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ORIGINAL PAPER

Antiplasmodial volatile extracts from *Cleistopholis patens* Engler & Diels and *Uvariastrum pierreanum* Engl. (Engl. & Diels) (Annonaceae) growing in Cameroon

Fabrice Fekam Boyom · Vincent Ngouana · Eugenie Aimée Madiesse Kemgne · Paul Henri Amvam Zollo · Chantal Menut · Jean Marie Bessiere · Jiri Gut · Philip Jon Rosenthal

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Abstract In a search for alternative treatment for malaria, plant-derived essential oils extracted from the stem barks and leaves of *Cleistopholis patens* and *Uvariastrum pierreanum* (Annonaceae) were evaluated in vitro for antiplasmodial activity against the W2 strain of *Plasmodium falciparum*. The oils were obtained from 500 g each of stem barks and leaves, respectively, by hydrodistillation, using a Clevenger-type apparatus with the following yields: 0.23% and 0.19% for *C. patens* and 0.1% and 0.3% for *U. pierreanum* (*w*/*w* relative to dried material weight). Analysis of 10% (*v*/*v*) oil in hexane by gas chromatography and mass spectrometry identified only terpenoids in the oils, with over 81% sesquiterpene hydrocarbons in *C. patens* extracts and *U. pierreanum* stem bark oil, while the leaf oil from the latter species was found to contain a majority of monoterpenes. For *C. patens*, the major components were α-copaene, δ-cadinene, and germacrene D for the stem bark oil and β-caryophyllene, germacrene D, and germacrene B for the leaf oil. The stem bark oil of *U. pierreanum* was found to contain mainly β-bisabolene and α-bisabolol, while α- and β-pinenes were more abundant in the leaf extract. Concentrations of oils obtained by diluting 1-mg/mL stock solutions were tested against *P. falciparum* in culture. The oils were active, with IC_{50} values of 9.19 and 15.19 μg/mL for the stem bark and leaf oils, respectively, of *C. patens* and 6.08 and 13.96 μg/mL, respectively, for those from *U. pierreanum*. These results indicate that essential oils may offer a promising alternative for the development of new antimalarials.

F. F. Boyom (✉) · V. Ngouana · E. A. M. Kemgne · P. H. A. Zollo
Department of Biochemistry, Faculty of Science, University of Yaoundé I, P.O. Box 812, Yaoundé, Cameroon
e-mail: fabrice.boyom@fulbrightmail.org

C. Menut
Laboratoire de Chimie Biomoléculaire, UMR 5032, ENSCM, Montpellier, France

J. M. Bessiere
Laboratoire de Phytochimie, ENSCM, Montpellier, France

J. Gut · P. J. Rosenthal
Division of Infectious Diseases, Department of Medicine, University of California, San Francisco, San Francisco, CA, USA

Introduction

A multitude of biological activities have been described for essential oils. The physicochemical properties of these substances, including ready diffusion across cell membranes (acting as drug transport enhancers; Cornwell and Barry 1994) and other specific actions on parasite metabolism, e.g., modulators of P-glycoprotein drug efflux activity (Calcabrini et al. 2004; Munoz-Martinez et al. 2004) and protein isoprenylation (Moura et al. 2001), are likely to explain their biological activities.

Malaria is one of the most prevalent infections in the world and constitutes one of the main causes of death in much of the tropics (Walther and Walther 2007; Rowe et al. 2007). *Plasmodium falciparum* is increasingly resistant to available antimalarial agents, and so, the identification of new compounds that are active against the parasite is an urgent priority.

Nonvolatile natural products have been widely investigated for antiplasmodial activity, with encouraging results

(Kaur et al. 2009). Although recent studies have underlined the potential biological activities of essential oils against malaria parasites (Tchoumbougnang et al. 2005; Boyom et al. 2003a) and as insecticides against the mosquito vector (Senthilkumar and Venkatesalu 2010; Pitasawat et al. 2007), the antimalarial properties of these substances have been little studied. This paper describes the phytochemical composition and antiplasmodial activity of essential oils obtained from two Annonaceae species growing in Cameroon (*Cleistopholis patens* and *Uvariastrum pierreanum*).

C. patens is a tree, up to 27 m high, with horizontal branches. The infusion of its leaves is used as febrifuge and vermifuge. Apart from a novel monoterpene derivative isolated by Ngnokam et al. (2003), no previous investigation has been reported on the essential oils of this species. Nevertheless, nonvolatile extracts from its stem bark have been evaluated for antiplasmodial activity (Addae-Kyereme et al. 2001).

U. pierreanum Engl. (Engl. & Diels) is a forest tree, about 27 m high, which grows in Southern Nigeria, Cameroon, and Gabon (Hutchinson and Dalziel 1954). Apart from the investigation of the chemical composition and radical scavenging activity of the leaf and stem bark oils by Boyom et al. (2003b), no work has been published on the essential oils of this species.

Materials and methods

Collection and extraction of plant material

The stem bark and leaves of *C. patens* and *U. pierreanum* were collected in Mount Kalla (Yaoundé area) in December 2008. The plant samples were identified, and voucher specimens were deposited at the National Herbarium (Yaoundé), with the following identification numbers: 23169/SRF/Cam for *C. patens* and 23162/SRF/CAM for *U. pierreanum*. Air-dried leaves and stem barks were ground using a blender. Batches of 500 g of plant material were subjected to hydrodistillation for 3 h using a Clevenger-type apparatus, according to Boyom et al. (2003b). The resulting essential oils were dried over anhydrous sodium sulfate and subsequently used for further experiments.

Phytochemical analysis of the essential oils

Gas chromatography (GC) analyses were performed on a Varian CP-3380 gas chromatograph with flame ionization detectors fitted with a fused silica capillary column (30 m× 0.25 mm i.d.) coated with DB1 phase, with a film thickness of 0.25 μm. GC/mass spectrometry (MS) was performed using a Hewlett-Packard apparatus equipped with an HP1 fused silica column (30 m×0.25 mm i.d.; film thickness 0.25 μm) and interfaced with a quadrupole detector (Model 5970), with the mass spectrometer operated at 70 eV. These experiments were carried out according to Boyom et al. (2003b).

Evaluation of the biological activities

Evaluation of the erythrocytes' susceptibility to plant extracts

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), with some modifications. The essential oils were dissolved in DMSO to afford 1-mg/mL stock solutions that were subsequently diluted serially in 96-well culture plates using RPMI 1640. The final concentrations of DMSO in the wells were lower or equal to 0.5% and showed no effect on parasites, as previously shown by Abiodun et al.

Table 1 Extraction yields and antiplasmodial activities of the essential oils

Essential oil sample		Extraction yield[a] (% *w*/*w*)	IC_{50}[b] (μg/mL) ± SD
C. patens	Stem bark	0.23	9.19 ± 0.08
	Leaf	0.19	15.19±0.13
U. pierreanum	Stem bark	0.10	6.08±0.01
	Leaf	0.30	13.96 ± 0.05

IC_{50} values are means from triplicate experiments

SD standard deviation

[a] Essential oils were obtained from plant materials by hydrodistillation, and the yields were calculated in percentage relative to the weight of starting material

[b] The oils were evaluated against the W2 strain of *P. falciparum*

Table 2 Chemical composition of the essential oils

RI[a]	Compounds	*C. patens*		*U. pierreanum*	
		Cp1 (%)	Cp2 (%)	Up1 (%)	Up2 (%)
913	α-Thujene		0.16		
920	α-Pinene		0.12		22.80
933	Camphene		0.10		0.50
955	Sabinene		0.10		
959	β-Pinene	0.28	0.10		23.00
981	Myrcene		2.00		2.50
999	δ-3-Carene		0.15		
1006	α-Terpinene		0.10		
1011	*p*-Cymene		0.22		0.80
1014	Limonene		1.15		8.50
1028	(*Z*)-β-ocimene				0.10
1038	(*E*)-β-ocimene				4.00
1058	γ-Terpinene	0.24	0.21		
1068	Terpinolene				0.10
1134	Neo-allo-ocimene				0.10
1072	Linalool	0.65	0.22		0.80
1156	Borneol				0.10
1161	Terpinen-4-ol		0.10		0.30
1172	α-Terpineol		0.10		4.00
1220	Geraniol				0.10
1334	δ-Elemene	2.44	0.86		
1347	α-Cubebene	1.33	0.10		0.10
1356	α-Ylangene	0.42	0.11		
1361	α-Copaene	16.90	1.12	5.70	7.90
1374	β-Elemene	2.44	1.57	0.10	0.10
1387	Cyperene	1.66			
1396	*Z*-α-bergamotene	0.43	0.10	5.20	0.88
1397	α-Cedrene			1.00	0.10
1411	α-Santalene			4.50	
1413	β-Caryophyllene	2.58	27.54	0.90	0.10
1417	γ-Elemene + β-cubebene	2.91	3.2		
1423	(*E*)-α-bergamotene	1.10	0.10	9.00	2.50
1427	*E*-β-farnesene	0.80	0.20	3.00	1.70
1453	α-Humulene	0.90	2.77	0.10	
1455	Alloaromadendrene	0.80	1.27		
1455	γ-Muurolene	1.80	0.42		
1458	α-Farnesene			5.70	1.80
1461	Germacrene D	3.80	16.14		
1472	α-Muurolene	1.19	0.70		
1474	Ar-curcumene			8.60	0.80
1487	β-Bisabolene	0.94	1.00	28.20	4.30
1490	*Z*-γ-bisabolene	0.87	0.80		
1492	γ-Cadinene	0.33	0.70		
1494	Bicyclogermacrene	2.81	4.23		
1505	δ-Cadinene	28.70	1.60	0.70	2.60
1506	Cadina-1,4-diene		0.70		
1513	(*Z*)-calamenene			3.00	0.30

Table 2 (continued)

RI[a]	Compounds	*C. patens*		*U. pierreanum*	
		Cp1 (%)	Cp2 (%)	Up1 (%)	Up2 (%)
1514	(*E*,*Z*)-germacrene B	4.91	1.97		
1514	β-Sesquiphellandrene			5.30	
1517	*E*-calamenene			0.70	
1538	Germacrene B	7.40	15.98		
1537	(*E*)-nerolidol			0.80	1.22
1551	Spathulenol	2.52	0.78		
1555	Caryophyllene oxide	2.90	3.80	1.20	0.10
1568	γ-Eudesmol			1.00	0.50
1595	Cubenol	2.46	0.43		
1602	Epi-α-cadinol			0.10	0.10
1607	Epi-α-muurolol	1.92	2.39		
1638	α-Cadinol	0.30	2.42	0.70	0.10
1666	β-Bosabolol			2.30	0.20
1674	α-Bisabolol			11.50	4.56
1680	*E*,*E*-farnesol				1.22
Monoterpene hydrocarbons		0.52	4.41		62.40
Oxygenated monoterpenes		0.65	0.42		5.30
Sesquiterpene hydrocarbons		87.46	83.18	81.70	23.10
Oxygenated sesquiterpenes		10.10	9.82	17.60	8.00
Total		98.73	97.83	99.30	98.80

Components were identified based on RI and GC/MS data and listed according to their order of elution on DB1 (50 m)

Cp1 stem bark oil of *C. patens*, *Cp2* leaf oil of *C. patens*, *Up1* stem bark oil of *U. pierreanum*, *Up2* leaf oil of *U. pierreanum*, *%* percent peak area of essential oil constituents

[a] Retention indices

(2010). Each concentration was incubated in triplicate, with erythrocytes (at 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 1,500 rpm for 5 min, and the supernatant was discarded. MTT solution (1.5 mL) with 250 μg PMS was added to the pellets. The controls contained no erythrocytes. The tubes were thereafter incubated for 45 min at 37°C and 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. The tubes containing the most viable erythrocytes produced more formazan (highest OD). From the results obtained, the highest drug concentrations producing minimal damage to the cells were considered as starting points for further drug dilutions.

Evaluation of the antiplasmodial activity

P. falciparum strain W2, which is resistant to chloroquine and other antimalarials, 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; Lambros and Vanderberg 1979) and studied at 1% parasitemia, as described by Boyom et al. (2003a).

Parasitemias of the treated and control cultures were compared using a Becton-Dickinson FACSort flow cytometer to count the nucleated (parasitized) erythrocytes. Data acquisition was performed using CellQuest software. These data were normalized to percent control activity and 50% inhibitory concentrations (IC_{50}) calculated using Prism 4.0 software (GraphPad) with data fitted by nonlinear regression to the variable slope sigmoidal dose–response formula $y = 100/\left[1 + 10^{(\log IC_{50} - x)H}\right]$ where H is the hill coefficient or slope factor (Boyom et al. 2003a).

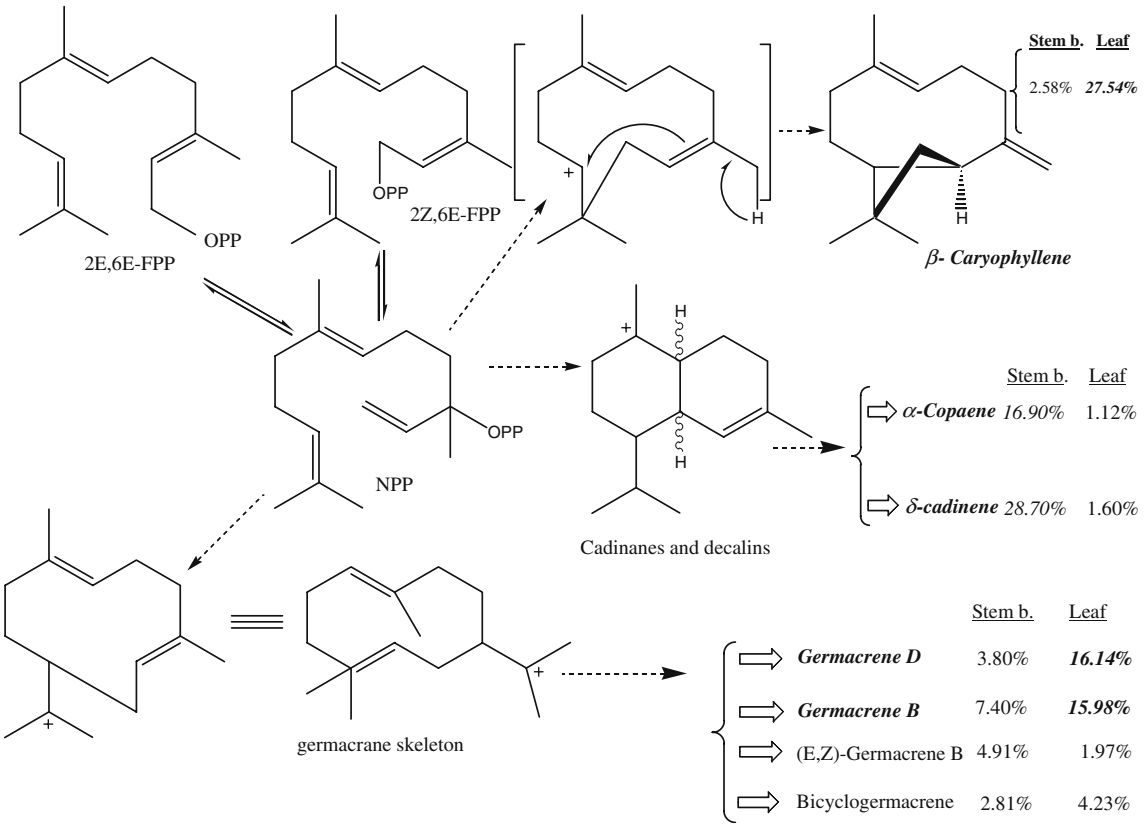

Fig. 1 GC/MS analyses of C.p. oils led to the identification of α-copaene and δ-cadinene as chemical markers of the stem bark oil, while germacrene D, germacrene B, and β-caryophyllene were more abundant in the leaf oil. The most active metabolic pathways start respectively from the cadinane and germacrene skeletons

Results and discussion

Extraction yields and phytochemical analysis of essential oils

The oils were obtained respectively from the stem barks and leaves with the following yields (*w*/*w*): 0.23% and 0.19% for *C. patens*; 0.10% and 0.30% for *U. pierreanum* (relative to the weight of the starting material; Table 1). The extraction yields from *U. pierreanum* were slightly higher compared to the previous results (Boyom et al. 2003b).

The chemical compositions of the essential oils are given in Table 2. From these results, the two essential oils from *C. patens* were found to contain only terpenoids, with 97.56% and 93.00% of sesquiterpenes for the stem bark and leaf oils, respectively. Sesquiterpene hydrocarbons were more represented, with 87.46% and 83.18%, respectively. The major constituents were found to be δ-cadinene (28.70%), α-copaene (16.90%), and germacrene B (7.40%) for the stem bark oil and β-caryophyllene (27.54%), germacrene D (16.14%), and germacrene B (15.98%) for the leaf oil. More importantly, compounds derived from the germacrene skeleton (Fig. 1) were found to constitute an important fraction of the leaf oil (38.32%), compared to 18.92% for the stem bark. This indicates that metabolic pathways from the germacrene substrate were very active in the leaves and, to a lesser extent, in the stem bark of *C. patens*. On the other hand, α-copaene and δ-cadinene, derived from the cadinane skeleton, were found to be abundant in the stem bark oil (45.60%) and to represent only 2.72% of the leaf oil, indicating an active biogenesis of these compounds primarily in the stem bark.

The oils from *U. pierreanum* were found to be qualitatively comparable to those studied previously by Boyom et al. (2003b), with modest quantitative variations. The main chemical markers were found to be the same as in the previous study. The stem bark extract was found to contain only sesquiterpenoids, with up to 81.70% hydrocarbon derivatives and 17.60% oxygenated compounds. The major components of this oil were β-bisabolene (28.20%), α-bisabolol (11.50%), (*E*)-α-bergamotene (9.00%), ar-curcumene (8.60%), and α-copaene (5.70%).

The leaf oil of *U. pierreanum* was found to contain a majority of monoterpene hydrocarbons (62.40%), with α-pinene (22.80%) and β-pinene (23.00%) being the most abundant. Oxygenated monoterpenes (8.30%) were identified in this extract, with α-terpineol (4.00%) as the major

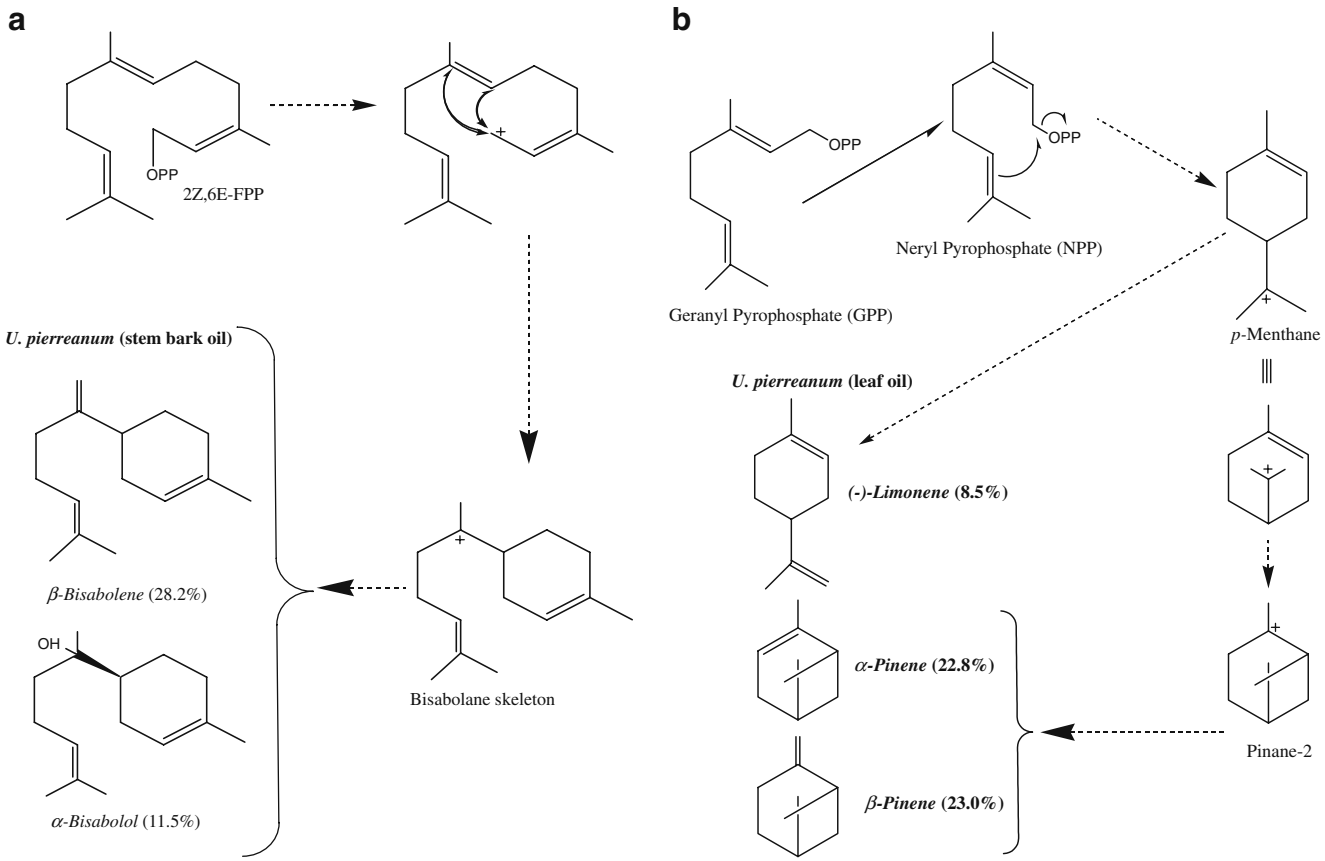

Fig. 2 **a** GC/MS analyses of the stem bark oil led to the identification of β-bisabolene and α-bisabolol as the main constituents. The major constituents of the oil were formally derived from 2Z,6E-FPP via the bisabolane skeleton. *Double-headed arrows* indicate bond linkages, not electron flow. **b** GC/MS analyses of the leaf oil led to the identification of α- and β-pinenes and limonene as the more abundant constituents. They are built up through the menthane and pinane skeletons

constituent. Sesquiterpenoids (31.10%) were also identified in this extract, with α-copaene (7.90%), β-bisabolene (4.30%), and α-bisabolol (4.56%) being the most abundant.

From this study, it appears that the main metabolic pathway in the stem bark of *U. pierreanum* led to sesquiterpenoids and, more importantly, to hydrocarbon derivatives. β-Bisabolene (28.20%) and α-bisabolol (11.50%) were found to be the privileged products in this extract (Fig. 2a). In contrast, the leaves of this species showed a tendency to synthesize more monoterpenoids, the most active metabolic pathway being that leading to pinenes (α and β) and, to a lesser extent, to limonene (Fig. 2b).

Biological activities of essential oils

The preliminary toxicological screening allowed the setting of lytic concentration thresholds of the essential oils. To start with, initial experiments showed that essential oils led to toxic effects on adjacent cultures at high concentrations (>0.6 mg/mL, many orders of magnitude above concentrations with antiplasmodial activity), presumably due to effects of volatile components.

P. falciparum parasites were found to be sensitive to the four essential oils in culture, the most potent being the stem bark extract of *U. pierreanum*, with an IC_{50} value of 6.08 μg/mL. The other extracts also showed potency, but to a lesser extent, with IC_{50} values of 9.19 and 15.19 μg/mL, respectively, for the stem bark and leaf oils of *C. patens* and 13.96 μg/mL for the leaf extract of *U. pierreanum* (Table 1). For the extracts from *C. patens* and those from the stem bark of *U. pierreanum*, the results obtained might be attributable to their high sesquiterpene content (>81%). Indeed, sesquiterpenoids are credited with various biological actions. Furthermore, it has been shown that sesquiterpenes are promising skin penetration enhancers (Williams and Barry 1991; Cornwell and Barry 1994; Santoro et al. 2007) and specific P-glycoprotein modulators that can reverse cellular multidrug resistance by inhibiting the drug efflux process (Munoz-Martinez et al. 2004). In a less recent study, Lopes et al. (1999) identified nerolidol (an acyclic oxygenated sesquiterpene) as an active ingredient against malaria parasites. Limonene might be contributing to the activity exerted by the *U. pierreanum* leaf extract, in which it represents 8.5%. Indeed, this compound, as well

as nerolidol, has been shown to impair the activity of the *P. falciparum* isoprenoid pathway, which biosynthesizes isoprenic chains of coenzyme Q. Parasites treated with nerolidol or limonene showed a decreased ability to synthesize coenzyme Q in the parasites' intraerythrocytic stages (Moura et al. 2001; De Macedo et al. 2002). Apart from their biological activities, the physical properties of essential oils, including low density (~0.94 g/mL) and ready diffusion across cell membranes, might enhance the targeting of the intracellular malaria parasites. Although further investigations are necessary to identify the specific components that elicit antiplasmodial activity, our results suggest that essential oils offer a new potential alternative for antimalarial chemotherapy. Important goals will be to identify the active components of essential oils with antimalarial activity and to determine the mechanisms by which these compounds exert their biological activities.

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