrations).

- Cover the plates and incubate for 72 hours at 37°C in a candle jar or in an incubator with CO2-enriched atmosphere using a gas mixture of 5% CO2, 5% O2, and 90% N2 or candle jar. If possible, re-gas and gently agitate the plates every 24 hrs.

- After 72 hours incubation, prepare another thick and thin blood film to determine adequate parasite growth, defined as a 4 to 10 fold increase in parasite density over the time period. If there was little or no increase in parasite density, discard sample and re-check culture conditions, if possible.

Sample haemolysis:

- After the 72-hour incubation period, plates are removed from the incubator and stored at or below –20°C until all the wells are completely frozen. A simple household freezer may be also be used. Freezing may take from 60 minutes to 24 hours, depending on the equipment used. A minimum overnight period is recommended.

- Once completely frozen, plates may either be processed immediately (if an ELISA plate reader is available), or stored and transported frozen to laboratory facilities equipped to perform ELISA tests.

- Thaw the plates when ready to perform the ELISA assay. If the RBCs are not completely haemolysed i.e. all wells look completely clear on visual inspection, repeat the freezing and thawing cycle as above at least once until complete hemolysis is achieved.

3.17.2 HRP2 ELISA

The HRP2 ELISA measures the quantity of histidine-rich protein 2 (HRP2) produced by *P. falciparum* during the 72 hour incubation and its inhibition by antimalarial drugs.

Step 1: Preparation of antibody-coated ELISA plates

- Antibody-coated, 96-well, high-binding ELISA plates are ideally prepared in batches. They may be stored at -20°C for up to 12 months.

- Dilute primary IgM antibody (MPFM-55A, Immunology Consultants Laboratories, Inc, Newberg, OR, USA or equivalent) to a final concentration of **1.0 µg/mL in PBS.**

- Add 100 µl of diluted antibody to each well of an ELISA plate, using a multichannel pipette.

- Seal the plate and incubate at 4°C overnight. Using a pipette, remove and discard the diluted antibody from the ELISA plate, inverting and rapping the plate onto a paper towel to remove the remaining liquid (bang dry).

- Freshly prepare a blocking solution of 2% bovine serum albumin (BSA) solution in PBS. For each plate dissolve 0.4 g of BSA in 19.6 mL PBS.

- Add 200 µl of blocking solution to each well, cover and incubate at room temperature for 2 hours. Discard the blocking solution and bang dry as before.

- Prepare 0.05% Tween® 20 washing solution by adding 0.5 ml Tween® 20 to 999.5 mL PBS.

- Wash each well by adding and discarding 200µl 0.05% Tween® 20 washing solution. Bang dry as before. Repeat the wash step one more time. Seal each plate into an airtight plastic bag and store frozen at -20°C for up to 12 months.

Sample dilution

- The HRP2 ELISA method described in this procedure is extremely sensitive and able to assay reproducibly the drug sensitivity of fresh clinical blood samples at pre-culture parasite densities as low as **0.002% (c. 100 parasites/µL)**.

- Samples from patients with pre-culture parasite densities of **≤0.1% (approx. 5000 parasites/µL)** may be assayed directly following culture (i.e. the culture samples can directly be transferred to the ELISA plates).

- If the parasite density in the patient's blood was **>0.1% (approx. 5000 parasites/µL),** and the parasite density was **not** adjusted by dilution with uninfected RBCs prior to culture, the test samples should be diluted **to a final**

volume of 100 µL per well and the equivalent of 0.05% pre-culture parasite density after culture directly on the ELISA plate as follows.

▪**Example:** a patient blood sample with a pre-culture parasite density of 0.25% should be diluted 1 in 5 (0.25%/0.05%) to obtain the equivalent of a 0.05% pre-culture parasite density. To do this, add 80µL of distilled water to each well of the ELISA plate. Using a multichannel pipette, and after thorough mixing, transfer 20µL sample from each well of the culture plate to the equivalent well of the ELISA plate and mix again. Start transferring samples from the wells containing the highest drug concentration. This way a single set of pipette tips can be used for an entire plate.

- Add at least one well of an external positive control to each plate. A positive control could be, for example, a culture sample with known, very high parasitaemia. This sample will provide information on the maximum 450 nm absorbance – see 6.4.4 - that can be obtained in the ELISA. The positive control should always give a higher absorbance than the drug-free controls.

Step 3: Adding samples to ELISA plates and incubation

- Transfer 100 µL of sample, diluted if necessary (see 6.4.2), from the culture plate to an antibody-coated ELISA plate, previously warmed to room temperature.

- Incubate for 1 hour at room temperature in a humidified chamber.

- Wash 2 times in PBS/Tween (200 µl/well) and bang dry.

Step 4: Dilute the second antibody conjugate and add to ELISA plate

- Prepare the second antibody conjugate diluent, 2% BSA/1% Tween®20 in PBS, by adding 2 gm BSA and 1 mL Tween®20 to 97 mL PBS and adjusting to pH 7.4 if possible. 10 mL of diluent is needed for every test plate.

- Prepare a 200x stock solution (10 – 40 µg/mL) of second antibody conjugate (MPFG-55P or equivalent) using 2% BSA/1% Tween®20 in PBS (as above). Store the stock solution at 4oC for a maximum four weeks.

Note: The required second antibody concentration, which determines the ELISA sensitivity, may vary in the range from **0.05 to 0.2 µg/mL** because the conjugate

activity will vary from batch to batch and will decrease if the antibody is stored unfrozen for extended periods of time. Each new batch of second antibody should be titrated to determine the appropriate conjugate concentration to use with test samples. This is done by carrying out a preliminary test using serial dilutions of a culture sample with known parasite density titrated against a range of second antibody concentrations from 0.05 to 0.2µg/mL.

- For each test plate, freshly prepare 1x antibody conjugate by adding 50 µL of 200x antibody stock solution to 10 mL of diluent. Mix carefully.

- Using a multichannel pipette, add100 µl of 1x antibody conjugate to each well of the test plate.

- Cover the plate and incubate for 1 hour at room temperature in a humidified chamber.

- Wash the plate two times using 200 µl 0.05% Tween®20 in PBS for each well, banging dry after each wash.

Step 5: Add substrate and read sample absorbance

- Add 100 µl per well of **TMB chromogen**and incubate for **2 to 10 minutes** at room temperature in the dark.

    - The duration of exposure to TMB depends on the activity of the TMB as well as the parasite density. Stop the reaction as soon as the controls (in which the HRP2 concentration is highest) have turned bright blue and the wells with the highest drug concentration are very pale blue.

- Stop the reaction by adding 50 µl of 1 M sulphuric acid to each well.

    - Prepare the stop solution by adding 10 mL of sulphuric acid to 90 mL of distilled water. SAFETY NOTE: always add sulphuric acid to water, never water to sulphuric acid.

- Using an ELISA plate reader, measure the absorbance at 450 nm.

- **Note:** if all wells turn dark blue then either:

a) the parasite density may be too high
   Action: dilute the culture samples to lower density and repeat; or
b) the ELISA is too sensitive.
   Action: reduce the concentration of the second antibody; or
c) the incubation with TMB was too long
   Action: reduce the TMB incubation time

Chapter Four
RESULTS

4.1. Different concentration of standard (Chloroquine) and plant extract.

Table 2: Different concentration of standard (Chloroquine) and plant extract (*Clerodendrum infortunatum* Linn).

Drug conc. (µg/ml)	**Plant dilutions (µg/ml)**	**Plant dilutions(µg /ml)**
6.25×10^{-6}	0.35	0.2
1.25×10^{-06}	0.116666	0.04
2.5×10^{-07}	0.038866	0.008
5×10^{-08}	0.012955	1.6×10^{-3}
1×10^{-08}	0.004318	3.2×10^{-4}
2×10^{-09}	1.43×10^{-3}	6.4×10^{-5}
4×10^{-10}	4.79×10^{-4}	1.28×10^{-6}
8×10^{-11}	1.59×10^{-4}	2.56×10^{-7}
1.6×10^{-11}	5.3×10^{-5}	5.12×10^{-8}
3.2×10^{-12}	1.76×10^{-05}	1.02×10^{-8}

4.2. ELISA (enzyme-linked immunosorbent assay) reading at 450nm.

Table 3: Inhibition of *Plasmodium falciparum* 3D7 strain (Chloroquine resistant) after treatment with different log concentrations of plant extract.

Plant extract (log conc. ; µg/ml)	**Absorbance of HRP2 at 450nm**
-0.455931956	0.103
-0.933055692	0.184
-1.410430154	0.598
-1.887562583	0.655
-2.364717362	0.561
-2.841939206	0.596
-3.319664487	0.545
-3.798602876	0.571
-4.27572413	0.633
-4.754487332	0.53
-5.252588192	0.596
-5.730487056	0.697

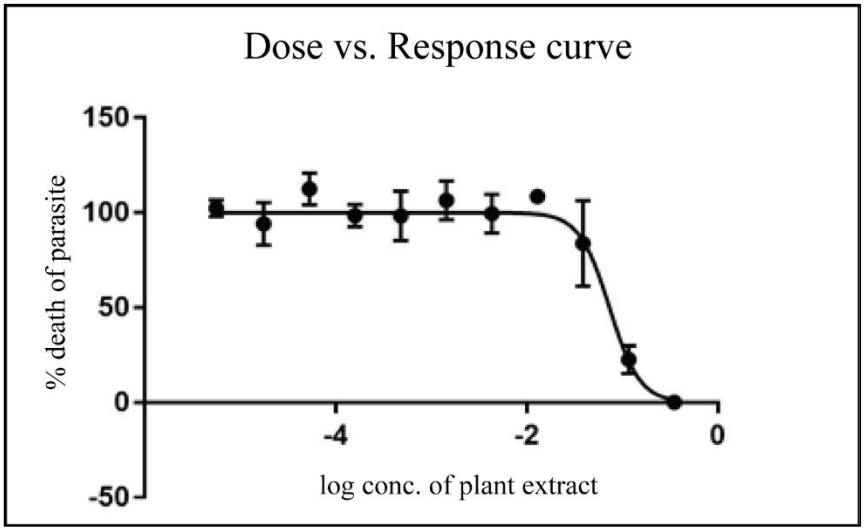

Figure 13: Dose vs. Response curve

The dose vs. response curve represent that 100% death of parasite occurred in seven lower log concentration of pant extract. And percent death of parasite was gradually reduced at the higher log concentration of plant extract.

4.3. Inhibitory concentration (IC50):

IC50 is the concentration of an inhibitor where the response (or binding) is reduced by half.

Table 4. *In vitro* antimalarial activity (IC50 values) of the plant extract against *Plasmodium falciparum* 3D7.

Non-lin fit of Normalize of Transform of dose vs. response: Table of results	
log(inhibitor) vs. normalized response -- Variable slope	
Best-fit values	
LogIC50	-1.134
HillSlope	-2.74
IC50	0.07342

Std. Error	
LogIC50	0.0511
HillSlope	0.5939
95% CI (profile likelihood)	
LogIC50	-1.242 to -1.029
HillSlope	-5.029 to -1.834
IC50	0.05725 to 0.09345
Goodness of Fit	
Degrees of Freedom	20
R square	0.9355
Absolute Sum of Squares	1860
Sy.x	9.644
Number of points	
# of X values	33
# Y values analyzed	22

Mean IC50 values were calculated from dose-response curves (percentage of schizonts vs. logarithm of plant extract concentration) by linear interpolation. For the log concentration of plant extract range of IC50 value was 0.05725 to 0.09345 and the best fit value of IC50 0.07342 was calculated.

4.4. ELISA (enzyme-linked immunosorbent assay) reading at 450nm.

Table 5: Inhibition of *Plasmodium falciparum* 3D7 strain (Chloroquine resistant) after treatment with different log concentrations of plant extract.

Plant extract (log conc. ; µg/ml)	**Absorbance of HRP2 at 450nm**
-0.698970004	0.111
-1.397940009	0.312
-2.096910013	0.601

-2.795880017	0.627
-3.494850022	0.561
-4.193820026	0.509
-4.89279003	0.596
-5.591760035	0.541
-6.290730039	0.633
-6.991399828	0.583

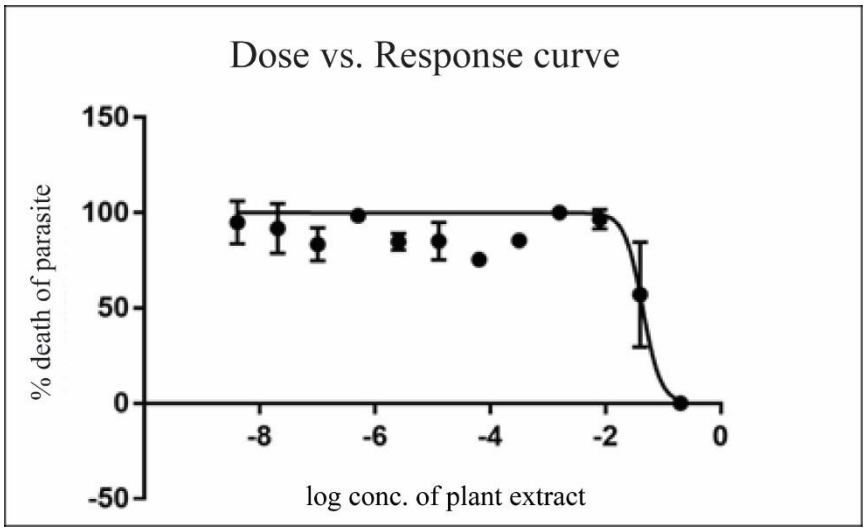

Figure 14: Dose vs. Response curve

The dose vs. response curve represent that 100% death of parasite occurred in one, two and fourth log concentrations of pant extract. And percent death of parasite was gradually reduced at the higher log concentration of plant extract.

4.5. Inhibitory concentration (IC50):

IC50 is the concentration of an inhibitor where the response (or binding) is reduced by half.

Table 6. *In vitro* antimalarial activity (IC50 values) of the plant extract against *Plasmodium falciparum* 3D7.

Nonlin fit of Normalize of Transform of dose vs. response :Table of results	
log(inhibitor) vs. normalized response -- Variable slope	
Best-fit values	
LogIC50	-1.354
HillSlope	-2.772
IC50	0.04422
Std. Error	
LogIC50	0.08585
HillSlope	3.707
95% CI (profile likelihood)	
LogIC50	-1.535 to -1.149
HillSlope	-1.013
IC50	0.02916 to 0.07099
Goodness of Fit	
Degrees of Freedom	22
R square	0.7465
Absolute Sum of Squares	4583
Sy.x	14.43
Number of points	
# of X values	36
# Y values analyzed	24

Mean IC50 values were calculated from dose-response curves (percentage of schizonts vs. logarithm of plant extract concentration) by linear interpolation. For the log concentration of plant extract range of IC50 value

was 0.02916 to 0.07099 and the best fit value of IC50 0.04422 was calculated.

4.6. ELISA (enzyme-linked immunosorbent assay) reading at 450nm.

Table 7: Inhibition of *Plasmodium falciparum* 3D7 strain (Wild type) after treatment with different log concentrations of plant extract.

Plant extract (log conc. ; µg/ml)	**Absorbance of HRP2 at 450nm**
-0.455931956	0.066
-0.933055692	0.148
-1.410430154	0.617
-1.887562583	
-2.364717362	0.68
-2.841939206	0.652
-3.319664487	0.769
-3.798602876	0.707
-4.27572413	0.726
-4.754487332	0.759
-5.252588192	0.696
-5.730487056	0.716

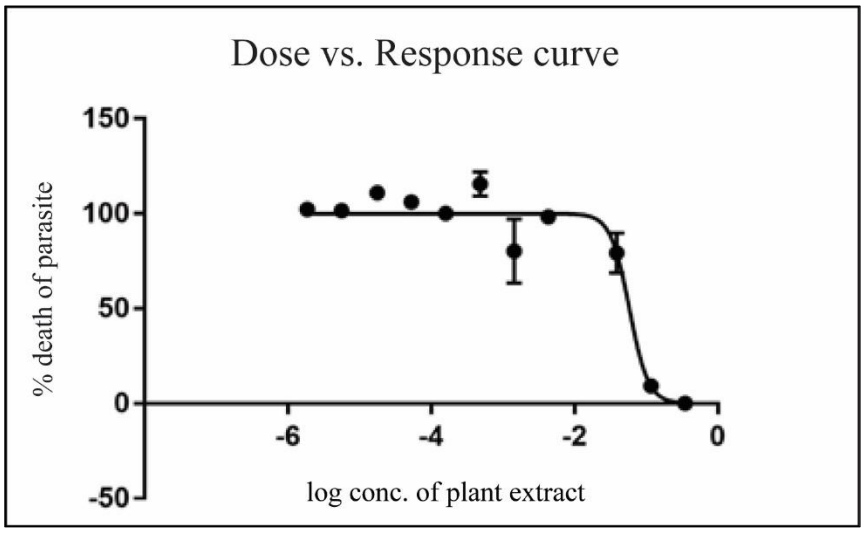

Figure 15: Dose vs. Response curve

The dose vs. response curve represent that 100% death of parasite occurred in six lower log concentrations of pant extract. And percent death of parasite was gradually reduced at the higher log concentration of plant extract.

4.7. Inhibitory concentration (IC50):

IC50 is the concentration of an inhibitor where the response (or binding) is reduced by half.

Table 8. *In vitro* antimalarial activity (IC50 values) of the plant extract against *Plasmodium falciparum* 3D7 (Wild type).

Nonlin fit of Normalize of Transform of dose vs. response:Table of results	
log(inhibitor) vs. normalized response -- Variable slope	
Best-fit values	
LogIC50	-1.234
HillSlope	-3.294
IC50	0.0583

Std. Error	
LogIC50	0.05531
HillSlope	0.8744
95% CI (profile likelihood)	
LogIC50	-1.119
HillSlope	-1.98
IC50	0.07595
Goodness of Fit	
Degrees of Freedom	20
R square	0.936
Absolute Sum of Squares	2073
Sy.x	10.18
Number of points	
# of X values	36
# Y values analyzed	22

Mean IC50 values were calculated from dose-response curves (percentage of schizonts vs. logarithm of plant extract concentration) by linear interpolation. For the log concentration of plant extract IC50 value was 0.07595 and the best fit value of IC50 0.0583 was calculated.

Chapter Five

DISCUSSION AND CONCLUSION

DISCUSSION AND CONCLUSION

The *in vitro* activity against *Plasmodium falciparum* of crude extracts was evaluated by means of the Mark III test, as developed by the WHO. Briefly, the 3D7 *P. falciparum* strains were cultured in human RBC at 5% hematocrit in complete medium composed of RPMI 1640 medium supplemented with 25 ml HEPES buffer (pH 7.4) and 10% AB human serum. Growth inhibition was expressed as percent of the number of schizonts for each concentration, compared with untreated controls. Mean IC50 values were calculated from dose-response curves (percentage of schizonts vs. logarithm of drug concentration) by linear interpolation. The methanolic extract of *Clerodendrum infortunatum* Linn. (0.35, 0.116, 0.038, 0.012, 0.004, 1.43×10^{-3}, 4.79×10^{-4}, 1.59×10^{-4} and 5.3×10^{-5} µg/ml) showed antimalarial activity against 3D7 (Chloroquine resistance) strain of *P. falciparum.* As well as (0.2, 0.04, 0.008, 1.6×10^{-3}, 3.2×10^{-4} and 6.4×10^{-5} µg/ml) concentration of extract of *Clerodendrum infortunatum* Linn. showed antimalarial activity against 3D7 (chloroquine resistance) strain of *P. falciparum.* The methanolic extract of *Clerodendrum infortunatum Linn.* (0.35, 0.116, 0.038, 0.012, 0.004, 1.43×10^{-3}, 4.79×10^{-4}, 1.59×10^{-4} and 5.3×10^{-5} µg/ml) showed antimalarial activity against 3D7 (Wild type) strain of *P. falciparum*.

Plate cultures are prepared with a 5% hematocrit and about 1% parasitemia. The lower the initial parasitemia is, the greater the increase in numbers of parasites that will occur during *in vitro* growth. To obtain 20- to 50-fold increase in parasite numbers our starting parasitemia was 0.1%. Parasitemia of cultures can be increased to about 20% by changing medium in cultures every 8 h. Monitoring of parasitemia is accomplished by preparing blood films, staining with Giemsa stain following methanol fixation, and counting infected red blood cells microscopically.

While the simplest system for cultivation of parasites uses petri or Linbro plates in a candle jar, this system is requiring constant attention and daily changes of medium in order to maintain parasite growth. In this static system infected erythrocytes settle out to form a layer, producing microenvironments high in lactic acid in the region of the proliferating parasites. This may lead to conditions unfavorable for schizont development and penetration of merozoites into uninfected erythrocytes. Optimal parasite yields occurred with an extracellular pH of 7.2 to 7.45. Continuous flow of medium through

growth vessels, with a controlled gas phase of 2 to 5% CO_2, 3 to 18% O_2, and N_2 are required for maintaining cell culture.

However, parasite resistance to antimalarial has been documented in malaria species known to affect humans *P. falciparum.* Parasite resistance results in a delayed or incomplete clearance of parasites from the patient's blood when the person is being treated with an antimalarial. The problem of antimalarial drug resistance is compounded by cross resistance, in which resistance to one drug confers resistance to other drugs that belong to the same chemical family or which have similar modes of action. Therefore, it is essential to establish the efficacy and safety of traditional medicinal plants which are used to fight the disease. *In vitro* biological tests for the detection of antimalarial activity in plant extracts are currently available and some measure of specificity of action can be obtained by monitoring their cytotoxicity in mammalian cells. *In vitro* drug sensibility using an HRP2 assay have a pivotal role in strategies aimed at discovering new antimalarial compounds or monitoring the emergence of drug resistance in field isolates.

Clerodendrum infortunatum is a perennial shrub belonging to the family Lamiaceae. It is the type species among ~400 species of *Clerodendrum*. It is one of the most well-known natural health remedies in traditional practices. In many traditional practices the leaves and root are widely used in treatment of skin diseases, diabetes, gravel, malaria, scabies, sore, spasm, scorpion sting, snake bite and tumor. The antibacterial and antifungal properties of *Clerodendrum infortunatum* against antagonistic pathogens have been scientifically proved. The species is also used in the treatment of anaemia, cluster headache, diabetes, diarrhoea, fever, food poisoning, gallstone, gastric ulcer, general weakness, gout, headache, impotence, indigestion, itching, jaundice, malaria, obstructive labor, osteoarthritis, paralysis, piles, rheumatism (gout), scabies, stomachache, tuberculosis and vaginitis *(Uddin, 2006)*; chest pain and cough *(Kirtikar et al., 1935)*; insect bites, skin diseases, scorpion sting and tumor *(Ghani, 2003)*; asthma, cough, malarial fever and snake bite *(Chopra et al., 1956)*; skin diseases *(Moldenke and Moldenke, 1983).* Ascaris and liver pain were treated with this extract *(Partha and Hossain, 2007).* The species is also used for the treatment of diabetes and sores *(Roy et al., 2008).* Abdominal pain, dental caries, pain, roundworms with indigestion and vomiting were also treated with this species *(Rahman et al., 2007).* It was also used to treat fever and to heal cut injury *(Uddin et al., 2006).* Asthma, chest complaint, cough, impotency, jaundice, malaria, scabies, skin diseases and tumors were also treated with

this species *(Yusuf et al., 2009, Motaleb et al. 2013).* Our result also showed that methanolic plant extract of *Clerodendrum infortunatum* Linn. has antimalarial activity.

In-vitro assay of antimalarial activity appears promising antimalarial properties of *Clerodendrum infortunatum* Linn. against *Plasmodium falciparum*. Further investigation is required for determining the minimum effective concentration, maximum effective concentration of plant extract against malaria parasite. The plant merit further scientific research towards possible discovery of novel compounds that can be used to successfully treat malaria with less undesirable side effects.

Chapter Six
REFERENCES

6. REFERENCES

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