The Antiplasmodial Activity of Isolates from *Ajuga remota*

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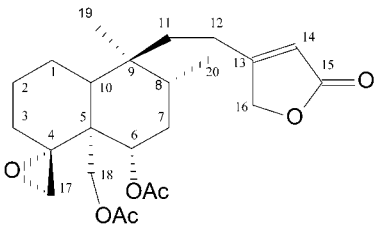
Ajuga remota is the most frequently used medicinal herb for malaria treatment in Kenya. Its two known isolates ajugarin-1 (**1**) and ergosterol-5,8-endoperoxide (**3**) and a new isolate 8-*O*-acetylharpagide (**2**) were evaluated for their *in vitro* antiplasmodial activity. Ajugarin-1 was moderately active, with an IC_{50} of 23.0 ± 3.0 μM, as compared to chloroquine (IC_{50} = 0.041 ± 0.003 μM) against the chloroquine-sensitive (FCA 20/GHA) strain of *Plasmodium falciparum*. Ergosterol-5,8-endoperoxide was about 3× as potent (IC_{50} = 8.2 ± 1.1 μM), while 8-*O*-acetylharpagide, whose structure was established by spectroscopic evidence, was inactive. Both ajugarin-1 and ergosterol-5,8-endoperoxide did not exhibit cytotoxicity against A431 (skin carcinoma) cell line, but 8-*O*-acetylharpagide was significantly cytotoxic. This iridoid glucoside, which has been formerly isolated from *Ajuga decumbens*, was identified in *A. remota* for the first time.

Malaria is a major risk for more than 2 billion human beings on earth currently,[1] and it is responsible for between 1.5 and 2.7 million deaths each year: an average of one person (often a child aged less than 5 years) every 12 s. In addition, 300 to 500 million people contract the disease each year, with unmeasured impact on local economies, human health in general, and longevity. In Africa alone, more than a million children less than 5 years old die of malaria each year. In Kenya, the disease accounts for 30% of all the outpatient cases and 19% of all admissions, 5.1% of whom die, and 72 children die daily before the age of 5 years.[1−3] Accessibility to traditional herbal medicine practices in Kenya is easy both in the rural and urban areas, as a consequence of which many patients visit the herbalists' practices for the treatment of malaria.

The declining efficacy of classical medication in relation to the rapid extension of *Plasmodium falciparum* chloroquine-resistant strains means that the development of new efficient antimalarial drugs is a real emergency.[4,5] Our attention was focused upon *Ajuga remota* Benth (Labiatae), the herb that is most frequently prescribed for malaria by the herbalists.[6] The purpose of our work is to link "field" practice with laboratory investigation in order to confirm possible interesting antiplasmodial properties of *A. remota*. Finding clinically useful antiplasmodial compounds in plant extracts could provide significant medical and economic benefits. The current study describes the isolation of **1**, ajugarin-1 (a known isolate), and of **2**, 8-*O*-acetylharpagide (a new isolate), and the synthesis of **3**, ergosterol-5,8-endoperoxide (a known isolate), followed by their *in vitro* assessment for antiplasmodial and cytotoxic activities. The iridoid glucoside **2** has been formerly isolated from *Ajuga decumbens* and was identified in *A. remota* for the first time.



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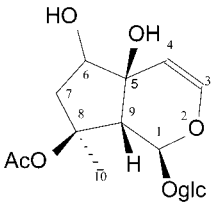

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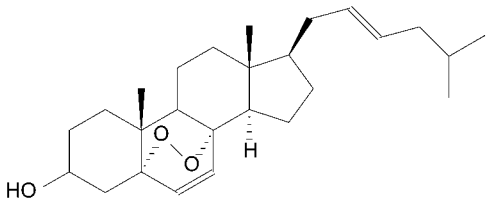

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Selection of *A. remota* for this study was based on its reputed use in traditional medicine in Kenya.[6] The isolated ajugarin-1 was identified mainly by LCMS and NMR spectroscopy and by comparison with previously published data.[7] The NMR data (Table 1) presented here are now much more complete and agree otherwise very well with the published values.[7] The structure was deduced in a completely independent way from ^1H and ^{13}C spectra, using 2D experiments such as COSY spectra (homonuclear correlation spectroscopy) and inverse heteronuclear C,H-correlation spectra (GHSQC and GHMBC, see also for 8-*O*-acetylharpagide) for making unambiguous assignments. Overlapping signals could be unraveled by 1D-TOCSY

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Table 1. ^{13}C and ^{1}H NMR Data for Ajugarin-1 in $CDCl_3$ Solution

no.	^{13}C	^{1}H (multiplicity, *J*'s)[a]
1	21.00	A: 1.653 (dq, $J = 4.7$ and 13.5 Hz)
		B: 1.561 (qd, $J = 3.3$ and 14.0 Hz)
2	25.03	A: 1.992 (ttd, $J = 2.3$, 4.6 and 13.4 Hz)
		B: 1.425 (tq, $J = 4.6$ and 13.3 Hz)
3	32.53	A: 2.148 (ddt, $J = 2.5$, 4.2 and 13.4 Hz)
		B: 1.060 (ddd, $J = 2.1$, 2.8 and 13.2 Hz)
4	64.98	
5	45.15	
6	72.16	4.710 (dd, $J = 4.9$ and 10.6 Hz)
7	32.90	A: 1.625 (obs)
		B: 1.535 (obs)
8	34.72	1.54 (obs)
9	38.33	
10	48.15	1.412 (dd, $J = 3.5$ and 12.6 Hz)
11	34.68	1.59 (2H, obs)
12	21.91	A: 2.271 (dddd, $J = 1.5$, 4.5, 13.3 and 17.1 Hz)
		B: 2.100 (dddd, $J = 1.7$, 5.2, 12.5 and 17.0 Hz)
13	169.64	
14	115.41	5.842 (p, $J = 1.7$ Hz)
15	173.55	
16	72.89	: 4.741 (2H, d, $J = 1.8$ Hz)
17	48.46	A: 2.997 (dd, $J = 2.3$ and 4.0 Hz).
		B: 2.224 (d, $J = 4.0$ Hz)
18	61.77	A: 4.831 (d, $J = 12.1$ Hz)
		B: 4.376 (d, $J = 12.1$ Hz)
19	17.31	0.781 (s, CH_3)
20	15.27	0.838 (d, $J = 6.2$ Hz, CH_3)
18-OAc	21.14	2.107 (s, CH_3)
	170.79	
6-OAc	21.10	1.958 (s, CH_3)
	169.98	

[a] Multiplicities are indicated as singlets (s), doublets (d), triplets (t), quadruplets (q), pentuplets (p), or obscured by overlapping (obs).

experiments (total correlation spectroscopy) with selective excitation. Configurations and steric problems could be solved by considering the vicinal H,H-coupling constants or by performing 1D-NOEDIF (nuclear Overhauser difference spectra) experiments which correlate spatially close protons and, thus, give information about the stereostructure of the compound.

Ajugarin-1 caused a concentration-dependent growth inhibition of the chloroquine-sensitive strain of *P. falciparum* (FCA 20/GHA). It had an IC_{50} of 23.0 ± 3.0 μM as compared to 0.041 ± 0.003 μM for chloroquine as a positive control. Cytotoxicity test of ajugarin-1 against the human skin carcinoma cell line (A431) did not show any detectable cytotoxicity even at a concentration of 88 μM, which was also the highest test concentration used for antiplasmodial activity. Its ED_{50} was therefore >88 μM. The antineoplastic agent, fluorouracil, functioned well as a positive control. It caused a concentration-dependent inhibition of cell proliferation with an ED_{50} of 44 μM.

Ajugarin-1 is interesting in view of its unusual chemical structure (epoxide ring) when compared to conventional antimalarial compounds. Such a compound can be considered as a lead compound to synthesize new pharmaceutically important derivatives with possibly higher antimalarial activities.

The second isolated and purified substance was identified as 8-*O*-acetylharpagide, a compound that has already been isolated before from *A. decumbens* and shown to be a potent antitumor promoter (a potential cancer chemopreventive agent).[8] The detailed structure of the compound was deduced from NMR, using the usual 2D techniques for unambiguous assignment of all the peaks and couplings (see Table 2). The presence of a β-glucose moiety was clearly indicated by its typical ^{13}C and ^{1}H responses and

Table 2. ^{13}C and ^{1}H NMR Data for 8-*O*-Acetylharpagide in D_2O Solution

no.	^{13}C	^{1}H (multiplicity, *J*'s)[a]
1′	101.38	4.751 (d, $J = 8.3$ Hz)
2′	75.21	3.331 (t, $J = 8.7$ Hz)
3′	78.16	3.513 (t, $J = 9.0$ Hz)
4′	72.32	3.410 (t, $J = 9.2$ Hz)
5′	78.84	3.490 (dd, $J = 2$ and obs)
6′	63.39	A: 3.943 (dd, $J = 2.0$ and 12.6 Hz)
		B: 3.750 (dd, $J = 5.9$ and 12.6 Hz)
1	96.57	6.076 (d, $J = 0.8$ Hz)
3	145.32	6.456 (d, $J = 6.4$ Hz)
4	107.59	5.009 (dd, $J = 1.5$ and 6.4 Hz)
5	74.98	
6	78.96	3.841 (d, $J = 4.1$ Hz)
7	47.02	A: 2.173 (d, $J = 15.9$ Hz).
		B: 2.026 (dd, $J = 4.2$ and 15.9 Hz)
8	90.58	
9	55.83	2.864 (s)
10	23.97	1.450 (s, Me)
OAc	24.36	2.059 (s, Me)
	176.84	
2′-OH		4.954 (d, $J = 4.1$ Hz)[b]
3′-OH/4′-OH		4.914 (m, 2H)[b]
6′-OH		4.416 (t, $J = 5.9$ Hz)[b]
6′-OH		4.31 (d, $J = 2.6$ Hz)[b]
5′-OH		4.49 (s)[b]

[a] d = doublet, t = triplet, s = singlet, and obs = obscured by overlapping. [b] As measured in DMSO-d_6 solution.

confirmed by a 1D-TOCSY experiment with selective excitation of the anomeric proton H-1′ at 4.751 ppm. The large coupling of this proton (8.0 Hz) indicated that the glucose unit was present in the β-configuration. The other signals and coupling patterns of the ^{1}H NMR spectra were elucidated by 2D-correlated spectroscopy (COSY) or by homonuclear decoupling techniques. The ^{13}C assignments leading to a molecular formula of $C_{17}H_{26}O_{11}$ were made by edited DEPT spectra for differentiation of CH-, CH_2-, and CH_3-carbons and by using inverse 2D-heterocorrelation experiments such as GHSQC (gradient heteronuclear single quantum correlation spectroscopy) and GHMBC (gradient heteronuclear multiple bond correlation spectroscopy) to indicate one-bond H,C-correlations or two-bond and three-bond H,C-correlations, respectively. In this way it was proven that the glucose unit was attached glycosidically to C-1 of the aglycone and also that the acetyl group was located at 8-OH. By taking the spectra in DMSO-d_6 we were also able to directly observe the six free hydroxyl protons and to locate them on the molecule. Taking all these data together, the structure of the compound was proven to be 8-*O*-acetylharpagide. The carbon spectrum (but not the ^{1}H spectrum) had been already published by Chaudhuri et al.,[9] and the values and assignments corresponded very well with our data, confirming the proposed structure.

The iridoid glucoside **2**, which we isolated for the first time from the East African *A. remota*, did not exhibit any antiplasmodial activity even at the highest concentration of about 500 μM used against the chloroquine-sensitive strain of *P. falciparum* (FCA 20/GHA). However, the compound exhibited in vitro cytotoxicity against the A431 human cell line. It caused a concentration-dependent inhibition of cell proliferation with an ED_{50} of 310 ± 116 μM, which is indicated to be approximately 7× less potent than the standard antineoplastic agent (fluorouracil) used in the test against the A431 human skin carcinoma cell line.

Ergosterol-5,8-endoperoxide was isolated for the first time from the aerial parts of *A. remota* by Cantrell et al.[10] and shown to have some potent antimycobacterial activity.

Table 3. Antiplasmodial and Cytotoxic Activity of the Various *A. remota* Isolates against *P. falciparum* (FCA 20/GHA) and Human Skin Carcinoma (A431) Cell Line (mean ±SD where $n \geq 6$)

A. remota isolate	antiplasmodial activity (FCA 20/GHA)[a] IC_{50} (μM) [max. conc tested]	cytotoxicity (A431) ED_{50} (μM) [max. conc tested]	cytotoxicity/ antiplasmodial activity ratio
ajugarin-1	23.0 ± 3.0 [88]	>88 [88]	≫4
8-*O*-acetylharpagide	NIL [500]	310 ± 116 [500]	
ergosterol-5,8-endoperoxide	8.2 ± 1.1 [23]	>23 [23]	≫3

[a] Chloroquine $IC_{50} = 0.041 \pm 0.003$ μM.

For the purpose of the present studies, the compound was synthesized starting from ergosterol by using the method described by the same investigators with some modification as follows: the packed VLC column was eluted with 50 mL portions of chloroform and gradient mixtures of chloroform and ethyl acetate instead of propyl acetate. Pure ergosterol-5,8-endoperoxide (a white crystalline powder: 70 mg) eluted with chloroform−ethyl acetate (90:10). It was identified mainly by comparison of MS and NMR spectral data with previously reported values.[11,12] The ergosterol-5,8-endoperoxide caused a concentration-dependent growth inhibition of the chloroquine-sensitive strain of *P. falciparum* (FCA 20/GHA) with an IC_{50} of 8.2 ± 1.1 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, 2001]. Compared to ajugarin 1, ergosterol-5,8-endoperoxide is indicated to be approximately 3× as potent.

A cytotoxicity test of ergosterol-5,8-endoperoxide against the human skin carcinoma cell line (A431) did not show any detectable cytotoxicity at a concentration of 23 μM, which was also the highest test concentration used for antiplasmodial activity. The ED_{50} is therefore >23 μM, and the cytotoxicity/antiplasmodial activity ratio is therefore >3, implying ergosterol-5,8-endoperoxide to be nontoxic at the tested dose levels (Table 3). Ergosterol-5,8-endoperoxide, which is shown here for the first time to have more antiplasmodial activity with reference to the other *A. remota* isolates tested, could serve as a template in search of new antimalarial drugs.

Endoperoxydes such as artemisinin are being used widely in areas with multidrug-resistant *P. falciparum* malaria such as Southeast Asia and increasingly in Africa.[13,14] Heme or Fe^{2+} catalyzes the opening of the peroxide bridge in artemisinin, leading to the formation of free radicals, killing the parasite.[15] In the case of the steroid derivative ergosterol-5,8-endoperoxide, still other mechanisms might be involved. Dehydroepiandrosterone (DHEAS) contributes to the development of resistance against *P. falciparum*. Increased DHEAS was associated with a lower mean parasitemia, a lower frequency of parasitemia, and longer time to reappearance of parasitemia.[16] The androstane-regulating compound HE2000 has been used to correct the immune system dysregulation in HIV-positive patients. It also showed benefits in preclinical studies as compared to chloroquine against chloroquine-sensitive and chloroquine-resistant *P. falciparum* strains. It will be further tested for its prophylactic and therapeutic potential.[17,18]

Ethnopharmacological use of plants can be a basis for phytochemical and phytopharmacological investigation. Isolation of pure compounds is useful in the standardization of herbal medicinal preparations. Recent work on African plants used in the treatment of malaria is very encouraging. It is striking how many different plants are reported by herbalists to cure malaria. When we compare antimalarial species being used in Kenya with those recently reported to be used in Ivory Coast, Madagascar, and Sudan, only *Azadirachta indica* (Neem tree) is shared.[19−21] The challenge will be to translate herbal medicine practice with these plants into an evidence-based monotherapy or combined therapy as suggested by Rasoanaivo et al.[20]

The case of *A. remota* is one of the many examples to consider because everything starts with the use of unpurified 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. Nevertheless, pharmacological evaluation should always go hand in hand with the search for pure compounds. In the future, isolation and testing of other possible isolates of *A. remota* will be performed. In this testing immunogenicity will be one of the points of interest.

Experimental Section

General Experimental Methods. UV spectra were run on a Philips PU 8740 UV/vis scanning spectrophotometer. HPLC was carried out with a LC system consisting of a L-6200 intelligent pump (Merck-Hitachi, Darmstadt, Germany), an Autosampler SpectraSeries AS 100 (Thermo Separation Products, Freemont, CA), a variable-wavelength spectra 100 UV−vis detector (Thermo Separation Products, Freemont, CA) set at 211 nm, and a Hewlett-Packard integrator model HP 3396A series (Hewlett-Packard, Avondale, PA). A Hypersil BDS C-18 5 μm (100 × 4.6 mm i.d.) (Shandon, Runcorn, UK) column maintained at 30 °C was used for Ajugarin-1, while a Hypersil BDS C-18 3 μm (100 × 4.0 mm i.d.) (Shandon, Runcorn, UK) column maintained at 50 °C was used for 8-*O*-acetylharpagide. The mobile phase (acetonitrile−0.2 M potassium phosphate buffer pH 4.3−water [5:10:85] for 8-*O*-acetylharpagide and acetonitrile−water [35:65] for Ajugarin-1) was delivered at a rate of 1.0 mL/min. LCMS spectra were run using a Finnigan LCQ ion trap mass spectrometer (Finnigan MAT, San Jose, CA) with an Atmospheric Pressure Chemical Ionization probe (APCI probe) in the positive ion mode. The LC apparatus consisted of a SpectraSYSTEM P1000XR quaternary pump, a SpectraSERIES AS100 autosampler equipped with a 20 μL loop, and a variable-wavelength spectra 100 UV−vis detector set at 215 nm, all from Thermo Separation Products (Freemont, CA), and an integrator Model 3390A (Hewlett-Packard, Avondale, PA). The XTerra RP C18 column (3.2 μm, 100 × 2.1 mm i.d.) (Waters, Milford, MA) was immersed in a waterbath at 30 °C. The mobile phase used was methanol−water (50:50), and the sample was dissolved in methanol. ^{1}H and ^{13}C NMR spectra were recorded with a Varian UNITY-500 spectrometer operating at 125.591 MHZ for ^{13}C and at 499.40 MHZ for ^{1}H NMR and using the standard pulse sequences and software (VNMR version 6.1b). Spectra were obtained at 27 °C in 5 mm tubes in the solvent indicated, and the chemical shifts are reported in ppm relative to tetramethylsilane (TMS) as internal reference or in the absence of TMS to the HOD resonance in D_2O set at 4.70 ppm.

Plant Material. Authenticated *Ajuga remota* Benth plant material was collected from Chiromo campus grounds (University of Nairobi) in October and November 1999, and a voucher specimen has been deposited in the herbarium of the

Faculty of Pharmacy, University of Nairobi, Kenya (deposit number 1999/11/1/Aj/pharmNK). The fresh aerial parts were dried in the open air and reduced to powder by use of a mortar and pestle, and after being sifted on the spot, the powder was sifted once more in the laboratory using sieve no. 18 (PS Standard Sieve Series, Chicago, IL).

***In Vitro* Parasite Cultures.** *In vitro* cultures of *P. falciparum* (chloroquine-sensitive FCA/20GHA strain) were maintained at 1% parasitemia in 1% human erythrocytes (O^+ or A^-) suspended in complete RPMI 1640 medium (ICN Biomedicals Inc., Asse-Relegem, Belgium) with L-glutamine without sodium bicarbonate and 10% human serum or plasma (A^+ or A^-) supplemented with D-glucose, 1.77 g/L (UCB, Belgium) $NaHCO_3$, 2.0 g/L (ICN Biomedicals Inc. Asse-Relegam, Belgium), and HEPES buffer 8.3 g/L (ICN Biomedicals Inc. Asse-Relegam, Belgium).

***In Vitro* Cell Cultures.** Toxicity was evaluated *in vitro* in one human cell line (A431, skin carcinoma) obtained from the American Type Culture Collection (Rockville, MD). The cell line was grown at 37 °C in humidified 5% CO_2 and 95% air atmosphere in minimum essential medium (MEM) with Earl's salt containing 2 mM L-glutamine, nonessential amino acids (100×), penicillin (100 IU/mL), streptomycin (100 μg/mL), tyrocin (60 μg/mL), amphotericin B (0.25 μg/mL), and 10% fetal calf serum (FCS) all obtained from Gibco BRL, UK.

Extraction and Isolation. Extraction of Ajugarin-1. *A. remota* aerial parts powder was extracted with chloroform. The extract thereof was 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 and spectroscopically identified as ajugarin-1.

Extraction of 8-*O*-Acetylharpagide. Aerial dried plant material (100 g) was macerated with 2 L of 80% v/v ethanol for 5 days. The extract was evaporated and redissolved in 100 mL of distilled water and partitioned between chloroform (BDH), ethyl acetate (BDH), and *n*-butanol (BDH) (5 × 100 mL portions each). TLC of the *n*-butanol fraction (major 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 sulfuric acid, giving a brick reddish spot. The *n*-butanol fraction was dissolved in a minimum amount of methanol and adsorbed onto silica gel for column chromatography. The methanol was then removed under vacuum at 40 °C to yield a free-flowing powder. This powder was then added to the top of a column packed with silica gel and eluted with ethyl acetate−methanol (70:30), and the combined fractions were combined and further purified by shaking it with a small quantity of activated charcoal. The fraction yielded an amorphous powder. TLC of the powder in methanol using ethyl acetate−methanol (60:40) as the mobile phase revealed it to be one compound when sprayed with concentrated sulfuric acid having an R_f 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-*O*-acetylharpagide through MS and NMR spectroscopy.

Ajugarin-1: white crystalline powder; mp 155−157 °C; UV absorption maxima 211 ± 1 nm in acetonitrile−water (1:1). The compound had a purity > 99% as determined by LC. Its molecular weight of 434 (M+), formula ($C_{24}H_{34}O_7$), and stereostructure were determined on the basis of LCMS and NMR spectra.

8-*O*-acetylharpagide: amorphous white powder, mp 225−228 °C; UV absorption maxima at 196 nm in distilled water. TLC on silica gel 60 F254 (Merk) using ethyl acetate−methanol (90:10) as the mobile phase revealed the compound after spraying with concentrated sulfuric acid as a brick reddish spot. The compound had a purity > 98% as determined by LC. Its molecular weight of 406 (M+) and structure were determined on the basis of MS and NMR spectra.

Synthesis of Ergosterol-5,8-endoperoxide. Ergosterol (200 mg) and methylene blue (20 mg) were dissolved in 50 mL of dichloromethane and placed in a 100 mL three-necked flask. Oxygen was bubbled through the ice-cooled solution under constant magnetic stirring, and the solution was irradiated with a tungsten filament light (100 W) placed 2 feet above the flask. This corresponds to the method of Cantrell et al.[10] with slight modifications for chromatography. The reaction was monitored by TLC (chloroform−ethyl acetate, 95:5). Completion of the reaction after 2 h was shown by spraying the TLC plate with concentrated sulfuric acid. 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 (vacuum liquid chromatography) 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 evaporated under vacuum at 40 °C, yielding 70 mg of product.

Ergosterol-5,8-peroxide: white crystalline powder, mp 163−174 °C (lit. mp 171−174 °C); $[\alpha]^{20}_D$ −25.7° (*c* 0.04, $CHCl_3$) (lit. $[\alpha]^{25}_D$ −41.9° (*c* 0.04, $CHCl_3$)).[11,12] This difference might be due to impurities, although the compound 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.[11,12]

***In Vitro* Antiplasmodial Activity Test.** The antiplasmodial activities of the isolated ajugarin-1,8-*O*-acetylharpagide and the synthesized ergosterol-5,8-endoperoxide were assessed against a chloroquine-sensitive (FCA/20GHA) and -resistant (W2) strains of *P. falciparum* maintained in continuous culture. This was done according to the method of Trager and Jensen[22] modified by Osisanya et al.[23] and Fairlamb et al.[24] Parasitemia was evaluated after 72 h, by an enzyme assay method (Malstat assay) described by Makler et al.[25] based on the measurement of the lactate dehydrogenase activity of *P. falciparum*. Antiplasmodial activity was measured by the IC_{50} representing the concentration of drug that caused a 50% decrease in the parasite lactate dehydrogenase (pLDH) activity compared with the control culture, and chloroquine was tested for standard antiplasmodial activity.

***In Vitro* Cytotoxic Activity Test.** Cellular viability in the presence and absence of experimental agents was determined using the standard 3-(4,5-dimethylthiazo-2,5-dimethyltetrazolium bromide (MTT- Sigma) microtiter assay.[26] Cytotoxicity was measured by the ED_{50} representing the concentration of drug that caused a 50% decrease in cell proliferation compared with the control cell culture, and the antineoplastic agent, fluorouracil (Pharmacia & Upjohn N.V./S.A. Rijksweg, Puurs, Belgium), was tested for standard cytotoxic activity.

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