

## Antimalarial Compounds from the Stem Bark of *Vismia laurentii*

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A phytochemical study of the stem bark of *Vismia laurentii* resulted in the isolation of a tetracyclic triterpene, tirucalla-7,24-dien-3-one (**1**), and seven other known compounds: 3-geranyloxyemodin (**2**), vismiaquinone A (**3**), vismiaquinone B (**4**), bivismiaquinone (**5**), epifriedelinol (**6**), betulinic acid (**7**) and stigmasta-7,22-dien-3-ol (**8**). The structure of all these compounds was elucidated by spectroscopic means. The stem bark extract and compounds **1** and **3** showed good antimalarial activity against the W2 strain of *Plasmodium falciparum*.

*Key words:* *Vismia laurentii*, Tetracyclic Triterpene, Antimalarial Activity

### Introduction

*Vismia laurentii* De Wild. (Guttiferae) is a small tree that grows in many parts of the tropical region of Cameroon. According to Hutchinson and Dalziel (1954), this species belongs to the Vismieae tribe, subfamily Hypericoideae, family Guttiferae. The mainly fifty species of the *Vismia* genus consist of shrubs, small trees or large trees inhabiting the tropical regions of the world (Delle Monache, 1997). They are used in folk medicine as purgative, tonic or febrifugal agents and also for the treatment of skin diseases (Kerharo, 1974; Macfoy and Sama, 1983; Nagem and Faustino de Oliveira, 1997). Phytochemical investigations of some *Vismia* species led to the isolation of benzophenones, xanthenes, anthranoids and triterpenoids (Seo *et al.*, 2000; Botta *et al.*, 1983; Ngue-meving *et al.*, 2006). Cytotoxic and antifeedant activities have also been reported for some *Vismia* constituents (Seo *et al.*, 2000; Simmonds *et al.*, 1985; Hussein *et al.*, 2003). As a part of our ongoing search for novel antimalarial agents of plant origin (Ngouela *et al.*, 2006), the stem bark

of *V. laurentii* was subjected to phytochemical investigations, since hexane and ethyl acetate extracts showed good antiplasmodial activity against the W2 strain of *Plasmodium falciparum*. In the present paper, we report the isolation of eight known compounds, some of them being potential antimalarial agents.

### Material and Methods

#### *Plant material*

The stem bark of *Vismia laurentii* was collected from the banks of the Nyong River near Nkolmaka Lake (Endome) in Center Province, Cameroon on 17<sup>th</sup> of October 2004 by Mr. Nana Victor. A voucher specimen (No. 1882/SRFK) has been deposited in the National Herbarium, Yaoundé, Cameroon.

#### *Extraction and isolation*

Plant material was subjected to the following procedures: Dried stem bark (2.4 kg) was ground and exhaustively extracted by maceration successively with 3 solvents of increasing polarity

[hexane, ethyl acetate (EtOAc), and methanol (MeOH), respectively]. In each extraction 3 × 5 L of solvent were used for a period of 3 × 24 h. The extracts obtained were concentrated to give green hexane (56.3 g), brown EtOAc (35.6 g) and brown MeOH (62.1 g) crude residues. On the basis of TLC profiles, the hexane and EtOAc extracts were combined. The hexane/EtOAc extract mixture (55 g) was subjected to flash chromatography over silica gel 60 (0.063–0.200 mm, Merck, 500 g) as a stationary phase eluted with hexane/EtOAc/MeOH mixtures of increasing polarity. 64 fractions of 250 mL each were collected and grouped on the basis of TLC analysis to afford six main fractions (I–VI). Fraction II (5.7 g) was chromatographed on a silica gel column, using as eluent gradient mixtures of hexane and EtOAc, and yielded 4 fractions (IIa–IId). Fraction IIb (2.2 g) was subjected to further column chromatography to afford, after filtration, tirucalla-7,24-dien-3-one (**1**) (90 mg) from sub-fractions 3–10. 3-Geranyloxyemodin (**2**) (40 mg) crystallized from sub-fractions 15–22. Vismiaquinone A (**3**) (35 mg) was obtained as crystals from fraction IIc. Chromatography of fraction III (1 g) gave epifriedelinol (**6**) (20 mg). Finally, fraction V (11 g) was eluted with a gradient of mixtures of hexane, EtOAc and MeOH to afford 5 fractions (Va–Ve). Fraction Vb (1.5 g) yielded a sterol (stigmasta-7,22-dien-3-ol, **8**) (30 mg). Elution of fraction Vc (4 g) gave vismiaquinone B (**4**) (50 mg) and bivismiaquinone (**5**) (20 mg) from sub-fractions 8–15, while betulinic acid (95 mg) (**7**) was isolated from sub-fractions 20–27.

#### *Physical and spectroscopic measurements*

Melting points were measured on a Büchi apparatus and are uncorrected. Optical rotations were measured with a Perkin-Elmer 341 polarimeter. IR spectra were recorded using FT-IR spectroscopy on a Thermo-Nicolet 380 instrument, using the solid compound directly pressed on a diamond, which is considered to be a “universal ATR material”. NMR spectra were recorded using a Bruker Avance 300 (300 MHz for <sup>1</sup>H and 75 MHz for <sup>13</sup>C) and Avance 500 spectrometer (500 MHz for <sup>1</sup>H and 125 MHz for <sup>13</sup>C). 2.5 mm or 5 mm, Dual or BBI, probe heads were used depending on the NMR results. For NMR analysis all the compounds were dissolved in 0.5 mL or 0.2 mL of CDCl<sub>3</sub> or MeOD. Spectra were cali-

brated on the residual nondeuterated solvent, so CHCl<sub>3</sub> or MeOH were used as internal standard, and the temperature was stabilized at 25 °C for all experiments. One- and two-dimensional experiments were performed using the Bruker software library and modified pulse program. Mass spectra were obtained on a Kratos MS50TC mass spectrometer. Silica gel [Merck, Kieselgel 60 (0.063–0.200 mm)] was used for column chromatography. Silica gel plates (Merck, Kieselgel 60 F<sub>254</sub>) were used for TLC.

#### *Evaluation of erythrocyte susceptibility of crude extracts and purified compounds*

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 already described by Cedillo-Rivera *et al.* (1992) with some modifications. The drugs were serially diluted in 96-well culture plates, and each concentration was incubated in triplicate with erythrocytes (2% hematocrit) in a final 100 μL culture volume [at 37 °C, in a 3% O<sub>2</sub>, 5% CO<sub>2</sub> and 91% N<sub>2</sub> atmosphere, in the presence of RPMI (Roswell Park Memorial Institute) 1640 medium, 25 mM HEPES (1-piperazineethanesulfonic acid), 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 supernatants were discarded. The pellets were re-suspended in 0.75 mL of 0.04 M HCl 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 antiplasmodial activity*

*P. falciparum* strain W2, which is resistant to chloroquine and other antimalarials (Singh and Rosenthal, 2001), was cultured in sealed flasks at 37 °C, in a 3% O<sub>2</sub>, 5% CO<sub>2</sub> and 91% N<sub>2</sub> atmosphere, in RPMI 1640 medium, 25 mM HEPES, pH

7.4, supplemented with heat-inactivated 10% human serum and human erythrocytes to achieve 2% hematocrit. Parasites were synchronized at the ring stage by serial treatment with 5% sorbitol (Sigma) (Lambros and Vanderberg, 1979) and studied at 1% parasitemia.

Compounds were prepared as 1 mg/mL 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. Negative controls contained equal concentrations of DMSO. Positive controls contained 1  $\mu$ M chloroquine phosphate (Sigma). Cultures were incubated at 37 °C

for 48 h (one 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  $\mu$ L) of each culture were added to 5 mL round-bottom polystyrene tubes containing 0.5 mL 0.1% Triton X-100 and 1 nM YOYO nuclear dye (Molecular Probes) in PBS, and parasitemias of treated and control cultures were compared using a Becton-Dickinson FACSsort 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 the Prism 3.0 software (GraphPad) with data fitted by nonlinear regression to the variable slope sigmoidal dose-response

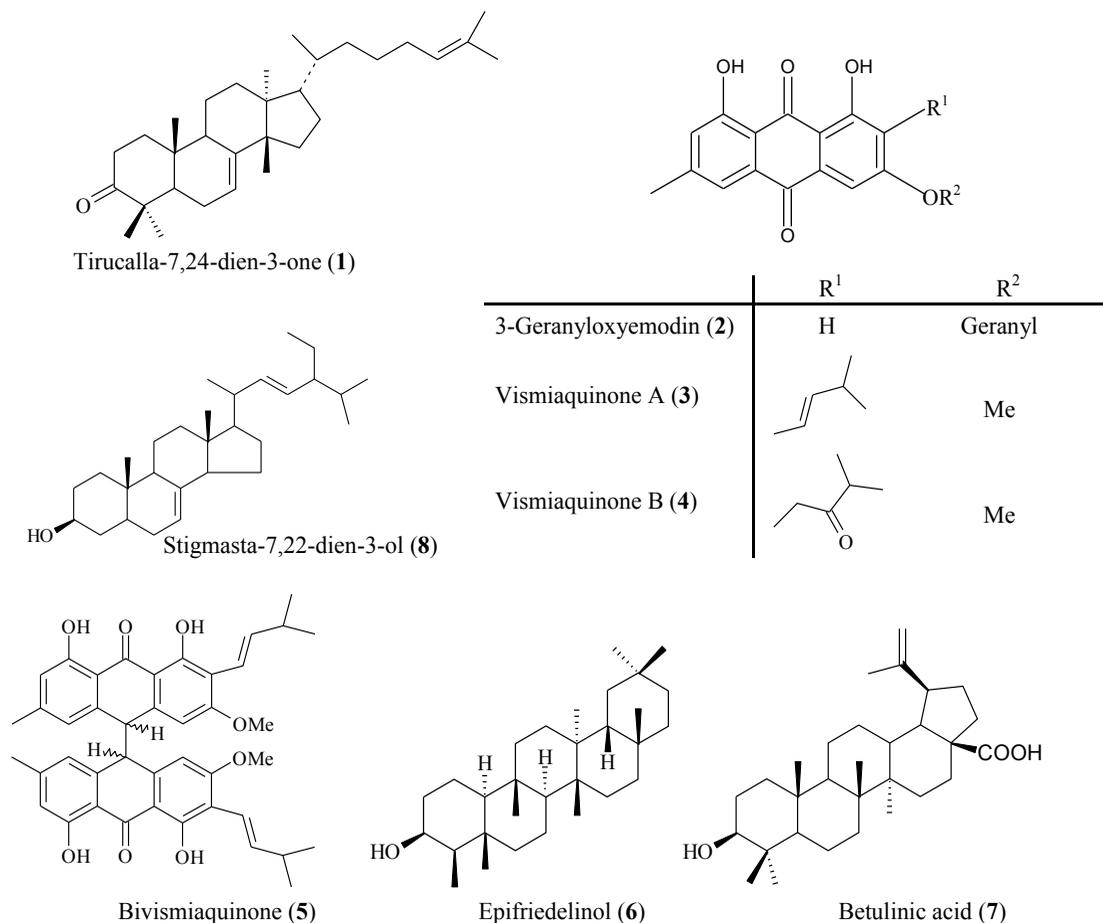


Fig. 1. Chemical structures of compounds 1–8.

Table I. Antiplasmodial activity of *Vismia laurentii* crude extracts and purified compounds against chloroquine-resistant (W2) *P. falciparum* strain.

Compound/Extract	IC <sub>50</sub>	Erythrocyte susceptibility
Fruit: hexane extract	>10 µg/mL	
Fruit: EtOAc extract	>10 µg/mL	
Leaf: EtOAc extract	>10 µg/mL	
Stem bark: hexane/EtOAc extract	(4.6 ± 0.09) µg/mL	> 2 mg/mL
Tirucalla-7,24-dien-3-one ( <b>1</b> )	(1.18 ± 0.05) µM	
Vismiaquinone A ( <b>3</b> )	(1.42 ± 0.03) µM	
3-Geranyloxyemodin ( <b>2</b> )		–
Epifriedelinol ( <b>6</b> )		–
Vismiaquinone B ( <b>4</b> )		–
Chloroquine	0.13 µM	

– No activity (insoluble saturated solution).

formula:  $y = 100/[1 + 10^{(\log IC_{50} - x)^H}]$ , where  $H$  is the Hill coefficient or slope factor (Singh and Rosenthal, 2001).

## Results and Discussion

Air-dried and ground stem bark of *V. laurentii* was successively extracted with hexane, ethyl acetate and methanol. The TLC profiles of hexane and ethyl acetate extracts were quite similar and were therefore combined. Repeated column chromatography of the combined extract on silica gel led to the isolation of eight compounds named: tirucalla-7,24-dien-3-one (**1**), 3-geranyloxyemodin (**2**), vismiaquinone A (**3**), vismiaquinone B (**4**), bivismiaquinone (**5**) (Nagem and Faustino de Oliveira, 1997; Hussein *et al.*, 2003; Marston *et al.*, 1986; Miraglia *et al.*, 1981), epifriedelinol (**6**), betulinic acid (**7**) (Venkatraman *et al.*, 1994; Nougoue *et al.*, 2007), and stigmasta-7,22-dien-3-ol (**8**) (Nguemeving *et al.*, 2006) (Fig. 1).

The tested extracts showed toxicity to erythrocytes at concentrations above 2 mg/mL, *i.e.* many orders of magnitude above the concentrations of compounds with antimalarial activity. The compounds were tested for their antiplasmodial activity against the W2 strain of *P. falciparum*. The stem bark crude extract exhibited a greater potency against *P. falciparum* parasites (4.6 µg/mL, Table I), compared to the fruit and leaf extracts. The silica gel-based chromatographic fractionation of the stem bark extract afforded eight pure compounds, amongst which five were evaluated for their antiplasmodial activity (Table I). From

this evaluation, only two compounds exhibited good antiplasmodial activity (IC<sub>50</sub> = 1.18 µM for **1** and IC<sub>50</sub> = 1.42 µM for **3**, Table I).

Compound **1** was 4- to 7-times more potent than betulinic acid (IC<sub>50</sub> = 5.10 µM) and friedelan-3-one (IC<sub>50</sub> = 7.70 µM) which were evaluated against the same parasite strain in a previous study (Lenta *et al.*, 2007). This result highlights these derivatives as an eventual source of lead compounds for the development of new antimalarials.

Furthermore, a related plant species, *Vismia guineensis* (Linn.) Choisy, was previously investigated for its antiplasmodial activity. One of its constituents, vismione H, exhibited a very good activity *in vitro* against *P. falciparum* (NF 54, clone A1A9), with an IC<sub>50</sub> value of ~0.23 µM (Francois *et al.*, 1999).

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