In vitro antiplasmodial activity of extract and constituents from *Esenbeckia febrifuga*, a plant traditionally used to treat malaria in the Brazilian Amazon

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Abstract

*Esenbeckia febrifuga* (Rutaceae) is a plant traditionally used to treat malaria in the Brazilian Amazon region. Ethanol extract of stems displayed a good antiplasmodial activity against *Plasmodium falciparum* strains W-2 (IC\textsubscript{50} = 15.5 ± 0.71 μg/ml) and 3D7 (IC\textsubscript{50} = 21.0 ± 1.4 μg/ml). Two coumarins (bergaptene 1 and isopimpinellin 2), five alkaloids (flindersiamine 3, kokusaginine 4, skimmiamine 5, \( \gamma \)-fagarine 6 and 1-hydroxy-3-methoxy-N-methylacridone, 7), besides a limonoid (rutavine 8), have been isolated for the first time from this species. Antiplasmodial activity of compounds 3, 5–8 has been evaluated \textit{in vitro} against *P. falciparum* strains (W-2 and 3D7) and the furoquinolines 5 and 6 were the most potent displaying IC\textsubscript{50} values < 50 μg/ml; flindersiamine (3) showed a weak activity while alkaloid 7 and rutavine (8) were inactive (IC\textsubscript{50} > 100 μg/ml).

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Keywords: *Esenbeckia febrifuga*; Rutaceae; Antiplasmodial activity

Introduction

Malaria is one of the major parasitic diseases in the tropical and subtropical regions of the world and its aetiological agents are protozoans of the genus *Plasmodium*. It is responsible for over 1 million deaths each year and approximately 3.2 billion people, living in 107 countries, are presently at risk. Over 80% of malaria deaths occur in Africa and 15% in Asia. In the Americas, 14% of the population is at risk although the mortality is relatively low in this region. The emergence of chloroquine-resistant strains of *P. falciparum*, the most deadly species of malaria parasites, the resistance of vectors (*Anopheles* spp.) to insecticides, in combination with poverty and lack of good quality health care, are the main causes for the increase of malaria morbidity and mortality (WHO, 2005).

In general, Brazil reports approximately 40% of the total number of malaria cases in the Americas, of which almost 99% occurs in the Legal Amazon Region, where 12% of the population of the country lives. An increase in the number of cases began in the 1980s and a peak of 610,878 cases has been reported in 2000. An improvement in the epidemiological situation in 2006 has been related to the Plan for Intensification of Control Measures in the Amazon (PICAM), initiated in 2000.
In 2003, a National Program for Malaria Control (PNCM) was created by the Ministry of Health, an effort to strengthen the health services to provide conditions for rapid diagnosis, adequate treatment of the cases, control of vectors, fast detection of outbreaks and thus push on the measures to control malaria in the country (WHO, 2005). In 2001, a malaria network, the RAVREDA (Rede Amazônica de Vigilância da Resistência às Drogas Antimaláricas), was created and has gathered several American malarious countries, including Brazil. Besides monitoring the antimalarial drug resistance in the region, this network is also including Brazil. The Malaria Laboratory at Evandro Chagas Institute, state of Pará, under the coordination of Dr. Marinete M. Póvoa, is participating in this programme for evaluation of parasites’ drug resistance, diagnosis methods and quality, entomology and control of malaria vectors (MS/SVS, 2007).

There is a consensus that new drugs to treat malaria are urgently needed. Many approaches to antimalarial drug discovery are available (Ridley, 2002; Rosenthal, 2003; Fidock et al., 2004). Investigation of plant-derived compounds is a valid strategy and this approach can benefit from traditional knowledge of populations from malarious regions. Natural products afforded two of the most important currently available drugs to treat malaria falciparum, quinine and artemisinin. The first one, a quinoline alkaloid, was isolated from Cinchona species used for treatment of fevers and/or malaria by South America Peruvian Indians and has been a template for the synthesis of chloroquine, the most widely used antimalarial drug. Artemisinin is responsible for the antimalarial activity of Artemisia annua, a species of millenar traditional use in China. The development of artemisinin derivatives has been a major advance in the chemotherapy of malaria (Wright, 2005).

It is estimated that 80% of the world’s population depends on herbal remedies for treatment of diseases. Indeed, in malaria endemic areas, plant remedies are still widely used but mostly without assurance of their efficacy. Validation of traditionally used plants to treat malaria is important and requires clinical trials (Wright, 2005; Willcox and Bodeker, 2004) which must be preceded by phytochemical and toxicological studies that are necessary to guarantee efficacy and safety of herbal preparations (phytomedicines). Furthermore, knowledge of active compounds of a medicinal plant is important for development of standardized preparations for pre-clinical and clinical assays.

The genus Esenbeckia Kunth. (family Rutaceae, sub-family Rutidoideae) includes ca. 30 species native to the tropical Americas (Dreyer et al., 1972). Previous chemical studies on species of this genus revealed the presence of typical rutaceous metabolites like coumarins, alkaloids, flavonoids, limonoids and terpenoids (Dreyer et al., 1972; Dreyer, 1980; Bevalot et al., 1984; Oliveira et al., 1996; Rios et al., 2002; Trani et al., 2004).

Esenbeckia febrifuga (A. St.-Hil.) A. Juss. ex Mart., popularly known in Brazil as “quina-do-mato” and “tres folhas”, is used for the treatment of fever and/or malaria by inhabitants of the Brazilian Amazon region. An aqueous bark/stalk extract of this species has been previously assayed in vivo against Plasmodium berghei-infected mice, at a dose of 1.0 g/kg, and was shown to be partly active, causing 43% inhibition of parasite multiplication (Carvalho et al., 1991; Brandão et al., 1992).

In this paper we report on the phytochemistry of E. febrifuga and the in vitro evaluation against P. falciparum of an ethanol extract from stems of this species, as well of five out of the eight compounds isolated (Fig. 1). The susceptibilities were assessed against both chloroquine-sensitive (CQS) (3D7) and chloroquine-resistant (CQR) (W-2) strains of P. falciparum.

**Experimental section**

**Isolation of chemical constituents:** Stems of a tree growing at Campus Pampulha–UFMG, Belo Horizonte, state of Minas Gerais, Brazil, were collected and dried at 50 °C, in an oven with circulating air. A voucher specimen is deposited at the BHCB–UFMG (number 3825). Powdered stems (1.3 kg) were exhaustively extracted by percolation with EtOH; the combined extracts were concentrated in a rotavapor and dried under vacuum to afford 92 g of the crude extract. Chromatography of this extract (75 g), on a silica gel column (Merck 60; 0.040–0.063 mm) eluting initially with hexane–chloroform (80:20) and then increasing the proportions of chloroform followed by chloroform, then chloroform with increasing proportions of methanol, and finally with methanol, led to fractions which were combined according to the similarity on TLC (Merck q60 G) profiles. Repetition of the chromatographic separations and crystallization led to the isolation of compounds 1–8 (Fig. 1) which were identified by analysis of their spectrometric data. Mass spectra were recorded at 70 eV on a Kratos MS80 RFA (Manchester, UK). NMR spectra were recorded on chloroform-d (compounds 1–7) and on DMSO-d6 (8) on a Bruker AM-360 (360.136 MHz) and on a JEOL GSX 400 N (3999.65 MHz), at Fakultät für Chemie und Pharmazie der Ludwig-Maximilians-Universität, Munich, Germany.

**Antiplasmodial assay:** Parasite strains were kept in continuous cultures in human erythrocytes suspended in RPMI 1640 supplemented with 10% human serum according to the method described by Trager and Jensen (1976). The antiplasmodial activity of the extract and test compounds was performed in 96-well tissue culture plates as described by Rieckman (1980) with...
modifications reported by Carvalho et al. (1991). Twofold serial dilutions of test samples dissolved in sterile methanol were placed in microtiter plates and diluted with culture medium (RPMI 1640 plus 10% human serum). A suspension of parasitized erythrocytes (0.5–1% parasitaemia, 2.5% haematocrit) containing mainly trophozoites was added to the wells to give a final volume of 100 μl. Chloroquine was used as positive control and uninfected and infected erythrocytes were included as negative controls. The plates were incubated at 37 °C and after 24 and 48 h the culture medium was replaced with fresh medium with or without test samples. Samples were taken 24 h later, smeared, Giemsa stained and microscopically examined to determine the percentage of parasitaemia by counting 5000 erythrocytes. The results were expressed as the mean IC_{50} of three independent experiments for each sample. The Student t test was used to compare the inhibition of the two different P. falciparum strains.

**Results and discussion**

Phytochemical investigation of an ethanol extract from stems of *E. febrifuga*, collected at the municipality of Belo Horizonte, state of Minas Gerais, Brazil, led to the isolation of two coumarins (bergaptene 1 and isopimpinellin 2), four furoquinoline alkaloids (flindersiamine 3, kokusaginine 4, skimmiamine 5 and γ-fagarine 6), one acridone (1-hydroxy-3-methoxy-N-methylacridone, 7) besides a limonoid (rutaevine, 8). These compounds, identified by comparison of physical
and spectroscopic data with those reported in the literature, are described for the first time for this species. Recently, a new coumarin, named aurapten (7-geranyloxycoumarin), isolated from this species, showed significant in vitro growth inhibition (IC$_{50}$ of 30 μM) of Leishmania major promastigotes (Napolitano et al., 2004).

The in vitro antiplasmodial activities of the crude ethanol extract and of isolated compounds against chloroquine-sensitive (CQS) (3D7) and chloroquine-resistant (CQR) (W-2) strains of *P. falciparum* are depicted in Table 1, together with those of the control drugs chloroquine and mefloquine. For comparison purposes, in the table are also included data previously reported for furoquinoline and acridone alkaloids from rutaceous plant species. Earlier studies by Basco et al. (1994) on the in vitro activity of furoquinoline and acridone alkaloids reported the results on IC$_{50}$ values expressed in μM and has considered as inactive compounds showing IC$_{50}$ > 100 μM, of limited (moderate?) activity, compounds with IC$_{50}$ of 1–20 μM and of low activity those acridones displaying IC$_{50}$ of 20–60 μM. In this paper, the criteria considered by Basco et al. (1994) are being adopted although the IC$_{50}$ values are being expressed in μM.

Kokusagine (4) and the coumarins 1 and 2 were not tested. Of the five compounds assayed, skimmiamine (5) was the most active against W-2 strain (CQR) with an IC$_{50}$ value of 75.3 ± 2.74 μM (19.5 ± 0.71 μg/ml) which is higher than that reported previously for the same strain (IC$_{50}$ 54.4 μM or 14.1 μg/ml), by the $^3$H-hypoxanthine method (Basco et al., 1994). γ-Fagarine (6) was more active against the 3D7 clone (CQS) (IC$_{50}$ 109.8 ± 18.3 μM or 25.0 ± 4.2 μg/ml), γ-Fagarine (6) and skimmiamine (5) displayed moderate activities against both W-2 and 3D7 strains (IC$_{50}$ 157.2 ± 12.2 and 166.0 ± 5.4 μM or 36.0 ± 2.8 and 43.0 ± 1.41 μg/ml, respectively) while flindersiamine (3) showed low activity against both clones (IC$_{50}$ 348.0 ± 35.8 and 265.6 ± 12.8 μM or 95.0 ± 4.2 and 72.5 ± 3.5 μg/ml, respectively). Acridone (7) and rutaevine (8) were inactive (IC$_{50}$ > 100 μg/ml against the assayed *P. falciparum* clones. The most active furoquinolines 5 and 6 showed relatively weak antiplasmodial effect compared to chloroquine and mefloquine (Table 1).

Furoquinoline and acridone alkaloids have been isolated from plants belonging to the Rutaceae family (Waterman, 1999). Earlier studies reported the activity of several acridone alkaloids against *P. yoelli* both in vitro and in vivo (Fujioka et al., 1989, 1990) as well the in vitro activity of furoquinolines and acridones against *P. falciparum* (Basco et al., 1994).

### Table 1. In vitro activities of extract and compounds 3, 5–8 from *Esenbeckia febrifuga* against *Plasmodium falciparum* strains and some literature data

<table>
<thead>
<tr>
<th>Activity against <em>P. falciparum</em>, IC$_{50}$ (μM)</th>
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<tr>
<td><strong>Plasmodium falciparum</strong> strains</td>
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<tr>
<td>3D7$^a$</td>
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<tr>
<td><strong>E. febrifuga extract</strong></td>
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<tr>
<td>21.0 ± 1.4$^e$</td>
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<tr>
<td><strong>Compounds</strong></td>
</tr>
<tr>
<td>Flindersiamine 3</td>
</tr>
<tr>
<td>Kokusagine 4</td>
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<tr>
<td>Skimmiamine 5</td>
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<tr>
<td>g-Fagarine 6</td>
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<tr>
<td>Alkaloid 7</td>
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<tr>
<td>Rutaevine 8</td>
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<tr>
<td>Dictamine 9</td>
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<tr>
<td>Acronymine 10</td>
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<tr>
<td>Arborinine 11</td>
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<td>Melicopicine 12</td>
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<td>Normelicopicine 13</td>
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<td>Chloroquine</td>
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$^a$This paper.
$^b$Basco et al. (1994).
$^c$Muriithi et al. (2002).
$^d$Randrianarivelojosia et al. (2003).
$^e$μg/ml.
The results obtained for the furoquinolines 3, 5 and 6 are consistent with those reported by Basco et al. (1994) and other recent publications on the antiplasmodial activity of rutaceous alkaloids. Skimmimiane (5), γ-fagarine (6) and dictamine (9) were isolated from Zanthoxylum tsihanimposa, a bitter Rutaceae endemic to Madagascar, whose leaf and bark decoctions are used alone or in mixture with other plants for treatment of malaria. The IC$_{50}$ values of these alkaloids against the FcM29 strain of P. falciparum (CQR) were of 134.3, 98.4 and 332.1 µM (30.0, 25.0 and 66.0 µg/ml), respectively, 5 and 6 being thus significantly more potent than 9 (Randrianarivelosojosa et al., 2003). In comparison with data for the FcM29 strain, lower IC$_{50}$ values have been observed for skimmimiane (5) against W-2 strain (CQR), in the present investigation (IC$_{50}$ 75.3±2.74 µM or 19.5±4.2 µg/ml), and earlier by Basco et al. (1994) (IC$_{50}$ 54.4 µM or 14.1 µg/ml) while γ-fagarine (9) displayed a higher IC$_{50}$ value (157.2±12.2 µM or 360.±2.8 µg/ml) and the 3D7 strain showed similar susceptibility as FcM29 (IC$_{50}$ 109.8±8.3 versus 98.4 µM or 25.0±4.2 versus 25.5 µg/ml). Skimmimiane (5) was one of the antiplasmodial alkaloids from Tellea trichocarpa (Rutaceae), a species used in Kenyan traditional medicine for various purposes, including treatment of malaria. It showed a moderate activity against HB3 (CQS) and K1 (CQR) strains of P. falciparum (IC$_{50}$ 47.5±0.42 and 59.0±0.32 µM, respectively) (Murithi et al., 2002).

So far, the most potent antiplasmodial furoquinoline alkaloid yet described is acronydine (10) which showed an IC$_{50}$ of 7.0 µM (2.18 µg/ml) against W-2 strain. It is proposed that the pyran ring would be important for enhancing the antiplasmodial activity of furoquinolines, similarly as it is considered for acridone alkaloids (Basco et al., 1994).

The only acridone alkaloid isolated from E. febrifuga, in the present investigation, was 1-hydroxy-3-methoxy-N-methyl-acridone (7), obtained previously from callus cultures of Ruta graveolens (Rutaceae) (Baumert et al., 1982; Kuzovkina et al., 2004) and whose occurrence is reported in Mexican Esenbeckia species (Dreyer, 1980) besides Fagara macrophylla (Rutaceae) (Spatafora and Tringali, 1997). This acridone (8) was inactive against 3D7 and W-2 strains (IC$_{50}>100$ µg/ml) which is a surprise when compared to arborinine (11) which presents only one more methoxy group and showed a good activity against HB3 and K1 strains of P. falciparum (IC$_{50}$ 3.85±0.11 and 9.34±0.37 µM or 0.98±0.39 and 2.38±1.30 µg/ml, respectively). However, melicopicine (1,2,3,4-tetramethoxy-N-methylacridone) (12) was inactive and normelicopicine (13), the 1-demethyl derivative of melicopicine, has shown good activity against both the strains (IC$_{50}$ 8.25±0.12 and 14.7±0.26 µM, respectively) which led the authors to suggest that the presence of a 1-hydroxyl group in these compounds is essential for the antiplasmodial activity (Murithi et al., 2002). An opposite effect was observed for a series of pyranoacridones for which the presence of a chelated hydroxyl group resulted in a complete loss of antiplasmodial activity and the methoxy group in the same position was crucial for the activity (Basco et al., 1994). As previously observed, these contradictory observations indicate that structure–activity relationships in acridone alkaloids are rather complex (Randrianarivelosojosa et al., 2003).

**Conclusion**

This study reports for the first time the phytochemistry of E. febrifuga which was shown to contain both furoquinoline (3–6) and acridone (7) alkaloids, besides coumarins (1–2) and a limonoid (rutaevine) (8) and the evaluation of the antiplasmodial in vitro activity of an ethanol extract from the stems of this species as well of compounds 3, 5–8. The antiplasmodial furoquinoline alkaloids skimmimiane (5), γ-fagarine (6), flindersiamine (3) and kokusaginine (4), the last one not tested but its activity was previously described (W-2 strain, 29.7 µM; HB3 strain, 89.2 µM) (Basco et al., 1994), are responsible, at least in part, for E. febrifuga activity, and would explain the traditional use of this plant to treat malaria in the Brazilian Amazon region. However, it does not preclude the presence of other active constituents in the aqueous extract which has been shown to be partly active in vivo against P. berghei-infected mice (Carvalho et al., 1991). Therefore, non-isolated chemical constituents may have higher activity and/or synergistic effects would be taking place. As far as we are concerned the evaluation of the antiplasmodial activity of 7 and 8 is reported here for the first time. Some acridone alkaloids occurring in other rutaceous plants disclosed good antiplasmodial activity but 7, the only one isolated from E. febrifuga, was assayed for the first time and was inactive. Rutaevine (8), a limonoid, occurs in some species of Esenbeckia (Dreyer, 1980). Further studies are needed for validation of this Brazilian traditional remedy and the knowledge on its antiplasmodial constituents will be then useful for standardization of phytomedicines.

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References


Bevalot, F., Fournet, A., Moretti, C., Vaquette, J., 1984. Identification of chemical constituents from E. febrifuga stems was carried out at Fakulta¨tf u¨r Chemie und Pharmazie der Ludwig-Maximillians-Universitat, Munich, Germany, as part of an International Cooperation Project between H. Wagner (Germany) and A. Braga de Oliveira (Brazil) within the CNPq (Brazil)/DLR (Germany) agreement.

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References


