



## CHAPTER 3

### ANTIPLASMODIAL BIOACTIVITY OF CRUDE EXTRACT AND ISOLATED COMPOUNDS

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## CHAPTER 3

### ANTIPLASMODIAL BIOACTIVITY OF CRUDE EXTRACT AND ISOLATED COMPOUNDS

#### 3.1 Introduction

Among many and different maladies that afflict mankind, malaria appears to be making a strong comeback (Van der Westhuyzen; Parkinson, 2005). Cases of malaria are on the increase in the world, more especially, in the tropical and sub-tropical regions. The World Health Organization estimates that 300-500 million new cases of malaria are reported annually, causing the death of about 2.5 million people (Hyde, 2002; Van der Westhuyzen; Parkinson, 2005). The increasing prevalence and distribution of malaria has been attributed to a number of factors, one of them being the emergence and spread of drug resistant parasites. The search for new methods to combat the disease has become imperative as commonly available cheap drugs are no longer effective for treatment. Efforts are now being directed towards the discovery and development of new chemically diverse antimalarial agents (Clarkson *et al.*, 2004). The success of artemisinin and its derivatives has encouraged and supported the continued investigation of plant resources for novel antimalaria drugs (Willcox, 2004).

Before 1976 drug sensitivity testing was done only *in vivo*, making the procedure very expensive and difficult. Since the development of *in vitro* assays, drug sensitivity testing has become easier, faster and more efficient (Noedl *et al.*, 2003). Although *in vitro* antiplasmodial testing is a lot easier than *in vivo* methods, bio-guided isolation of antiplasmodial compounds using *in vitro* methods remains a long and difficult process. The antibacterial screening test (direct bioautography) is the easiest and might be successful in isolating antiplasmodial compounds (Prozesky, 2004; Boonphong, 2007; Zdzislawa, 2007).

## 3.2 Methods

### 3.2.1 Culture medium and washed human erythrocytes

The wash medium was made up of 10.4 g RPMI 1640 (containing L-glutamine), 5.94 g HEPES, 4.0 g D-glucose, 44 mg hypoxanthine, 5% NaHCO<sub>3</sub> and 4 mg of gentamycin dissolved in 900 ml deionised sterile water. The culture medium was prepared by supplementing the wash medium with 5% Albumex II.

Erythrocytes for maintenance of the parasite cultures were obtained from the whole blood of a O<sup>+</sup> blood group donor, and then centrifuged in a Hermle Z 320 bench centrifuge at 500 g for 5 minutes. The plasma and buffy coat was removed. The erythrocytes were then re-suspended in wash medium and centrifuged at 500 g for 5 minutes. The supernatant was removed and the procedure was repeated three times. The washed erythrocytes were then stored in 10 ml wash medium at 4°C for up to 2 weeks (Trager and Jensen, 1976; Stoltz, 1992; Hoppe, 1993).

### 3.2.2 *In vitro* culturing of malaria parasites

Two chloroquine sensitive (D6 and D10) and two chloroquine resistant (Dd2 and W2) strains of *Plasmodium falciparum* was used in the bioassay (Stoltz, 1992). For continuous *in vitro* culturing a slightly modified version of the Trager and Jansen method was employed (Trager and Jensen, 1976; Hoppe, 1993).

The frozen malaria-isolates (~5% parasitemia) in cryotubes stored in liquid nitrogen, was quickly thawed in a water bath at 37°C. The content of the cryotube (~1 ml) was transferred under sterile conditions to a 10 ml centrifuge tube and 0.2 ml sterile 12% NaCl was slowly added to the thawed culture. It was then mixed well for (10-20) seconds after which a sterile 1.6% NaCl solution was slowly added and mixed for (10-20) seconds. The solution was centrifuged at 2 500 g for 5 min and the supernatant was removed. The parasite was then re-suspended in a 75 ml culture flask (Sterilin) containing

10 ml culture medium and supplemented with fresh uninfected human erythrocytes from the O<sup>+</sup> blood group.

The hematocrit in the parasite culture was adjusted and maintained at 5% by adding washed erythrocytes to the culture medium. The culture flask was then filled with a special gas mixture made up of 5% oxygen, 5% carbon dioxide and 90% nitrogen before being incubated in a Forma incubator at 37C. Daily the parasite culture was checked, with the thin blood smear (reported in section 3.2.3). The culture medium was changed, flask filled with the gas mixture from above and returned to the incubator. The hematocrit of the cultured parasites was continually maintained at 5% by adding fresh cells at least every 2-3 days.

### **3.2.3 Giemsa stained thin blood smear preparation**

A smear of parasitic culture was made by placing a drop of about 12 µl parasitic culture at one end of a slide and by using a second slide at an angle, the drop was evenly spread along the first slide and then allowed to dry. Methanol (analytical grade) was used to fixate the blood smear by allowing it to stand for 1 minute, after which the methanol was removed by decanting. The DNA intercalator Giemsa (Sigma) was used to stain the parasitic DNA (Wright, 1984). The Giemsa stain was formulated in glycerol and methanol as indicated by the supplier. A phosphate buffer, containing 9.5 g/l sodium dihydrogen phosphate at a pH of between 6.8 and 7.2 was used to dilute the Giemsa solution. Two drops of Giemsa solution were added for every 1 ml of phosphate buffer. The slide was covered with this solution for 5 minutes, rinsed in water and then allowed to dry in the open air. A drop of microscope oil immersion was placed on the slide and it was evaluated using a 100x oil objective of a Nikon phase contrast microscope.

### **3.2.4 *In vitro* synchronisation of malaria parasites**

The method described by Vernes *et al.* (1984) and modified by Hoppe (1993) was used for the *in vitro* synchronization of malaria parasites. Synchronisations were performed on malaria cultures consisting of 80% ring-phase parasites. The cultures were transferred from the culture flask to a 50

ml centrifuge tube, centrifuged at 500 g for 5 minutes and the supernatant removed. The pellet volume was about 0.5 ml when 10 ml of a parasitic culture (5% hematocrit) was centrifuged. Then 4 ml of a 15% D-sorbitol solution was added to every 0.5 ml of parasite pellet. After careful mixing, by inversion, the solution was incubated at 37°C for 5 minutes, where after, 8 ml of a 0.1% D-glucose solution was added per 0.5 ml of parasite pellet, followed by mixing by tube inversion. The parasite solution was again incubated at 37°C for 5 minutes, centrifuged at 500 g for 5 minutes and the supernatant containing lysate erythrocytes, was removed. The pellet ring-phase infected and uninfected erythrocytes were re-suspended in 10 ml of the culture medium and returned to the culture flasks. The hematocrite was adjusted to about 5% by adding washed erythrocytes, filled with the special gas mixture described in 3.2.2 and returned to the incubator. This procedure was repeated until the cultures consisted of about 95% ring-phase parasites.

### **3.2.5 Preparation of microculture plates**

Microculture plates were prepared in the same way for all the antiplasmodial bioassays. Final concentrations of the extracts were made by appropriate dilutions of stock solutions with the culture medium. 20 µl extract/compound in duplicate and at different concentrations were added to the flat-bottom wells of a 96-wells microculture plate, as well as 80 µl of a 5% hematocrit of 0.5-1.0% parasitized cells (95-100% rings) in supplemented RPMI 1640 medium. Microcultures were incubated for 48 hours in a modular incubator chamber at 37°C in a gas mixture of 5% oxygen, 5% carbon dioxide and 90% nitrogen for the assessment of antiplasmodial activity. After 24 hours, 50µl of the medium from each well was removed and replaced by 10 µl of the extract/compound and 40 µl of the fresh culture medium (Makler *et al.*, 1995).

### **3.2.6 Determination of antiplasmodial activity with the Malstat method**

The method used was a slightly modified version of the lactate dehydrogenase assay (Makler *et al.*, 1995). The experiment was done in duplicate at 1% parasitemia and 5% hematocrite as described in section 3.2.5 After 48 hours, the 96-well microculture plate was prepared by adding 100 µl

Malstat reagent (133 ml Triton X-100, 1.33 g lactate, 0.44 g Tris buffer and 44 mg 3-actylpyrimidine adenine dinucleotide (APAD) made up to 200 ml) to each well together with 25  $\mu$ l developing dye solution (160 mg nitroblue tetrazolium (NBT) and 8 mg phenazine ethosulphate (PES) to 100 ml Millipore water) and 10  $\mu$ l from the incubated plate. The plate was then incubated for 20 min in the dark and read with an ELISA plate reader at 620 nm.

### **3.2.7 Determination of antiplasmodial activity with the microfluorimetric method**

The method used was based on the detection of parasitic DNA by the intercalation of PicoGreen (Corbett *et al.*, 2004). Synchronized ring form cultures (2% hematocrit and 1% parasitemia) were used to determine the activity of pure compounds in 96-well microculture culture plates. The cultures of *P. falciparum* were grown and synchronized as described in section 3.2.2 and 3.2.4. The microculture plates were prepared as described in section 3.2.5. Compounds were dissolved in DMSO and tested in duplicate at the final concentration of 10.0, 2.0 and 0.4  $\mu$ g/ml and re-evaluated at higher or lower concentration when necessary. The final dilution in the wells contains less than 0.1% DMSO, which had no measurable effect on the parasite survival in this system. The negative control was 0.1% DMSO in RPMI 1640 culture media which represent 100% parasitic viability. The positive control consisted of chloroquine at concentrations of 100.0, 10.0, and 1.0 nM that provided a measure of susceptibility of the parasite to known antimalarial drugs (Corbett *et al.*, 2004).

#### **3.2.7.1 Fluorimetric susceptibility test**

After 48 hours, 150  $\mu$ l of the culture described in 3.2.7 was transferred to a new 96 well microculture flat bottom plate. Fifty micro-litres of the fluorochrome mixture, which consisted of PicoGreen (Molecular Probes, Inc., EUGENE, OR), 10 mM Tris-HCl, 1 mM EDTA, pH 7.5 (TE buffer), and a 2% Triton X-100 dilution with double distilled DNase-free water, was then added to liberate and label the parasitic DNA. The plates were then incubated for 5-30 minutes in the dark. The fluorescence signal, measured as relative

fluorescence units (RFU) was quantitated with a fluorescence microplate reader (FL<sub>x</sub> 800; Bio-Tek Instruments, Inc., Winooski, VT) at 485/20 nm excitations and 528/20 nm emissions. Simultaneously, the RFU from the samples (compounds, negative and positive control) were obtained, stored, and analysed (Corbett *et al.*, 2004).

The data analyses were performed with a programmed calculus sheet on Microsoft (Redmond, WA) Excel 2000 that processes the relative fluorescence units exported through the KC junior software from the microplate fluorimeter. The calculus sheet consists of 1) a formula that calculates the mean of the two replicates per sample condition, 2) the subtraction of the respective colour background of each dilution of the plant extract, 3) the conversion of the mean RFU value to a percentage of the response, taking as 100% the mean of the negative control, and 4) the conversion of the percentage to the 50% inhibitory concentration (IC<sub>50</sub>) by log regression. To adjust for the potential contribution of the haemoglobin pigments from the erythrocytes and the possible fluorescence from the intrinsic pigments present in some plant extracts, control wells were used that consisted of non-infected erythrocytes alone, samples of the diluted drugs or extracts with non-infected erythrocytes.

### **3.2.7.2 Synergistic activity**

The chloroquine reversal effect of compounds with chloroquine was determined by measuring the activity of each of the compounds alone and in combination with chloroquine. Two strains of *P. falciparum* were used, a sensitive strain (D6) and resistant strain (W2). They were cultured and synchronised as described in sections 3.2.2 and 3.2.4. The *In vitro* antiplasmodial test was done with the microfluorimetric method described in section 3.2.7. The compounds at concentrations of 10.0, 2.0 and 0.4 µg/ml were combined with chloroquine at concentrations of 100.0, 10.0 and 1.0 nM respectively.

### 3.3 Results and Discussion

The antiplasmodial activity of the compounds isolated are shown in Table 3.1. Two of the flavonoids (tamarixetin and eriodictyol) isolated were not tested because they were obtained in insufficient quantities. Quercetin showed a very good antiplasmodial activity against D6 (0.34  $\mu\text{g/ml}$ ) and Dd2 (0.73  $\mu\text{g/ml}$ ). Antimalarial activity of quercetin was also reported by Bylka *et al.* (2004), Tona *et al.*, (2004) and Prozesky (2004). The activity shown by steenkrotin A is moderate and promising. This is because it is more active against resistant strains (3.00 and 3.10  $\mu\text{g/ml}$  for W2 and Dd2 respectively) than the sensitive strain D10 (5.20  $\mu\text{g/ml}$ ). It did not show any activity against D6 at the concentration in which it was tested. This compound was first isolated and described as an antiplasmodial agent by Prozesky (2004).

**Table 3.1:** Antiplasmodial activity of compounds and extract.

Extract/Compounds	IC <sub>50</sub> ( $\mu\text{g/ml}$ )			
	Sensitive strains		Resistant strains	
	D6	D10	W2	Dd2
Ethanol extract	ND	8.60	ND	5.80
Eriodictyol	ND	ND	ND	ND
Indane	>10.00	>10.00	>10.00	>10.00
Quercetin	>10.00	0.34	>10.00	0.73
Steenkrotin A	>10.00	5.20	3.00	3.10
Steenkrotin B	>10.00	>10.00	>10.00	>10.00
Steenkrotin B acetate	ND	>10.00	ND	>10.00
Tamarixetin	ND	ND	ND	ND
Chloroquine (Q)	0.01	0.01	0.03	0.07
Indane + Q	>10.00	ND	2.00	ND
Quercetin + Q	8.00	ND	3.00	ND
Steenkrotin A + Q	12.00	ND	2.00	ND
Steenkrotin B + Q	3.00	ND	3.00	ND

ND = not done

Q = chloroquine

Steenkrotin B, steenkrotin B acetate and the indane were not active (compounds with  $IC_{50} < 10 \mu\text{g/ml}$  is said to be active) against the strains of *P. falciparum* at the concentrations at which they were tested. Steenkrotin B and its acetate were expected to be active because they contain an endoperoxide which has been linked to antiplasmodial activity of artemisinin, a potent antimalarial agent (van der Westhuyzen; Parkinson, 2005). The inactivity of these two compounds against *P. falciparum* seem to indicate that there are severe structural constraints involved in them that prevent either the access of the radicals to the membranes of the parasite, or are involved in some mode of deactivation of the material itself. Studies on structural modifications of these compounds are needed to determine the activity of the endoperoxide in them. Furthermore, there is no report on the antimalarial activity of an indane in literature.

When the indane, steenkrotin A and steenkrotin B were spiked with chloroquine and tested against D6 and W2, the  $IC_{50}$  of the compounds improved a little bit. However, Prozesky (2004) reported synergistic activity of steenkrotin A (called crotrene A) with chloroquine on *P. falciparum* (RB1). He also reported a similar observation that the compound is more potent against a resistant strain than a sensitive one.

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