



HAL
open science

The antiplasmodium effects of a traditional South American remedy: *Zanthoxylum chiloperone* var. *angustifolium* against chloroquine resistant and chloroquine sensitive strains of *Plasmodium falciparum*

Gerardo Cebrian Cebrian-Torrejon, Kevin Spelman, Karine Leblanc, Katalina Muñoz-Durango, Sandra Torijano Gutierrez, Maria Elena Ferreira, Antonieta Rojas De Arias, Bruno Figadère, Alain Fournet, Alexandre Maciuk, et al.

► **To cite this version:**

Gerardo Cebrian Cebrian-Torrejon, Kevin Spelman, Karine Leblanc, Katalina Muñoz-Durango, Sandra Torijano Gutierrez, et al.. The antiplasmodium effects of a traditional South American remedy: *Zanthoxylum chiloperone* var. *angustifolium* against chloroquine resistant and chloroquine sensitive strains of *Plasmodium falciparum*. *Revista Brasileira de Farmacognosia*, 2011, 21 (4), pp.652-661. 10.1590/S0102-695X2011005000104 . mnhn-02867451

HAL Id: mnhn-02867451

<https://mnhn.hal.science/mnhn-02867451v1>

Submitted on 22 May 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial - NoDerivatives 4.0 International License

Article

Received 29 Nov 2010
Accepted 8 Jan 2011
Available online 17 Jun 2011

Keywords:

alkylamides
avicennol
canthinone alkaloids
Heme-drug adducts
Plasmodium falciparum
Zanthoxylum chiloperone

ISSN 0102-695X
doi: 10.1590/S0102-695X2011005000104

The antiplasmodium effects of a traditional South American remedy: *Zanthoxylum chiloperone* var. *angustifolium* against chloroquine resistant and chloroquine sensitive strains of *Plasmodium falciparum*

Gerardo Cebrián-Torrejón,^{1§} Kevin Spelman,^{2§} Karine Leblanc,¹ Katalina Muñoz-Durango,¹ Sandra Torijano Gutiérrez,¹ Maria Elena Ferreira,³ Antonieta Rojas de Arias,⁴ Bruno Figadère,¹ Alain Fournet,^{*5} Alexandre Maciuk,¹ Philippe Grellier,⁶ Nadja B. Cech,⁷ Erwan Poupon¹

¹Laboratoire de Pharmacognosie associé au CNRS, UMR 8076 BioCIS, Faculté de Pharmacie, Université Paris-Sud, France,

²Bioanalytical Chemistry and Drug Discovery Section, Laboratory of Clinical Investigation, National Institute on Aging, National Institute Health, USA,

³Department of Tropical Medicine, Instituto de Investigaciones en Ciencias de la Salud Asunción, Universidad Nacional de Asunción, Paraguay,

⁴Centro para el Desarrollo de la Investigación Científica (CEDIC/FMB/Diaz Gill Medicina Laboratorial), Asunción, Paraguay,

⁵IRD UMR217, Laboratoire de Pharmacognosie, Faculté de Pharmacie, France,

⁶Muséum National d'Histoire Naturelle, FRE 3206 CNRS, Département Régulations, Développement et Diversité Moléculaire, France,

⁷Department of Chemistry and Biochemistry, University of North Carolina Greensboro, USA.

Abstract: *Zanthoxylum chiloperone* var. *angustifolium* Engl., Rutaceae, is used in traditional medicine to treat fungal and protozoal infections in the central area of South America. Considering the increasing resistance of *Plasmodium falciparum* in malarial ridden areas, we explored the anti-plasmodial effects of three compounds isolated from *Z. chiloperone*. The pyranocoumarin *trans*-avicennol and the canthinone alkaloids, canthin-6-one and 5-methoxycanthin-6-one, were found to have IC₅₀ on chloroquine/mefloquine resistant and sensitive strains of *P. falciparum* of 0.5-2.7, 2.0-5.3 and 5.1-10.4 µg/mL, respectively. Moreover, the formation of heme adducts by these compounds is described by a novel alternative method based on MS-CID methods. The alkylamide sanshool was also identified, for first time in this plant, in the dichloromethanic and ethanolic extracts and the extracts were found to be notably non-toxic and displayed good anti-plasmodial effects.

Introduction

In the majority of countries that cope with high malaria infection, medicinal plants contribute significantly to primary health care due to cultural traditions, and lack of access and affordability of pharmaceuticals. In addition, natural products are potential sources of new and selective agents for the treatment of important tropical diseases caused by protozoans (Karioti et al., 2009; Froelich et al., 2007; Luize et al., 2005). For example, *Zanthoxylum*

chiloperone var. *angustifolium* Engl. (syn. *Fagara chiloperone* Engl. Ex Chod. & Hassl.), Rutaceae, is a dioic tree indigenous to the central and southern continent of South America, which is called “tembetary hu” and “mamicão” (Spichiger & Stutz de Ortega, 1987; Tabanez et al., 2005). A decoction of *Z. chiloperone* root and stem bark has been used in traditional medicine to treat malaria and for its emmenagogue and antirheumatic properties (Ferreira et al., 2007; Milliken, 1997). Recent studies have shown that the crude extract of the stem bark and leaves have

activity against *Trypanosoma cruzi* (Ferreira et al., 2011; Ferreira et al., 2002) and antifungal activity *in vitro* (Thouvenel et al., 2003). Further investigations demonstrate that canthinone type alkaloids, canthin-6-one and 5-methoxycanthin-6-one, are antifungal (Soriano-Agatón et al., 2005; Thouvenel et al., 2003) and effective *in vivo* against *Leishmania amazonensis* and *Trypanosoma cruzi* (Ferreira et al., 2011; Ferreira et al., 2007; Ferreira et al., 2002). Canthin-6-one has been suggested to be an inexpensive and safe treatment for use in long-term oral treatment as well as a good candidate against drug resistant strains of *T. cruzi*.

Other compounds were isolated from species of *Zanthoxylum* including the pyranocoumarin avicennol and alkylamides, such as the sanshools (Jang et al., 2008; Soriano-Agatón et al., 2005; Xiong et al., 1997; Yang, 2008). To date, there is a paucity of research on the biological activity of avicennol, which has been previously identified in *Z. elephantiasis* Macfad (Gray et al., 1977). Recent work with avicennol reports an induction of UDP-glucuronosyltransferases (UGT), specifically UGT1A1, which detoxifies xenobiotics (Chlouchi et al., 2007).

The alkylamides are a class of compounds which are well known in Asia, North America and Europe due to the tingling sensation on the tongue after ingestion of Sichuan pepper and *Echinacea purpurea* (Spelman et al., 2010). While many *Zanthoxylum* spp. are known to contain alkylamides, to the best of our knowledge there are no reports of the occurrence or identity of alkylamides in *Z. chiloperone*. Notably, these compounds have also been shown to be antiparasitic (Lozano et al., 1984), as well as insecticidal (Jacobson, 1948). Recent reports have shown *in vitro* and *in vivo* anti-plasmodial activity of alkylamides identical to, or similar to, the alkylamides occurring in other *Zanthoxylum* spp. (Spelman et al., 2010).

One of the mechanisms of action of antimalarial drugs is based on their interference with the heme detoxication pathway (Weissbuch & Leiserowitz, 2008). As a toxic, non-peptidic by-product of hemoglobin digestion, free heme must be sequestered in the digestive vacuole. The biomineralization process involves the heme dimerization and the subsequent aggregation of dimers into hemozoin, an insoluble pigment. Antimalarial drugs are known to form adducts with heme, thus impairing heme polymerization and leading to the death of parasites at the trophozoite stage. Artemisinin for instance has been showed to form a covalent complex between heme and the alkylating species generated by the endoperoxide ring opening through Fe(II)-mediated catalysis (Robert et al., 2002). The free heme in its commercial form (hemin or hematin) is known to be suited for testing heme polymerization inhibition (Tekwani & Walker, 2005).

Most of the protocols are based on spectrophotometry or radioactivity monitoring. (Garavito et al., 2007; Kurosawa et al., 2000; Rush et al., 2009). A novel method based on mass spectrometry can be used as an alternative method to detect heme-drug adducts (Figadère et al. 2010). It has been used to detect adducts between Fe(III)-heme and quinine, artemisinin and its derivatives. (Bilia et al., 2002; Pashynska et al., 2004). Our laboratory used mass spectrometry and in-source collision-induced dissociation (CID) to assess the stability of the adducts. In our method, heme is incubated with the compound and analyzed by mass spectrometry using an increasing fragmentor voltage to dissociate the formed adduct. A relative assessment of the adduct stability can be obtained and compared to one reference compound, like quinine.

Thus far there has been limited exploration of *Zanthoxylum chiloperone*'s chemistry and biological activity. The experiments continued herein describe the isolation of two alkaloids and a pyranocoumarin, the identification of an alkylamide and the anti-malarial activity of the crude extracts and the isolated compounds. In addition, using mass spectrometry and in-source CID to study the formation and stability of the heme adducts with avicennol and the canthinone alkaloids, we propose a possible basis for the antiplasmodial activity of these *Zanthoxylum* compounds.

Material and Methods

General experimental procedures

Yields refer to chromatographically and spectroscopically homogeneous materials, unless otherwise stated. Extraction was monitored by TLC carried out on Merck Kieselgel silica gel plates (60F-254) using UV light as visualizing agent and sulfuric vanillin or Dragendorff reagent and heat as developing agent. Merck Kieselgel silica gel (60, particle size 40-63 μm) was used for flash chromatography. NMR spectra were recorded on an AM-400 Bruker spectrometer and calibrated using undeuterated solvent as an internal reference. IR spectra were recorded on Vector 22 Bruker spectrometer and values are reported in cm^{-1} units. Mass spectra were recorded at "Service d'Analyse des Médicaments et Métabolites", SAMM, Université Paris-Sud, Châtenay-Malabry, France and University of North Carolina, Greensboro, North Carolina, U.S.A.

Plant material

Stem bark of *Zanthoxylum chiloperone* var. *angustifolium* Engl., Rutaceae, was collected by Maria Elena Ferreira, in Paraguay near Piribebuy, Department

of Cordillera and identified by N. Soria (Department of Botany, National University of Asuncion, Paraguay). A voucher specimen (AF 917) has been deposited at the Herbarium of Chemical Sciences Faculty, San Lorenzo, Paraguay.

Extraction and isolation

Powdered dried stem bark of *Z. chiloperone* var. *angustifolium* (1.3 kg) was basified with ammonia, extracted in a Soxhlet apparatus with CH_2Cl_2 and MeOH separately and successively for three days to afford 23 and 30 g of crude extracts, respectively. The yields of the dichloromethane and the methanol extracts were approximately 1.7 and 2.3%, respectively, of the plant powder. Both extracts were evaluated for antiplasmodial activity and the CH_2Cl_2 extract showed greater antimalarial activity. Therefore, the CH_2Cl_2 extract was subjected to silica gel flash column chromatography, eluted with CH_2Cl_2 /ethyl acetate (8:2), to obtain 42 fractions. Fractions 10-18 were combined providing 3.5 g of *trans*-avicennol (**1**) a yellow crystal (yield of 15.2 and 0.3% respectively from the dichloromethane extract and plant powder). Fractions 21-30 were combined obtaining 3 g of canthin-6-one (**2**), a yellow crystalline powder (yield of 13 and 0.2%, respectively). Fractions 33-39 were combined yielded 1.5 g of 5-methoxycanthin-6-one (**3**), a brown crystalline powder (with a yield of 6.5 and 0.11%, respectively). Physical and spectral data were used to determine the chemical structure of the compounds and compared to reference samples and literature values (^1H , ^{13}C NMR, MS, HRMS, IR).

The powered stem-bark (5 g) was basified with ammonia, extracted by ethanol maceration over two days, changing the solvent three times, for a yield of 2.3%. The compositions of the ethanol and the dichloromethane extracts were studied by HPLC-UV-MS method.

Analysis by HPLC-UV-MS and MS/MS methods

HPLC-UV-MS qualitative study

A LC/MSD 1100 series + 1100 series (Agilent Technologies) spectrometer was utilized in the HPLC study of the extracts by using a 150 mm x 4.6 mm stainless-steel column Sunfire (Waters) C18. The eluents were (A) H_2O +TFA 0.1% and (B) MeOH+0.1% TFA. Separations were performed at room temperature by solvent gradient elution from 50% A/50% B to 20% A/80% B in 20 min then to 100% B in 12 min at a flow rate of 1 mL/min.

The UV measurement was made at 240 and 260 nm. The injection volume was 5 μL . Peak identification

was performed by comparison of the retention time (t_r), UV absorption and mass study at the different wavelengths with the compounds *trans*-avicennol (**1**), canthin-6-one (**2**), 5-methoxy-canthin-6-one (**3**) as references (Figure 1).

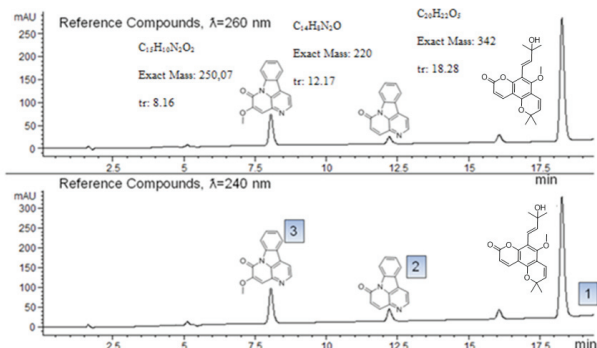


Figure 1. HPLC-UV Chromatogram of reference compounds ($\lambda=260$ and 240 nm).

Figure 2 illustrates the HPLC-UV chromatogram of the dichloromethane and alcohol extracts of stem bark extract of the plant *Z. chiloperone*.

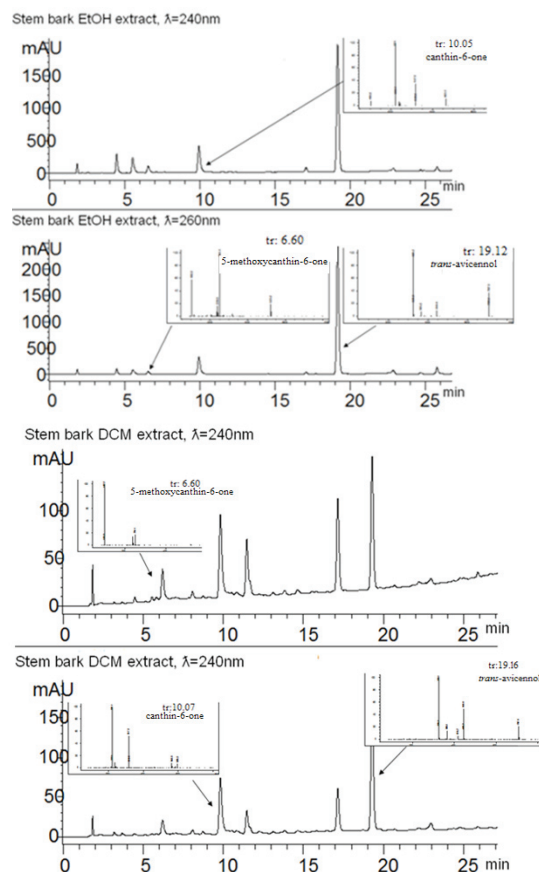


Figure 2. HPLC-UV Chromatogram of extracts of *Z. chiloperone*. The chromatographic profile (HPLC-UV-MS) of ethanol and dichloromethane extracts of *Z. chiloperone* obtained through maceration and soxhlet extraction illustrate the peaks for the canthinones and avicennol before 20 min.

HPLC-UV quantitative study

The quantification analysis was performed by external standard method, using a calibration curve constructed utilizing solutions containing different concentrations of the three reference samples (1-3). The stock solutions were prepared in a mixture MeOH+DMSO and then diluted with the mobile phase in various proportions. The calibration curves of the different samples represent the concentration "C" of each reference molecule *versus* the area under the peak "S"; this graphic presents a linearity behaviour ($C = a + bS$) in the interval of concentrations from 0.01 to 0.3 mg/mL.

All the samples for HPLC analysis were operated directly without any previous purification. A modular system comprised of 600 E controller + pump and a 2996 PDA UV (Waters) detector was utilized in the HPLC-UV quantification experiment.

Reference compounds stock solutions of 10, 50, 100, 150, 200, 300 µg/mL were utilized. Each determination was carried out in triplicate. The calibration curve showed the linearity of the detector over the tested range (10-300 µg/mL). The R^2 of the calibration curve is

linear and 0,999. The quantitative evaluation of the peaks previously identified in the crude extracts was made by integration of the peak area and comparison with the area of the corresponding reference peak (Table 1).

Table 1. Concentrations of compounds in the ethanol and dichloromethane extracts.

(mg/g extract)	1	2	3
Stem bark DCM	63.2	64.7	29.4
Stem bark EtOH	85.6	85.6	19.1

MS/MS study

To elucidate the presence of alkylamides, LC-ESI-MS/MS analysis was conducted on the crude extracts of *Z. chiloperone* ramulus cortex using the previously established structures of alkylamides identified in related *Zanthoxylum* spp. and the results of a previously validated LC-ESI-MS/MS method for the identification of alkylamides (Spelman et al., 2009). An ion trap mass spectrometer with electrospray ionization source (LCQ Advantage, ThermoFisher, San Jose, CA) was employed.

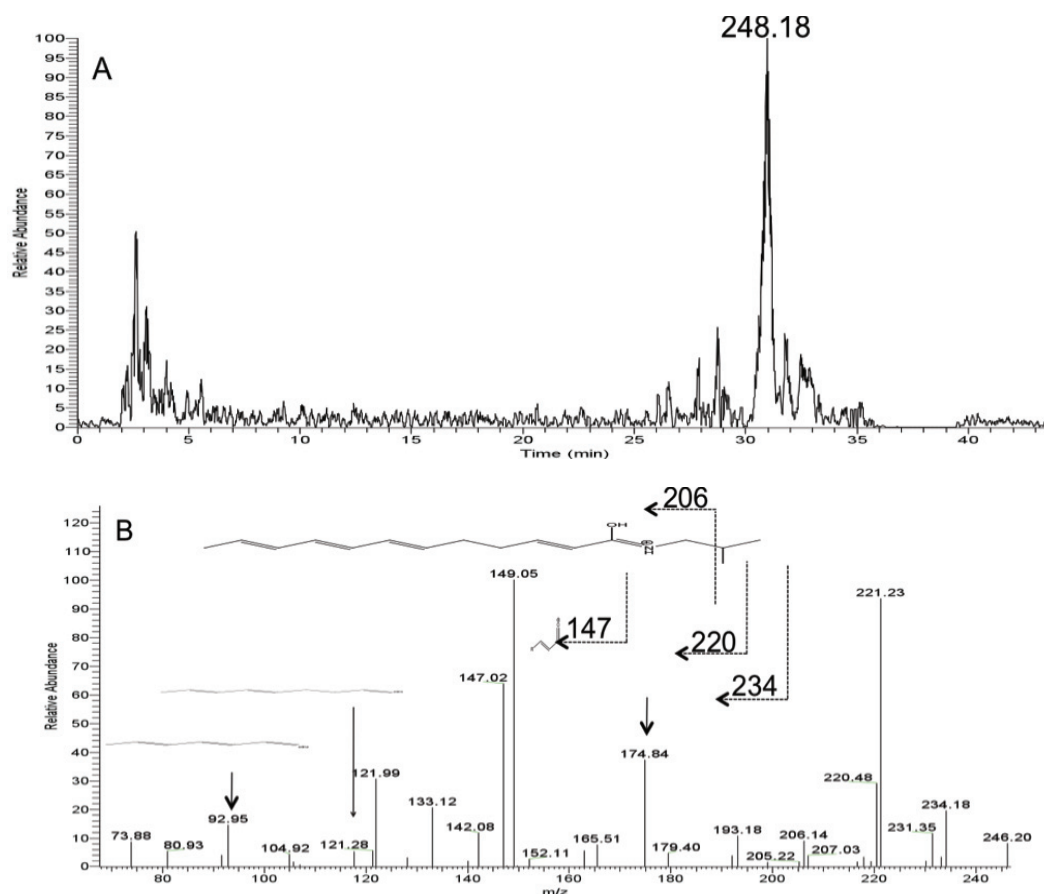


Figure 3. Selected ion chromatogram and MS/MS Spectra of dodeca-2,6,8,10-tetraenoic acid isobutylamide (4). **A.** The selected ion chromatogram illustrates the M^+ ion at m/z 248. **B.** The MS/MS spectra of the 248 M^+ ion identifies the compound as the alkylamide sanshool (4), dodeca-2,6,8,10-tetraenoic acid isobutylamide although the stereochemistry cannot be identified. The structure shown is that of the protonated (M^+) form of sanshool 4.

The solvent gradient, which was a minor variation on that previously published (Spelman et al., 2009), was as follows, where solvent A is aqueous acetic acid (17 mM, original pH 2.74) and solvent B is neat HPLC grade acetonitrile. For $t=0$ to 4 min, a constant composition of A-B (90:10 v/v); for $t=4$ to 15 min, a linear gradient from A-B (90:10, v/v) to A-B (60:40, v/v); for $t=15$ to 30 min, a linear gradient from A-B (60:40, v/v) to A-B (40:60, v/v); for $t=30.1$ to 35 min, a constant composition of A-B (0:100, v/v); for $t=35.1$ to 43 min, a constant composition of A-B (90:10, v/v). The mass spectrometer was operated in the positive ion mode with a scan range of 50.00-2000.00. Spray, capillary, and tube lens offset voltages were 4.5 kV, 3 V and -60 V, respectively (Figure 3).

MS-CID study of the heme-compounds (**1**, **2**, **3**) adducts

Methanol HPLC grade was obtained from VWR (Fontenay-sous-Bois, France) and deionized water from a SynergyUV deionizer from Millipore (Molsheim, France). DMSO was obtained from Sigma-Aldrich (Lyon, France). Hemin was obtained from Alfa Aesar (Schiltigheim, France). Bulk solutions (5 mM) were made for avicennol **1**, canthin-6-one **2**, 5-methoxycanthin-6-one **3**, quinine in methanol and for heme in $\text{NH}_4\text{OH}:\text{H}_2\text{O}$ (1:7). Using methanol as dilution solvent, a mixture of each compound (250 μM) and heme (250 μM) in a final volume of 100 μL was incubated 30 min in a water bath at 37 °C. The incubates (2 μL) were injected using $\text{MeOH}:\text{H}_2\text{O}$ (3:1) as mobile phase at 200 $\mu\text{L}/\text{min}$ in an Agilent LC/MSD 1100 Series HPLC-mass spectrometer system (Santa Clara, USA) using electrospray positive mode ionization, drying gas at 350 °C, 10 L/min and 35 psig and capillary voltage of 3000 V. The mass spectra were recorded in the mass range m/z 100-2000. In-source CID was performed in single ion monitoring mode by applying an increasing fragmentor voltage from 160 to 400 V in a flow injection analysis sequence with 30 V increment steps (Figure 4). Data are plotted so as to show the ion abundance of the adduct at the maximum of the injection peak versus the fragmentor voltage, and fitted by a sigmoidal curve using the GraphPad Prism software (Figure 6).

Biological assays

Anti-*Plasmodium falciparum* resistant and sensitive strains activity

After isolation of *trans*-avicennol **1**, canthin-6-one **2** and methoxycanthin-6-one **3**, the half maximal inhibitory concentrations (IC₅₀) of extracts and the previously listed compounds were assessed by determining the concentration inhibiting 50% of parasite growth (Table 2, Figure 5). The IC₅₀ determined against the Colombian

chloroquine-resistant FcB1 strain, the Brazilian mildly chloroquine-resistant strain PFB, the multi-drug and chloroquine-resistant strain K1 from Thailand, and the chloroquine-sensitive, but mefloquine resistant Tanzanian strain F32 were determined using a previously established method (Desjardins et al., 1979).

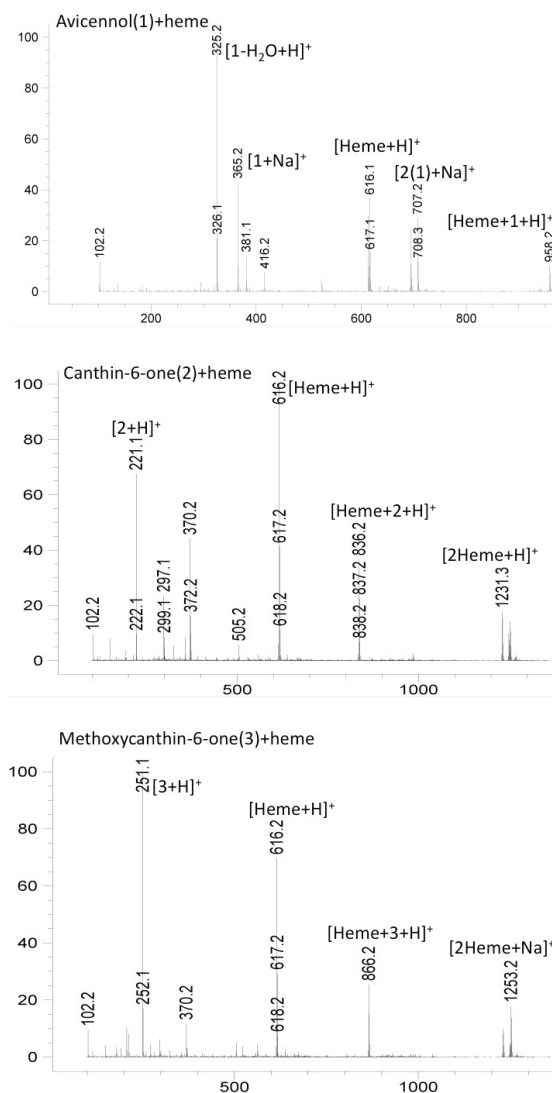


Figure 4. Mass spectra of incubates of heme and *trans*-avicennol (**1**), canthin-6-one (**2**) and 5-methoxycanthin-6-one (**3**).

Hemolytic activity

In order to confirm that the antimalarial activities measured by [³H] hypoxanthine incorporation are due to the intrinsic antiparasite property of the experimental compounds and not due to hemolytic activity, a standard hemolysis assay was run. After 48 h incubation time in the presence of the various test compounds, the hemolysis of non-infected erythrocytes was measured at 530 nm

incubated for 48 h at concentrations up to 100 µg/mL.

Table 2. IC₅₀ of *Z. chiloperone* isolated compounds and extracts.

Compounds	<i>P. falciparum</i> strain IC ₅₀ (µg/mL)			
	F32	K1	PFB	FcB1
<i>Trans</i> -avicennol (1)	0.5	2.7	1.2	2.2
Canthin-6-one (2)	2.0	5.3	3.2	4.0
5-methoxycanthin-6-one (3)	10.4	5.1	Nt	Nt
Extracts				
DCM	8.9	8.9	Nt	Nt
EtOH	10.5	9.3	Nt	Nt
MeOH	89.5	>100	Nt	Nt
Positive Control				
Chloroquine	*2.6	*77.4	*27.8	*62.8

*Chloroquine concentrations are in nM; nt: not tested

Cytotoxic activity

The cytotoxicity of the plant extracts was determined using the colorimetric methylthiazolotetrazolium (MTT) assay (Mosmann, 1983). MRC5 cells were incubated for an extended period (five days) in the presence of *trans*-avicennol **1**, canthin-6-one **2**, 5-methoxycanthin-6-one **3**, and the dichloromethane, ethanol and methanol extracts of *Z. Chiloperone* (Table 3), and scored as a percentage of absorbance reduction at 540 nm of treated cultures versus untreated control cultures. IC₅₀ values on cell growth were obtained from the drug concentration-response curves and expressed as the means±the standard deviations determined from triplicate independent experiments.

Table 3. Cell survival of MCR5 cells incubated with *Z. chiloperone* isolated compounds and extracts.

Experimental compound(s)	IC ₅₀ (µg/mL)
<i>Trans</i> -avicennol (1)	4.4
Canthin-6-one (2)	9.4
MeOH extract	>100
DCM extract	12.3
EtOH extract	13.0

DCM: dichloromethane; EtOH: ethanol, MeOH: methanol.

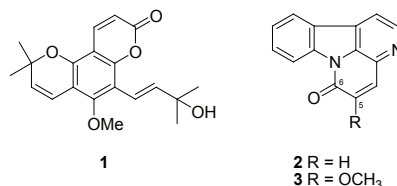
Results and Discussion

Qualitative and quantitative analysis of *Z. chiloperone* stem bark dichloromethane and ethanol extracts

Analysis using HPLC-UV-MS methods

The composition of each organ of *Z. chiloperone*

was analyzed by HPLC-UV-MS study of the ethanol and dichloromethane extracts of stem bark. The qualitative studies identified the main compounds in *Z. chiloperone*, such as the coumarin *trans*-avicennol **1** and canthin-6-one alkaloids, *i.e.* canthin-6-one **2**, 5-methoxy-canthin-6-one **3**. The compounds were identified by their retention times (*t_r*) and by their mass spectra respectively (see also Figures 1 and 2: chromatographic profiles of the reference compounds and profiles of the respective ethanol and dichloromethane extracts).



The quantitative analysis was performed by the external standard method, using a calibration curve constructed utilizing solutions containing different concentrations of the three reference samples, *trans*-avicennol **1**, canthin-6-one **2** and 5-methoxy-canthin-6-one **3**. The concentrations of *trans*-avicennol **1** in the dichloromethane and ethanol extract of the stem bark are 63.2 and 85.6 mg/g extract respectively, while the concentrations of canthin-6-one **2** in the same extracts are 64.7 and 85.6 mg/g extract respectively, finally the concentrations of 5-methoxycanthin-6-one **3** are 29.4 and 19.1 mg/g of extract respectively (Table 1).

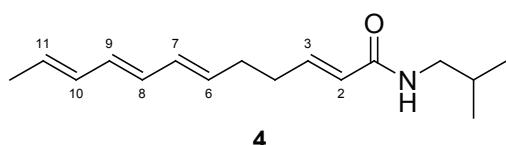
MS/MS of *Z. chiloperone* demonstrates the presence of alkylamides

Previous reports have shown a substantial profile of alkylamides occurring in *Zanthoxylum* spp. (Jang et al., 2008; Xiong et al., 1997; Yang, 2008). However, to our knowledge, there have been no reports on the presence of alkylamides in *Z. chiloperone*. To elucidate the presence of alkylamides, LC-ESI-MS/MS analysis was conducted on the crude extracts of *Z. chiloperone ramulus* cortex using a previously validated method (Spelman et al., 2009). Figure 3A illustrates a selected ion chromatogram obtained from analysis of the 1:2 dry stem bark of *Z. chiloperone* extract. The ion corresponds to the *m/z* of the protonated (M^+) forms of one of the alkylamides in *Z. chiloperone* of MW 247. Figure 3B illustrates the MS/MS spectrum obtained by (CID) of the M^+ ion. For ease of reference, Figure 3B and Table 4 illustrate the primary fragments obtained. These major groups of fragments are consistent with those previously observed for alkylamides (Spelman et al., 2009). They confirm the presence of compound **4**, dodeca-2,6,8,10-tetraenoic acid isobutylamide, also known as sanshool, in *Z. chiloperone*. Note that it is not possible to distinguish E/Z isomers with the LC-MS method employed here.

Table 4. Fragments formed by Collisionally Induced Dissociation (MS-MS) of the M⁺ ion of various *Z. chiloperone* alkylamides.

Designation, name, <i>m/z</i>	group i ⁴ RI ³ 37%	group ii ⁵ RI 64%	group iii ⁶ RI 100%	group iv ⁷ RI 9%	group v ⁸ RI 19-29%	group vi ⁹ RI 5-14%
248 ¹ dodeca-2,6,8,10-tetraenoic acid isobutylamide	175	147	149	206	220, 234	81, 93, 121, 133

¹The number beneath the letter designation indicates the *m/z* value for the M⁺ ion; ²Proposed structure for compound E based on retention time and MS/MS fragmentation; ³RI corresponds to relative intensity; ⁴The group i fragments correspond to acylium ions as shown in Figure 3; ⁵The group ii fragments are carbocations that correspond to the alkyl chain of the alkylamide and are formed by loss of the amide portion (isobutylamide or 2-methylbutylamide); ⁶The group iii fragments correspond to the alkyl chain of the alkylamide and are formed by the loss of the amide portion of the molecule and saturation of one of the double bonds on the alkyl chain. The *m/z* value of these fragments is +2 greater than the group ii fragments; ⁷The group iv fragments correspond to the protonated alkylamide minus the N-alkyl group; ⁸The group v fragments correspond to the protonated alkylamide minus various portions of the N-alkyl group (see Fig. 3 for the bonds at which fragmentation occurs); ⁹The group vi fragments correspond to the varying carbocation fragments formed after loss of various portions of the C-alkyl group.



In vitro biological activity of isolated compounds and extracts of *Z. chiloperone* towards *P. falciparum*

After isolation of *trans*-avicennol **1**, canthin-6-one **2** and 5-methoxycanthin-6-one **3**, the half maximal inhibitory concentrations (IC₅₀) of extracts and the previously listed compounds were assessed by determining the concentration inhibiting 50% of parasite growth. The IC₅₀ were determined against the Colombian chloroquine-resistant FcB1 strain, the Brazilian mildly chloroquine-resistant strain PFB, the multi-drug and chloroquine-resistant strain K1 from Thailand, and the chloroquine-sensitive, but mefloquine resistant Tanzanian strain F32. Figure 5 illustrates the IC₅₀ for avicennol **1**, canthin-6-one **2** and 5-methoxycanthin-6-one **3** on the *Plasmodium falciparum* strain F32. These data show that F32 is the most sensitive strain to avicennol **1** and canthin-6-one **2** (0.5 and 2.0 µg/mL, respectively). Further testing of additional *P. falciparum* strains demonstrated that these compounds all had approximately the same activity on K1, PFB and FcB1 strains. Table 2 shows the results on parasite growth in tabulated form. Note that the crude extracts of *Z. chiloperone* demonstrated robust activity. This is possibly due to the combination of the canthin-6-one type compounds, the pyranocoumarin, as well as the alkylamide(s), previously shown to be active against *P. falciparum* in vitro and *P. yoelii yoelii* in vivo (Spelman et al., 2010). Considering that traditional extracts of *Z. chiloperone* are used to treat Chagas disease, this is an intriguing finding and suggest that there may be various modes of *Plasmodium* inhibition due to the presence of multiple active compounds and possible unknown compounds as well.

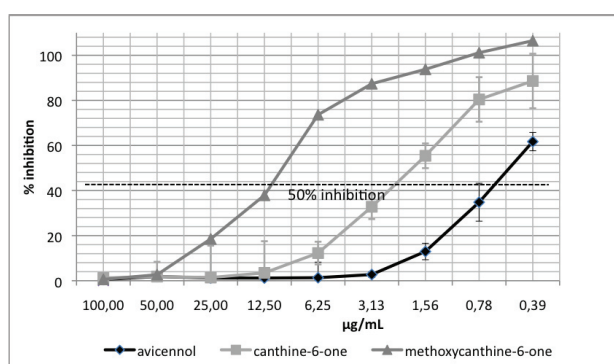


Figure 5. IC₅₀ of isolated compounds from *Z. chiloperone* against *P. falciparum* F32. Avicennol shows an IC₅₀ of 0.5 µg/mL; canthin-6-one and 5-methoxycanthin-6-one show IC₅₀ of 2.0 and 10.4, respectively.

IC₅₀ values for the positive control chloroquine are also listed in Table 2. Note that the IC₅₀ for our K1 clone of *P. falciparum* were considerably less than those published by Elueze et al. (1996) and Fivelman et al. (2007), but higher than Sharrock et al. (2008).

Study of hemolytic activity of compounds isolated from, and extracts of, *Z. chiloperone*

In order to confirm that the antimalarial activities measured by [³H]-hypoxanthine incorporation are due to the intrinsic antiparasite property of the experimental compounds and not due to hemolytic activity, a standard hemolysis assay was run. After 48 h incubation time in the presence of the various test compounds, the hemolysis of non-infected erythrocytes was measured at 530 nm. Results demonstrated that *trans*-avicennol **1**, canthin-6-one **2** and 5-methoxycanthin-6-one **3**, as well as the dichloromethane, ethanol and methanol extracts of *Z. chiloperone* incubated for 48 h at concentrations up to 100 µg/mL, had no haemolytic effect on erythrocytes.

Cytotoxicity assay

To probe for cytotoxicity, MRC5 cells were incubated for an extended period (five days) in the presence of *trans*-avicennol **1**, canthin-6-one **2** and 5-methoxycanthin-6-one **3**, and the dichloromethane, ethanol and methanol extracts of *Z. chiloperone*. Table 3 shows the results of these assays. *Trans*-avicennol **1** had the most cytotoxic effect on MRC5 cells at an IC50 of 4.4 µg/mL. The canthinone alkaloids, canthin-6-one **2** and 5-methoxycanthin-6-one **3**, were observed to have IC50 at 12.1 and 12.3 µg/mL, respectively. Supporting the frequent use of this plant species in traditional medicine, the crude extracts demonstrated less cytotoxicity with the DCM extract and the ethanol extract showing 12.5 and 13.0 µg/mL IC50, respectively. The hydrophilic methanol extract had no detectable effect on cell survival up to 100 µg/mL.

MS-CID detection of the heme-compound (1, 2, 3) adducts

The formation of adducts between compounds and heme is an indication of the ability of the compound to interfere with hematin formation process and thus to show a potential antimalarial activity. Mass spectra from the incubates with heme (Figure 4) show a clear adduct for each of the compounds. The stability of the adducts between heme and the different isolated compounds were assessed by submitting the adducts to in-source CID and by comparing the results to the quinine adduct used as reference (Figure 6). *Trans*-avicennol **1** shows a relatively more stable adduct with heme than the canthin derivatives, which can be correlated with the differences of results in the in vitro assays. A slight difference of stability is observed between avicennol **1** and the canthin derivatives, suggesting a distinct level of binding to heme of the two skeletons. These data give an indication of the ability of these compounds to bind with heme as a major target for antimalarial compounds, although there is no yet established correlation between adduct stability under these conditions and biological activity.

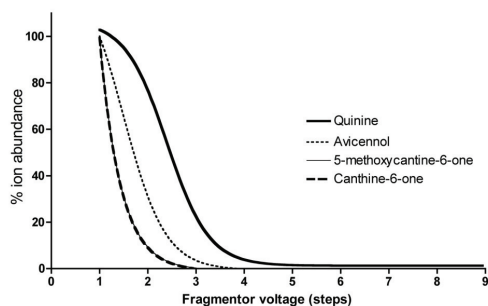


Figure 6. Stability of the heme-compound adducts vs fragmentor voltage.

Conclusion

The studies herein report the quantization, identification and isolation of compounds new to *Zanthoxylum chiloperone* including the pyranocoumarin avicennol **1** and the canthinone alkaloids canthin-6-one **2** and 5-methoxycanthin-6-one **3**. In addition, we identified sanshool, dodeca-2,6,8,10-tetraenoic acid isobutylamide **4**, in this species. We report a good antiplasmodial effect on chloroquine resistant and sensitive strains of *P. falciparum* for the pyranocoumarin avicennol **1** (LD50 of 0.5-2.7 µg/mL) and the canthinone alkaloids (LD50 of 2.0-5.3 µg/mL and 5.1-10.4 µg/mL) for canthin-6-one **2** and 5-methoxycanthin-6-one **3**, respectively. The generation of adducts, heme-avicennol and heme-canthinones, suggests the basis that this activity may be at least partly explained through the heme detoxification pathway. Intriguingly, the dichloromethane and ethanol extracts showed robust effects on the chloroquine-resistant strain K1, and the chloroquine-sensitive, but mefloquine resistant Tanzanian strain F32 of *P. falciparum*. Considering these data, future improvements in antimalarial chemotherapy based on avicennol and the canthinone alkaloids could generate analogs with increased selectivity and lowered resistance index, and opens new horizons for targeting the increased mortality due to drug-resistant malaria.

Acknowledgements

The authors thank the European Community for the financial support (Marie Curie Early Stage Training Fellowship of the European Community's Sixth Framework Programme: contract BioMedChem (for G. CT., K. S. and K. MD.)). Thanks are due to Mrs. A. Solgadi (SAMB) for MS experiments and Dra Nashira Campos Vieira (University of Brasilia) for the portuguese traduction.

References

- Bilia AR, Lazari D, Messori L, Taglioli V, Temperini C, Vincieri FF 2002. Simple and rapid physico-chemical methods to examine action of antimalarial drugs with hemin: its application to *Artemisia annua* constituents. *Life Sci* 70: 769-778.
- Chlouchi A, Girard C, Bonet A, Viollon-Abadie C, Heyd B, Mantion G, Martin H, Richert L 2007. Effect of chrysin and natural coumarins on UGT1A1 and 1A6 activities in rat and human hepatocytes in primary culture. *Planta Med* 73: 742-747.
- Desjardins RE, Canfield CJ, Haynes JD, Chulay JD 1979. Quantitative assessment of antimalarial activity in vitro by a semiautomated microdilution technique. *Antimicrob Agents Chemother* 16: 710-718.
- Elueze EI, Croft SL, Warhurst DC 1996. Activity of pyronaridine and mepacrine against twelve strains of *Plasmodium falciparum* in vitro. *J Antimicrob Chemother* 37: 511-518.

- Ferreira ME, Cebrián-Torrejón G, Corrales AS, de Bilbao NV, Rolón M, Gomez CV, Leblanc K, Yaluf G, Schinini A, Torres S, Serna E, Rojas de Arias A, Poupon E, Fournet A 2011. *Zanthoxylum chiloperone* leaves extract: first sustainable Chagas disease treatment. *J Ethnopharmacol* 133: 986-993.
- Ferreira ME, Nakayama H, de Arias AR, Schinini A, Serna E, Lagoutte D, Soriano-Agaton F, Poupon E, Hocquemiller R, Fournet A 2007. Effects of canthin-6-one alkaloids from *Zanthoxylum chiloperone* on *Trypanosoma cruzi*-infected mice. *J Ethnopharmacol* 109: 258-263.
- Ferreira ME, Rojas De Arias A, Torres De Ortiz S, Inchausti A, Nakayama H, Thouvenel C, Hocquemiller R, Fournet A 2002. Leishmanicidal activity of two canthin-6-one alkaloids, two major constituents of *Zanthoxylum chiloperone* var. *angustifolium*. *J Ethnopharmacol* 80: 199-202.
- Figadère B, Munoz-Durango K, Maciuk A, Londono J, Arrango Acosta GJ 2010. New method for detecting antimalarial drugs. PCT Int. Appl., 97pp. WO 2010103025 (A1) 2010-09-16.
- Fivelman QL, Adagu IS, Warhurst DC 2007. Effects of piperazine, chloroquine, and amodiaquine on drug uptake and of these in combination with dihydroartemisinin against drug-sensitive and -resistant *Plasmodium falciparum* strains. *Antimicrob Agents Chemother* 51: 2265-2267.
- Froelich S, Onegi B, Kakooko A, Siems K, Schubert C, Jennet-Siems K 2007. Plants traditionally used against malaria: phytochemical and pharmacological investigation of *Momordica foetida*. *Rev Bras Farmacogn* 17: 1-7.
- Garavito G, Monje MC, Maurel S, Valentin A, Nepveu F, Deharo E 2007. A non radiolabeled heme-GSH interaction test for the screening of antimalarial compounds. *Exp Parasitol* 116: 311-313.
- Gray AI, Waigh RD, Waterman PG 1977. Cis-avicennol, a new pyranocoumarin from the root bark of *Zanthoxylum elephantiasis*. *Phytochemistry* 16: 1017-1018.
- Jacobson M 1948. Herculol, a pungent insecticidal constituent of Southern prickly ash bark. *J Am Chem Soc* 70: 4234-4237.
- Jang KH, Chang YH, Kim DD, Oh KB, Oh U, Shin J 2008. New polyunsaturated fatty acid amides isolated from the seeds of *Zanthoxylum piperitum*. *Arch Pharm Res* 31: 569-572.
- Karioti A, Skaltsa H, Kaiser M, Tasdemir D 2009. Trypanocidal, leishmanicidal and cytotoxic effects of anthecotulide-type linear sesquiterpene lactones from *Anthemis auriculata*. *Phytomedicine* 16: 783-787.
- Kurosawa Y, Dorn A, Kitsuji-Shirane M, Shimada H, Satoh T, Matile H, Hofheinz W, Masciadri R, Kansy M, Ridley RG 2000. Hematin polymerization assay as a high-throughput screen for identification of new antimalarial pharmacophores. *Antimicrob Agents Chemother* 44: 2638-2644.
- Lozano R, Chitwood DJ, Lusby WR, Thompson MJ, Svoboda JA, Patterson GW 1984. Comparative effects of growth inhibitors on sterol metabolism in the nematode *Caenorhabditis elegans*. *Comp Biochem Physiol C Comp Pharmacol* 79: 21-26.
- Luize PS, Tiunan TS, Morello LG, Maza PK, Ueda-Nakamura T, Dias Filho BP, Cortez DAG, De Mello JCP, Nakamura CV 2005. Effects of medicinal plant extracts on growth of *Leishmania* (L.) *amazonensis* and *Trypanosoma cruzi*. *Braz J Pharm Sci* 41: 85-94.
- Milliken W 1997. Malaria and antimalarial plants in Roraima, Brazil. *Trop Doct* 27 Suppl 1: 20-25.
- Mosmann T 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 65: 55-63.
- Pashynska VA, Van den Heuvel H, Claeys M, Kosevich MV 2004. Characterization of noncovalent complexes of antimalarial agents of the artemisinin-type and FE(III)-heme by electrospray mass spectrometry and collisional activation tandem mass spectrometry. *J Am Soc Mass Spectrom* 15: 1181-1190.
- Robert A, Coppel Y, Meunier B 2002. NMR characterization of covalent adducts obtained by alkylation of heme with the antimalarial drug artemisinin. *Inorganica Chim Acta* 339: 488-496.
- Rush MA, Baniecki ML, Mazitschek R, Cortese JF, Wiegand R, Clardy J, Wirth DF 2009. Colorimetric high-throughput screen for detection of heme crystallization inhibitors. *Antimicrob Agents Chemother* 53: 2564-2568.
- Sharrock WW, Suwanarusk R, Lek-Uthai U, Edstein MD, Kosaisavee V, Travers T, Jaidee A, Sriprawat K, Price RN, Nosten F, Russell B 2008. *Plasmodium vivax* trophozoites insensitive to chloroquine. *Malar J* 7: 94.
- Soriano-Agaton F, Lagoutte D, Poupon E, Roblot F, Fournet A, Gantier JC, Hocquemiller R 2005. Extraction, hemisynthesis, and synthesis of canthin-6-one analogues. Evaluation of their antifungal activities. *J Nat Prod* 68: 1581-1587.
- Spelman K, Depoix D, McCray M, Mouray E, Grellier P 2010. The traditional medicine *Spilanthes acmella*, and the alkylamides spilanthol and undeca-2E-ene-8,10-diyonoic acid isobutylamide, demonstrate *in vitro* and *in vivo* antimalarial activity. *Phytother Res In press*, PTR-10-0925.
- Spelman K, Wetschler MH, Cech NB 2009. Comparison of alkylamide yield in ethanolic extracts prepared from fresh versus dry *Echinacea purpurea* utilizing HPLC-ESI-MS. *J Pharm Biomed Anal* 49: 1141-1149.
- Spichiger R, Stutz de Ortega L 1987. Fagara. In Spichiger R, Stutz de Ortega L (eds.) *Flora de Paraguay: Rutaceae*. St. Louis: Ed Conservatoire et Jardin botaniques de la Ville de Genève, p. 19-36.
- Tabanez MF, Durigan G, Keuroghlian A, Barbosa AF, de Freitas CA, da Silva CEF, da Silva, DA, Eaton DP, Brisolla G, de Faria HH, Mattos IFdA, Lobo MT, Barbosa MR, Rossi M, de Souza MdG, Machado RB, Pfeifer RM, Ramos VS, Andrade WJ D, Contieri WA 2005. In Florestal I. *Plano de Manejo da Estação Ecológica dos Caetetus*. Ed São Paulo, p. 1-103.
- Tekwani BL, Walker LA 2005. Targeting the hemozoin synthesis pathway for new antimalarial drug discovery: Technologies for *in vitro* β -hematin formation assay. *Comb Chem High Throughput Screen* 8: 63-79.
- Thouvenel C, Gantier JC, Duret P, Fournet C, Hocquemiller R, Ferreira ME, Rojas de Arias A, Fournet A 2003. Antifungal compounds from *Zanthoxylum chiloperone* var. *angustifolium*. *Phytother Res* 17: 678-680.
- Weissbuch I, Leiserowitz L 2008. Interplay between malaria, crystalline hemozoin formation, and antimalarial drug

- action and design. *Chem Rev* 108: 4899-4914.
- Xiong Q, Shi D, Yamamoto H, Mizuno M 1997. Alkylamides from pericarps of *Zanthoxylum bungeanum*. *Phytochemistry* 46: 1123-1126.
- Yang X 2008. Aroma constituents and alkylamides of red and green huajiao (*Zanthoxylum bungeanum* and *Zanthoxylum schinifolium*). *J Agric Food Chem* 56: 1689-1696.

***Correspondence**

Alain Fournet
Laboratoire de Pharmacognosie associé au CNRS, UMR 8076
BioCIS, Faculté de Pharmacie, Université Paris-Sud
5, rue Jean-Baptiste Clément, 92296 Châtenay-Malabry CEDEX,
France
alain.fournet@ird.fr
Tel.: +33 (0)1 46 83 59 69
Fax: +33 (0)1 46 83 53 99