In vitro Antiplasmodial Investigation of Medicinal Plants from El Salvador[§]

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In vitro antiplasmodial activities of extracts from *Albizia saman*, Fabaceae, *Calea tenuifolia (C. zacatechichi)*, Asteraceae, *Hymenaea courbaril*, Fabaceae, *Jatropha curcas*, Euphorbiaceae, *Momordica charantia*, Cucurbitaceae, and *Moringa oleifera*, Moringaceae were evaluated. From the lipophilic extract of *C. tenuifolia* five active flavones were obtained. 4′,5-Dihydroxy-7-methoxyflavone [genkwanin] and 5-hydroxy-4′,7-dimethoxyflavone [apigenin 4′,7-dimethylether] exhibited the strongest antiplasmodial activity against a chloroquine-sensitive strain (poW) and a chloroquine-resistant strain (Dd2) of *Plasmodium falciparum* (IC_{50} values: 17.1–28.5 μM). Furthermore octadeca-9,12-dienoic acid [linoleic acid] {IC_{50} values of 21.8 μM (poW) and 31.1 μM (Dd2)} and octadeca-9,12,15-trienoic acid (α-linolenic acid) were isolated.

Introduction

Malaria is still the most dangerous parasitic infectious disease which causes millions of deaths every year. In many countries where it is endemic the traditional medical methods hold a strong part in the public health care system. For safety reasons phytochemical investigations on medicinal plants traditionally used as antimalarials are urgently needed. In this context we are evaluating several species from El Salvador. Results of a bioassay-guided fractionation of *Exostema mexicanum* (Rubiaceae) were already described previously (Köhler *et al*., 2000). In the present study we investigated another six traditional medicinal plants used as antimalarial or antipyretic remedies (Morton *et al*., 1981): *Albizia [Samanea] saman* (Fabaceae), *Calea tenuifolia* (Asteraceae), *Hymenaea courbaril* (Fabaceae), *Jatropha curcas* (Euphorbiaceae), *Momordica charantia* (Cucurbitaceae), and *Moringa oleifera* (Moringaceae). Uses in traditional medicine and previously isolated classes of constituents from these species are given in Table I. In our screening program a crude extract from the leaves of *Calea tenuifolia* showed the most promising antiplasmodial activity. Thus, a bioassay-guided fractionation was carried out in order to isolate and characterize the major antiprotozoan principles. Phytochemical and pharmacological investigations of the other active extracts will be part of further studies.

Calea tenuifolia Kunth is the correct name for the species commonly designated as *C. zacatechichi* Schlecht. It is a plant species of extensive popular medicinal use in Mexico (Díaz *et al.*, 1976). "Zacatechichi" (Nahuatl language) means "bitter grass". It is also known as "dream herb", "zacate de perro" (Spanish for dog's grass), "hoja de dios" (God's leaf), and thle-pela-kano (Chontal) (Rätsch, 1998). The shrub, 1–1.5 m in height, is native to dry forests from central Mexico to Costa Rica at 1500–1800 m (Morton, 1981). The leaves of *C. tenuifolia* are famed as a febrifuge, e.g. aqueous decoctions are given to patients in hospitals (Martinez, 1959). Mixe Indians are using such preparations against haemorrhage and malaria (Heinrich, 1989); it is also a popular remedy

[§] Part 3 in the series 'Herbal remedies traditionally used against malaria', for Part 2 see Kraft *et al.*, 2000.

against bilharziose and diarrhoea (Baytelman, 1979). Furthermore, this species is used by the Chontal Indians to produce or to enhance dreams of a divinatory nature (Mayagoitia *et al.*, 1986).

Experimental

General experimental procedures

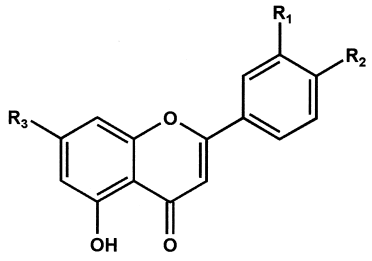
For fractionation, a column containing reversed phase material (LiChroprep® RP-18, 40-63 μm) was used. Preparative high performance liquid chromatography (HPLC) was performed on a Knauer Eurochrom 2000 equipped with an Eurosphere 100 C-18 (10 μm, 22 × 250 mm) column. For preparative thin layer chromatography (TLC) aluminum sheets (20 × 20 cm) coated with silica gel 60 F_{254} were used. Mass spectra were determined with a Finnigan MAT CH7A (220 °C, ionisation 70 eV) and ^{1}H NMR spectra were obtained using acetone-d_6 and MeOD as solvents with a Bruker AVANCE DPX 400 (400 MHz, TMS as internal standard). To evaluate the bioassays we used an Inotech cell harvester and for determination of IC_{50} values a liquid scintillation counter Wallac 1450 MicroBeta plus.

Plant material

Albizia saman, leaves collected May 7, 1995, at the roadside in San Pedro Mashuat, La Paz, El Salvador; voucher specimens deposited in the herbaria B, LAGU, MEXU, and MO; duplicate at MEXU authenticated by M. Sousa. *Calea tenuifolia*, leaves collected October 27, 1996, La Palma, Chalatenango, El Salvador, authenticated by González. *Hymenaea courbaril,* bark and leaves collected June 24, 1995, cantón Calderitas, Apastepeque, San Vicente, El Salvador, authenticated by González. *Jatropha curcas*, leaves collected in April, 1996, roadside at Rosario de Mora, San Salvador, El Salvador; voucher specimens authenticated by J. C. González were deposited in the herbaria B, ITIC, LAGU, and MO. *Momordica charantia,* stems with leaves collected June 8, 1996, at Laguna de Chanmico, San Juan Opico, La Libertad, El Salvador, voucher specimens authenticated by González were deposited in the herbaria B, ITIC, LAGU, and MO. *Moringa oleifera*, leaves, collected February 1, 1998, at Comalapa, road to International Airport, La Paz, El Salvador, voucher specimens authenticated by Hernández were deposited at the herbarium LAGU.

Extraction and isolation

In a screening program, the air dried plant material (20 g) was crushed and extracted three times for 2 h with 150 ml petrol-EtOAc (1:1, V/V) at room temperature to gain the lipohilic extracts. Afterwards the plant material was again air dried and treated three times with 150 ml MeOH-H_2O (8:2) to afford the hydrophilic extracts. Additionally, air dried plant material of *Calea tenuifolia* was extracted for 2 h with 150 ml H_2O under reflux to gain the aqueous extract. For further investigation of *C. tenuifolia*, air dried leaves (300 g) were extracted with petrol-EtOAc (1:1, V/V) and MeOH. The oily residue from the lipophilic extraction was subjected to column chromatography on RP 18 material and sequentially eluted with MeOH-H_2O mixtures of decreasing polarity (up to 90% MeOH), MeOH, and $CHCl_3$. Fractions, eluting with MeOH-H_2O 8:2 and 9:1, proved to be most active in the antiplasmodial assay and were further purified by preparative HPLC with MeOH-H_2O mixtures. Comparison of its spectroscopic data with literature values led to the identification of **1** as 5,7-dihydroxy-3′,4′-dimethoxyflavone (luteolin 3′,4′-dimethyl ether) (Nakanishi *et al.*, 1985). Compound **2** was identified as 4′,5,7-trihydroxyflavone (apigenin) by ^{1}H-NMR and MS spectra and comparison with an authentic natural sample. Spectroscopic data from **3** were identical with literature data for 4′,5-dihydroxy-7′-methoxyflavone



Flavone	R_1	R_2	R_3
5,7-Dihydoxy-3′,4′-dimethoxyflavone (**1**)	OCH_3	OCH_3	OH
4′,5,7-Trihydroxyflavone (**2**)	H	OH	OH
4′,5-Dihydroxy-7-methoxyflavone (**3**)	H	OH	OCH_3
5-Hydroxy-3′,4′,7-trimethoxyflavone (**4**)	OCH_3	OCH_3	OCH_3
5-Hydroxy-4′,7-dimethoxyflavone (**5**)	H	OCH_3	OCH_3

(genkwanin) (Brieskorn *et al.*, 1968). Compound **4** was identified as 5-hydroxy-3′,4′,7-trimethoxyflavone (luteolin 3′,4′,7-trimethyl ether) (Nakanishi *et al.*, 1985), **5** as 5-hydroxy-4′,7-dimethoxyflavone (apigenin 4′,7-dimethylether) (Silva *et al.*, 1971), **6** as octadeca-9,12,15-trienoic acid (α-linolenic acid) (Bhacca *et al.*, 1963), and **7** as octadeca-9,12-dienoic acid (linoleic acid) (Gunstone, 1995). Fractionation of the inactive aqueous extract by column chromatography on RP 18 material with MeOH-H_2O mixtures of decreasing polarity (up to 80% MeOH) led to active fractions, which contained compounds **1** and **3**.

Antiplasmodial activity

The antiplasmodial assay was performed by means of the microculture radioisotope technique as described previously (Jenett-Siems *et al.*, 2000). The concentration at which growth was inhibited by 50% (IC_{50}) was estimated by interpolation. IC_{50} values > 50 μg/ml for extracts and IC_{50} values > 25 μg/ml for fractions, respectively, were considered inactive (O'Neill *et al.*, 1985).

Results and Discussion

Of the six plant species tested, lipohilic crude extracts of *C. tenuifolia*, *H. coubaril*, *M. oleifera*, and *M. charantia* showed significant antiplasmodial activity *in vitro* with IC_{50} values between 6

Table I. *In vitro* antiplasmodial activity of plant extracts against *Plasmodium falciparum.*

Plant family/Species	Local uses	Previously isolated compounds	Part used	Extract	Mean IC_{50} values [μg/ml][a]	
					poW	Dd2
Asteraceae						
Calea tenuifolia Kunth (syn.: *C. zacatechichi* Schlecht.)	febrifuge (Martinez, 1959) haemorrhage, malaria (Heinrich, 1989) bilharziose, diarrhoea (Baytelman, 1979) enhancing dreams (Mayagoita *et al.*, 1986)	sesquiterpene lactones (Ortega *et al.*, 1970; Bohlmann *et al.*, 1981; Quijano *et al.*, 1977 and 1978; Herz *et al.*, 1980) flavonoids (Herz *et al.*, 1980)	leaves	lipophilic methanolic aqueous	10.4 19.7 > 50	24.3 19.5 > 50
Cucurbitaceae						
Momordica charantia L.	antimalarial (Muñoz *et al.*, 2000) hypoglycemic (Matsuda *et al.*, 1998)	oleanolic acid glycosides (Matsuda *et al.*, 1998)	whole plant	lipophilic methanolic	10.3 > 50	9.4 > 50
Euphorbiaceae						
Jatropha curcas L.	molluscicidal activity (Liu *et al.*, 1997) febrifuge (Morton, 1981)	phorbol esters (Liu *et al.*, 1997)	aerial parts	lipophilic methanolic	> 50 > 50	> 50 > 50
Fabaceae						
Samanea saman (Jacq.) Merr. (syn.: *Albizia saman* (Jacq.) F.v. Muell.)	antimalarial use of the related species *Albizia amara* Boiv. (Watt *et al.*, 1962)	terpenoids (Varshney *et al.*, 1985)	leaves bark	lipophilic methanolic lipophilic methanolic	> 50 46.4 > 50 35.5	> 50 10.0 > 50 7.3
Fabaceae						
Hymenaea courbaril L.	substitute for quinine, rheumatism (Morton, 1981)	terpenoids (Khoo *et al.*, 1973)	stems	lipophilic methanolic	11.8 > 50	11.8 > 50
Moringaceae						
Moringa oleifera Lam.	antibiotic (Faizi *et al.*, 1995) antitumor (Murakami *et al.*, 1998)	isothiocyanates (Faizi *et al.*, 1995) thiocarbamate (Murakami *et al.*, 1998)	flowers leaves stems	lipophilic methanolic lipophilic methanolic lipophilic methanolic	6.0 > 50 7.8 > 50 > 50 > 50	16.3 > 50 15.4 > 50 > 50 > 50

[a] Tested in triplicates, internal standard: see Table II.

and 25 μg/ml (Table I). Methanolic crude extracts of *C. tenuifolia* and *S. saman* displayed an activity with IC_{50} values ranging from 7 to 36 μg/ml. Bioactivity-guided fractionation of the lipophilic extract of *C. tenuifolia* led to the isolation of five flavones (**1**-**5**). To the best of our knowledge, these flavones were isolated from *C. tenuifolia* for the first time. All compounds showed activities against *P. falciparum*, with IC_{50} values in a range between 4 and 40 μM (Table II). From all active fractions we isolated flavones. Certain active fractions additionally contained fatty acids as by-products. Two of them could be isolated and characterized as **6** and **7**. The antimalarial properties of unsaturated fatty acids were described previously: linoleic acid and α-linolenic acid seem to inhibit parasite growth in culture and *in vivo* (Krugliak *et al.*,1995). Results of another study demonstrated that the antiplasmodial activity of the fatty acids is dependent in part on the degree of unsaturation (Kumaratilake *et al.*, 1992). Since flavones were isolated from all active fractions of *C. tenuifolia*, we assume, that this class of compounds represents the major antiprozoan principle. Thus, our results may represent a rational explanation for a potential antimalarial effect of the leaves of *C. tenuifolia*. Furthermore, fractionation of the aqueous extract led to the detection of the flavones **1** and **3** in active fractions. This result can account for the ethnomedicinal use, because an aqueous leaf decoction is used in traditional Central American medicine.

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Table II. *In vitro* antiplasmodial activity of compounds isolated from the leaves of *Calea tenuifolia* against *Plasmodium falciparum*.

Compound	Mean IC_{50} values[a]			
	poW		Dd2	
	[μg/ml]	[μM]	[μg/ml]	[μM]
5,7-Dihydroxy-3′,4′-dimethoxyflavone (**1**) [luteolin 3′,4′-dimethyl ether]	13.6	43.3	10.5	33.4
4′,5,7-Trihydroxyflavone (**2**) [apigenin]	14.6	54.1	25.0	92.6
4′,5-Dihydroxy-7-methoxyflavone (**3**) [genkwanin]	5.4	19.0	8.1	28.5
5-Hydroxy-3′,4′,7-trimethoxyflavone (**4**) [luteolin 3′,4′,7-trimethyl ether]	5.9	18.0	n.d.	n.d.
5-Hydroxy-4′,7-dimethoxyflavone (**5**) [apigenin 4′,7-dimethylether]	6.0	20.1	5.1	17.1
Octadeca-9,12,15-trienoic acid (**6**) [α-linolenic acid]	13.8	49.6	39.5	142.0
Octadeca-9,12-dienoic acid (**7**) [linoleic acid]	6.1	21.8	8.7	31.1
Artemisinin	0.0008	0.003	0.004	0.015
Choroquine × 2 H_3PO_4	0.008	0.015	0.073	0.14

[a] Tested in triplicate; n.d.: not determined.

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