Anti-plasmodial Activity of Some Constituents of the Root Bark of *Harungana madagascariensis* Lam. (Hypericaceae)

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Bazouanthrone (1), a new anthrone derivative, has been isolated from the root bark of *Harungana madagascariensis*, together with known compounds, feruginin A (2), harunganin (3), harunganol A (4), harunganol B (5), friedelan-3-one (6) and betulinic acid (7). The structure of the compound (1) was assigned as 3,5,8,9-tetrahydroxy-2,4,4-tri-(3,3-dimethylallyl)-6-methyl-1-(4*H*)-anthracenone, by means of spectroscopic analysis. The anti-plasmodial activity of the isolated compounds was evaluated in culture against W2 strain of *Plasmodium falciparum*. All the compounds were found to be active against the *Plasmodium* parasites with bazouanthrone (1) showing particular potency (IC_{50}=1.80 μM).

Key words *Harungana madagascariensis*; Hypericaceae; anthrone; *Plasmodium falciparum*; anti-plasmodial

Malaria remains the one of the most important infectious diseases in the world. It constitutes a public health problem in more than 90 countries, inhabited by about 40% of the world's population. The World Health Organisation estimates that there are 300—500 million malaria cases annually, causing 2—3 million deaths, mostly in children under five years old.[1)] Africa accounts for over 90% of malaria mortality.[1)] Malaria also has significant economic impacts in endemic countries, costing Africa $ 12 billon in lost gross domestic product every year and consuming 40% of all public health spending.[2)] Most countries where *Plasmodium falciparum* malaria, the most severe and dangerous form of this disease, is endemic face significant parasite resistance to frequently used antimalarial drugs, in particular chloroquine and antifolates. Furthermore, resistance to artemisinins, which are the most promising new antimalarials, has been induced in rodent malarial models[3)] and identified rarely in clinical isolates. If resistance to artemisinins emerges, no drug will be available to reliably offer protection against malaria in all parts in the world and the efficacy of new artemisinin-based combination therapies (ACTs), which are highly effective, but expensive, will be jeopardized. For these reasons, new effective and affordable antimalarials are badly needed.[4)] In this regard research on traditional medicinal plants for their antimalarial constituents is important, to facilitate utilization of available botanical resources and to provide potentially active lead anti-plasmodial compounds, perhaps with new mechanisms of action. In Africa, the use of indigenous plants still plays an important role in the treatment of malaria.[5,6)]

Some anthrone derivatives from plant sources have demonstrated interesting anti-plasmodial activity.[7,8)] They are also naturally occurring competive inhibitors of adenosine–triphosphate-citrate-lyase.[9)] Plants of the Hypericaceae family are well known to produce bioactive anthraquinones and anthrones. They are distributed in tropical and sub-tropical regions.[10)] *Harungana madagascariensis*, a plant from this family, is used for various medicinal purposes in Africa.[11,12)] Leaf extract of this plant exhibited antibacterial activity.[13)] In the West province of Cameroon, the roots and bark are used by traditional healers to treat malaria. Various phenolic compounds have been isolated from this plant, among them anthraquinones, xanthones, biflavonoids, coumarins and anthrone derivatives.[14—16)] These compounds have demonstrated a wide range of biological activities.[16—18)]

Although some phytochemical and pharmacological studies have been carried out on *H. madagascariensis*, no investigation has been reported, to the best of our knowledge, on the anti-plasmodial activity of its constituents. In our continuing interest in new anti-plasmodial metabolites from plant sources, we investigated the MeOH–CH_2Cl_2 (1 : 1) root bark extract of this plant which showed anti-plasmodial activity (IC_{50}=25.12 μg/ml) against W2 strain *P. falciparum*. In this paper, we report the isolation and structure elucidation of a new anthrone derivative and the anti-plasmodial activity of the isolated compounds.

The air dried root bark of *Harungana madagascariensis* was extracted at room temperature with a mixture of MeOH–CH_2Cl_2 (1 : 1). The extract was concentrated to dryness under vacuum and the residue subjected to repeated column chromatography to yield compound (**1**) along with the known feruginin A (**2**), harunganin (**3**), harunganol A (**4**), harunganol B (**5**), friedelan-3-one (**6**) and betulinic acid (**7**). Compound (**1**) was obtained as an orange powder, mp 178—179 °C. It gave positive ferric chloride and Gibbs tests, indicating its phenolic nature. The molecular formula $C_{30}H_{36}O_5$ was deduced from HR-ESI-TOF mass spectrometry which showed a pseudomolecular ion peak at *m*/*z* 477.6089, corresponding to 15 degrees of unsaturation. The IR spectrum exhibited strong absorption bands due to conjugated carbonyl (1615 cm^{-1}) and chelated hydroxyl group(s) (3261 cm^{-1}). The UV absorptions at λ_{max} 208, 236, 263, 388 nm, combined with IR spectral data, were suggestive of an anthranoid

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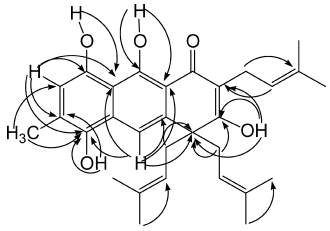
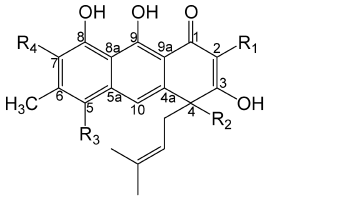


Fig. 1. HMBC Correlations in Bazouanthrone (**1**)



1: R_1= Prenyl, R_2= Prenyl, R_3=OH, R_4=H
2: R_1= H, R_2= Prenyl, R_3=H, R_4=Prenyl
3: R_1= H, R_2= Prenyl, R_3=Prenyl, R_4=H
4: R_1= H, R_2= H, R_3=Prenyl, R_4=Prenyl
5: R_1= H, R_2= H, R_3=Prenyl, R_4=H

Fig. 2. Structure of Compounds **1**—**5**

skeleton.[19,20] The broad band decoupled ^{13}C-NMR spectrum of compound (**1**) (Table 1) displayed 30 carbon signals, which were sorted by Jmod and HSQC techniques into seven methyls, three methylenes, five methines and fifteen quaternary carbons, including a carbonyl group at δ 192.0. The ^{1}H-NMR spectrum of (**1**) revealed signals for two chelated hydroxyl groups at δ 9.90 and 16.20, two D_2O exchangeable protons as broad singlets at δ 12.20 and 5.70, two aromatic protons at δ 6.79 (1H, s) and 7.40 (1H, s) and one aromatic methyl group at δ 2.39 (3H, s). The downfield chemical shift of the highly chelated hydroxyl is characteristic for an 8,9-dihydroxyanthrone structure.[19—21] The ^{1}H-NMR spectrum of (**1**) also showed characteristic resonances of three 3,3-dimethylallyl moieties [two triplets for olefinic protons at δ 4.61 (2H, J=7.2 Hz), 5.05 (1H, J=7.2 Hz), a doublet at δ 3.65 (1H, J=7.2 Hz, one of methylene protons), two doublets of doublets for methylene diastereotopic protons at δ 2.99 (Ha, Ha′, J=6.8, 13.6 Hz) and 2.66 (Hb, Hb′, J=6.8, 13.4 Hz), and four singlets for olefinic methyls at δ 1.44, 1.45 (2×3H, each), 1.65, 1.90]. This was further supported by ^{13}C-NMR signals (Table 1) indicative of three methylenes δ 27.5, 40.7 (×2) , three methines δ 118.4 (×2), 123.4 and six methyls δ 17.9, 18.8 (×2), 25.5, 25.6 (×2). This spectrum also showed the presence of an aromatic methyl group at δ 27.5.

The HMBC spectrum of (**1**) showed correlations between the methyl protons at δ 2.39 and C-7 (δ 112.8) and C-5 (δ 140.3), suggesting its location at C-6 (δ 125.3), as expected on biogenetic grounds.[22] The methylene protons of two prenyl groups at δ 2.99 and 2.66 showed cross peaks with the saturated carbon at δ 50.5 (C-4), suggesting clearly their attachment to C-4. The proton of the free hydroxyl group at δ 5.70 had cross peaks with C-2 (δ 110.0), C-3 (δ 181.0), C-4 (δ 50.5) and the two aromatic protons at δ 6.79 (1H, s) and 7.40 (1H, s) correlated with C-8 (δ 154.9), C-6 (δ 125.3), C-5 (δ 140.3) and C-8a (δ 110.0), C-9a (δ 108.0), C-5 (δ 140.3), C-4a (δ 139.0), respectively. All these findings

Table 1. ^{13}C- and ^{1}H-NMR of Bazouanthrone **1** in $CDCl_3$

No.	^{13}C[a] (m)	^{1}H[b] (m, J: Hz)	HMBC correlations
1	192.0 (s)	—	—
2	110.0 (s)	—	—
3	181.0 (s)	—	—
4	50.05 (s)	—	—
4a	139.0 (s)	—	—
5	140.3 (s)	—	—
6	125.3 (s)	—	—
7	112.8 (d)	6.79 (s)	C-6, C-8, C-5
8	154.9 (s)	—	—
8a	111.0 (s)	—	—
9	162.0 (s)	—	—
9a	108.0 (s)	—	—
10	112.7 (d)	7.40 (s)	C-4a, C-10a, C-5
10a	112.2 (s)		
11, 11′	40.7 (t)	2.66 (dd, 6.8, 13.4, H-11b, 11′b)	C-3, C-4, C-13
		2.99 (dd, 6.8, 13.6, H-11a, 11′a)	C-4, C-4a, C-12′, C-13′
12, 12′	118.4 (d)	4.61 (t, 7.2)	C-4, C-13
13, 13′	134.2 (s)	—	—
14, 14′	18.8 (q)	1.44 (s)	C-12, C-13
15, 15′	25.6 (q)	1.45 (s)	C-12, C-13,
16	27.5 (t)	3.65 (d, 6.0)	C-2, C-19
17	123.4 (d)	5.05 (t, 7.2)	C-2
18	131.3 (s)	—	—
19	17.9 (q)	1.65 (s)	C-19
20	25.5 (q)	1.90 (s)	C-18
21	20.8 (q)	2.39 (s)	C-7, C-5
3-OH	—	5.70 (s)	C-2, C-3
5-OH	—	12.20 (s)	C-6
8-OH	—	9.90 (s)	C-8a
9-OH	—	16.20 (s)	C-8, C-9a, C-9

a) ^{13}C-NMR carried out at 100.6 MHz. *b*) ^{1}H-NMR carried out at 400.13 MHz.

clearly indicate that the hydroxyl group (δ 5.70) is located at C-3 (δ 180.0) and the two aromatic protons at C-7 (δ 112.8) and C-10 (δ 112.7). The position of the last free hydroxyl group was deduced to be C-5 (δ 140.3) from the cross peak between the broad singlet proton at δ 12.20 (1H, exchangeable to D_2O) and C-5. Thus the structure of **1** was characterized as 3,5,8,9-tetrahydroxy-2,4,4-tri-(3,3-dimethylallyl)-6-methyl-1-(4*H*)-anthracenone. The compound was given the trivial name bazouanthrone. In addition to **1**, six known compounds were isolated and identified as ferruginin A(**2**), harunganin (**3**), harunganol A (**4**), harunganol B (**5**), friedelan-3-one (**6**) and betulinic acid (**7**).[14,19,20,23,24]

Test compounds (**1**—**7**) showed toxicity to erythrocytes at concentrations above 20 mM, about three orders of magnitude above concentrations with antimalarial activity. Compounds were tested for their antiplasmodial activity against the W2 strain of *P. falciparum*, which is resistant to chloroquine and other antimalarials (Table 2). These compounds exhibited good anti-plasmodial activities, with bazouanthrone (**1**) showing the best potency (IC_{50}=1.80 μM). Concerning the anthranoids, compounds **1**—**3** and **5** possess three prenyl groups, against two for compound **4**; all of these compounds had IC_{50}<8 μM against cultured parasites.

Based on skeletal features, the isolated anthranoids can be classified into two groups: i) compounds **1**—**3** and ii) compounds **4** and **5**. In the first group, the position of the prenyl groups is critical for the activity levels. Indeed, it appears from the results that compound **1** (the most active) has three prenyl groups at C-2 and C-4. In addition, it bears a hydroxyl at C-5. The activity of compound **1** (IC_{50}=1.80 μM) is 1.5

Table 2. Anti-plasmodial Activities of Compounds **1**—**7** against W2 Strain *P. falciparum*

Compound	IC_{50} (μM)	Erythrocyte susceptibility
Bazouanthrone (**1**)	1.80	
Ferruginin A (**2**)	5.00	
Harunganin (**3**)	2.70	
Harunganol A (**4**)	3.70	>20 mM
Harunganol B (**5**)	3.70	
Friedelan-3-one (**6**)	7.70	
Betulinic acid (**7**)	5.10 (0.04[a], 0.06[b])[30]	
Chloroquine	0.13	
Extract	25.12 μg/ml	

a) Against chloroquine resistant (K1) *P. falciparum*. *b*) Against chloroquine sensitive (T9-96) *P. falciparum*.

times greater than that of compound **3** (IC_{50}=2.7 μM) in which the prenyl at C-2 was transferred at C-5 in substitution of the hydroxyl.

Comparatively to **2** (IC_{50}=5.00 μM) in which the prenyl at C-5 was transferred at C-7, the activity of compound **3** is about 1.9 times greater. This indicates that the prenyl at C-2, or the hydroxyl at C-5 or both are important for the enhancement of the activity. But at this stage, the interacting effects of the prenyl and hydroxyl groups (cumulative or synergistic) in **1** remain unknown.

In the second group, it appears that the prenyl at C-7 has no influence on the activity level, since both compounds (**4**, **5**) have the same potency (IC_{50}=3.7 μM).

Structurally closely related anthranoids have already been evaluated for anti-plasmodial activity, with demonstration of high potencies.[25] Of note, Vismione H isolated from *Vismia guineensis* was found to be very active *in vitro* against *P. falciparum* (NF 54, clone A1A9), with an IC_{50} of *ca.* 0.23 μM. From these prior results, it appears that multiple prenylations are not the only requirements for strong anti-plasmodial activity, as was demonstrated by the potency of Vismione H.[25]

The two triterpenoids (**6**, **7**) evaluated in this work displayed moderate activities (IC_{50}s of 7.7 μM and 5.1 μM respectively). Betulinic acid has already been evaluated for antiplasmodial activity in a previous work.[30] Structurally related compounds (lupeol fatty acid ester derivatives) were recently evaluated by Fotie *et al.* (2006) against chloroquine sensitive 3D7 and resistant FCR-3 strains of *P. falciparum*. They demonstrated only modest antiplasmodial activities (IC_{50}s>105 μM against 3D7 and IC_{50}s>83 μM against FCR-3).[26]

The interesting results obtained in this work with anthranoids highlight this class of compounds as a potential source for the discovery of new leads for antimalarial drug development

Experimental

General Melting points were determined on a Buchi melting point apparatus B-545. UV spectra were determined on a Shimadzu spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were run on a Bruker spectrometer equipped with 5 mm ^{1}H and ^{13}C probes operating at 400.1 and 100.6 MHz respectively, with TMS as internal standard. Silica gel 230—400 mesh (Merck) and silica gel 70—230 mesh (Merck) were used for flash and column chromatography, while percolated aluminium silica gel 60 F_{254} sheets were used for TLC with a mixture of cyclohexane, ethyl acetate, and methanol as eluents; spots were visualised under UV lamps (254 nm) and (365 nm) or by MeOH–H_2SO_4 reagent.

Plant Material The root bark of *Harungana madagascariensis* was collected in November 2003 at Bazou in the western province of Cameroon. The plant was identified by Mr. Nana, botanist at the National Herbarium of Cameroon where a voucher specimen No 4224/SRFK-HNC has been deposited.

Extraction and Isolation Root bark of *Harungana madagascariensis* (1.8 kg) was extracted with a mixture of CH_2Cl_2/MeOH (1 : 1) at room temperature for 24 h. The solvent was evaporated under reduced pressure and the residue (83 g) was fractionated by flash chromatography over silica gel (70—230 mesh, Merck), eluting with hexane–EtOAc of increasing polarity to yield two main fractions F_1 and F_2.

Fraction F_1 (28.5 g) was subjected to successive column chromatography over silica-gel (70—230 mesh, Merck), eluting with a hexane–EtOAc mixture (85 : 15) to yield friedelan-3-one (**6**, 46 mg) and a mixture which was rechromatographed over Sephadex LH-20, eluting with methanol, to yield harunganol A (**4**, 3 mg), harunganol B (**5**, 43 mg) and harunganin (**3**, 2 mg).

Fraction F_2 (15.0 g) was subjected to flash chromatography. Forty-two fractions (200 ml) were obtained and grouped on the basis of TLC to yield three sub-fractions labelled F_{21}, F_{22}, and F_{23}. Further chromatography of fraction F_{21}, eluting with hexane–EtOAc (80 : 20), yielded harunganin (**3**, 13 mg) and betulinic acid (**7**, 80 mg). Similarly, fraction F_{22} yielded bazouanthrone (**1**, 7 mg) and ferruginin A (**2**, 4 mg) on elution with CH_2Cl_2/MeOH (8 : 2).

Bazouanthrone (**1**): Orange crystals. mp 178—179 °C. IR (KBr) cm^{-1}: 3450—3150, 2980, 1660, 1450, 1267, 1017, 868, 718. UV (EtOH) λ_{max} nm: 208, 236, 268, 388. ^{1}H- and ^{13}C-NMR, see Table 1. HR-TOF-MS *m*/*z* 477.6089 $[M+H]^+$ for $C_{30}H_{38}O_5$. EI-MS *m*/*z* (rel. int.): 207 (28), 151 (100), 69 (9).

Evaluation of Erythrocyte Susceptibility to Compounds 1—7 *in Vitro* A preliminary toxicological assessment was carried out to determine the highest drug concentrations that can be incubated with erythrocytes without any significant damage. This was done according to the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide/phenazine methosulfate (MTT/PMS) colorimetric assay described by Cedillo-Rivera *et al.* (1992),[27] with some modifications. The drugs were serially diluted in 96 well culture plates, and each concentration incubated in triplicate with erythrocytes (2% hematocrit) in a final 100 μl culture volume (at 37 °C, in a 3% O_2, 5% CO_2 and 91% N_2 atmosphere, in the presence of RPMI 1640, 25 mM HEPES, pH 7.4 for 48 h). At the end of the incubation period, the cultures were transferred into polypropylene microcentrifuge tubes and centrifuged at 1500 rpm for 5 min, and the supernatant was discarded. 1.5 ml MTT solution with 250 μg PMS were added to the pellets. Controls contained no erythrocytes. The tubes were thereafter incubated for 45 min at 37 °C, then centrifuged, and the supernatant was discarded. The pellets were re-suspended in 0.75 ml of HCl 0.04 M in isopropanol to extract and dissolve the dye (formazan) from the cells. After 5 min, the tubes were vigorously mixed and centrifuged, and the absorbance of the supernatant was determined at 570 nm.

Evaluation of Anti-plasmodial Activity *P. falciparum* strain W2, which is resistant to chloroquine and other antimalarials,[28] was cultured in sealed flasks at 37 °C, in a 3% O_2, 5% CO_2 and 91% N_2 atmosphere in RPMI 1640, 25 mM HEPES, pH 7.4, supplemented with heat inactivated 10% human serum and human erythrocytes to achieve a 2% hematocrit. Parasites were synchronized in the ring stage by serial treatment with 5% sorbitol (Sigma)[29] and studied at 1% parasitemia.

Compounds were prepared as 10 mM stock solutions in DMSO, diluted as needed for individual experiments, and tested in triplicate. The stock solutions were diluted in supplemented RPMI 1640 medium so as to have at most 0.2% DMSO in the final reaction medium. An equal volume of 1% parasitemia, 4% hematocrit culture was thereafter added and gently mixed thoroughly. Negative controls contained equal concentrations of DMSO. Positive controls contained 1 μM chloroquine phosphate (Sigma). Cultures were incubated at 37 °C for 48 h (1 parasite erythrocytic life cycle). Parasites at the ring stage were thereafter fixed by replacing the serum medium by an equal volume of 1% formaldehyde in PBS. Aliquots (50 μl) of each culture were then added to 5 ml round-bottom polystyrene tubes containing 0.5 ml 0.1% Triton X-100 and nM YOYO nuclear dye (Molecular Probes) in PBS, and parasitemias of treated and control cultures were compared using a Becton-Dickinson FACSort flow cytometer to count nucleated (parasitized) erythrocytes. Data acquisition was performed using CellQuest software. These data were normalized to percent control activity and 50% inhibitory concentrations (IC_{50}) were calculated using Prism 3.0 software (GraphPad) with data fitted by non linear regression to the variable slope sigmoidal dose–response formula, $y=100/1+10^{(\log IC_{50}-x)H}$ where H is the hill coefficient or slope factor.[28]

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