Vol. 6(2003) 26 -30

In vitro Antimalarial Activity of *Ajuga remota* Benth (Labiatae)

K. A.M. KURIA[1], K. O. ABUGA[1], W. MASENGO[1], C. GOVAERTS[2], E. ROETS[2], R. BUSSON[2], P. DE WITTE[2], I. ZUPKO[2], G. HOORNAERT[3], J. HOOGMARTENS[2] AND G. LAEKEMAN[2]

[1]*Faculty of Pharmacy, University of Nairobi, P.O. Box 19676, 00202, Nairobi, Kenya*
[2]*Faculty of Pharmaceutical Sciences, Katholieke Universiteit Leuven, E.Van Evenstraat 4,B-3000 Leuven, Belgium.*
[3]*Faculty of* Sciences, Katholieke Universiteit Leuven, E. Van Evenstraat 4, B-3000 Leuven, Belgium.

***Ajuga remota* Benth is the most frequently used plant to treat malaria by Kenyan herbalists. Both crude extracts and pure isolates of the plant were tested for their *in vitro* antimalarial properties. The activity was assessed by an enzyme assay method based on the measurement of the parasite lactate dehydrogenase activity. The IC_{50} of the most active A. *remota* extract (ethanol macerate) was 71 and 69 µg/ml against the chloroquine sensitive (FCA/20GHA) and resistant (W2) strains of *Plasmodium falciparum* respectively. Ajugarin-1 was moderately active with IC_{50} of 23.0 ± 3.0 µM as compared to chloroquine (IC_{50} = 0.041 ± 0.003 µM) against the chloroquine-sensitive strain of *Plasmodium falciparum.* Ergosterol-5, 8-endoperoxide was about 4x as potent (IC_{50} = 5.4 ± 1.9 µM) while 8-0- acetylharpagide, a new isolate of *A.remota* and whose structure was established by spectroscopic evidence, was inactive.**

Keywords: *Ajuga remota, in vitro* antimalarial activity, pure isolates.

INTRODUCTION

Plants are recognized as important tools in the practice of traditional African medicine [1]. *Ajuga remota* Benth (Labiatae) is an erect rhizomatous pubescent herb found growing in the seasonally flooded grasslands of Kenya and other parts of East Africa. The herb is not eaten by animals, birds or insects. This is probably due to the very bitter taste of almost all its parts. The leaves of *A. remota* are known to relieve tooth ache, while a decoction or infusion from the leaves is prescribed by Kenyan herbalists for severe stomachache, treatment of malaria [2] and oedema associated with protein-calorie malnutrition disorders in infants when breast feeding is terminated [3]. The plant is also used for the treatment of pneumonia and liver problems [4]. Recently it was reported that a methanolic extract of *A. remota* lowered blood pressure in experimentally hypertensive rats [5]. More recently, ergosterol -5,8 - endoperoxide was isolated from the aerial parts of *A. remota* and shown to have some potent antimycobacterial activity [6]. The purpose of our work is to evaluate the traditional use of *A .remota* for its treatment of malaria by *in vitro* laboratory experiments.

MATERIALS AND METHODS

Collection of plant material

Authenticated *Ajuga remota* Benth (Labiatae) plant material (aerial parts) was collected in plastic bags from the area around Doonholm Estate, Nairobi, Kenya. The fresh plants were dried in the open air and reduced to powder by using a mortar and pestle. The powder was sifted again through sieve N^{o} 18 in the laboratory. The plant material as well as the sifted powder were kept at room temperature until required.

In vitro parasite cultures

Continuous cultures of *Plasmodium falciparum* (FCA/20GHA, chloroquine sensitive and W2, chloroquine resistant strains) were maintained at an initial 1% parasitemia in 1% human erythrocytes (O+ or A-) suspended in complete RPMI 1640 culture medium (ICN Biomedicals

* Author to whom correspondence may be addressed.

Inc., Asse-Relegem, Belgium) with L-Glutamine without Sodium bicarbonate and 10% human plasma (A+ or A-) supplemented with 1.77 g/L D - Glucose (UCB, Belgium), 2.0 g/L $NaHCO_3$ (ICN Biomedicals Inc., Asse-Relegem, Belgium) and 8.3 g/L HEPES buffer (ICN Biomedicals Inc., Asse-Relegem, Belgium). This was according to the method of Trager and Jensen [7], modified by Osisanya *et al.* [8] and Fairlamb *et al.* [9].

Extraction and isolation of ajugarin-1

A. remota aerial parts powder (100 g) was extracted with chloroform (3 x 700 ml) for three days under regular stirring. The extract thereof was concentrated under vacuum (30 °C) to dryness (6.4 g), re-dissolved in 80 % ethanol, decolorized with activated charcoal and the clear solution obtained was further concentrated to a small volume and left in a silica gel desiccator. The needle crystals formed were subjected to four successive crystallizations in methanol at –18 °C yielding 26 mg (0.026% w/w) of crystals whose purity (>99 %) was confirmed by liquid chromatography. The structure of the isolated ajugarin-1 was established by spectroscopic evidence.

Extraction and isolation of 8-acetylharpagide

100 g of *Ajuga remota* powder was extracted by maceration with 2000 ml of 80 % ethanol (BDH) for 5 days under regular magnetic stirring. The extract thereof was separated from the marc by decanting followed by filtration (sintered glass N^o 3). The extract was dried under vacuum at 40^o C (Rotovap Büchi) and a yield of 27.03 g was realized. 25 g of the dark green extract was redissolved in 100 ml distilled water and partitioned consecutively using 5 x 100 ml portions of chloroform (BDH), ethyl acetate (BDH) and n-butanol (BDH). These fractions were dried under vacuum. The chloroform fraction yielded 400 mg the ethyl acetate yielded 420 mg while the yield from n-butanol fraction was 7.03 g and it was therefore the major component.

TLC of the n-butanol fraction in methanol on silica gel 60 F254 (Merk) using ethyl acetate - methanol (90: 10) as the mobile phase revealed the presence of three compounds. Two of the minor compounds were UV active (blue spots) while the major compound was detected by spraying the plate with concentrated sulphuric acid giving a brick reddish spot. Further phytochemical work on the n-butanol fraction was carried out so as to isolate the major compound. 2 g of the n-butanol fraction material was dissolved in the minimum amount of methanol and adsorbed onto 2 g of silica gel for column chromatography. The methanol was removed under vacuum at 40 °C to yield a free flowing powder. A vacuum liquid chromatography (VLC) comprising of a 4 cm i.d. sintered glass N^o 3 filter was packed with 15 g silica gel with the aid of petroleum ether which was added to the column and sucked dry severally by application of vacuum to give an evenly packed column. The extract powder was then added to the top of the column evenly after which it was covered with a thin layer of cotton wool to facilitate even distribution of the elution solvents. The column was subjected to gradient elution with 50 ml portions of solvents with increasing polarities and the fractions were monitored by TLC and those with similar profiles were pooled together. A further 2 g of the n-butanol extract was processed in a similar manner and the two ethyl acetate-methanol (70: 30) fractions were combined and further purified by shaking it with a small quantity of activated charcoal (Vel) after which it was filtered (sintered glass N^o.3) and dried under vacuum at 40 °C yielding 60 mg of an amorphous powder. TLC of the powder using ethyl acetate methanol (60: 40) as the mobile phase revealed one compound when sprayed with concentrated sulphuric acid having an Rf value of 0.36. Probably the other two previously detected minor compounds got removed by adsorption onto the activated charcoal. The compound was identified to be 8-0-acetylharpagide (an iridoid glucoside) through MS and NMR spectroscopy.

Synthesis of ergosterol -5,8-endoperoxide

Ergosterol (200 mg) and methylene blue 20 ml were dissolved in 50 ml of dichloromethane (BDH,) Poole, England and placed in a 100-ml three necked flask. Oxygen held in a balloon was bubbled through the ice-cooled solution under

constant magnetic stirring and the solution was irradiated with a tungsten filament light (100 Watts) placed 2 feet above the flask. This was according to the method of *Cantrell et al* [6] with slight modifications. The reaction was monitored by TLC and stopped after 2 h once complete. (For this purpose, advantage was made of the property that ergosterol is UV active but the endoperoxide is not. It was visualized by spraying the TLC plate with concentrated sulphuric acid). The mobile phase consisted of chloroform - ethyl acetate (95: 5). The crude reaction mixture was concentrated under vacuum and adsorbed onto 300 mg of silica gel and placed on a 2 cm i.d. and 10 cm long VLC column that had been previously packed with 6 g of silica gel. The column was eluted with 50 ml portions of chloroform and increasing polarity mixtures of chloroform and ethyl acetate. The chloroform ethyl - acetate (90 : 10) fraction was dried under vacuum at 40 °C yielding 70 mg of a white crystalline powder whose TLC gave a single spot using chloroform methanol (99 : 1) as the mobile phase. The compound was identified to be ergosterol-5,8-endoperoxide mainly by comparison of MS and NMR spectral data with previously reported values [10,11].

In vitro testing of the antimalarial activity

To test for antiplasmodial activity of the plant isolates (ajugarin-1 and 8-O-acetylhypergide) and the synthesized ergosterol-5,8-endoperoxide the method based on measurement of the parasite lactate dehydrogenase activity (pLDH) described by Makler and Hinrichs [12] was used. Antimalarial activity (after 72 h incubation at 37 °C) was measured by the IC_{50} representing the concentration of drug that caused a 50% decrease in the parasite lactate dehydrogenase activity compared with the control culture and chloroquine was tested for standard antimalarial activity.

RESULTS

Antiplasmodial activity of isolates

Ajugarin-1 caused a concentration-dependent growth inhibitation of the chloroquine sensitive strain of *Plasmodium falciparum* (FCA 20/GHA). The IC_{50} was 23.0 ± 3.0 µM as compared to 0.041 ± 0.003 µM for chloroquine as a positive control and an IC_{50} of >88 µM (the highest test concentration) against the chloroquine resistant strain of *Plasmodium falciparum* (W2).

The iridoid glucoside which was isolated for the first time from the East African *Ajuga remota* did not exhibit any antiplasmodial activity even at the highest concentration of 500 µM used against the chloroquine-sensitive strain of *Plasmodium falciparum* (FCA 20/GHA).

Ergosterol-5,8 -endoperoxide was isolated for the first time from the aerial parts of *A. remota* by Cantrell *et al.* [6] and shown to have some potent antimycobacterial activity. For the purpose of the present studies, the compound was synthesized according to the method described by the same investigators with some modification as previously described.

The ergosterol -5,8 endoperoxide caused a concentration-dependent growth inhibition of the chloroquine sensitive strain of *Plasmodium falciparum* (FCA 20/GHA) with an IC_{50} of 5.4 ± 1.9 µM as compared to 0.041 ± 0.003 µM for chloroquine as a positive control. It has also been shown to have equal potencies against both the chloroquine sensitive (FCA 20/GHA) and resistant (W2) strains of the parasite [TIBOTEC: personal communication]. Compared to ajugarin-1, ergosterol-5,8-endoperoxide is indicated to be approximately 4x as potent.

Table 1: Antiplasmodial activity of the various *A. remota* isolates against *Plasmodium falciparum* (FCA 20/GHA) determined by the pLDH activity method

***A.remota* isolates**	**Antiplasmodial activity (FCA 20/GHA) IC_{50} (µM)**
Ajugarin-1	23.0 ± 3.0
8-O-acetylharpagide	NIL
Ergosterol-5,8-endoperoxide	5.4 ± 1.9

IC_{50} =50% inhibiting concentration. Values are the mean ± SD ($n \geq 3$) * Chloroquine-sensitive strain (Chloroquine IC_{50} = 0.041 ± 0.003µM).

DISCUSSION

The present study was undertaken to evaluate the antiplasmodial activity of Ajuga *remota*. Ethnopharmacological data has been one of the common useful ways for the discovery of biologically active compounds from plants [13,14]. The big advantage of the ethnopharmacological information is that the extensive literature may already allow for some rationalization with respect to the biological potential of a reputed use [13]. Ethnopharmacological use of plants can therefore be a basis for phytochemical and phytopharmacological investigation. The case of *Ajuga remota* is one of the many examples to consider. Everything starts with the use of whole extracts as complex mixtures. This is the easiest way to medical practice in countries where patients cannot afford the current use of chemically synthesized drugs. This is an important point although pharmacological evaluation should go hand in hand with the search for pure compounds. Some herbalists prescribe 40 ml of a cold water infusion with *Ajuga remota* powder in a concentration of one teaspoon in 700 ml. We should be critical towards this dosage as it is low compared to the inhibiting concentration or as shown in the previous [15] work. We might also expect more active substances to be extracted when hot water (decoction) or alcohol is used as was the case with the ethanol macerate which was the most active [15]. For several reasons, the subject of *Ajuga remota* should not be abandoned at this stage. First, *Ajuga remota* is used as an admixture, antimalarial activity of other plants has to be considered. Second, as already mentioned, the plant has a very bitter taste. Concentrating it too much will result in an aversion by the patient.

Isolation of pure compounds is useful in the standardization of herbal medicinal preparations. Ajugarin-1 is interesting in view of its unusual chemical structure (epoxide ring) when compared to conventional antimalarial compounds. Such a compound might lead to synthesis of pharmaceutically important derivatives with probably higher antimalarial activities. Ergosterol-5,8-endoperoxide is shown to have more promising antiplasmodial activity with reference to the other *A. remota* isolates tested and could serve as a template in search of new antimalarial drugs.

ACKNOWLEDGEMENTS

The Belgium Ministry of Foreign Affairs (Co-operation projects) supported this project under grant N^{o} JH/107. We are grateful to I. Knockaert for the regular blood donations and to TIBOTEC for the supply of the *Plasmodium falciparum* cultures used in this study.

REFERENCES

[1] D.T. Okpako.,TIPS, 20 (1999) 482-485.

[2] J.O. Kokwaro, Medicinal Plants of East Africa, 2nd Ed., Kenya Literature Bureau, Nairobi, Kenya, (1993) 118.

[3] K.A.M. Kuria, and G. Muriuki, East African Med. J. 61 (1984) 533-538.

[4] F.N. Gachathi, Kikuyu Botanical Dictionary of Plant Names and Uses, AMREF, Nairobi, Kenya, (1993) 118.

[5] M. Odek-Ogunde , M.S. Rajab, G.J. Migwi and J.M. Ndegwa, Planta Med. 59 (1993) 573-574.

[6] C.L. Cantrell, M.S. Rajab, S.G. Franzblau, *et al.* Planta Medica, 65 (1999) 732-734.

[7] W. Trager, and J.B. Jensen, Science, 193 (1976) 673-675.

[8] J.O. Osisanya, S. Gould, and D.C. Warhurst, Ann. Trop. Med. Parasitol. 75 (1981) 107-109.

[9] A. Fairlamb, D.C. Warhurst, and W. Peters, Ann. Trop. Med. Parasitol. 79 (1985) 379-384.

[10] S. Matsueda, and Y. Katsukara, Che. Ind. 12 (1985 411.

[11 B.Voigt, J. Schmidt, and G. Adam, Z. Chem. 25 (1985) 40406.

[12] M.T. Makler, and D.J. Hinrichs, The American Journal of Tropical Medicine and Hygiene, 48 (1993) 205-210.

[13] G.A. Cordell, C.W. Beecher, J.M. Pezzuto, J. Ethnopharmacol. 32 (1991) 117-133.

[14] G.M. Cragg, M.R Boyd, J.H. Casrdellina II, D.J. Newmann, K.M. Snader, T.G. McCloud, Ethnobotany and Search for New Drugs, Ciba Foundation Symposium 185.Wiley, Chichester, (1994) 178-196.

[15] T. Mosman, J. Immunol. Methods, 65 (1983) 55-63.