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MASTERARBEIT

Titel der Masterarbeit

Activity of Eurycoma analogs and Artemisinin and their interaction in *Plasmodium falciparum* in vitro

Verfasserin

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In this, O Nature, yield I pray to me.
I pace and pace, and think and think, and take
The fever'd hands, and note down all I see,
That some dim distant light may haply break.
The painful faces ask, can we not cure?
We answer, No, not yet; we seek the laws.
O God, reveal thro' all this thing obscure
The unseen, small, but million-murdering cause.
(Sir Ronald Ross 1895)

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1 Zusammenfassung

Die vorliegende Studie wurde zwischen Juni und August 2010 in Mae Sot, einer Stadt im Nordwesten von Thailand an der Grenze zu Myanmar durchgeführt. Dieses Gebiet ist bekannt für seine multiresistenten *P. falciparum* Stämme. Das Ziel dieser in-vitro Studie war die Aktivität, Sensibilität sowie die Interaktion von Artemisinin, *E. longifolia* Extrakten und Kombinationen aus beiden gegen *P. falciparum* zu testen.

Für die Untersuchungen wurde der WHO Standard in-vitro Test Mark III zur Messung der Schizontenreifungshemmung verwendet. Während der Studienzeit wurden 36 frische Parasitenisolate positiv auf *P. falciparum* getestet. Von diesen konnten 29 Isolate in die Studie aufgenommen werden.

Bei keiner der getesteten Substanzen konnte eine komplette Schizontenreifungshemmung festgestellt werden.

Die relative Schizontenreifungshemmung (SMI%) für Konzentrationen von 30 nmol/l bis 3000 nmol/l war für Artemisinin zwischen 20,31% und 92,32%, for Eury-1 zwischen 14,71% und 81,05%, für Eury-2 zwischen 14,47% und 82,02%, für Eury-3 zwischen 12,38% und 85,31%, für Eury-1-Art zwischen 18,96% und 92,34%, für Eury-2-Art zwischen 18,28% und 90,90% und für Eury-3-Art zwischen 18,69% und 93,03%.

Die durchschnittlichen EC_{50} Werte für Artemisinin, Eury-1, Eury-2, Eury-3, Eury-1-Art, Eury-2-Art und Eury-3-Art waren 27,8586 nmol/l, 113,4614 nmol/l, 96,9067 nmol/l, 102,3605 nmol/l, 50,7239 nmol/l, 36,0345 nmol/l und 35,6274 nmol/l. Die durchschnittlichen EC_{90} Werte für die selben Substanzen in der gleichen Reihenfolge waren 1277,0471 nmol/l, 13874,2892 nmol/l, 7763,3631 nmol/l, 5323,0386 nmol/l, 2621,9225 nmol/l 1706,2443 nmol/l und 1415,4586 nmol/l. Die durchschnittlichen EC_{99} Werte für die selben Substanzen in der gleichen Reihenfolge waren 28865,3737 nmol/l, 697799,1449 nmol/l, 276600,7487 nmol/l, 133347,3393 nmol/l, 65359,5184 nmol/l, 39,599,0921 nmol/l und 28472,0333 nmol/l.

Sowohl der Vergleich der EC Werte mit den EC Werten aus 2009 als auch die Regressionsparameter dieser zwei Jahre führte zu der Erkenntnis, dass die Wirksamkeit von Artemisinin in diesem Gebiet nachgelassen hat.

In der Varianzanalyse zeigten die Substanzen Eury-1-Art und Eury-2-Art einen signifikanten Synergismus. Die Hemmung bei dem kombinierten Eury-3-Art Substrat war höher als bei den einzelnen Komponenten Art mit Eury-3. Dieses Ergebnis könnte durch die schwächere Wirkung von Artemisinin beeinflusst worden sein.

2 Abstract

This study was carried out in Mae Sot, a town in north western Thailand on the border to Myanmar between June and August 2010. This area is known for its multi-drug-resistant strains of *P.falciparum*. The aim of this in vitro study was the assessment of the activity and sensitivity of Artemisinin, *E. longifolia* analogs, combinations of both and their interaction in *P. falciparum*.

The examinations were based on the WHO standard in vitro micro-test Mark III for determining the inhibition of schizont maturation. Fresh blood isolates from 36 patients were tested positive of *P. falciparum*. Out of them, 29 isolates could be included in the study.

A complete cut-off concentration could not be calculated for any of the tested substances.

The observed inhibition of schizont maturation (SMI%) was for Artemisinin between 20,31% and 92,32%, for Eury-1 between 14,71% and 81,05%, for Eury-2 between 14,47% and 82,02%, for Eury-3 between 12,38% and 85,31%, for Eury-1-Art between 18,96% and 92,34%, for Eury-2-Art between 18,28% and 90,90% and for Eury-3-Art between 18,69% and 93,03% for concentration from 3 nmol/l up to 3000 nmol/l.

The mean EC_{50} values for Artemisinin, Eury-1, Eury-2, Eury-3, Eury-1-Art, Eury-2-Art and Eury-3-Art were 27,8586 nmol/l, 113,4614 nmol/l, 96,9067 nmol/l, 102,3605 nmol/l, 50,7239 nmol/l, 36,0345 nmol/l and 35,6274 nmol/l. The mean EC_{90} values for the same substances in the same order were 1277,0471 nmol/l, 13874,2892 nmol/l, 7763,3631 nmol/l, 5323,0386 nmol/l, 2621,9225 nmol/l 1706,2443 nmol/l and 1415,4586 nmol/l. The mean EC_{99} values for the same substances in the same order were 28865,3737 nmol/l, 697799,1449 nmol/l, 276600,7487 nmol/l, 133347,3393 nmol/l, 65359,5184 nmol/l, 39,599,0921 nmol/l and 28472,0333 nmol/l.

The comparison of the EC values with EC values from 2009 as well as the regression parameters of this two years lead to the assumption that there is a decrease in effectiveness of Artemisinin in this area.

In the variance analysis the isolates showed in Eury-1-Art and Eury-2-Art a significant

synergism. The inhibition under Eury-3-Art was higher than under separate compounds. This might be influenced from the reduced activity of Artemisinin.

3 Introduction

The human malaria parasites belong to the Domain: Eukaryota, Kingdom: Chromalveolata, Superphylum: Alveolata, Phylum: Apicomplexa, Class: Aconoidasida, Order: Haemosporida, Family: Plasmodiidae, Genus: *Plasmodium* (Wehner and Gehring, 2007).

The Genus *Plasmodium* (Marchiafava and Celli, 1885) can be further divided into two subgenera: *Plasmodium* in the strict sense, where *P. vivax* (Grassi and Feletti, 1890), *P. ovale* (Stephens, 1922) and *P. malariae* (Grassi and Feletti, 1890) belong to and *Laverania* with *P. falciparum* (Welch, 1897) as the only agent. (Garnham, 1988)

3.1 History of malaria

Human being and the malaria parasites have a common evolutionary history. There are different theories from where and how *Plasmodium* may have evolved. According to Garnham (1966) the quartan malaria parasite appeared and evolved together with the first prosimian in the Eocene, the tertian group in the Oligocene together with the main simians. The subgenera *Laverania* at the time when higher apes and early man separated. (Bruce-Chwatt, 1988)

With this theory it seams that host-parasite relation started in tropical Africa when humans still lived as nomads. This way of life limited the malaria transmission. In the Neolithic revolution with settlements, agriculture and migration along river valleys in India, south China, Mesopotamia and the Nile valley the population began to grow. Together with the warm climate and good breading conditions for mosquitoes due to periodic inundations of the rivers the infection started to spread, so malaria endemicity began. Along with human migration also malaria conquered other parts of continents. Whereas the spread of malaria on the American continent is not fully clear, the rather cold climate in Europe was limiting the malaria transmission to warm seasons. (Bruce-Chwatt, 1988)

Evidence of malaria parasites in humans can be found in ancient texts and human findings. So seamed to exist malaria in Thailand 4000 years ago. This is based on examination of human bones, where severe anemia was observed, which could be a sign of chronic malaria. In ancient texts from Egypt different types of fever sometimes accompanied with enlarged spleen were described, which lead to the assumption of malarial origin. Enlarged spleens have also been found in Egyptian mummies about 3000 years old. (Poser and Bruyn, 1999)

In China different types of fever with enlarged spleen were already mentioned in documents 2700 BC. For treatment, together with acupuncture, exorcisms and other plants, they used Ch'ang shan (*Dichroa febrifuga*) which is known to have some antimalarial effect. The first evidence for the use of Qinghaosu (*Artemisia annua*), which is now the standard treatment of *P. falciparum* malaria, can be found in Chinese documents 340 AD. (Bruce-Chwatt, 1988)

The first clear description of the different fever attacks together with other symptoms like splenomegaly, cachexia and bilious complexions is found in Hippocratic book of endemics of the fourth century BC. But not only the clear description, also a coherence between the fever, marshes and even division of low, moist and hot districts and the risk involved is documented. Even though Hippocrates thought the disease was caused by drinking the stagnant water. (Poser and Bruyn, 1999)

The Roman history is strongly associated with malaria, even if the first appearance of the disease is not clear. But the intermittent fever related with swampy areas can be found in many Latin documents starting in the first century BC. At the time the malaria burden became so severe that they built a temple for the goddess *Febris Magna* and might even play an important role at the fall of the Roman Empire. (Bruce-Chwatt, 1988)

In following centuries malaria stayed a great burden almost all over Europe without treatment and caused many deaths. Before the discovery of the Cinchona bark, many different, for present times scurrilous ways of treatment for the intermittent fever are suggested in papers all over the world. There are also different stories from whom and when and how the Cinchona bark was discovered and used first. The only thing which seams to be clear, is the finding of it in the late sixteenth or early seventeenth century somewhere in Peru. And that it was brought to Europe and promoted around 1730 by Jesuits, thats why it was also called 'Cardinal's Powder' and 'Jesuits' Bark'. The active agent of the Cinchona bark is known today as Quinine. (Poser and Bruyn, 1999)

Despite this effective medicine of the Cinchona bark against malaria, the disease was

still widely distributed all over the world in the nineteenth and beginning of the twentieth century, even up to Canada and the Arctic Circle in Europe, pictured in Fig. 3.1. Almost 90% of the world population was living in malaria endemic areas (Wernsdorfer and Wernsdorfer, 2003; Poser and Bruyn, 1999).

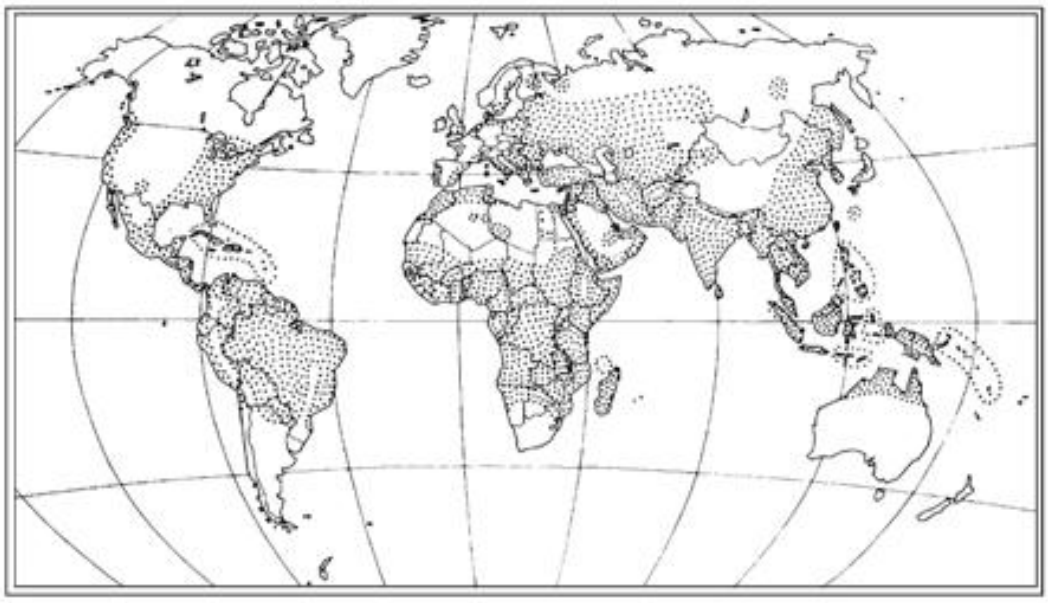

Figure 3.1: Malaria distribution in the mid-19th century
Source: Wernsdorfer and Wernsdorfer, Malaria at the turn from the 2nd to the 3rd millennium

The term 'Malaria' origins from Italian and means 'bad air', was coined in the nineteenth century and referred to the fact that this periodic fever occurred in Europe mainly in marshy areas and was caused by the 'bad air' of these swamps (Poser and Bruyn, 1999).

In the nineteenth century the theory that malaria may be caused by a 'contagium vivum' a living microorganism and not by 'bad air' was formulated. In 1880 Alphonse Laveran, a French surgeon, discovered the actual parasite of malaria. But he falsely thought that there was only one type of parasite causing malaria. Camillo Golgi proclaimed in 1885, that there are distinct species causing tertian and quartan malaria. Up to that point the parasites could only be seen in fresh blood, the development of staining by Metchnikoff in 1886 and further developed by Dimitri Romanowsky in 1891 helped to differentiate the plasmodial species, the further differentiation of the parasite species, the life-cycle and the transmission through anopheline mosquito bites was discovered in 1897 by the British military doctor Ronald Ross. This was the final breakthrough in malaria research. (Poser and Bruyn, 1999; Bruce-Chwatt, 1988)

In the twentieth century malaria could be eradicated in some parts like Canada and Sweden by changing the land use. This did not happen in other parts of the world. At the end of the second world war dichloro-diphenyl-trichloroethane (DDT) was discovered as a cheap, safe and potent insecticide. Together with the synthesized chloroquine group of drugs it seamed to be the breakthrough in eradicating malaria from the world. This method was working in USA, Europe, Australia and parts of northern Asia and led to a reduction of endemicity in north Africa, southern Asia, western Oceania and other parts of America (Gramiccia and Beales, 1988). By 1975 around 50% of the world population lived in malaria free areas (Wernsdorfer and Wernsdorfer, 2003).

Between 1975 and 2000 due to resistance to DDT and antimalarial drugs and also as an effect of the reduction in research and control efforts, malaria started to increase again (Gramiccia and Beales, 1988). Even though malaria was eliminated from large areas of China and by 2000 about 60% of the world population lived in malaria free areas, in many parts of the world, especially sub-saharan Africa and civil war afflicted regions, an improvement of the malaria situation cannot be seen in the near future. (Wernsdorfer and Wernsdorfer, 2003; Gramiccia and Beales, 1988)

3.2 Epidemiological facts

In low-income countries malaria is one of the main causes of morbidity and mortality (WHO, 2011).

According to the world malaria report released by the WHO (World Health Organization) in 2010, in 106 countries and areas malaria is endemic. Fig. 3.2 shows the geographical distribution of malaria in 2007. In these countries an estimated 225 million cases of malaria occurred among 3,3 billion people at risk in 2009. Compared with the figures of 2005 this is a drop of 19 million reported cases. This seamed to be due to impact of malaria control (WHO, 2010d). But these estimations are only based on numbers of national malaria control programs and probably far from complete. The incidence may probably be much higher.

The main malaria case reduction of 86% can be found in the European Region followed by American Regions with 42%. In Rwanda, Sao Tome and Principe and Zambia an increase of cases was reported, the reason is not clear. “The vast majority of cases in 2009 (78%) were in the African Region, followed by the South-East Asia (15%) and Eastern Mediterranean Regions (5%).” (WHO, 2010d, p. 60)

More than 90% of the clinically manifest malaria cases are caused by *P. falciparum*,

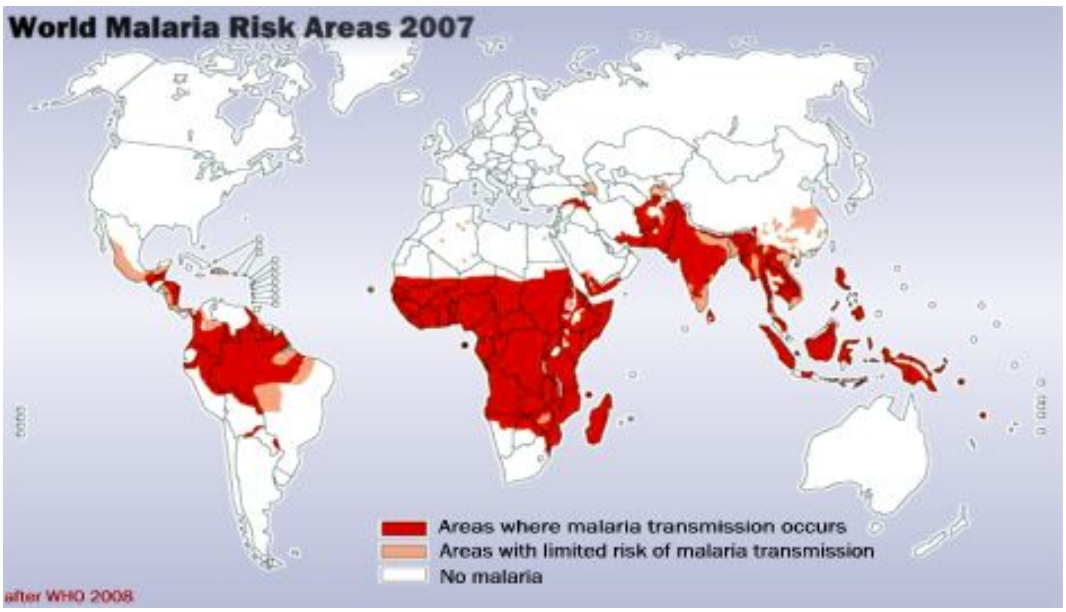

Figure 3.2: Malaria distribution 2007
Source: http://www.mosqguide.org.uk/images/Malaria.jpg

which can manifest in severe malaria with fatal outcome and therefore the reason of nearly one million deaths worldwide (Wernsdorfer and Wernsdorfer, 2003). The world malaria report estimated a decrease of 20% of malaria deaths between 2005 and 2009 with the largest reduction in the Region of America (WHO, 2010d). But again these are only numbers of national malaria control programs.
"It is estimated that 91% of deaths in 2009 were in the African Region, followed by the South-East Asia (6%) and Eastern Mediterranean Regions (2%). About 85% of deaths globally were in children under 5 years of age." (WHO, 2010d, p. 60)

The WHO and some partners started the roll back malaria program to reduce the malaria burden in the world. The main goal of this program is to reduce the number of malaria cases and deaths by 75% or more by 2015.
Strategies to attain this target are that: "countries must reach all persons at risk for malaria with an insecticide-treated mosquito net (ITN) or indoor residual spraying (IRS) and provide laboratory-based diagnosis for all suspected cases of malaria and effective treatment of all confirmed cases." (WHO, 2010d, p. xiii)

3.3 The Parasite

The species *Plasmodium* with around 100 subspecies is part of the suborder Protozoa. Out of these 100 only 6 are human pathogen, *P. falciparum*, *P. vivax*, *P. ovale* which are obligate human pathogen, *P. malariae* in human and other primates, *P.knowlesi* (Sinton and Mulligan, 1932) and *P. cynomolgi* (Mayer, 1907) usually in primates but facultative also in humans. The rest is hosted in simians, birds and reptiles. (Wernsdorfer and Wernsdorfer, 2010)

3.3.1 *Plasmodium falciparum*

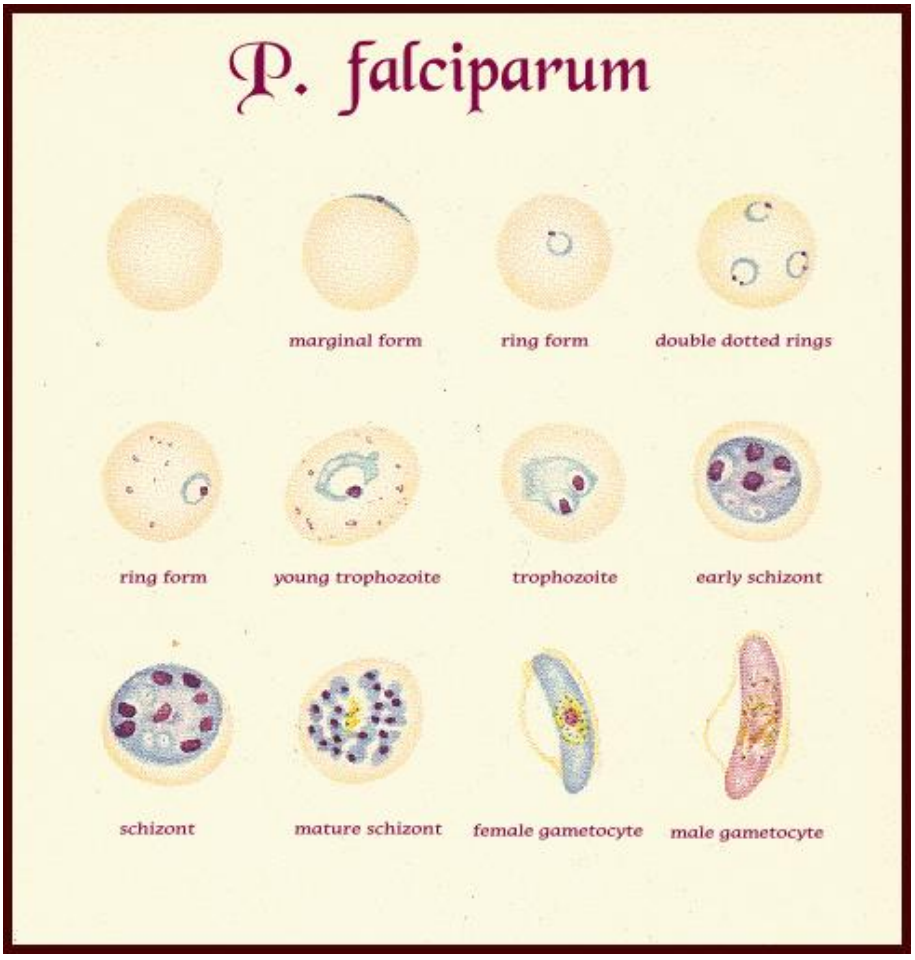

Figure 3.3: Different stages of *P. falciparum* in erythrocytes
(Source: http://hermes.ffn.ub.es/oscar/Biologia/Malaria/Malariafalcip.gif)

P. falciparum is the pathogenic agent of malaria tropica and is responsible for almost all severe cases and malarial deaths. If untreated usually 25% of *P. falciparum* infections

have a lethal outcome in nonimmune persons. The specially unpleasant and dangerous effect of *P. falciparum* malaria is its potential to invade all ages of Red Blood Cells (RBC), hence the parasitaemia can be up to 50%, its incubation period of 8-11 days and the occurrence of schizogony in deep capillaries. The infected RBC become physically altered and tend to adhere to the internal lining of the capillaries. Together with changes in the capillary walls themselves, this has the effect of producing microcirculatory arrest in different organs like stomach, brain, kidney, liver, lungs and can also lead to 'algid malaria'. (Lucius and Loos-Frank, 2008; Garnham, 1988)

That a parasite is killing his host leads to the hypothesis that *P. falciparum* is, in evolutionary terms, a relatively young parasite (about 10000 years old). Well adapted parasites tend not to kill their host, because they need them to survive themselves. (Lucius and Loos-Frank, 2008)

Compared to the other plasmodium species the incubation is relatively short, and one erythrocyte can be invaded by more than one parasite or often shows double chromatin. A mature schizont, which cannot be seen in the peripheral blood, contains 24-36 merozoites, the duration of the erythrocytic schizogony is 48 hours and is not synchronized. That is why the fever pattern is continuously with temperatures up to 40°C, but without periodic intervals. The gametocytogenesis takes 12 days, the gametocytes are ovoid and can appear in a blood film if the infection stays 8-10 days in the host. The different stages of *P. falciparum* can be seen in Fig. 3.3. An untreated primary attack usually lasts around 2-3 weeks. True relapses from the liver do not occur, and recurrences after a year are rare. The maximum duration of *P. falciparum* in the host is about 4 years. (Wernsdorfer and Wernsdorfer, 2010; Lucius and Loos-Frank, 2008)

The sporogony of *P. falciparum* in the mosquito needs a relative high temperature >18°C, hence why the distribution of *P. falciparum* malaria occurs mainly in the tropes. (Lucius and Loos-Frank, 2008; Garnham, 1988)

People living in a holoendemic area can acquire a partial immunity, which can be passive or active. New born get the passive immunity from their immune mother through transport through the placenta. This will last for the first months. Thereafter and till the age of five, the highest mortality rates can be found. Children at the age of five to six years develop an active immunity so the mortality rates decrease. Due to the acquired immunity the parasitaemia is lower and the clinical symptoms are less severe. Without repeated infections the immunity will disappear within a year. (Troye-Blomber et al., 1999)

3.3.2 *Plasmodium vivax*

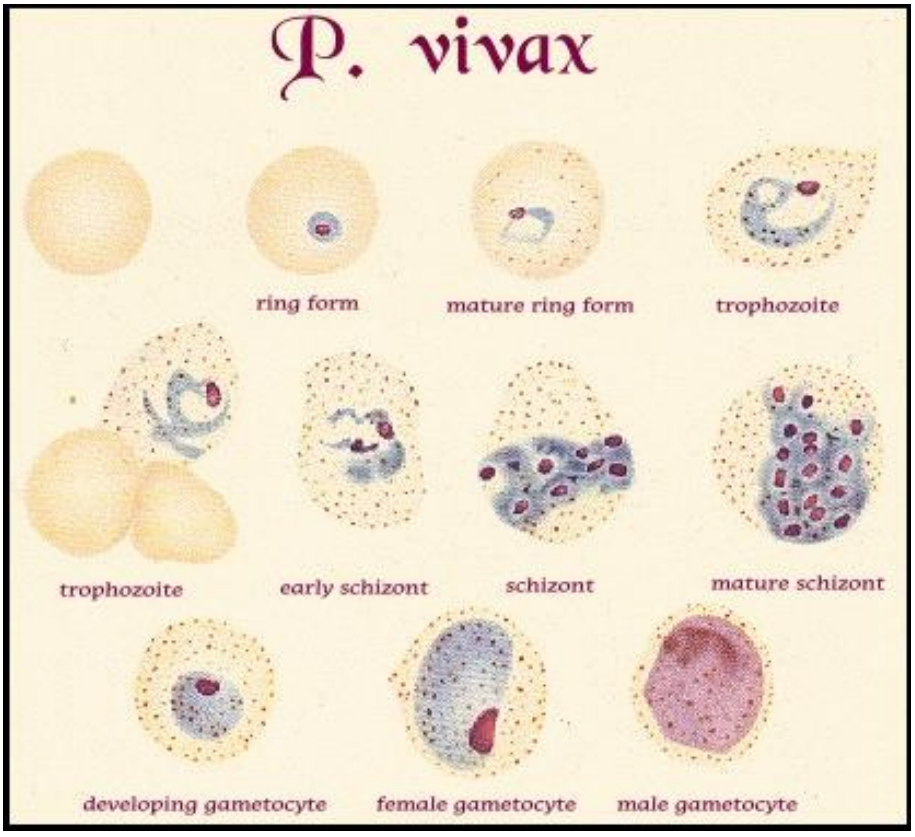

Figure 3.4: Different stages of *P. vivax* in erythrocytes
(Source: http://upload.wikimedia.org/wikipedia/commons/6/60/Plasmodiumvivax.jpg)

P. vivax is the pathogenic agent, next to *P. ovale* of the malaria tertiana. The distribution of *P. vivax* is worldwide due to relatively low average temperature of 16°C needed for the sporogony in the mosquito. It is predominant in India, Central and South America and eastern Asia, but it is absent in areas where people live with Duffy negative antigens on erythrocytes, which occur mainly in West Africa. (Lucius and Loos-Frank, 2008; Garnham, 1988)

The incubation period of *P. vivax* is 8-17 days. The merozoits can only enter reticulocytes, the young RBC. Therefore the parasitaemia is usually limited to 2%. The invaded RBCs are enlarged and have tiny reddish granules, so called "Schüffner dots". The erythrocytic schizogony takes 48 hours and is synchronized. Malaria tertiana has its name because of this erythrocytic schizogony, with fever attacks every 48 hours, according to the roman counting where day one is the day with the first fever attack. Mature schizonts contain 16-24 merozoits. The duration of the gametocytogenesis is 48-96 hours

and the gametocytes are round. Different development stages of the parasite can be seen in Fig. 3.4. As a speciality *P. vivax* can form hypnozoits, which can survive in the hepatocytes of the human liver and cause relapse after months to years. An untreated primary attack usually lasts around 12-15 cycles till cure. But due to the hypnozoits, relapse can appear up to 5 years, which is the maximum duration of the parasite in the host. (Lucius and Loos-Frank, 2008; Markell et al., 1999)

Even though *P. vivax* malaria is regarded to be 'benign', the attacks have a serious course, with fever up to 40,5℃, chills and rigor to last four to eight hours. Very seldom are severe cases with splenic rupture, pulmonary injury and even cerebral malaria. (Mendis et al., 2001)

3.3.3 *Plasmodium ovale*

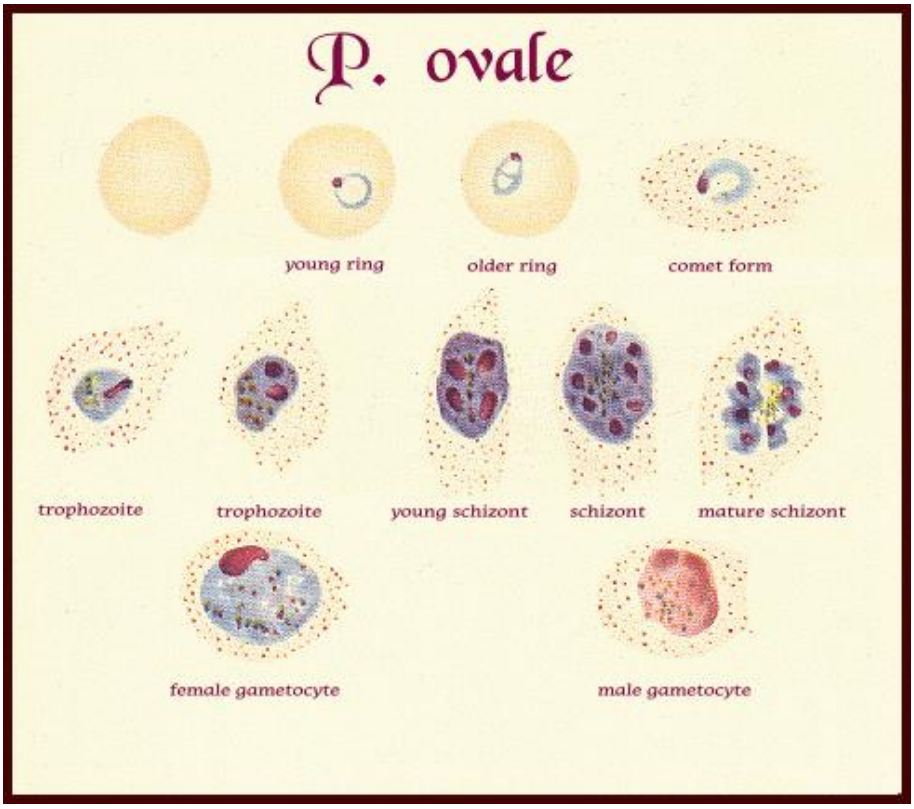

Figure 3.5: Different stages of *P. ovale* in erythrocytes
(Source: http://static.periscopepost.com/wpcontent/uploads/2010/04/Povale.gif)

The appearance of *P. ovale* is generally rare and its distribution mainly in the tropical Africa. Sometimes there are reported cases in South America and Asia. There are many

similarities in morphology and symptoms to *P. vivax*, but the clinical picture is not as severe. (Lucius and Loos-Frank, 2008; Markell et al., 1999)

Like *P. vivax*, the merozoits of *P. ovale* can only invade reticulocytes and also the incubation period is almost the same. The invaded RBCs are enlarged and show Schüffner dots as well, but have a typical ovoid form which gave the name to the parasite. The erythrocytic schizogony takes 48 hours and is synchronized, which again leads to a malaria tertiana. But compared to *P. vivax* there are less merozoits (8-12) in a mature schizont. With 48-96 hours the duration of the gametozytogenesis is the same as well. Different stages of *P. ovale* in RBCs can be seen in Fig. 3.5. An untreated infection can last up to 7 years in the host, the hypnozoits from the hepatocytes can cause relapses. (Markell et al., 1999; Garnham, 1988)

3.3.4 *Plasmodium malariae*

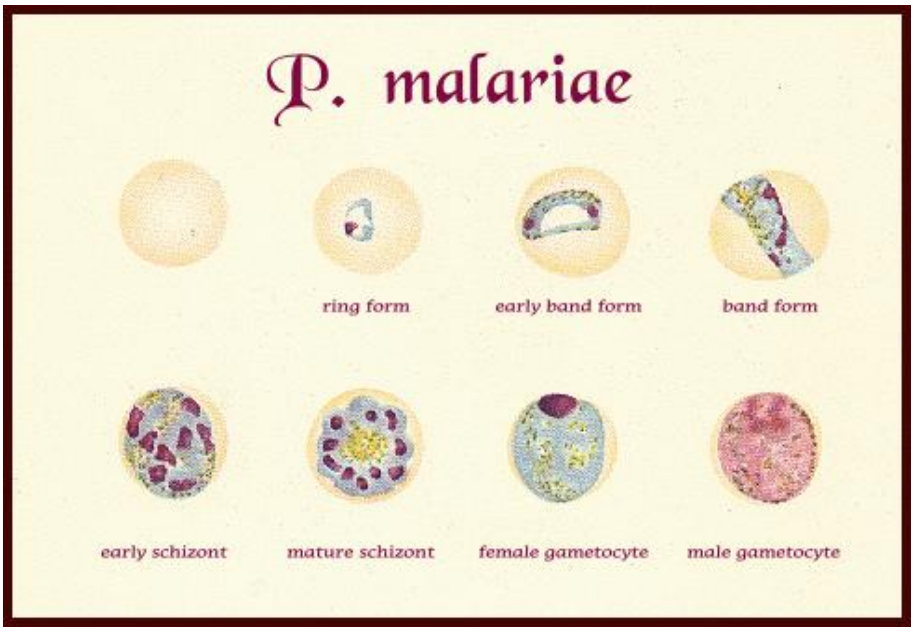

Figure 3.6: Different stages of *P. malariae* in erythrocytes
(Source: http://www.bioon.com/Article/ UploadFiles/200406/20040603215831151.gif)

P. malariae is the pathogenic agent of malaria quartana. The distribution of *P. malariae* malaria is mainly in western and eastern Africa and parts of India, but generally very rare. (Lucius and Loos-Frank, 2008; Markell et al., 1999)

The incubation period is 18-40 days. Merozoits from *P. malariae* can only invade old RBCs, therefore the parasitaemia is most of the time less than 1%. The RBCs

are not enlarged and have no Schüffner dots. The name malaria quartana, is owed to the fact that the fever attacks are every 72 hours, which is also the time for the synchronized erythrocytic schizogony. Trophozoites tend to form the characteristic band form. Shown, together with other stages in Fig. 3.6. A mature schizont contains 6-8 merozoits, which are typically arranged in a rosette, the so called 'daisy head'. The duration of the gametozytogenesis is 72-120 hours, but the round gametocytes usually appear some weeks after the initial fever attack. An untreated primary attack usually lasts for 3-24 weeks. The attacks are not as severe as *P. vivax* malaria, but last longer and sometimes nephrotic syndromes can occur as complications. *P. malariae* does not form hypnozoits, but it can still come to relapse even 50 years after primary infection. (Lucius and Loos-Frank, 2008; Markell et al., 1999; Garnham, 1988)

Due to the fact that *P. malariae* malaria is the most benign of all plasmodial infections, it seams that on a phylogenetic base it is the oldest and most adapted plasmodial species. But there are already evidences against this theory. (Garnham, 1988)

3.4 The Life cycle

The life cycle of plasmodia, pictured in Fig. 3.7, is divided in an asexual cycle in the human being and a sexual cycle in the female *Anopheles* (Meigen, 1818) mosquito (Nauck et al., 1967).

3.4.1 Liver phase

The target of the sporozoites, which are imported through an infected mosquito bite, are the liver cells. They reach the cells by circulating for a short time in the blood stream. In the hepatic cells they develop into exoerythrocytic schizonts within 5-15 days, depending on the plasmodial species (Fujioka and Aikawa, 1999), 5,5 days in *P. falciparum*, 8 days in *P. vivax*, 9 days in *P. ovale*, and 15 days in *P. malariae* (Aikawa, 1988). *P. cynomolgi*, *P. vivax* and *P. ovale* now also develop their dormant stage the hypnozoites, which can turn into exoerythrocytic schizonts and appear as relapse even after years (Fujioka and Aikawa, 1999). A mature exoerythrocytic schizont contains 2000-40000 merozoits depending on the plasmodial species (*P. malariae* $\sim$ 2000, *P. ovale* $\sim$ 15000, *P. vivax* $\sim$ 10000 and *P. falciparum* 20000-40000) (Wernsdorfer and Wernsdorfer, 2010). The hepatocytes rupture and merozoits are released to the blood stream and invade RBCs within 30 seconds. Now the erythrocytic cycle begins. (Fujioka

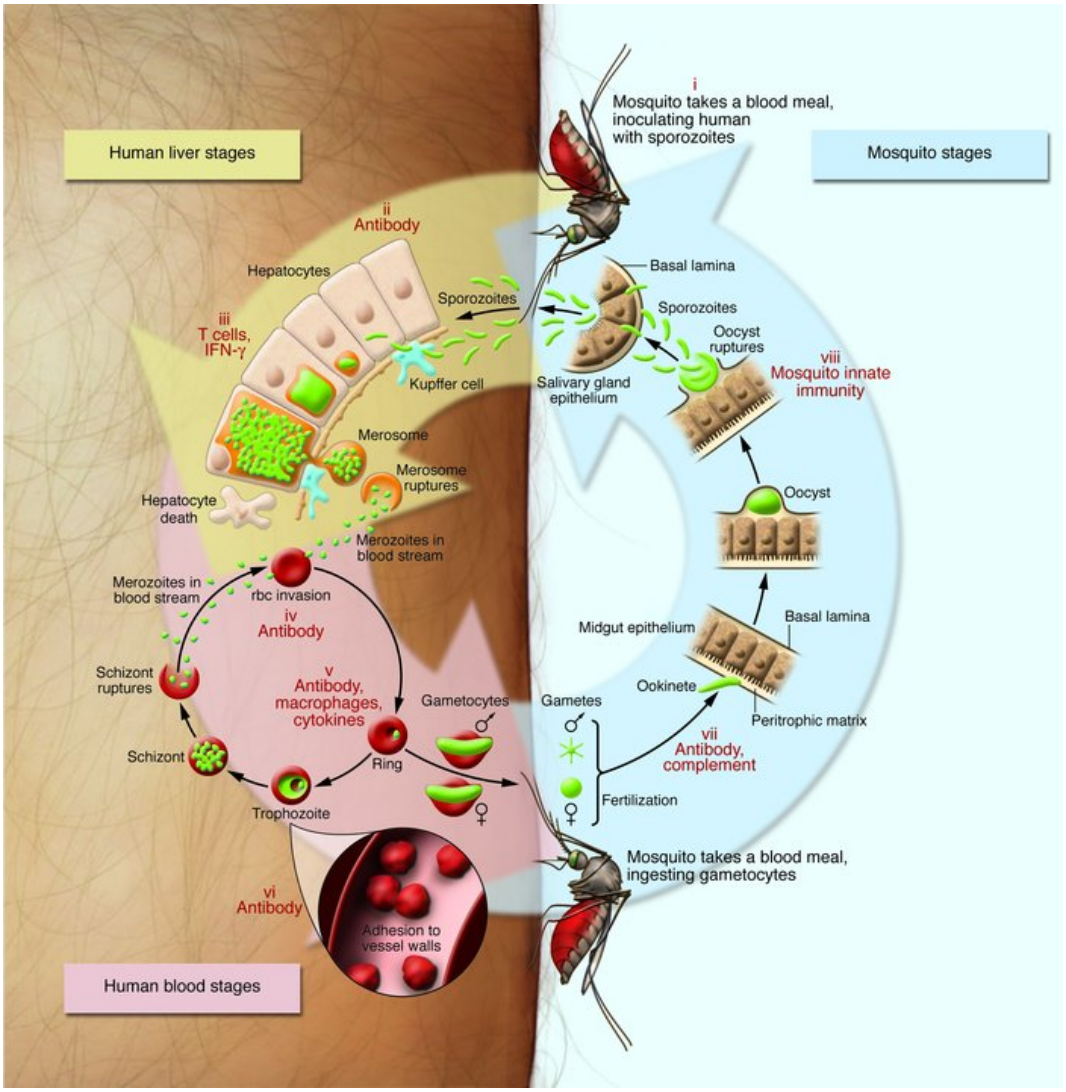

Figure 3.7: Life Cycle of Plasmodia
(Source: http://www.jci.org/articles/view/33996/files/JCI0833996.f1/medium)

and Aikawa, 1999)

3.4.2 Blood phase

The development in the RBCs from merozoits through ring, trophozoites and schizonts is called the erythrocytic schizogony (Fujioka and Aikawa, 1999). In most species the merozoits in the RBCs have only one nucleolus and cytoplasm. By digesting the haemoglobin and developing a vacuole they grow to trophozoits. Further on the nucleolus begins to divide and becomes a schizont (Aikawa, 1988). After maturation of the schizont with the species specific amount of merozoits, (mentioned in 3.3.1, 3.3.2, 3.3.3, 3.3.4) the

RBCs bust and the released merozoits invade again new RBCs. After several of these erythrocytic cyles, some of the merozoits turn into sexual forms, the female macrogametocytes and the male microgametocytes. This is called the gametocytogony. (Fujioka and Aikawa, 1999)

3.4.3 Mosquito phase

After intake of this sexual form via a blood meal in the mosquito midgut, the macrogametocytes leave the erythrocytes and form macrogametes. The microgametocytes exflagellate and form eight microgametes. After fertilization of a macrogamete with a microgamete a zygote is formed, which develops within 18-24 hours to an ookinet. The ookinet moves through the peritrophic membran and the epithelial cells. Beneath the basement membrane of the midgut epithelium they turn into an oocyst. An oocyst releases up to 10000 sporozoits, which move to the salivery glands and accumulate there. In case this infected mosquito takes another blood meal from a human being, the life cycle starts again. (Fujioka and Aikawa, 1999)

3.5 The Vector

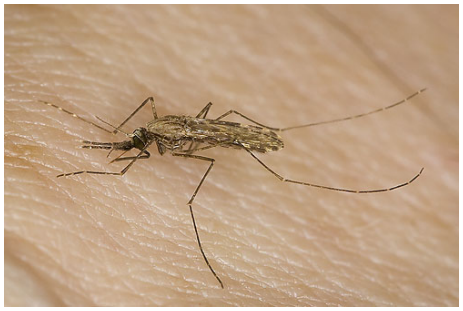

Figure 3.8: *Anopheles gambiae*
(Source: http://www.raywilsonbirdphotography.co.uk /Anophelesgambiae.jpg)

The obligate vector for all human pathogen plasmodium are the female anopheline mosquitoes. An example pictured in Fig. 3.8 with *A. gambiae*. Out of the currently known 420 different anopheline species, around 67 merit consideration as malaria vectors, but roughly half of them are responsible for the most transmissions. (Wernsdorfer and Wernsdorfer, 2010)

The distribution of the anopheline mosquitoes is not restricted to the tropics and subtropics, they are found worldwide, even in the arctic territories. But if a mosquito is able to transmit malaria, many factors have to accord. Some of these differences in malaria infection and transmission may be due to differences in preference for human blood (anthropophilism) or for animal blood (zoophilism). Another factor is the temperature, the sporogony in the mosquito can only take place at a temperature between 16℃ and 33℃. Therefore a temperature range between 20℃ and 30℃ and humidity form

the optimum conditions. Partially this is also depending on the different plasmodial species, e.g. *P. vivax* can stand lower temperatures whereas *P. falciparum* needs higher temperatures. Some other important conditions are the lifespan of the mosquito, the duration of blood meals (at least two are needed for transmission), the breeding and living preferences and the frequency of occurrence of female mosquitoes. (Wernsdorfer and Wernsdorfer, 2010; Lucius and Loos-Frank, 2008; Weyer, 1939)

3.6 Clinical symptoms of *P. falciparum*

P. falciparum malaria or malaria tropica can roughly be divided into uncomplicated and severe malaria (Harinasuta and Bunnag, 1988).

3.6.1 Uncomplicated malaria

The first symptoms appear 8-12 days after the bite of an infected mosquito and are not specific. With headaches, fatigue, muscle and joint pain, nausea, diarrhea followed by chills, fever and vomiting, it is difficult to distinguish from influenza or other viral infections. In the early stage of the infection the fever pattern is continuous remittent but without periodicity. Mild anemia and enlarged spleen might also be found, especially in patients with constant re-infections. With proper treatment in this stage, the infections can be fully cured. Without treatment the primary attack can lead to severe malaria and can be fatal to non immune people. (WHO, 2010b; Harinasuta and Bunnag, 1988)

3.6.2 Severe malaria

Although about one percent of all malaria cases turn into severe malaria, it is still responsible for around one million deaths each year in sub-Saharan Africa (Mackintosh et al., 2004).

The reason for the manifestation of severe malaria is the fact that infected RBCs tend to adhere in the small blood vessels and block the blood supply to organs. This can appear suddenly and at any stage of the infection. (Harinasuta and Bunnag, 1988)

Definition of severe *P. falciparum* malaria according to WHO

"In a patient with *P. falciparum* asexual parasitaemia and no other obvious cause of symptoms, the presence of one or more of the following clinical or laboratory features

classifies the patient as suffering from severe malaria:

Clinical features:

- impaired consciousness or unrousable coma
- prostration, i.e. generalized weakness so that the patient is unable walk or sit up without assistance
- failure to feed
- multiple convulsions - more than two episodes in 24 h
- deep breathing, respiratory distress (acidotic breathing)
- circulatory collapse or shock, systolic blood pressure <70 mm Hg in adults and <50 mm Hg in children
- clinical jaundice plus evidence of other vital organ dysfunction
- haemoglobinuria
- abnormal spontaneous bleeding
- pulmonary oedema (radiological)

Laboratory findings:

- hypoglycaemia (blood glucose <2.2 mmol/l or <40 mg/dl)
- metabolic acidosis (plasma bicarbonate <15 mmol/l)
- severe normocytic anaemia (Hb <5 g/dl, packed cell volume <15%)
- haemoglobinuria
- hyperparasitaemia (>2% /100000/µl in low intensity transmission areas or >5% or 250000/µl in areas of high stable malaria transmission intensity)
- hyperlactataemia (lactate >5 mmol/l)
- renal impairment (serum creatinine >265 µmol/l)."

(WHO, 2010b, p. 35)

3.7 Diagnosis of malaria

3.7.1 Symptom-based diagnosis

As mentioned in 3.6 the clinical symptoms of malaria, e.g. headache, fever, chills and fatigue, are very general. Therefore they fit to quite a number of other diseases and also vary in different areas. A diagnosis of malaria based only on symptoms is low in sensitivity and specificity. Even if a detailed weighting and scoring system can improve this, it is still not accurate enough and might be too difficult to implement and supervise. On the one hand a symptom-based diagnosis of malaria may lead to an over diagnosis. On the other hand also to under diagnosis. Nevertheless to know the symptoms is important for the initial thought to get to the right diagnose (WHO, 2010b; Gilles, 1988).

3.7.2 Rapid diagnostic tests

According to the WHO "Rapid diagnostic tests (RDT) are immunochromatographic tests that detect parasite-specific antigens" (WHO, 2010b, p. 118). RDTs can be divided into four groups of antigen targets: histidin-rich protein 2 (HRP2), parasite lactone dehydrogenase (pLDH), *Plasmodium* aldolase and the fourth not further identified is specific to *P. vivax* (Murray et al., 2003). Many different tests from various producers are available to detect *P. falciparum*, most of them use HRP2 or pLDH or both (WHO, 2008).

Compared to microscopy, the advantage of RDTs is the simplicity in performance, interpretation and rapid results. To perform the test, there is no laboratory required, not even electricity and the health workers need to be trained but less skilled (WHO, 2010b).

But there are also limitations of this test method, for example the sensitivity. Most tests have limitations to detect low parasite density (200 parasite/µl), but good detection at 2000-5000 parasites/µl (WHO, 2008). Another limitation is the specificity, many of these test only differentiate *P. falciparum* and non-*P. falciparum*, but the non-*P. falciparum* are not clearer defined (Murray et al., 2003). In *P. falciparum* there are also difficulties to differ new infections from effectively treated infections in the last three weeks, due to the persistence of HRP2 in the blood after treatment (WHO, 2010b; Murray et al., 2003). There can also be false-positive results from patients with acquired immunity or rheumatoid factors (WHO, 2008; Murray et al., 2003)

However RDTs can be a good alternative to reduce anti-malarial overuse in areas with low transmission and poor settings. But it is important to ensure the good quality and storage of the tests and well trained health workers (WHO, 2010b; Murray et al., 2003).

3.7.3 Microscopic diagnosis

The light microscopy should be the 'field standard' in malaria diagnosis (WHO, 2010b). There are two ways of examination, a 'thick film' and a 'thin film'. In the thick film many layers of red and white blood cells upon each other, so the parasites are more concentrated and therefore easy to find and identify. This method is used to search for parasites. A thin film, if properly made, consists only of one layer of blood cells. This method is used to confirm the plasmodial species and only in exceptions for parasitic search (WHO, 1991).

Preparation of a thick film

Before starting, the patients data need to be recorded and the slide appropriately labeled. After disinfection and drying, the finger tip is pricked with a sterile lancet. Two or three large drops of blood are placed close together in the middle of the slide. To make an even thick film with a diameter of about one centimeter, the drops are joined with some strokes using the corner of another slide. Before staining, the slide needs to fully dry. (WHO, 1991)

Giemsa stain

Giemsa is the most commonly used stain for the diagnosis of malaria on blood smears (Shute, 1988).

> "Giemsa stain is an alcohol-based Romanowsky stain. Giemsa stain is a mixture of eosin, which stains parasite chromatin and stippling shades of red or pink, and methylene blue, which stains parasite cytoplasm blue. White-cell nuclei stain blue to almost black, depending on the type of white cell. During staining, the hemoglobin in the red cells dissolves (dehaemoglobinization)" (WHO, 1991, p. 32).

For the rapid staining a 10% solution of Giemsa in a buffered water (pH 7,2) is used, about 3-5 ml for each slide. Staining time is eight to ten minutes. After that the slides need to be washed gently with plain water and dried properly in a drying rack. (WHO, 1991; Shute, 1988)

Examination blood films for malaria parasites

The examination of blood films for malaria parasites is done by light optical microscope using a x10 paired oculars and x100 oil immersion objective (WHO, 1991).

> "Using the fine adjustment, focus on the cell elements and confirm that the portion of the film is acceptable for routine examination: 15-20 white blood cells per thick film field will give a satisfactory film thickness. Films with fewer white blood cells per field will require more extensive examination. Routine examination of a thick film is based on examination of 100 good fields; i.e. a slide can be pronounced negative only when a minimum of 100 fields have been carefully examined for the presence of parasites." (WHO, 1991, p. 72).

In a positive slide the parasite species needs to be identified and the density to be counted against white blood cells (WHO, 1991).

For microscopical diagnosis of malaria high costs in setting up, training and supervision are involved and the maintainability might be difficult. But if this is done, it is the method with the highest sensitivity and specificity. (WHO, 2010b)

3.8 Antimalarial drugs

Since malaria is a serious disease, a proper treatment is important (WHO, 2010b).

3.8.1 Classification of antimalarial drugs

Anti-malarial drugs can be roughly classified according to their biological activity in tissue schizontocides and blood schizontocides (Desjardins et al., 1988). This is listed in Table 3.1.

Blood schizontocide (erythrocytic schizontocide): "refers to a drug which destroys asexual parasites in the blood" (Desjardins et al., 1988, p. 828). Blood schizontocides are used as treatment as well as prophylaxis, but do not work against intrahepatic forms. Agents are: 4-Aminoquinolines, Arylaminoalcoholes and Artemisinin and its derivatives (Stuart et al., 2008).

Tissue schizontocide (exoerythrocytic schizontocide): "refers to a drug which destroys asexual parasites in the tissues" (Desjardins et al., 1988, p. 828), by inhibiting

Table 3.1: Summary and biological activities of anti-malarial drugs (0= no effect, + =effect, VMO=effective against *P. vivax*, *P. malariae*, *P. ovale*, F=against *P. falciarum*)

Class	Drug	Blood schizon-tocide	Tissue schizon-tocide	Mature gameto-cytes
4-Aminoquinolines	Chloroquine	++	0	VMO
Arylaminoalcohols	Quinine	++	0	VMO
	Quinidine	++	0	VMO
	Mefloquine	++	0	VMO
Phenoenthrene methanol	Halofantrine	++	0	0
Artemisinin and derivatives	Artemisinin	++	0	+
	Artemether	++	0	+
	Artesunate	++	0	+
Antimetabolites	Proguanil	+	+	0
	Pyrimethamine	+	0	0
	Sulfadoxine	+	0	0
Antibiotics	Tetracycline	+	F	0
	Doxycycline	+	+	0
	Clindamycin	+	+	0
8-Aminoquinoline	Primaquine	0	+	0

(Stuart et al., 2008; Ridley, 2002; Desjardins et al., 1988)

early stages or destroying hynozoites. Agents are: 8-Aminoquinoline, Proguanil and probable Pyrimethamine (Desjardins et al., 1988).

Gametocytocide: "refers to a drug which destroys the sexual forms of the parasite in the blood" (Desjardins et al., 1988, p. 828).

3.8.2 Treatment of malaria

Since malaria is a complex disease with different activators, the following aspects have to be considered before treatment is given:

- "The identity (species) of the *Plasmodium* with which the individual is infected or

to which exposure is anticipated

- The severity of an existing infection including level of parasitemia and presence of complications
- The anticipated level of immunity based on prior infection of exposure
- The likelihood of drug resistance based on geographical considerations
- The presence of special risk factors such as age, pregnancy and intercurrent illness
- The tolerability of specific drugs" (Desjardins et al., 1988, p. 828)

Treatment of uncomplicated *P. falciparum* malaria

For many years the standard therapy for uncomplicated *P. falciparum* malaria was Chloroquine. But now there is a resistance to Chloroquine in almost all parts of the world as well as to sulphadoxine/pyrimethamine. (Ridley, 2002)

The standard therapy recommended from the WHO is now a combination of two blood schizontocides with different way of action, to reduce the risk of resistance. One of this medications should be an artemisinin derivative (Stuart et al., 2008). This treatment regime is called Artemisinin-based combination therapy (ACT).
There are four oral ACTs recommended at this time:

- artesunate plus mefloquine (AS+MQ) in Asia
- artemether plus lumefantrine (AL) worldwide
- artesunate plus amodiaquine (AS+AQ) worldwide
- artesunate plus sulfadoxine-pyrimethamine (AS+SP)

(WHO, 2010b)

Artemisinin is contraindicated during the first trimester of pregnancy. In this period Quinine as a monotherapy or in combination with clindamycin should be given. (Stuart et al., 2008)

Treatment of severe *P. falciparum* malaria

Severe *P. falciparum* malaria is an emergency with a high mortality rate and needs to be treated in a hospital with prompt parenteral antimalarials and good medical care (WHO, 2010b).

There are two treatment regimes recommended from the WHO, intravenous Artemether or Artesunate or Quinine. Artesunate should be given in non-endemic areas with low to moderate transmission. In areas with high transmission the treatment of choice is intravenous Artemether or Quinine. (WHO, 2010b; Stuart et al., 2008)

Since Quinine causes a range of side effects it should only be given if other recommended parenteral antimalarials are not present (WHO, 2010b).

When the patients get better after the initial parenteral treatment and can tolerate oral medications, a full course of oral ACT should be given (WHO, 2010b).

Treatment of *P.vivax*, *P. ovale*, *P. malariae* malaria

The current WHO recommendation to treat the so called benign malarias is still Chloroquine. In areas with Chloroquine resistance, Amodiaquine or an Artemisinin derivative or Mefloquine should be given. The hypnozoites from *P. vivax* and *P. ovale* must be treated with Primaquine for radical cure. (Stuart et al., 2008)

"In pregnant patients with *P. vivax* or *P. ovale* infections, radical cure with Primaquine should be postponed until after delivery; Chloroquine at a dose of 600 mg weekly can be given until then." (Stuart et al., 2008, p. 189)

3.9 Antimalarial drug resistance

Resistance to antimalarial drugs has been defined by the WHO in 1965 as the "ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within tolerance of the subject". And was modified by Bruce-Chwatt L.J. 1981 that the drug must "gain access to the parasite or the infected red blood cell for the duration of the time necessary for its normal action" (Bloland, 2001, p. 12).

Malaria with its estimated one million deaths each year is one of the greatest disease burdens in the world, even more with antimalarial drug resistance (Wongsrichanalai et al., 2002). The distribution of resistance can be seen in Fig. 3.9.

Out of the four human pathogen plasmodial species, *P. falciparum* is the one with a comprehensive resistance to quite some drugs. A resistance to chloroquine in *P. vivax* can be found in some parts of the world. In *P. ovale* and *P. malariae* resistance is not documented yet. (Wongsrichanalai et al., 2002; Bloland, 2001)

Chloroquine resistant *P. falciparum* strains are documented, except of Central America and the Caribbean, in all areas where *P. falciparum* is endemic. In the late 1950s the first Chloroquine resistance to *P. falciparum* was reported at the Thai-Cambodian border and in Colombia, followed by Africa in 1978 in the east and covered this continent by 1983 and South America in the 1980s. By 1989 (with Asia and Oceania) the resistance was worldwide. (Wongsrichanalai et al., 2002)

P. falciparum resistance to sulfadoxine-pyrimethamine (SP) started again on the Thai-Cambodian border and is constantly increasing in Africa where it is used as a substitution for chloroquine (Wongsrichanalai et al., 2002; Bloland, 2001).

Up to now resistance from *P. falciparum* to mefloquine as well as artemisinin and derivatives occur only in some parts of south-east Asia and Africa (Wongsrichanalai et al., 2002; Bloland, 2001).

If a parasite is resistant and not only to the first two antimalarial drug classes (4-aminoquinolines e.g. chloroquine and antifolates e.g. SP) but also to a third class, it is called a Multi-drug-resistance (MDR) (Wongsrichanalai et al., 2002).

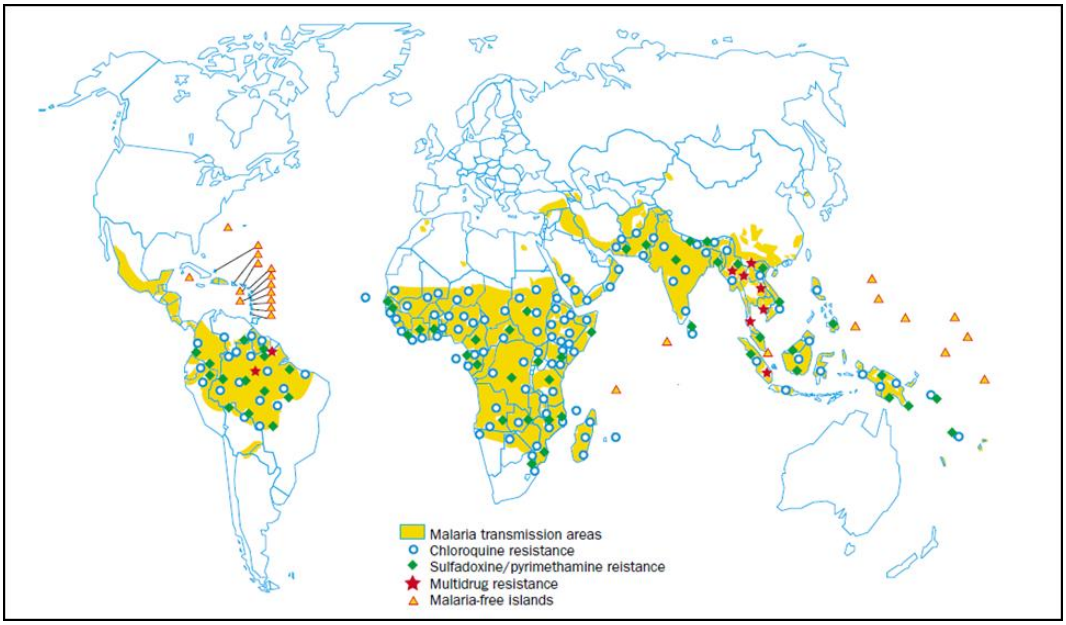

Figure 3.9: Antimalarial drug resistance to *P. falciparum* according to WHO
Source: Wongsrichanalai et al. (2002)

3.9.1 Mechanism of antimalarial resistance

There are different ways of how a parasite can acquire a resistance to drugs:
A resistance can occur through a spontaneous mutation, which makes the parasite less sensitive to a drug or drug class. Sometimes a single mutation is enough to make a parasite resist against a drug, for some drugs multiple mutations are needed. It does not necessarily mean that all parasites of a population have the same range of resistance, this can vary from completely sensitive to highly resistant. (Bloland, 2001)

Another way for example (chloroquine), that the parasite can increase his capacity to excrete the drug through his food vacuole, so that the drug does not reach the necessary level to inhibit the haem polymerization. If resistance to other quinolines develop with the same mechanism is not yet proved. (Bloland, 2001)

Also the half-life of a drug can influence resistance. Drugs with a long half-life (SP, MQ) decrease the duration of treatment and thus encourage the compliance, but also increase the possibility of the parasite to develop resistance. This can happen especially in areas with high transmission when the drug level drops below the level of full parasite growth inhibition but stays above 5% inhibition. There is still selective pressure but not enough to kill recrudescent parasites or from new infections. Due to this sub-therapeutic drug level parasites can build up resistance. The same can happen, if medication is under dosed either due to incorrect prescription or low patient compliance of dose or duration. (Bloland, 2001)

In vivo, in vitro, animal model studies, and molecular characterization are the four different ways how antimalarial resistance can be tested (Bloland, 2001).

3.9.2 Treatment failure

According to WHO "Treatment failure is defined as an inability to clear malarial parasitaemia or resolve clinical symptom despite administration of an antimalarial medicine." (WHO, 2010a, p. 9)

Not every treatment failure occurs due to drug resistance. There are many other reasons, like incorrect dosage of drugs, non compliance of patients in duration or dosing of drugs, poor absorption or fast elimination of drugs in very sick patients, poor quality or expired drugs or drug interaction. (WHO, 2010a; Bloland, 2001)

Treatment failures are classified by the WHO in 2009 as follows:

"Early treatment failure (ETF)

- danger signs or severe malaria on day 1, 2 or 3 in the presence of parasitaemia;
- parasitaemia on day 2 higher than on day 0, irrespective of axillary temperature;
- parasitaemia on day 3 with axillary temperature is 37.5℃; or
- parasitaemia on day 3 is 25% of count on day 0

Late clinical failure (LCF)

- danger signs or severe malaria in the presence of parasitaemia on any day between day 4 and day 28 (day 42) in patients who did not previously meet any of the criteria for early treatment failure; or
- presence of parasitaemia on any day between day 4 and day 28 (day 42) with axillary temperature >37.5℃ in patients who did not previously meet any of the criteria for early treatment failure

Late parasitological failure (LPF)

- presence of parasitaemia on any day between day 7 and day 28 (day 42) with axillary temperature <37.5℃ in patients who did not previously meet any of the criteria for early treatment failure or late clinical failure

Adequate clinical and parasitological response (ACPR)

- absence of parasitaemia on day 28 (day 42), irrespective of axillary temperature, in patients who did not previously meet any of the criteria for early treatment failure, late clinical failure or late parasitological failure" (WHO, 2010a, p. 90)

From a contemporary perspective, malaria is not going to be eradicated in the close future, consequently effective antimalarials are going to be needed. As long as drugs are in use, there is always a risk that parasites develop resistance. To minimize the risk of resistance drugs pressure needs to be reduced by correct diagnosis and prescription, good patients compliance and the use of drug combinations. (Bloland, 2001)

4 Methods and Material

4.1 Study area

The study was carried out in the Malaria Clinic in Mae Sot, Thailand. Mae Sot is a town in the Tak province, about 550km northwest of Bangkok on the border to Myanmar, shown in Fig. 4.1. The Moei River is the natural border between these two countries and the surroundings are forest hills where malaria transmission is high. (Knauer et al., 2003)

Tak province is one of the regions in Thailand which suffers most of malaria. One of the reasons for this situation is the heavy border traffic between Myanmar and Thailand due to labor migration. Most of the population is located in remote and rural areas surrounded by rice and cotton paddies and mountains with little access to health care. (WHO, 2007)

Figure 4.1: Map of Thailand
(Source:http://www.beepworld.de)

The main malaria vectors in this region and typical for forest areas are *Anopheles dirus* and *Anopheles minimus*. The malaria transmission period shows two peaks, one during rainy season between May and September and the second in November and December, but can generally occur at any time of the year. (Knauer et al., 2003) Malaria is endemic in this region, with *P. vivax* as the leading species (approximately 60%), followed by *P. falciparum* (approximately 40%). Mixed infections and other species such as *P. malariae* are rarely seen. (WHO, 2010c)

The standard treatment of uncomplicated *P. falciparum* malaria is an oral combination therapy of Mefloquine and Artesunate. Irrespective of their nationality, the treatment for all patients with malaria in Thailand is free of charge. This governmental

program aims at preventing the increase of drug resistance due to self treatment with incorrect use of drugs and low compliance. (WHO, 2010c)

The samples were taken between end of June 2010 and beginning of August 2010. The cases were diagnosed from the medical assistants of the local malaria clinic with Giemsa-stained thick blood film and microscope.

4.2 Patients

4.2.1 Including criteria

All patients came for treatment to the malaria clinic of Mae Sot. For inclusion in our study they had to be above 9 years old (excluding pregnant women) and suffering from *P. falciparum* mono infections with a parasitaemia between 1000 and 100000 asexual parasites/micro liter blood.

Exclusive criteria, apart from childhood and pregnancy, were severe and complicated malaria and a recent treatment with Mefloquin within the last 63 days all other anti-malarial drugs and antibiotics within the last 14 days.

All *P. falciparum* malaria patients were treated with Artesunate and Mefloquine, the standard therapy in Thailand, after confirmation of the diagnosis and blood sampling for the in-vitro test.

Before a blood sample was taken, an oral informed consent was required from the patients, including epidemiological data such as sex, age, nationality, occupation, place of residence, origin of infection and the registration number of the Mae Sot Malaria Clinic.

4.2.2 Epidemiology

During the period of the study in Mae Sot 36 patients were tested positive with *P. falciparum* malaria. Out of this group 29 patients could be included in the study. Out of this 29, 9 where female with an average age of 29,68 years. The remaining 20 male patients had an average age of 30,74 years.

18 out of the 29 patients in the study had their residence in Thailand (3 female and 15 male), the other 11 (6 female and 5 male) lived in Myanmar.

Out of the 29 patients from the study, only 13 got their infection in Thailand, while

the remaining 16 got infected in Myanmar. This might be due to the poor health system in Myanmar.

The most common occupation of the patients with *P. falciparum* malaria was farmer with 15 individuals, followed by 5 labourers (mainly woodcutters) and 5 workers. These findings are not uncommon since people with outdoor activities are more exposed to mosquitoes.

4.3 Substances tested

In the study Artemisinin (explained in 4.3.1) and extracts from *E. longifolia* (explained in 4.3.2) were tested. Also combinations of Artemisinin and extracts from *E. longifolia* were tested to prove an interaction.

4.3.1 Artemisinin

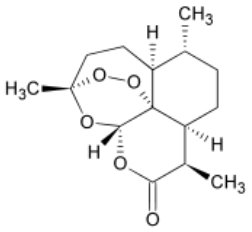

Figure 4.2: Artemisinin
(Source: http://www.york.ac.uk/org/cnap/artemisiaproject/images/artemsinin.gif)

Artemisinin, (structure shown in Fig. 4.2), was isolated from Chinese scientists in 1971 from the leaves of *Artemisia annua*. The indigenous plant, which grows throughout China, is locally called Qinghao and is in use as herbal medicine since more than 2000 years. (Wernsdorfer and Trigg, 1988)

Artemisinin is a very stable sespquiterpene lactone and almost insoluble in water, only sparingly soluble in oils, but dissolve in ethyl alcohol and dimethyl sulfoxide (DMSO). Because of the poor solubility of the parent compound, Artemisinin, some halve synthetic derivatives like artemether, artesunate, arteether, dihydroartemisinin and artelinic acid were metabolized. All of them are very potent and rapid acting blood schizontocides and can be administrated in many different ways, i.e. oral, intramuscular, intravenous and even rectal. (Krishna et al., 2004; Meshnick et al., 1996)

There is no difference of bioactivity between Artemisinin and its derivatives, because all are rapidly metabolized to di-hydroartemisinin (DHA) with a short half-live (Wernsdorfer and Wernsdorfer, 1991).

In *P. falciparum* Artemisinin and its derivatives are active against the broadest age range of the parasites, from tiny rings that have just invaded erythrocytes, up to mature trophozoites and even schizonts. Which also impacts the mosquito transmission. But they do not work against hepatic stages, therefore no prophylactic potential. Apart from *P. falciparum* they are killing all human pathogen Plasmodium species. (Woodrow et al., 2005)

The Artemisinins act faster than all other types of antimalarial, thus it is recommended by the World Health Organization (WHO) in oral Artemisinin combination therapy (ACT) as first line treatment for uncomplicated *P. falciparum* malaria. The combination is necessary because Artemisinin and its derivatives have a very short half-life, so a

treatment for seven days would be required. In combination with a slow elimination antimalarial the treatment can be shortened to three days. A benefit from the ACT is, that the partner compounds protect from resistance. (WHO, 2010b)

4.3.2 Extracts from *Eurycoma longifolia*

Eurycoma longifolia Jack is a plant in the family Simaroubaceae. It is an evergreen flowering tree, shown in Fig. 4.3 on the left side, indigenous to the jungles of Malaysia and Indonesia. In the local traditional medicine several products, especially from the roots pictured in Fig. 4.3 on the right side, locally known as Tongkat Ali or Pasak Bumi, are used as antipyretic, antimalarial and anabolic properties. (Wernsdorfer et al., 2009; Chan et al., 2004)

Figure 4.3: *E. longifolia*
(Source: http://www.corenberg.com)

In the late 20th century many substances from *E. longifolia* Jack have been tested in different areas, for example their anabolic properties (Muhamad et al., 2010) or their cytotoxic activity against tumor cells (Zakaria et al., 2009; Tee and Azimahtol, 2005).

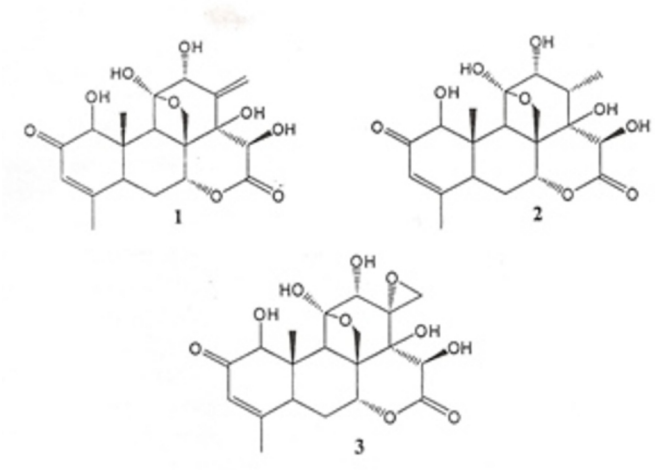

Figure 4.4: Structure of quassinoids from *E. longifolia* (1 = eurycomanone; 2 = 13,21-dihydroeurycomanone; 3 = 13α (21)-epoxyeurycomanone)
Source: Wernsdorfer et al. (2009)

In previous studies (Chan et al., 2004; Jiwajinda et al., 2002; O'Neill et al., 1986) several quassinoids from the roots of *E. longifolia* were tested for their antiplasmodial activity. Out of these, three standardized quassinoids, eurycomanone, 13,21- dihydroeuryco-

manone and 12α(21)-epoxyeurycomanone (structure shown in Fig. 4.4) were isolated at the University Sains Malaysia by Professor Kit Lam Chan and provided for our study.

The *E. longifolia* extracts where tested as followed:

1. Eury 1: eurycomanon (80%)
2. Eury 2: eurycomanon (80%) + 13,21- dihydroeurycomanone (4,9%)
3. Eury 3: eurycomanon (76%) + 13,21-dihydroeurycomanone (4,3%) + 12α (21)-epoxyeurycomanone (2,3%)

4.4 In vitro test system

The examinations are based on the WHO standard in vitro micro-test Mark III for the determination of drug sensitivity in *P. falciparum* . This test provides information about sensitivity and drug response by measuring the inhibition of schizont maturation in fresh blood isolates of *P. falciparum* . (WHO, 2001)

The pre-dosed test plates were prepared at the Institute of Specific Prophylaxis and Tropical Medicine, Medical University of Vienna.

For the test, the RPMI 1640 LPLF-Medium is prepared with sodium bicarbonate and Gentamycine, distributed in sterile plastic tubes à 900µl and stored at 4℃. After disinfecting the puncture site (finger tip or earlobe) with an alcohol swap and drying up, 100µl blood are taken in a sterile heparinized capillary tube, diluted into the serial number labeled plastic tube with the medium and gently agitated (n.b. this amount (1 ml) of blood-medium-mixture (BMM) is sufficient for two test columns). The BMM can be kept up to 4 hours in the incubator at nearly 37℃. For longer storage the BMM needs to be cooled down at 4℃, either in the refrigerator or on wet ice, without direct contact to the tube. (WHO, 2001)

At the same time a thick blood film is prepared to determine the initial parasitaemia of the patient to control the including criteria. This pre-incubation slides are read for the number of asexual parasites per at least 200 leukocytes and calculated with the following formula:

$$\text{Parasites/µl} = \frac{Number\ of\ parasites\ counted\ x\ 8000}{number\ of\ leukozytes\ counted}$$

(WHO, 1991)

Table 4.1: Micro titre plate and concentrations of the substances used in the study

	A	B	C	D	E	F	G	H
Eury 1	0	3 nM	10 nM	30 nM	100 nM	300 nM	1000 nM	3000 nM
Eury 2	0	3 nM	10 nM	30 nM	100 nM	300 nM	1000 nM	3000 nM
Eury 3	0	3 nM	10 nM	30 nM	100 nM	300 nM	1000 nM	3000 nM
Art	0	3 nM	10 nM	30 nM	100 nM	300 nM	1000 nM	3000 nM
Art+Eury1	0	3 nM	10 nM	30 nM	100 nM	300 nM	1000 nM	3000 nM
Art+Eury2	0	3 nM	10 nM	30 nM	100 nM	300 nM	1000 nM	3000 nM
Art+Eury3	0	3 nM	10 nM	30 nM	100 nM	300 nM	1000 nM	3000 nM

For the test the appropriate drugs are dosed on sterile micro titre plates with 12 columns of 8 wells each, sealed with a transparent sheet and labeled with the containing drug. The wells B to H are filled with increasing concentrations of the substances to be tested, well A is as a control free of drugs. An illustration of a micro titre plate is shown in table 4.1.

50µl aliquots of the BMM are added to well A-H of the scheduled test plates, beginning by well A to prevent contamination. The dosed plates are closed with a sterile lid and gently agitated in order to dissolve the drugs. The patients number, date and time of inoculation are inscribed on the lid. After that, the plates are placed in a candle jar (desiccator), containing a small container of water. The candle jar is sealed airtight, after the candle exhaust it is put in the incubator at 37,5℃ for 24 to 26 hours. (WHO, 2001; Wernsdorfer and Wernsdorfer, 1995)

After incubation the test plates are removed from the candle jar and 'harvested' by discharging the medium supernatant and preparing thick films from the sediments on microscopy slides. Each test column is placed in a set order of eight thick films on one slide, starting from well H up to well A. Each slide is labeled with patient number, substance and date of test. (WHO, 2001)

The slides are then dried at least 24 hours. After drying they are stained with Giemsa solution at pH 6,8 for 90 minutes. (Wernsdorfer and Wernsdorfer, 1995)

4.5 Evaluation and statistical analysis

The reading of the thick blood films is done by a light optical microscope with x10 paired oculars and x100 oil immersion objective (WHO, 1991).

In the evaluation, beginning with well A (control), the number of schizonts ($\geq$ 3 chromatin dots) are counted against other asexual forms to a total of 200 parasites. The slide is classified as valid, if the control shows either $\geq$ 20 schizonts in total or $\geq$ 10% schizonts of all asexual parasites in the count. Are these criteria fulfilled, the reading can proceed with well B to H. Usually the number of schizonts decreases with the increase of substanceconcentration, up to the point of total inhibition. The so-called cut-off point or cut-off concentration gives a rough indication of the parasite sensitivity to the testsubstance. (Wernsdorfer and Wernsdorfer, 1995)

The statistical evaluation is done by the classic method of Litchfield and Wilcoxon (1949), based on the assumption of a log-normal distribution of drug resistance in all parasite populations with no recent drug contact. The drug concentrations are transformed into logarithms and the inhibition in % in probits. For the calculation of the individual effective drug concentration ($EC_{50,\ 90,\ 99}$), the % inhibition of schizont maturation at given drug concentration (SMI%) and regression parameters, a computer model of Wernsdorfer and Wernsdorfer (1995) is used. With the EC_{50}, EC_{90} and EC_{99} of the individual samples the correlation coefficient between two substances is calculated. The interaction analysis, where the sensitivity of the individual substances are compared with the sensitivity of combinations is done with the Variance analysis (Sokal and F. James Rohlf, 1995) with the following formula:

$$t = \frac{x_1 - x_2}{\sqrt{(V1+V2) \cdot {}^{1}/_{n}}}$$

The term t is the probability that the difference is merely due to chance. x_1 and x_2 denote the difference of the incidences, n is the number of cases incorporated in the analysis and V1 as well as V2 the variances between the two sets of figures.

5 Results

The following tables and figures of the results are listed in a certain order

beginning with the % inhibition of schizont maturation (SMI%). This relative SMI% shows the maturation inhibition of schizonts with increasing drug concentration compared with the control well A and gives information about the relative activity of the tested substances.

The next table shows the regression parameters. Important in this table are the correlation of regression (r) and the function out of it the heterogeneity (χ^2), as well as the slope function (S). According to Litchfield and Wilcoxon (1949) a steep slope function (S <2) shows a full activity in a narrow concentration-range, whereas a flat slope function (S >8) could be a sign for decreasing sensitivity.

In the following tables are the calculated mean effective drug concentrations (EC) and the 95% confidence interval.

After that the regression analysis as well as the effective doses are presented graphically.

5.1 Activity of tested drugs

A cut-off concentration could not be calculated for any of the tested substances.

5.1.1 Activity of Artemisinin

Out of the 36 isolates, 29 (80,5%) have an adequate maturation of schizonts (more than 10% schizonts in well A).

The arithmetic mean schizont concentration in well A per 200 asexual parasites was 53,58%

The relative schizont maturation inhibition (SMI%) for Artemisinin at the concentration 3nmol/l is 20,31% increases constantly up to the concentration 3000nmol/l with 92,32% (shown in Tab. 5.1), but never reaches 100% the total maturation inhibition.

Table 5.1: Drug concentration (Artemisinin) and SMI%

Drug conc.	SMI%
3,0	20,31
10,0	35,22
30,0	55,35
100,0	68,63
300,0	80,26
1000,0	87,63
3000,0	92,32

Table 5.2: Regression parameters of Artemisinin*

n = 29	S = 19,4581	f_s = 2,7147
a = 3,8853	A = 4,0674	$f_{EC\text{-}50}$ = 1,9794
b = 0,3350	K = 7	$f_{EC\text{-}90}$ = 4,3358
r = 0,9942	N' = 145	$f_{EC\text{-}95}$ = 5,9518
χ^2 = 0,6576	R = 1000	$f_{EC\text{-}99}$ = 11,5216

*According to Litchfield and Wilcoxon (1949): n=number of isolates, a=intercept of regression, b=slope of regression, r=correlation of regression, χ^2=heterogeneity, S=slope function, A=intermediate term for f_s, K=number of drug concentration, N'= number of data points EC16 to EC84, R=highest/lowest drug concentration tested, f_s=factor of S, f_{EC}=multiplication/division factor for obtaining 95% confidence intervals of EC

Table 5.3: EC values: Artemisinin

EC	Mean	95% Confidence Intervals	
		Lower	Higher
EC_1	0,0269	0,0023	0,3098
EC_{16}	1,4317	0,7233	2,8340
EC_{50}	27,8586	14,0741	55,1441
EC_{84}	542,0753	273,8548	1072,9980
EC_{90}	1277,0471	294,5380	5536,9748
EC_{95}	3776,8250	634,5657	22479,0063
EC_{99}	28865,3737	2505,3266	332575,3264

The regression analysis of the inhibition of schizont maturation through Artemisinin is also represented graphically in Fig. 5.1, as well as the effective dose in Fig. 5.2.

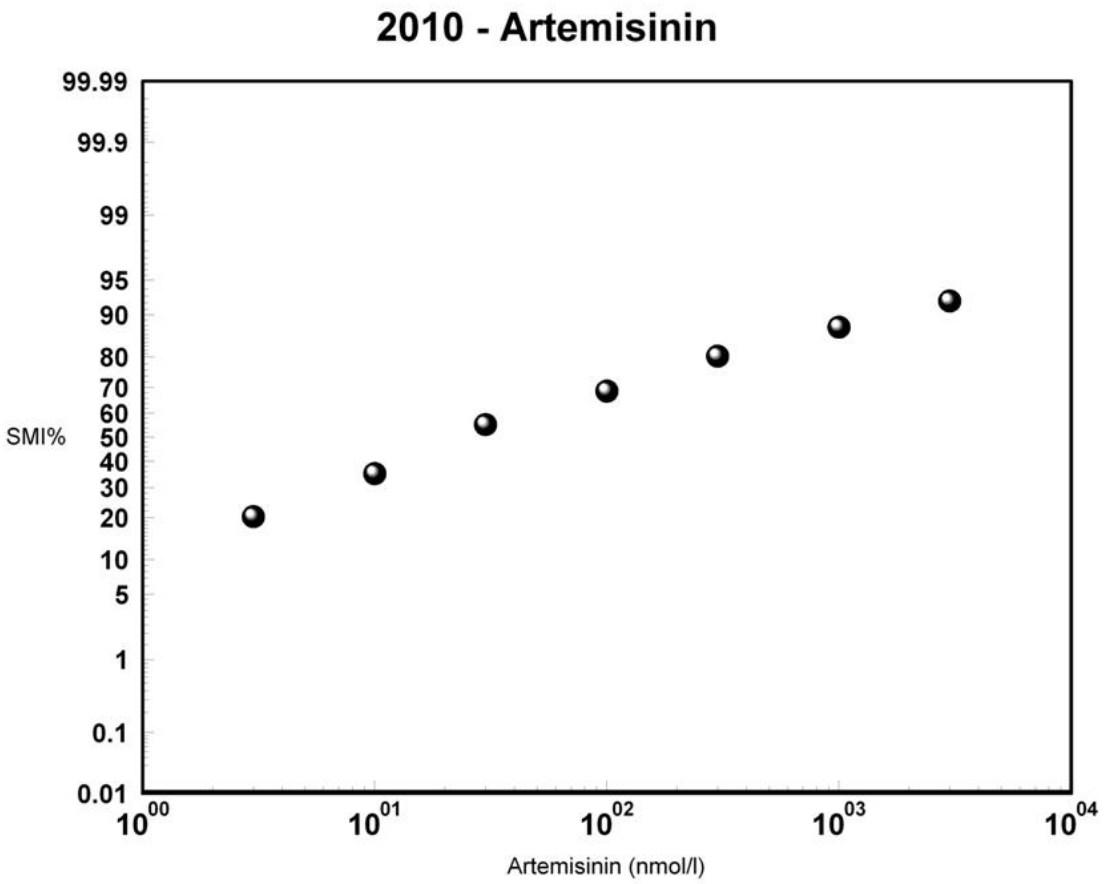

Figure 5.1: Regression analysis of Artemisinin

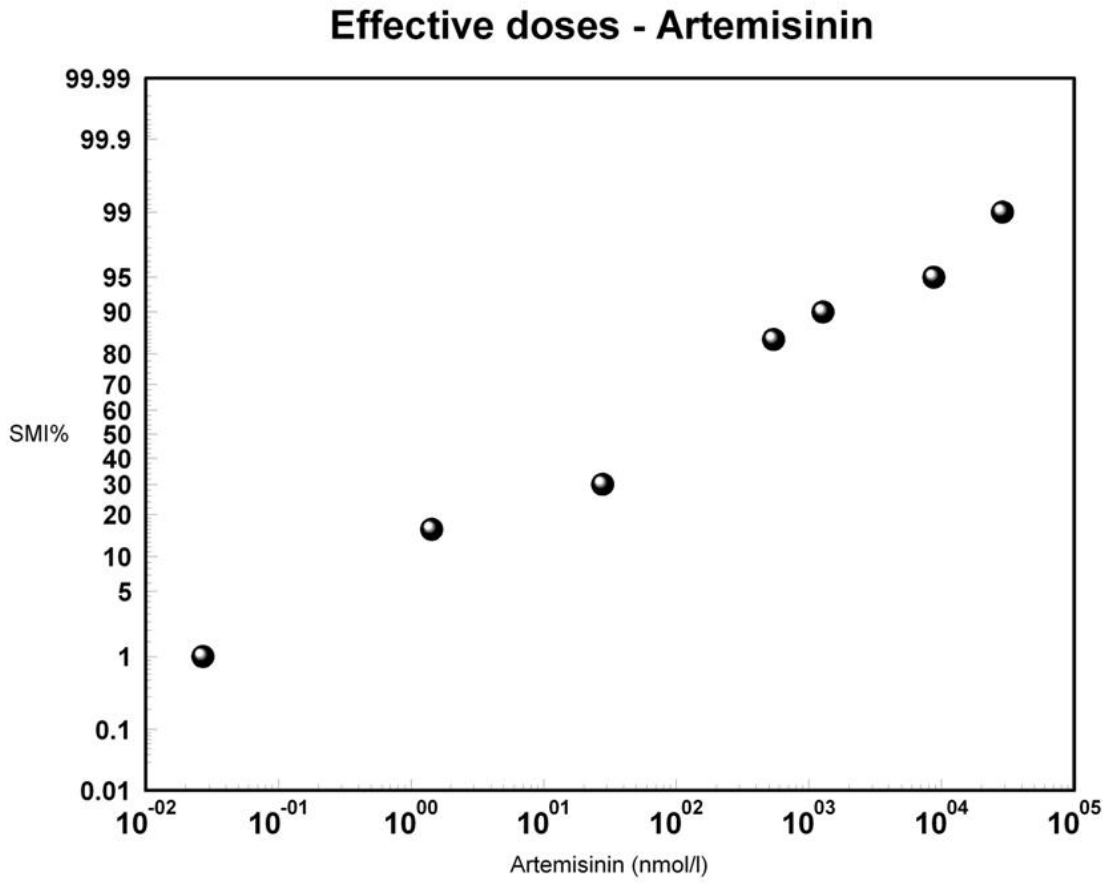

Figure 5.2: Effective doses of Artemisinin

5.1.2 Activity of Eury 1

Out of the 36 isolates, 29 (80,5%) have an adequate maturation of schizonts (more than 10% schizonts in well A).

The arithmetic mean schizont concentration in well A per 200 asexual parasites was 40,27%

The relative schizont maturation inhibition (SMI%) for Eury 1 at the concentration 3nmol/l is 14,71% and increases constantly up to the concentration 3000nmol/l with 81,05% (shown in Ta. 5.4), but never reaches 100% the total maturation inhibition.

Table 5.4: Drug concentration (Eury 1) and SMI%

Drug conc.	SMI%
3,0	14,71
10,0	28,53
30,0	38,97
100,0	45,00
300,0	60,75
1000,0	71,55
3000,0	81,05

Table 5.5: Regression parameters of Eury 1*

n = 29	S = 41,6637	f_s = 3,7908
a = 3,7384	A = 9,1621	$f_{EC\text{-}50}$ = 2,0649
b = 0,2666	K = 7	$f_{EC\text{-}90}$ = 6,5403
r = 0,9948	N' = 203	$f_{EC\text{-}95}$ = 10,1272
χ^2 = 0,4504	R = 1000	$f_{EC\text{-}99}$ = 24,8886

*According to Litchfield and Wilcoxon (1949): n=number of isolates, a=intercept of regression, b=slope of regression, r=correlation of regression, χ^2=heterogeneity, S=slope function, A=intermediate term for f_s, K=number of drug concentration, N'= number of data points EC16 to EC84, R=highest/lowest drug concentration tested, f_s=factor of S, f_{EC}=multiplication/division factor for obtaining 95% confidence intervals of EC

Table 5.6: EC values: Eury 1

EC	Mean	95% Confidence Intervals	
		Lower	Higher
EC_1	0,0184	0,0007	0,4592
EC_{16}	2,7233	1,3188	5,6234
EC_{50}	113,4614	54,9467	234,2906
EC_{84}	4727,2259	2289,2838	9761,4216
EC_{90}	13874,2892	2121,3590	90741,7865
EC_{95}	54190,5172	5350,9626	548800,7233
EC_{99}	697799,1449	28036,9133	17367234,4752

The regression analysis of the inhibition of schizont maturation through Eury 1 is also represented graphically in Fig. 5.3, as well as the effective dose in Fig. 5.4.

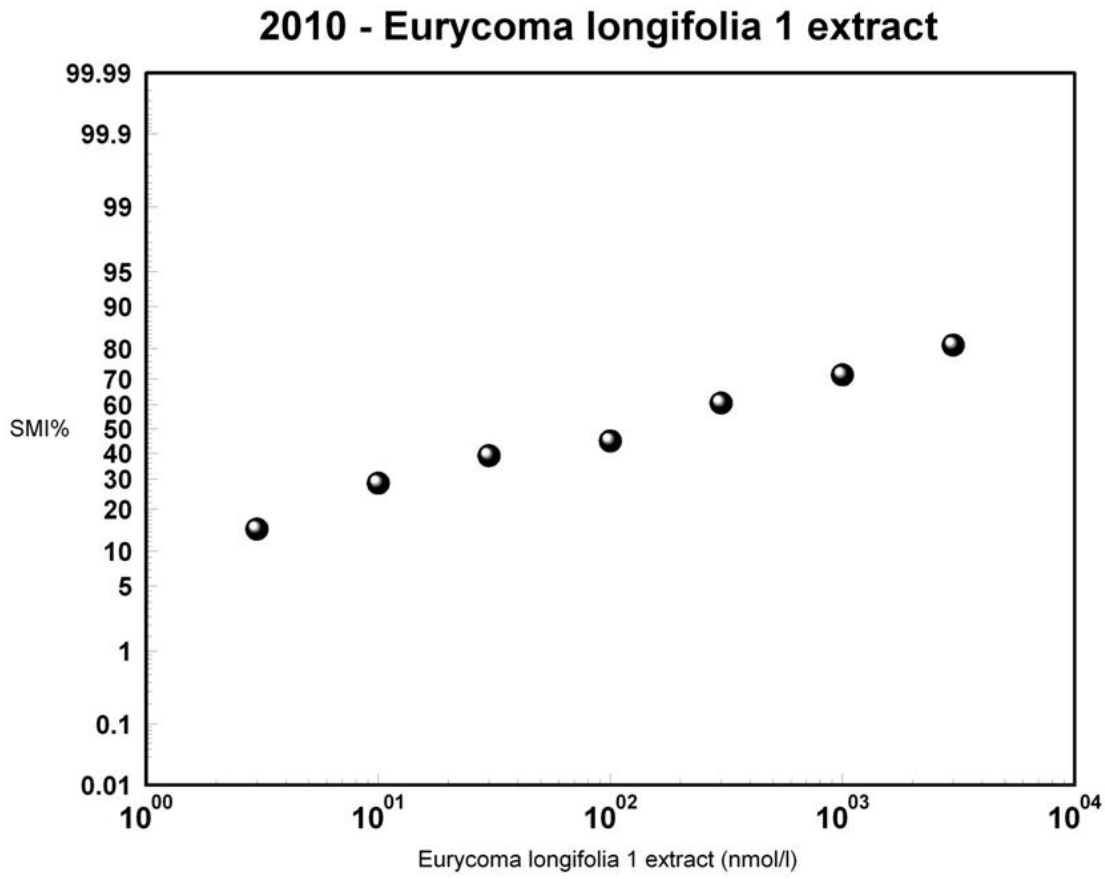

Figure 5.3: Regression analysis of *E. longifolia* 1 extract

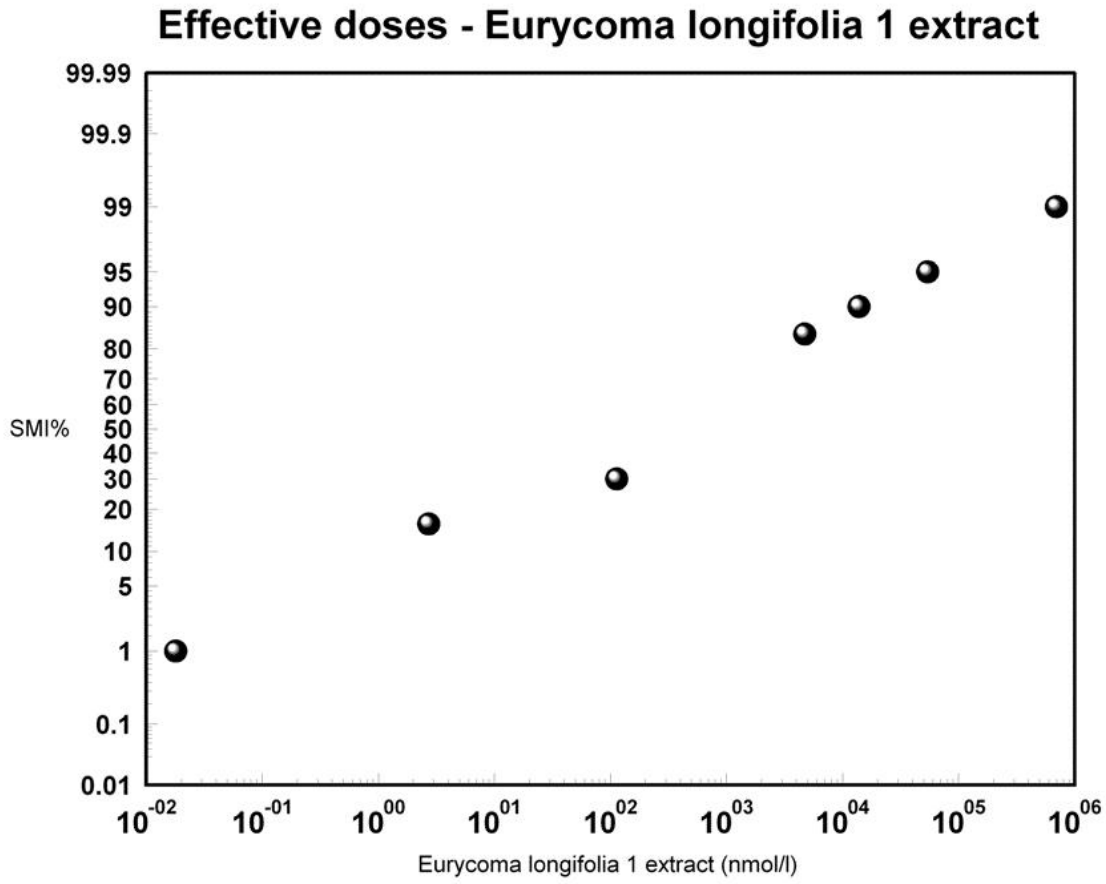

Figure 5.4: Effective doses of *E. longifolia* 1 extract

5.1.3 Activity of Eury 2

Out of the 36 isolates, 29 (80,5%) have an adequate maturation of schizonts (more than 10% schizonts in well A).

The arithmetic mean schizont concentration in well A per 200 asexual parasites was 43,10%

The relative schizont maturation inhibition (SMI%) for Eury 2 at the concentration 3nmol/l is 14,47% and increases constantly up to the concentration 3000nmol/l with 82,02% (shown in Tab. 5.7), but never reaches 100% the total maturation inhibition.

Table 5.7: Drug concentration (Eury 2) and SMI%

Drug conc.	SMI%
3,0	14,47
10,0	24,43
30,0	38,20
100,0	50,33
300,0	66,68
1000,0	75,63
3000,0	82,02

Table 5.8: Regression parameters of Eury 2*

n = 29	S = 30,0079	f_s = 3,7116
a = 3,6628	A = 6,3117	$f_{EC\text{-}50}$ = 2,1868
b = 0,2924	K = 7	$f_{EC\text{-}90}$ = 6,5266
r = 0,9964	N' = 145	$f_{EC\text{-}95}$ = 9,9845
χ^2 = 0,3539	R = 1000	$f_{EC\text{-}99}$ = 24,0399

*According to Litchfield and Wilcoxon (1949): n=number of isolates, a=intercept of regression, b=slope of regression, r=correlation of regression, χ^2=heterogeneity, S=slope function, A=intermediate term for f_s, K=number of drug concentration, N'= number of data points EC16 to EC84, R=highest/lowest drug concentration tested, f_s=factor of S, f_{EC}=multiplication/division factor for obtaining 95% confidence intervals of EC

Table 5.9: EC values: Eury 2

EC	Mean	95% Confidence Intervals	
		Lower	Higher
EC_1	0,0340	0,0014	0,8162
EC_{16}	3,2294	1,4767	7,0621
EC_{50}	96,9067	44,3135	211,9197
EC_{84}	2907,9698	1329,7575	6359,2711
EC_{90}	7763,3631	1189,5007	50668,1559
EC_{95}	26896,6276	2693,8322	268549,9745
EC_{99}	276600,7487	11505,9012	6649455,1797

The regression analysis of the inhibition of schizont maturation through Eury 2 is also represented graphically in Fig. 5.5, as well as the effective dose in Fig. 5.6.

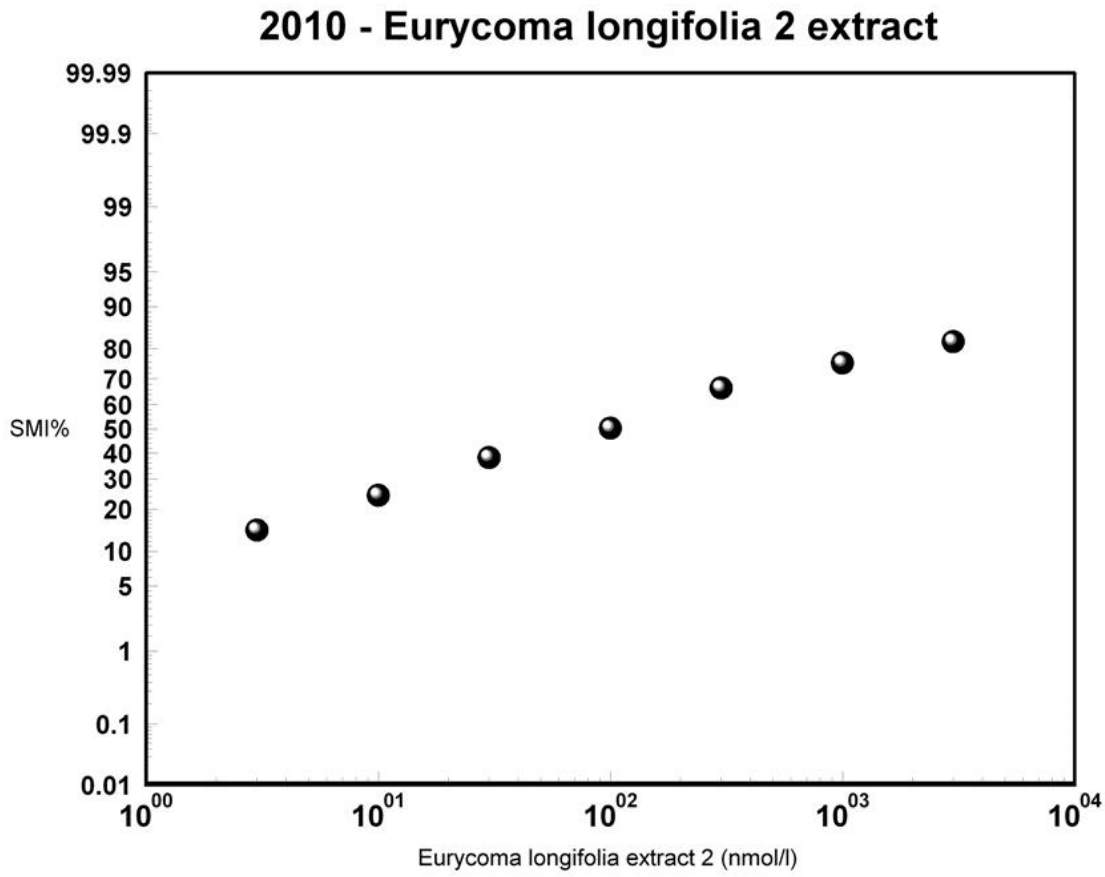

Figure 5.5: Regression analysis of *E. longifolia* 2 extract

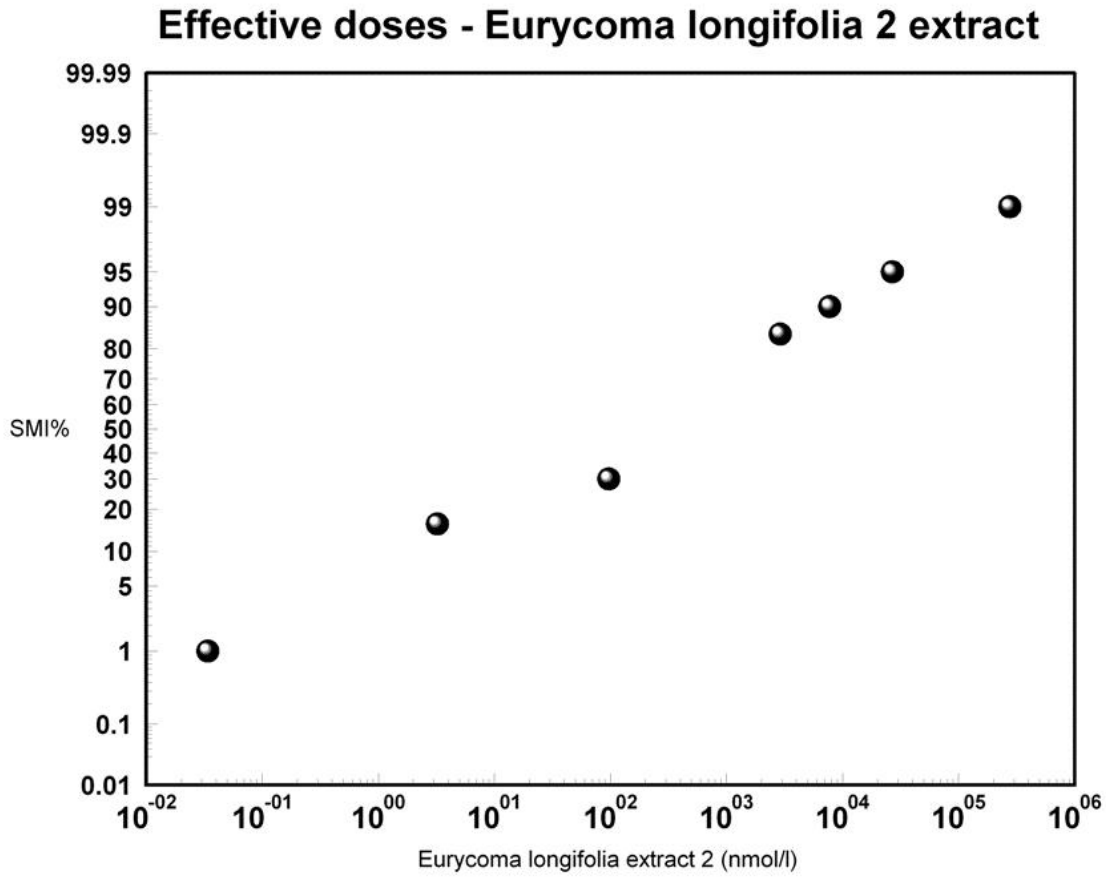

Figure 5.6: Effective doses of *E. longifolia* 2 extract

5.1.4 Activity of Eury 3

Out of the 36 isolates, 29 (80,5%) have an adequate maturation of schizonts (more than 10% schizonts in well A).

The arithmetic mean schizont concentration in well A per 200 asexual parasites was 40,48%.

The relative schizont maturation inhibition (SMI%) for Eury 3 at the concentration 3nmol/l is 12,38% and increases constantly up to the concentration 3000nmol/l with 85,31% (shown in Tab. 5.10), but never reaches 100% the total maturation inhibition.

Table 5.10: Drug concentration (Eury 3) and SMI%

Drug conc.	SMI%
3,0	12,38
10,0	23,92
30,0	34,17
100,0	44,86
300,0	67,12
1000,0	78,23
3000,0	85,31

Table 5.11: Regression parameters of Eury 3*

n = 29	S = 21,4589	f_s = 2,9027
a = 3,4988	A = 4,4685	$f_{EC\text{-}50}$ = 2,0245
b = 0,3243	K = 7	$f_{EC\text{-}90}$ = 4,7329
r = 0,9958	N' = 145	$f_{EC\text{-}95}$ = 6,6492
χ^2 = 0,5121	R = 1000	$f_{EC\text{-}99}$ = 13,4864

*According to Litchfield and Wilcoxon (1949): n=number of isolates, a=intercept of regression, b=slope of regression, r=correlation of regression, χ^2=heterogeneity, S=slope function, A=intermediate term for f_s, K=number of drug concentration, N'= number of data points EC16 to EC84, R=highest/lowest drug concentration tested, f_s=factor of S, f_{EC}=multiplication/division factor for obtaining 95% confidence intervals of EC

Table 5.12: EC values: Eury 3

EC	Mean	95% Confidence Intervals	
		Lower	Higher
EC_1	0,0786	0,0058	1,0597
EC_{16}	4,7701	2,3562	9,6570
EC_{50}	102,3605	50,5609	207,2288
EC_{84}	2196,5473	1084,9825	4446,9105
EC_{90}	5323,0386	1124,6793	25193,6182
EC_{95}	16315,8003	2453,8079	108486,6247
EC_{99}	133347,3393	9887,5680	1798370,7250

The regression analysis of the inhibition of schizont maturation through Eury 3 is also represented graphically in Fig. 5.7, as well as the effective dose in Fig. 5.8.

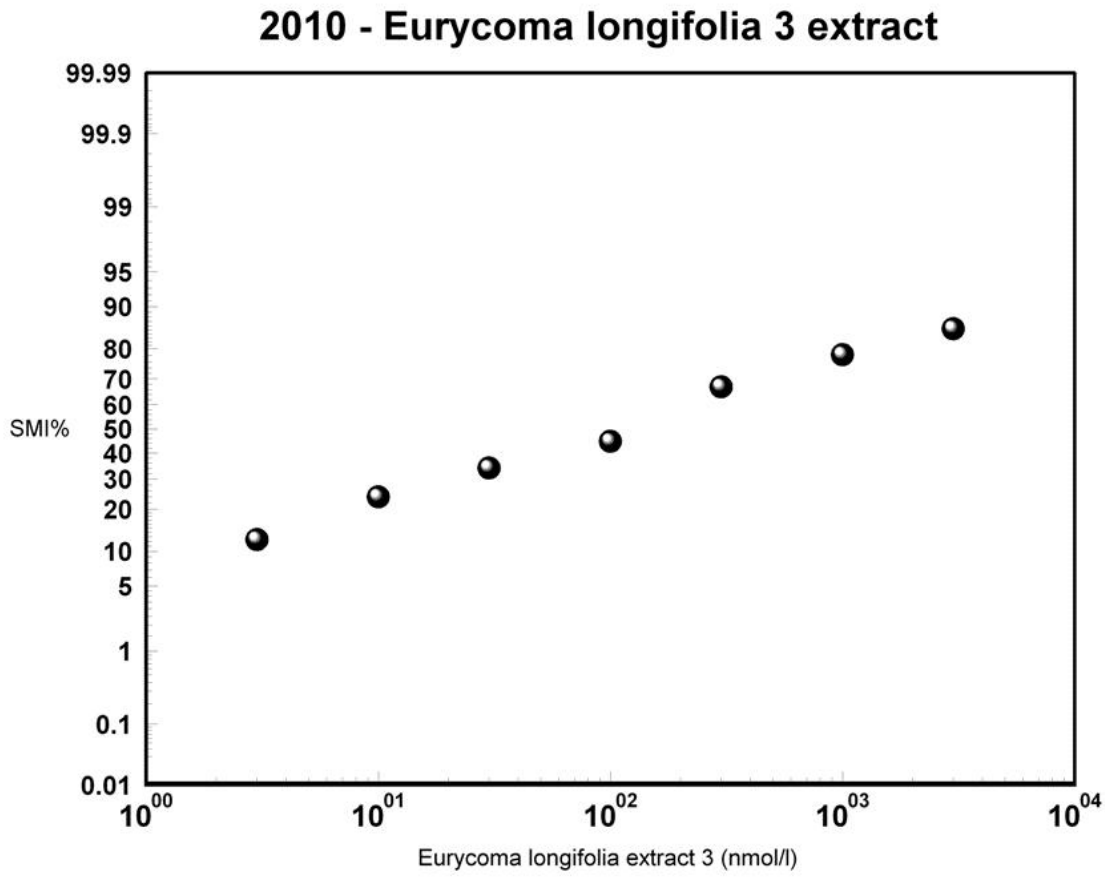

Figure 5.7: Regression analysis of *E. longifolia* 3 extract

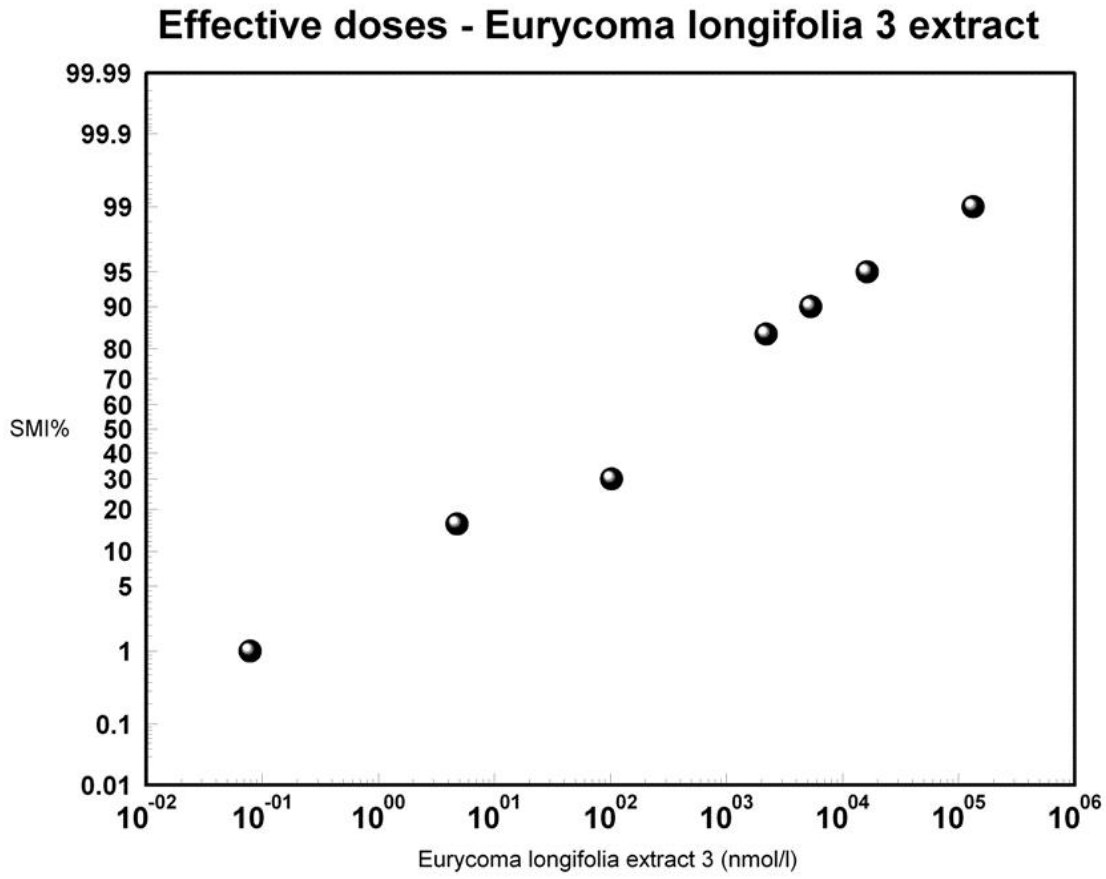

Figure 5.8: Effective doses of *E. longifolia* 3 extract

5.1.5 Activity of Eury 1 plus Artemisinin

Artemisinin was combined with Eury 1 (1:1) in order to test the interaction of these drugs in vitro.

Out of the 36 isolates, 29 (80,5%) have an adequate maturation of schizonts (more than 10% schizonts in well A).

The arithmetic mean schizont concentration in well A per 200 asexual parasites was 35,38%.

The relative schizont maturation inhibition (SMI%) for Eury 1 plus Artemisinin at the concentration 3nmol/l is 18,96% and increases constantly up to the concentration 3000nmol/l with 92,35% (shown in Tab. 5.13), but never reaches 100% the total maturation inhibition.

Table 5.13: Drug concentration (Eury 1 plus Artemisinin) and SMI%

Drug conc.	SMI%
3,0	18,96
10,0	30,97
30,0	44,08
100,0	52,66
300,0	70,93
1000,0	84,30
3000,0	92,34

Table 5.14: Regression parameters of Eury 1 plus Artemisinin*

n = 29	S = 21,3587	f_s = 2,6375
a = 3,7245	A = 4,4482	$f_{EC\text{-}50}$ = 1,9020
b = 0,3248	K = 7	$f_{EC\text{-}90}$ = 4,1174
r = 0,9948	N' = 174	$f_{EC\text{-}95}$ = 5,6098
χ^2 = 0,6092	R = 1000	$f_{EC\text{-}99}$ = 10,6762

*According to Litchfield and Wilcoxon (1949): n=number of isolates, a=intercept of regression, b=slope of regression, r=correlation of regression, χ^2=heterogeneity, S=slope function, A=intermediate term for f_s, K=number of drug concentration, N'= number of data points EC16 to EC84, R=highest/lowest drug concentration tested, f_s=factor of S, f_{EC}=multiplication/division factor for obtaining 95% confidence intervals of EC

Table 5.15: EC values: Eury 1 plus Artemisinin

EC	Mean	95% Confidence Intervals	
		Lower	Higher
EC_1	0,0394	0,0037	0,4203
EC_{16}	2,3749	1,2486	4,5169
EC_{50}	50,7239	26,6693	96,4749
EC_{84}	1083,3973	569,6211	2060,5798
EC_{90}	2621,9225	636,7947	10795,4374
EC_{95}	8022,7988	1430,1383	45006,3466
EC_{99}	65359,5184	6121,9852	697791,0812

The regression analysis of the inhibition of schizont maturation through Eury 1 plus Artemisinin is also represented graphically in Fig. 5.9, as well as the effective dose in Fig. 5.10.

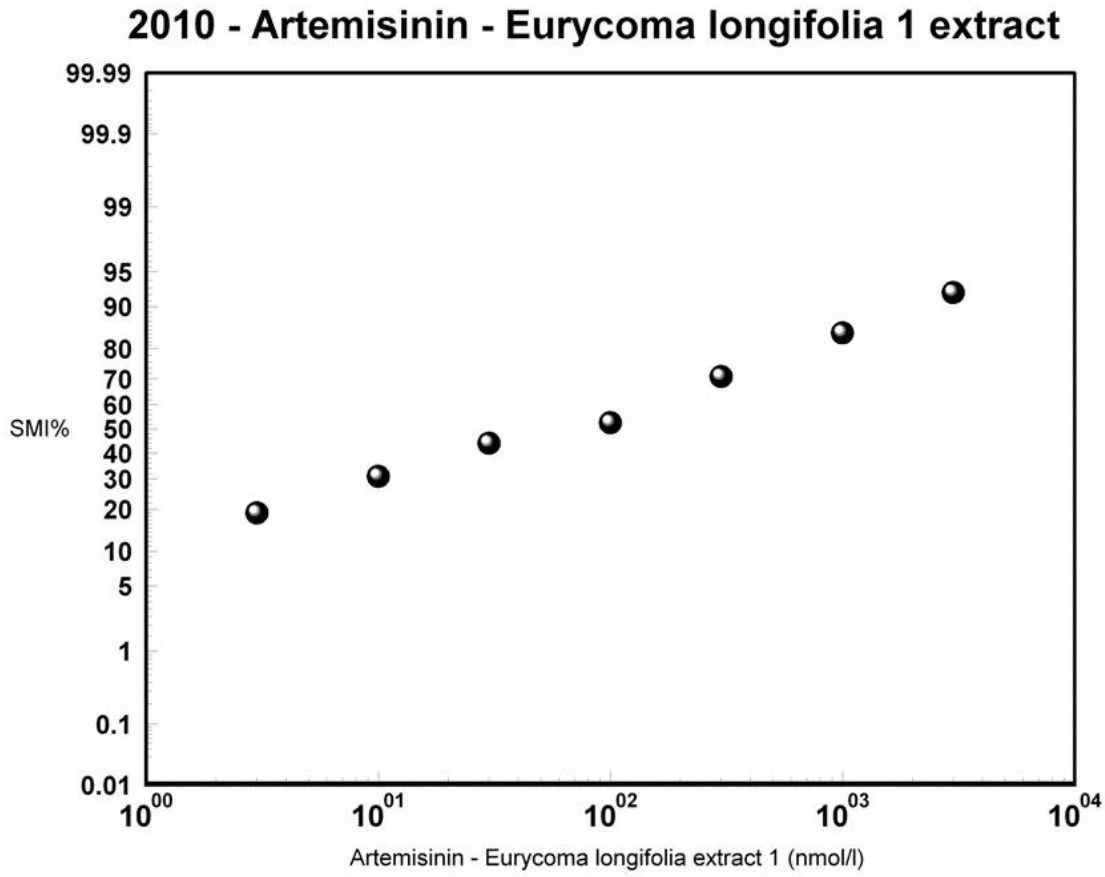

Figure 5.9: Regression analysis of Artemisinin plus *E. longifolia* 1 extract

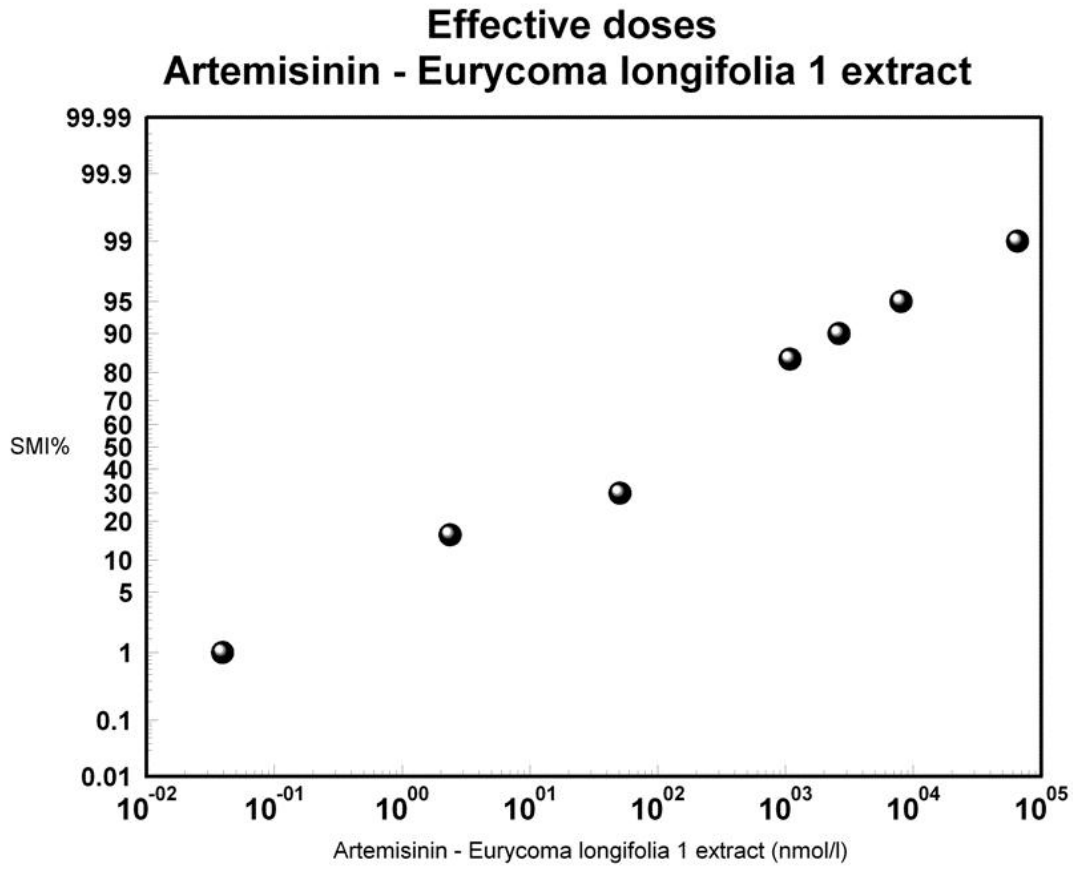

Figure 5.10: Effective doses of Artemisinin plus *E. longifolia* 1 extract

5.1.6 Activity of Eury 2 plus Artemisinin

Artemisinin was combined with Eury 2 (1:1) in order to test the interaction of these drugs in vitro.

Out of the 36 isolates, 29 (80,5%) have an adequate maturation of schizonts (more than 10% schizonts in well A).

The arithmetic mean schizont concentration in well A per 200 asexual parasites was 34,66%.

The relative schizont maturation inhibition (SMI%) for Eury 1 plus Artemisinin at the concentration 3nmol/l is 18,28% and increases constantly up to the concentration 3000nmol/l with 90,90% (shown in Tab. 5.16), but never reaches 100% the total maturation inhibition.

Table 5.16: Drug concentration (Eury 2 plus Artemisinin) and SMI%

Drug conc.	SMI%
3,0	18,28
10,0	33,58
30,0	48,80
100,0	65,48
300,0	79,03
1000,0	85,48
3000,0	90,90

Table 5.17: Regression parameters of Eury 2 plus Artemisinin*

n = 29	S = 19,9536	f_s = 2,7612
a = 3,8091	A = 4,1657	$f_{EC\text{-}50}$ = 1,9909
b = 0,3322	K = 7	$f_{EC\text{-}90}$ = 4,4333
r = 0,9951	N' = 145	$f_{EC\text{-}95}$ = 6,1216
χ^2 = 0,5378	R = 1000	$f_{EC\text{-}99}$ = 11,9915

*According to Litchfield and Wilcoxon (1949): n=number of isolates, a=intercept of regression, b=slope of regression, r=correlation of regression, χ^2=heterogeneity, S=slope function, A=intermediate term for f_s, K=number of drug concentration, N'= number of data points EC16 to EC84, R=highest/lowest drug concentration tested, f_s=factor of S, f_{EC}=multiplication/division factor for obtaining 95% confidence intervals of EC

Table 5.18: EC values: Eury 2 plus Artemisinin

EC	Mean	95% Confidence Intervals	
		Lower	Higher
EC_1	0,0328	0,0027	0,3932
EC_{16}	1,8059	0,9071	3,5954
EC_{50}	36,0345	18,0995	71,7415
EC_{84}	719,0197	361,1511	1431,5041
EC_{90}	1706,2443	384,8663	7564,3674
EC_{95}	5092,7366	831,9261	31175,8037
EC_{99}	39599,0921	3302,2740	474850,9971

The regression analysis of the inhibition of schizont maturation through Eury 2 plus Artemisinin is also represented graphically in Fig. 5.11, as well as the effective dose in Fig. 5.12.

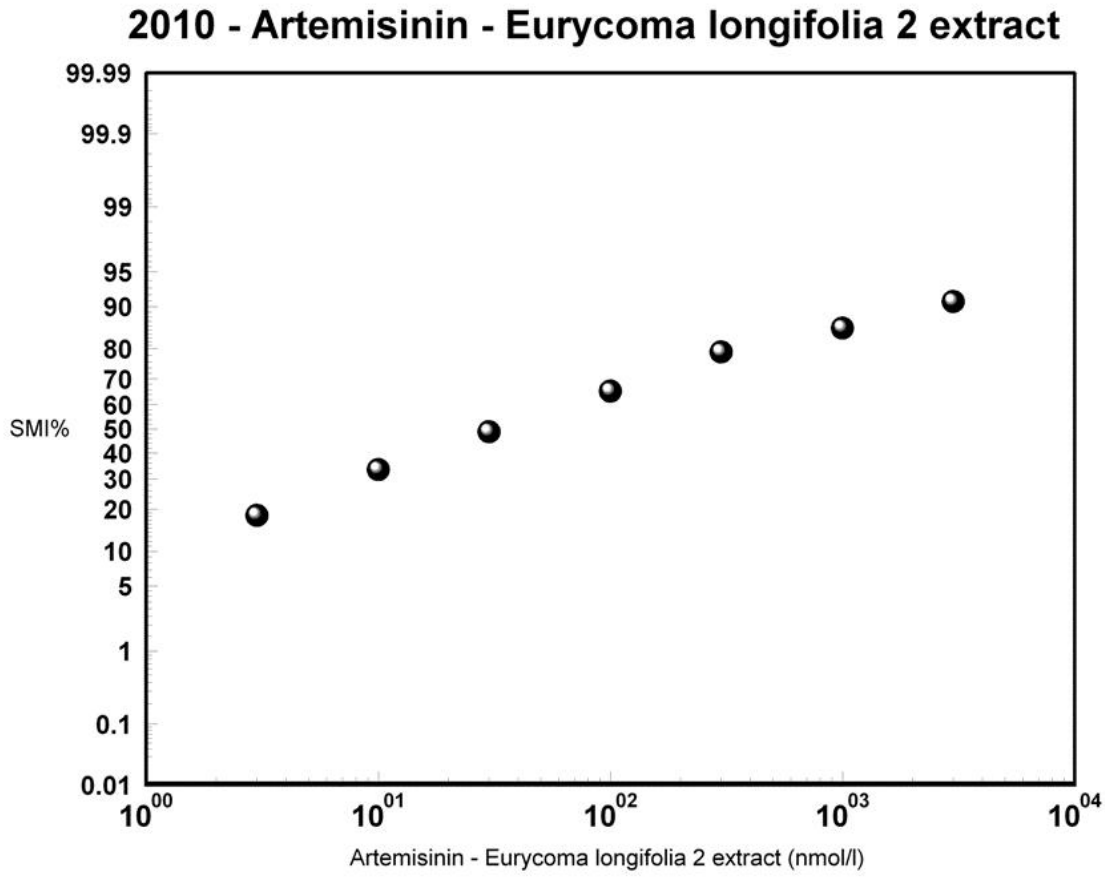

Figure 5.11: Regression analysis of Artemisinin plus *E. longifolia* 2 extract

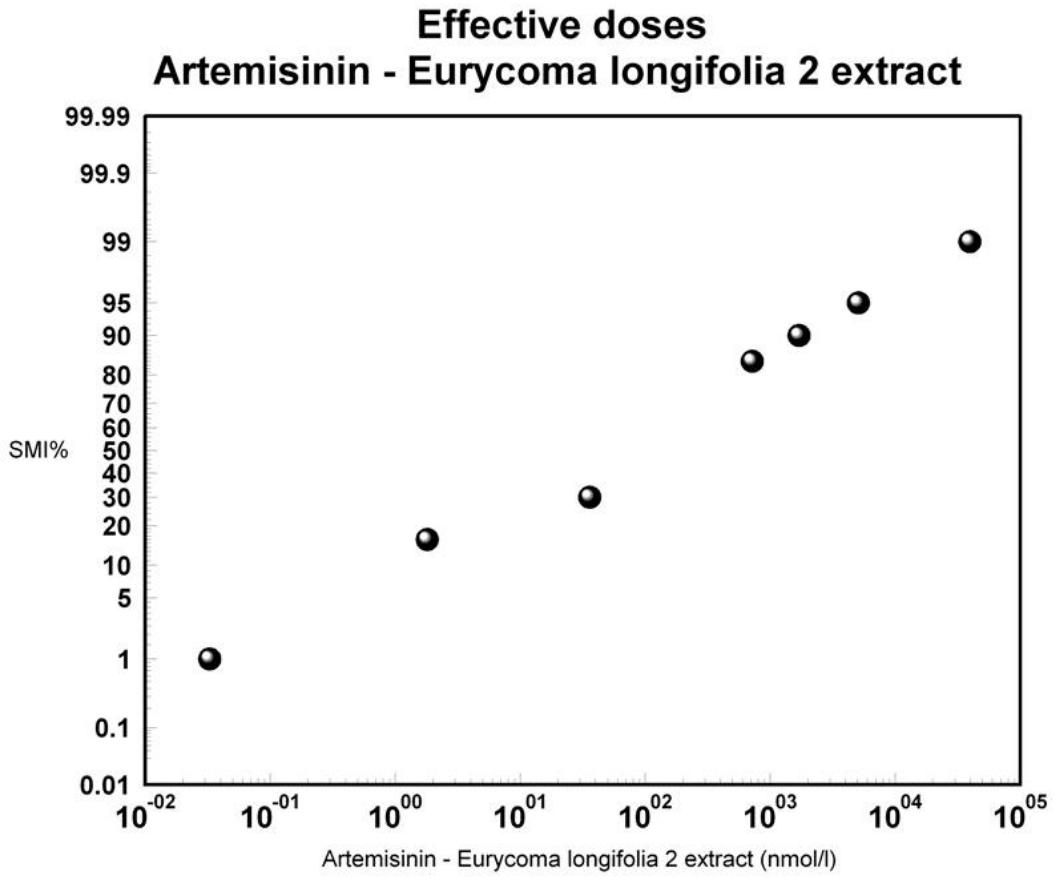

Figure 5.12: Effective doses of Artemisinin plus *E. longifolia* 2 extract

5.1.7 Activity of Eury 3 plus Artemisinin

Artemisinin was combined with Eury 3 (1:1) in order to test the interaction of these drugs in vitro.

Out of the 36 isolates, 29 (80,5%) have an adequate maturation of schizonts (more than 10% schizonts in well A).

The arithmetic mean schizont concentration in well A per 200 asexual parasites was 37,79%.

The relative schizont maturation inhibition (SMI%) for Eury 1 plus Artemisinin at the concentration 3nmol/l is 18,69% and increases constantly up to the concentration 3000nmol/l with 93,03% (shown in Tab. 5.19), but never reaches 100% the total maturation inhibition.

Table 5.19: Drug concentration (Eury 3 plus Artemisinin) and SMI%

Drug conc.	SMI%
3,0	18,69
10,0	34,08
30,0	48,78
100,0	60,37
300,0	79,68
1000,0	87,84
3000,0	93,03

Table 5.20: Regression parameters of Eury 3 plus Artemisinin*

n = 29	S = 17,4134	f_s = 2,5228
a = 3,7563	A = 3,6694	$f_{EC\text{-}50}$ = 1,9295
b = 0,3481	K = 7	$f_{EC\text{-}90}$ = 3,9386
r = 0,9968	N' = 145	$f_{EC\text{-}95}$ = 5,2716
χ^2 = 0,3670	R = 1000	$f_{EC\text{-}99}$ = 9,6968

*According to Litchfield and Wilcoxon (1949): n=number of isolates, a=intercept of regression, b=slope of regression, r=correlation of regression, χ^2=heterogeneity, S=slope function, A=intermediate term for f_s, K=number of drug concentration, N'= number of data points EC16 to EC84, R=highest/lowest drug concentration tested, f_s=factor of S, f_{EC}=multiplication/division factor for obtaining 95% confidence intervals of EC

Table 5.21: EC values: Eury 3 plus Artemisinin

EC	Mean	95% Confidence Intervals	
		Lower	Higher
EC_1	0,0446	0,0046	0,4323
EC_{16}	2,0460	1,0604	3,9477
EC_{50}	35,6274	18,4645	68,7437
EC_{84}	620,3960	321,5296	1197,0632
EC_{90}	1415,4586	359,3776	5574,9797
EC_{95}	4019,7928	762,5400	21190,6697
EC_{99}	28472,0333	2936,2345	276087,1714

The regression analysis of the inhibition of schizont maturation through Eury 3 plus Artemisinin is also represented graphically in Fig. 5.13, as well as the effective dose in Fig. 5.14.

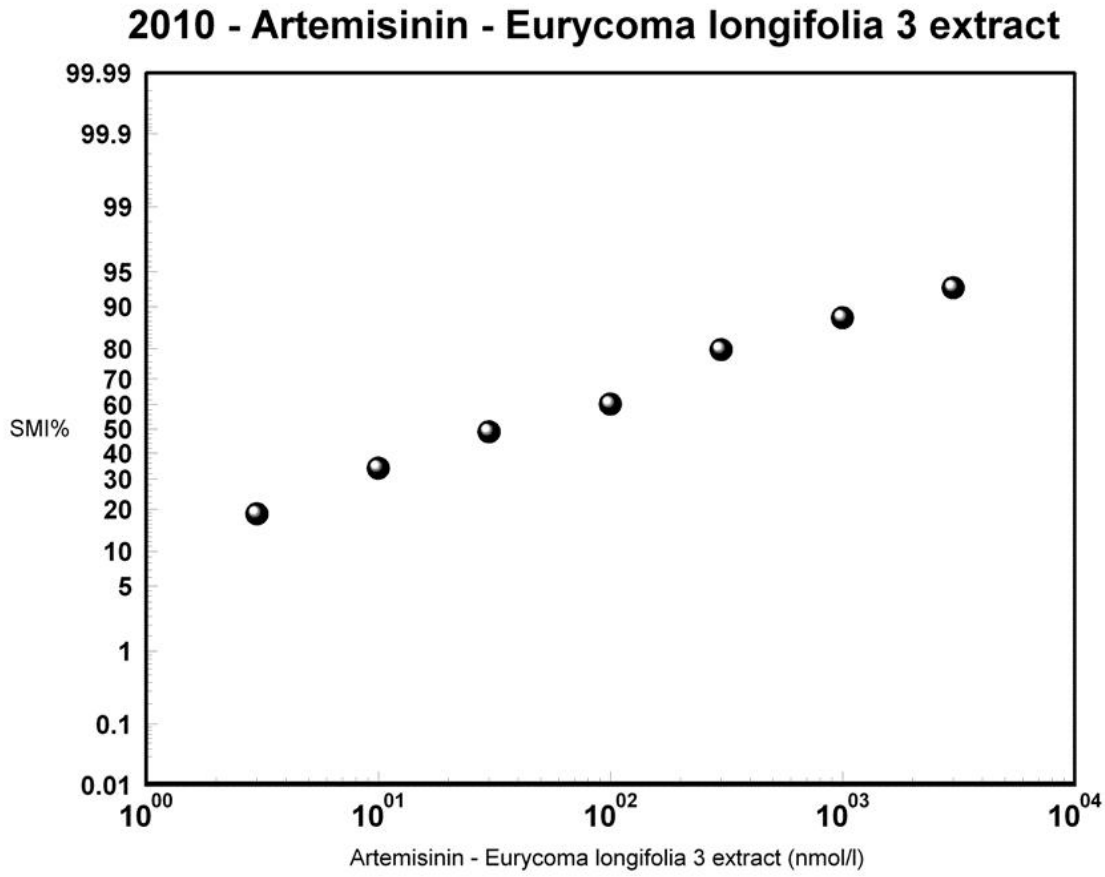

Figure 5.13: Regression analysis of Artemisinin plus *E. longifolia* 3 extract

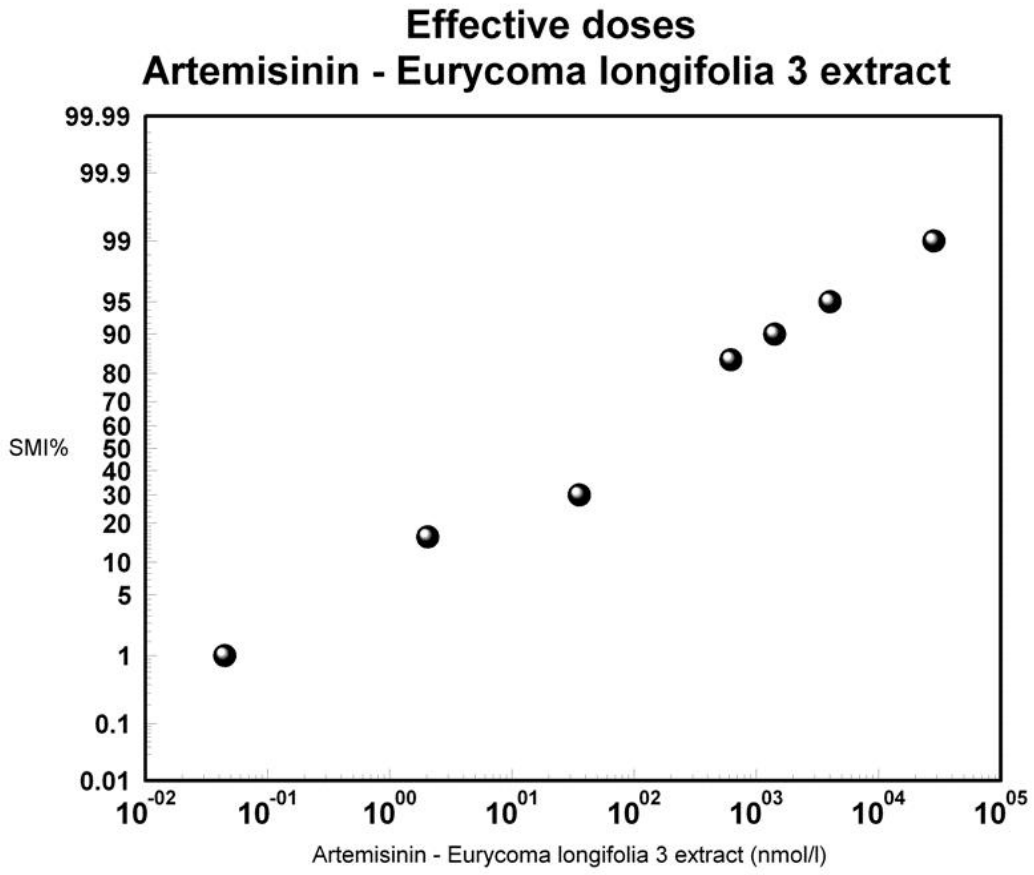

Figure 5.14: Effective doses of Artemisinin plus *E. longifolia* 3 extract

6 Discussion and Conclusion

6.1 Activity and Regression Analysis

6.1.1 Artemisinin

The evaluation of the activity of Artemisinin shows for the test isolates an EC_{50} of 27,8586 nmol/l, an EC_{90} of 1277,0471 nmol/l and an EC_{99} of 28865,3737 nmol/l. Compared with data from 2009 (Kerschbaumer et al., 2010), shown in table 6.1, there is a big increase in these values. This leads to the assumption that there is a decrease in effectiveness of Artemisinin in this area.

Table 6.1: Comparison of EC values 2009, 2010

Art	**2009**	**2010**
EC_{50}	8,1181	27,8586
EC_{90}	137,2319	1277,0471
EC_{99}	1375,6520	28865,3737

The regression lines of Artemisinin from 2009 and 2010, shown in table 6.2 are compared for parallelism and activity differences. The slope ratio of the regression lines, shown in table 6.3 can be considered as parallel, since f_{SR} >SR. Therefore the two could be compared for an efficacy difference, shown as well in table 6.3.

Table 6.2: Regression parameters of Artemisinin 2009/2010

Art	S	f_s
2009	8,9733	1,7836
2010	19,4581	2,7147

S = slope function, f_s = factor of S

Table 6.3: Comparison of regression parameters Artemisinin 2009/2010

Art 2009/ 2010	SR	f_{SR}	Slope of regression	PR	f_{PR}	
	2,1684	3,1714	parallel			
EC_{50}				3,4321	2,3810	significant
EC_{90}				9,3057	5,6588	significant
EC_{99}				20,9830	17,2484	significant

SR = slope ration, f_{SR} = factor of SR, PR = potency ratio, f_{PR} = factor of PR

The comparison of regression parameters confirm the assumption above that there is a loss of Artemisinin sensitivity between 2009 and 2010. In all three EC values (50, 90, 99) the potency ratio (PR) >factor of PR (f_{PR}) at the 95% confidence level.

6.2 Variance analysis of resulting data

6.2.1 Analysis for Interaction between Artemisinin and Eurycoma 1, 2, 3 in *P. falciparum*

Variance analysis was applied since the mixture of Artemisinin and the parallel administered Eurycomanons 1 and 2 charges resulted in *P. falciparum* always in a significantly lower antiplasmodial activity as compared to freshly prepared 0,5 Artemisinin and Eury 1 and 2 charges. This indicates that the Artemisinin+Eury charge prepared at the Institute of Specific Prophylaxis and Tropical Medicine, Medical University of Vienna has lost part of its activity due to long contact between Artemisinin and the Eurycomanone charge. With regard to Eurycomanon 3 it was probably an incompatibility between Artemisinin and the Eurycomanon. At the same time, the comparison between the two formulations indicated in freshly prepared Artemisinin-Eury-1 and Artemisinin-Eury-2 synergism at the EC_{50} and EC_{90} in *P. falciparum*.

Eury-1 - Art at EC_{50}

Result: 6,0993 $\longrightarrow$ significant synergism
Degree of Freedom 54 (=2,6908 at p=0,01)

Eury-2 - Art at EC_{50}

Result: 19,0742 $\longrightarrow$ significant synergism
Degree of Freedom 56 (= 2,6952 at p=0,01)

Eury-3 - Art at EC_{50}

Result: Inhibition under Eury-3 - Art higher than under separate compounds

Eury-1 - Art at EC_{90}

Result: 11,3260 $\longrightarrow$ significant synergism
Degree of Freedom 58 (=2,6864 at p=0,01)

Eury-2 - Art at EC_{90}

Result: 9,2170 $\longrightarrow$ significant synergism
Degree of Freedom 58 (=2,6952 at p=0,01)

Eury-3 - Art at EC_{90}

Result: Inhibition under Eury-3 - Art higher than under separate compounds

In *P. falciparum* the isolates showed in Eury-1-Art and Eury-2-Art a significant synergism, albeit at indices strongly reduced from the experience in *P. vivax*. This was obviously due to the reduced activity of Artemisinin.

Follow up on these observations it would be reasonable to investigate potential advantages of Eurycomanones together with Artemisinin in areas with Artemisinin-sensitive *P. falciparum* strains.

7 Annex

Table 7.1: Patient record form Mae Sot 2010, *P. falciparum*

DATE	PAT. NO.	PAT. REG. MC	SEX	AGE	OCCUPAT.	RESIDENCE COUNTRY	ORIGIN OF INFECTION COUNTRY	NO. DAYS SYMPT. BEFORE SEEK. TREATM.	COMMENTS
28.06.2010	1	68	M	22	Labour	Myanmar	Myanmar	2 d. Fever	
28.06.2010	2	71	F	35	Housewife	Myanmar	Myanmar	3 d. Fever	
29.06.2010	3	39	M	26	Labour	Thailand	Thailand	2 d. Fever	
30.06.2010	4	91	M	25	Farmer	Thailand	Thailand	9 d. Fever	
30.06.2010	5	93	F	30	Farmer	Myanmar	Myanmar	6 d. Fever	
01.07.2010	6	104	M	23	Farmer	Thailand	Thailand	1 d. Fever	
02.07.2010	7	116	F	48	Farmer	Myanmar	Myanmar	4 d. Fever	
02.07.2010	8	120	F	13	Student	Myanmar	Myanmar	3 d. Fever	
02.07.2010	9	126	F	47	Farmer	Myanmar	Myanmar	2 d. Fever	
02.07.2010	10	130	M	18	Farmer	Myanmar	Myanmar	2 d. Fever	
06.07.2010	11	84	M	16	Worker	Thailand	Thailand	6 d. Fever	
06.07.2010	12	171	M	16	Farmer	Thailand	Thailand	2 d. Fever	
07.07.2010	13	177	M	37	Farmer	Thailand	Thailand	3 d. Fever	
08.07.2010	14	191	M	41	Farmer	Thailand	Thailand	3 d. Fever	
09.07.2010	15	212	M	15	Student	Thailand	Thailand	1 d. Fever	
12.07.2010	16	227	M	25	Farmer	Thailand	Thailand	3 d. Fever	

Table 7.2: Patient record form Mae Sot 2010, *P. falciparum*

DATE	PAT. NO.	PAT. REG. MC	SEX	AGE	OCCUPAT.	RESIDENCE COUN-TRY	ORIGIN OF IN-FEC-TION COUN-TRY	NO. DAYS SYMPT. BEFORE SEEK. TREATM.	COMMENTS
14.07.2010	17	247	M	11	Monk	Myanmar	Myanmar	2 d. Fever	venous blood
14.07.2010	18	124	M	57	Farmer	Thailand	Thailand	5 d. Fever	
14.07.2010	19	251	F	42	Farmer	Myanmar	Myanmar	2 d. Fever	
15.07.2010	20	268	F	18	Worker	Thailand	Myanmar	2 d. Fever	venous blood
15.07.2010	21	269	M	24	Worker	Thailand	Myanmar	2 d. Fever	venous blood
16.07.2010	22	272	F	12	Student	Thailand	Myanmar	1 d. Fever	
19.07.2010	23	283	M	47	Farmer	Myanmar	Myanmar	14 d. Fever	
19.07.2010	24	152	M	45	Labour	Thailand	Myanmar	2 d. Fever	
19.07.2010	25	157	F	26	Worker	Thailand	Thailand	8 d. Fever	
20.07.2010	26	163	M	36	Labour	Thailand	Thailand	3 d. Fever	
20.07.2010	27	165	M	39	Farmer	Myanmar	Myanmar	3 d. Fever	
23.07.2010	28	31	F	32	Worker	Myanmar	Myanmar	3 d. Fever	mixed form?
28.07.2010	29	49	F	28	Worker	Thailand	Myanmar	20 d. Fever	
29.07.2010	30	62	M	18	Labour	Myanmar	Myanmar	2 d. Fever	
30.07.2010	31	56	M	47	Labour	Thailand	Thailand	6 d. Fever	
30.07.2010	32	76	M	39	Farmer	Thailand	Thailand	3 d. Fever	

Table 7.3: Patient record form Mae Sot 2010, *P. falciparum*

DATE	PAT. NO.	PAT. REG. MC	SEX	AGE	OCCUPAT.	RESIDENCE COUN-TRY	ORIGIN OF IN-FEC-TION COUN-TRY	NO. DAYS SYMPT. BEFORE SEEK. TREATM.	COMMENTS
03.08.2010	33	199	M	39	Farmer	Thailand	Myanmar	7 d. Fever	venous blood
05.08.2010	34	112	F	38	Worker	Myanmar	Myanmar	5 d. Fever	
06.08.2010	35	136	M	23	Farmer	Thailand	Thailand	3 d. Fever	
09.08.2010	36	147	M	46	Labour	Thailand	Thailand	2 d. Fever	

Table 7.4: Summary sheet Mae Sot 2010 *P. falciparum*

Isolate no.	As Par / ţl	% M+ troph	Well A Schiz/200	Result	Comments
1	14518	12 5	20	Valid	WHW
2	34225	20%	35	Valid	Good
3	8889	30%	32	Valid	WHW
4	9250	28%	61	Valid	Good
5	83429	59%	20	Valid	WHW
6	99999	35%	70	Valid	Good
7	69333	23%	75	Valid	Good
8	15059	10%	washed out	Invalid	OUT
9	29818	17%	20	Valid	WHW
10	44444	32%	97	Valid	Good
11	21053	15%	36	Valid	Good
12	17200	27%	20	Valid	WHW
13	1300	62%	23	Valid	WHW
14	19750	17%	3	Invalid	OUT
15	26233	20%	20	Valid	WHW
16	74812	15%	20	Valid	WHW
17	38400	35%	20	Valid	WHW
18	2576	40%	96	Valid	Good
19	9697	20%	53	Valid	Good
20	18963	25%	<4	Invalid	OUT
21	2791	10%	20	Valid	WHW
22	87999	10%	28	Valid	WHW
23	26371	20%	44	Valid	Good
24	42250	25%	51	Valid	Good
25	6154	25%	47	Valid	Good
26	49846	30%	23	Valid	Good
27	61429	20%	40	Valid	Good
28	6323	25%	62	Valid	WHW
29	2196	30%	56	Valid	WHW
30	30737	15%	<20	Invalid	OUT
31	17561	25%	20	Valid	WHW
32	22400	20%	57	Valid	WHW
33	9085	20%	59	Valid	WHW
34	2222	20%	63	Valid	WHW
35	32400	25%	<20	Invalid	OUT
36	2727	25%	55	Valid	GOOD

Table 7.5: Schizont count Pf 2010 Artemisinin

	A	B	C	D	E	F	G	H
Pat No	Control SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ
	0	3	10	30	100	300	1000	3000
1	100	70,37	51,85	33,33	25,93	14,81	7,41	3,7
2	100	82,5	25	17,5	5	2,5	0	0
3	100	100	86,21	74,14	36,31	17,24	Jan 00	5,17
4	100	81,25	75	39,58	43,75	25	25	2,08
5	100	89,29	80,36	16,07	3,57	1,79	0	0
6	100	93,65	93,65	93,65	57,14	34,92	26,98	15,87
7	100	81,25	81,25	18,75	10,42	4,17	2,08	0
9	100	70	27,5	17,5	10	7,5	5	2,5
10	100	85,11	65,96	29,79	34,04	23,4	19,15	19,15
11	100	75,25	68,75	62,5	37,6	18,75	12,5	3,13
12	100	76,75	58,14	48,84	39,54	23,26	13,95	4,65
13	100	83,96	70,76	49,06	33,96	26,42	20,75	15,09
15	100	59,09	36,36	27,27	22,73	13,64	9,09	0
16	100	67,74	45,16	25,81	9,68	3,23	0	0
17	100	71,88	51,56	32,81	21,88	18,75	15,63	12,5
18	100	94,83	67,24	37,93	24,14	15,52	10,34	3,45
19	100	60	50	40	23,33	23,33	10	0
21	100	90	80	53,33	30	23,33	20	13,33
22	100	100	100	72,33	52,78	22,22	8,33	2,78
23	100	65,79	68,42	52,63	50	52,63	28,95	28,95
24	100	62,07	48,28	31,03	20,69	17,24	17,24	23,79
25	100	79,41	58,82	58,82	50	44,12	23,53	23,53
27	100	78,57	60,71	35,71	10,71	3,57	0	0
28	100	80	66,67	46,67	33,33	16,67	6,67	0
29	100	76,36	58,18	38,18	25,54	9,09	3,64	0
31	100	76,32	57,89	39,47	26,32	18,42	13,16	7,89
32	100	94,44	88,89	59,26	38,89	11,11	3,7	1,85
33	100	94,74	89,47	76,32	65,79	13,16	2,63	0
36	100	70,37	66,67	66,67	66,67	66,67	44,44	33,33

Table 7.6: Schizont count Pf 2010 Eury-1

	A	B	C	D	E	F	G	H
Pat No	Control SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ
	0	3	10	30	100	300	1000	3000
1	100	85,72	71,43	61,9	52,38	42,86	33,33	9,52
2	100	93,33	62,22	60	42,22	40	13,33	6,67
3	100	102,78	102,78	66,57	41,67	25	13,89	5,56
4	100	103,45	84,49	77,59	70,69	65,52	55,17	55,17
5	100	80	64,44	60	53,33	15,56	4,44	2,22
6	100	81,4	84,88	81,4	79,07	75,59	72,09	16,28
7	100	68,25	53,96	38,09	34,92	23,81	22,22	3,17
9	100	81,82	63,64	63,64	63,64	18,18	4,55	0
10	100	80,3	80,3	63,64	68,18	50	36,36	9,09
11	100	91,31	82,61	69,57	60,87	52,17	47,83	39,13
12	100	91,43	62,86	48,57	31,43	25,71	20	14,29
13	100	89,71	80,88	63,24	48,53	44,12	41,18	38,24
15	100	62,86	40	25,71	17,14	11,43	8,57	5,71
16	100	95	90	85	85	55	35	15
17	100	97,87	96,76	72,34	66,32	44,68	36,17	31,92
18	100	121,05	103,05	108,77	105,26	52,63	19,3	7,02
19	100	64,29	42,86	28,57	28,57	14,29	10,71	7,14
21	100	81,54	67,69	55,39	46,16	30,77	21,54	13,85
22	100	83,05	67,8	57,63	50,85	20,34	8,47	3,39
23	100	65	45	40	40	40	40	35
24	100	92,59	86,19	77,78	70,37	66,67	44,44	44,44
25	100	85	70	55	95	45	40	35
27	100	100	100	100	80	64	12	0
28	100	86,21	75,86	62,07	48,28	31,03	17,24	6,9
29	100	71,43	51,43	48,57	45,72	42,86	42,86	37,14
31	100	58,62	34,48	27,59	17,24	10,34	6,9	3,45
32	100	81,58	65,79	57,9	52,83	36,84	26,32	15,79
33	100	77,78	61,11	33,33	19,44	13,89	11,11	8,33
36	100	100	80	80	80	80	80	80

Table 7.7: Schizont count Pf 2010 Eury-2

	A	B	C	D	E	F	G	H
Pat No	Control SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ
	0	3	10	30	100	300	1000	3000
1	100	70	50	35	25	15	10	0
2	100	82,86	85,72	58,57	48,57	32,86	32,86	20
3	100	100	100	64,29	42,86	43,86	42,86	28,57
4	100	100	97,78	97,78	95,55	66,67	46,67	31,11
5	100	77,09	60,42	50	41,67	16,67	6,25	2,08
6	100	100	89,59	70,84	52,09	38,54	20,83	7,29
7	100	73,17	56,1	56,1	56,1	9,76	4,88	4,88
9	100	85,71	71,43	66,66	57,14	47,62	42,86	38,1
10	100	96,72	93,45	92,81	42,62	8,2	3,29	1,64
11	100	65	40	35	30	20	15	10
12	100	69,23	46,15	46,15	42,31	42,31	42,31	38,46
13	100	90,91	81,82	63,64	48,49	39,39	33,33	27,27
15	100	84	68	60	52	32	20	8
16	100	90	85	70	55	30	15	5
17	100	97,56	92,68	68,29	48,78	34,15	21,95	12,2
18	100	84,62	69,23	53,65	42,31	34,62	19,23	11,54
19	100	70	45	40	25	0	0	0
21	100	94,74	89,47	84,21	81,6	36,84	15,79	7,89
22	100	100	100	77,5	60	27,5	12,5	5
23	100	100	100	95	95	90	85	85
24	100	92	88	76	68	64	64	64
25	100	75	60	50	45	40	30	25
27	100	100	100	55,56	22,22	14,81	0	0
28	100	90,91	81,82	63,64	51,52	39,39	27,27	15,15
29	100	66,67	45,1	41,18	39,22	33,33	29,41	25,49
31	100	70	50	30	20	10	6,67	3,33
32	100	97,5	92,5	70	52,5	35	22,5	12,5
33	100	97,73	93,18	61,36	40,91	18,18	9,09	4,55
36	100	59,09	59,09	59,09	59,09	45,46	27,27	27,27

Table 7.8: Schizont count Pf 2010 Eury-3

	A	B	C	D	E	F	G	H
Pat No	Control SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ
	0	3	10	30	100	300	1000	3000
1	100	65,22	43,48	39,13	39,13	17,39	8,7	4,35
2	100	92,13	82,02	74,16	34,83	14,61	8,99	2,25
3	100	66,67	42,86	38,1	33,33	28,57	23,81	19,05
4	100	100	100	100	93,94	34,85	22,73	16,67
5	100	96,3	88,89	85,19	81,48	25,93	7,41	3,7
6	100	100	100	84,16	84,16	50,5	30,69	7,92
7	100	100	95	90	85	20	20	0
9	100	75	55	50	45	30	20	10
10	100	96,55	72,41	60,34	50	3,45	0	0
11	100	66,67	43,33	30	20	13,33	10	6,67
12	100	88,89	80,56	66,67	58,33	52,78	47,22	41,67
13	100	88,71	77,42	59,68	46,77	35,48	24,19	17,74
15	100	94,74	89,47	84,21	78,95	44,74	26,32	10,53
16	100	89,29	78,57	46,43	28,57	17,86	10,71	3,57
17	100	78,57	61,43	41,43	28,57	18,57	11,43	4,29
18	100	100	100	102,94	102,94	102,94	32,35	32,35
19	100	85,71	71,43	61,9	42,86	19,05	14,29	9,52
21	100	64,44	41,11	32,22	25,56	16,67	10	6,67
22	100	83,67	71,43	53,06	38,78	20,41	10. Jan	4,08
23	100	100	100	100	85	80	80	80
24	100	100	100	100	100	68,18	59,09	54,55
25	100	85	70	45	40	40	40	25
27	100	78,06	73,17	70,73	19,51	12,2	4,88	0
28	100	92,5	85	67,5	55	25	12,5	5
29	100	80	65	62,5	60	45	35	25
30	100	92	80	52	32	16	8	4
32	100	85,37	73,17	65,85	58,54	34,15	19,51	7,32
33	100	95,45	95,45	90,91	90,91	40,91	18,18	9,09
36	100	100	70	55	40	25	15	15

Table 7.9: Schizont count Pf 2010 Eury-1-Art

	A	B	C	D	E	F	G	H
Pat No	Control SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ
	0	3	10	30	100	300	1000	3000
1	100	51,61	35,48	22,58	16,13	12,9	6,45	0
2	100	97,62	73,81	23,81	19,05	14,29	14,29	0
3	100	57,69	34,62	34,62	34,62	15,38	7,69	0
4	100	111,9	88,1	73,81	73,81	38,1	23,61	4,76
5	100	33,33	11,11	5,56	0	0	0	0
6	100	90,59	82,35	78,82	75,29	72,94	24,71	7,06
7	100	87,18	74,36	74,36	74,36	25,64	7,69	5,13
9	100	50	25	25	25	10	5	0
10	100	98,48	96,97	95,45	93,94	90,91	13,64	3,03
11	100	89,66	79,31	51,72	51,72	27,59	20,69	13,79
12	100	75,86	55,17	41,38	31,03	13,79	6,9	3,45
13	100	97,37	94,74	92,11	89,47	44,74	23,68	5,26
15	100	88,46	76,92	73,08	69,23	26,92	11,54	0
16	100	85,71	71,43	38,1	14,29	4,76	0	0
17	100	81,4	65,12	46,51	30,23	18,6	9,3	2,33
18	100	83,72	93,02	79,07	60,47	34,88	4,65	0
19	100	85,71	76,19	71,43	71,43	66,67	61,9	23,81
21	100	100	100	68,57	45,71	20	8,57	2,86
22	100	58,18	34,55	25,45	16,36	5,45	1,82	0
23	100	90,48	80,95	66,67	57,14	35,71	35,71	35,71
24	100	88	88	88	76	72	72	72
25	100	80	64	64	52	48	36	24
27	100	70,97	67,74	64,52	58,06	32,26	9,68	3,23
28	100	67,65	47,06	44,12	41,18	14,71	5,88	0
29	100	86,67	76,67	56,67	40	16,67	6,67	0
31	100	96,3	88,89	66,67	44,44	18,52	7,41	0
32	100	90,63	68,75	56,25	46,88	18,75	6,25	3,13
33	100	96,55	93,1	51,72	27,59	13,79	6,9	0
36	100	58,33	58,33	41,67	37,5	29,17	16,67	12,5

Table 7.10: Schizont count Pf 2010 Eury-2-Art

	A	B	C	D	E	F	G	H
Pat No	Control SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ
	0	3	10	30	100	300	1000	3000
1	100	68	44	36	32	16	8	4
2	100	93,1	89,66	48,28	41,38	37,93	27,59	3,45
3	100	80,95	66,67	38,1	23,81	14,29	9,52	4,78
4	100	92,86	88,1	69,05	50	38,1	14,29	2,38
5	100	72,41	37,93	10,34	0	0	0	0
6	100	81,05	74,74	68,42	42,11	30,53	24,21	2,11
7	100	71,43	66,67	45,24	7,14	4,76	4,76	2,11
9	100	77,27	59,09	31,82	18,18	13,64	9,09	4,55
10	100	115,38	98,46	64,62	24,62	24,62	24,62	24,62
11	100	100	100	100	50	30	20	20
12	100	96,55	62,07	41,38	27,59	24,14	20,69	17,24
13	100	66,67	26,19	19,05	11,9	4,76	0	0
15	100	84,62	69,23	42,31	26,92	19,23	11,54	0
16	100	68	48	36	24	8	0	0
17	100	75,61	56,1	43,9	34,15	19,51	9,76	4,88
18	100	94,29	71,43	68,57	65,71	14,29	11,43	0
19	100	75	60,71	50	39,29	28,57	17,86	17,86
21	100	97,22	91,67	86,11	83,33	25	8,33	2,78
22	100	70	50	34	22	14	10	4
23	100	74,07	55,56	51,85	51,85	51,85	51,85	51,85
24	100	88,24	73,53	61,76	55,88	44,12	44,12	44,12
25	100	62,5	64,17	41,67	37,5	33,33	33,33	25
27	100	80,65	80,65	67,74	19,35	9,68	0	0
28	100	78,79	60,61	48,48	39,39	15,15	6,06	0
29	100	78,95	63,18	52,63	42,11	15,79	5,26	0
31	100	71,43	50	32,14	21,43	14,29	7,14	0
32	100	57,58	33,33	30,3	27,27	12,12	6,06	3,03
33	100	97,14	94,29	90	37,14	14,29	5,71	0
36	100	100	90	75	45	30	30	25

Table 7.11: Schizont count Pf 2010 Eury-3-Art

	A	B	C	D	E	F	G	H
Pat No	Control SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ
	0	3	10	30	100	300	1000	3000
1	100	86,96	78,26	69,57	60,87	21,74	8,7	0
2	100	87,23	65,96	40,43	12,77	10,64	6,38	0
3	100	34,09	11,36	9,09	4,55	4,55	4,55	2,27
4	100	94,12	94,12	85,29	41,18	29,41	8,82	0
5	100	53,33	30	16,67	10	6,67	0	0
6	100	100	84,04	63,83	50	22,34	20,21	9,57
7	100	79,31	62,07	50	15,52	5,17	3,45	3,45
9	100	91,3	86,96	39,13	17,39	8,7	0	0
10	100	73,42	54,43	40,51	30,38	12,66	7,59	7,59
11	100	78,13	59,38	46,88	25	18,75	15,63	6,25
12	100	104,17	108,33	95,83	83,33	25	8,33	4,17
13	100	55,81	30,23	16,28	93,02	6,98	6,98	4,65
15	100	75	58,33	36,11	22,22	13,89	8,33	0
16	100	83,33	66,67	33,33	16,67	12,5	8,33	4,17
17	100	82,76	65,52	48,28	34,48	13,79	3,45	0
18	100	92,5	85	80	50	22,5	7,5	0
19	100	81,82	63,64	54,55	50	22,73	13,64	0
21	100	97,33	92	60	38,67	17,33	8	4
22	100	75,56	55,56	48,89	42,22	20	8,89	4,44
23	100	96	92	84	80	76	68	60
24	100	95,65	69,57	65,22	60,87	52,17	47,83	43,48
25	100	76	56	48	44	28	16	8
27	100	93,33	86,67	76,67	56,67	40	16,67	16,67
28	100	67,35	44,9	26,53	16,33	8,16	4,08	0
29	100	80	63,33	53,33	43,33	16,67	6,67	0
31	100	79,31	65,52	58,62	48,28	17,24	6,9	0
32	100	65,52	44,83	37,93	31,03	13,79	6,9	3,45
33	100	82,76	68,97	48,28	34,48	13,79	6,9	0
36	100	96	68	52	36	28	24	20

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