Herbal Remedies Traditionally Used Against Malaria in Ghana: Bioassay-Guided Fractionation of *Microglossa pyrifolia* (Asteraceae)[§]

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Different extracts from 11 West African plants traditionally used against malaria in Ghana were tested against both the chloroquine-sensitive strain PoW and the chloroquine-resistant clone Dd2 of *Plasmodium falciparum.* Due to the promising *in vitro* activity of the lipophilic extract [IC_{50}: 10.5 μg/ml (PoW); 13.1 μg/ml (Dd2)], *Microglossa pyrifolia* (Lam.) Kuntze (Asteraceae) was chosen for further phytochemical investigation. From active fractions 13 compounds were isolated; their structures were established on the basis of spectroscopic methods. 1-Acetyl-6*E*-geranylgeraniol-19-oic acid and sinapyl diangelate represent new natural compounds. The two diterpenes *E*-phytol [IC_{50}: 8.5 μM (PoW); 11.5 μM (Dd2)], and 6*E*-geranylgeraniol-19-oic acid [IC_{50}: 12.9 μM (PoW); 15.6 μM (Dd2)] proved to be the most active constituents in our test system.

Introduction

In continuation of our research on medicinal plants with antiplasmodial activity we investigated 11 plants from Ghana, where malaria causes many deaths per year, particularly in young children (Browne *et al.*, 2000). *Gomphrena celosioides* (Amaranthaceae), *Picralima nitida* (Apocynaceae), *Pergularia daemia* (Asclepiadaceae), *Emilia sonchifolia* (Asteraceae), *Microglossa pyrifolia* (Asteraceae), *Adansonia digitata* (Bombacaceae), *Euphorbia hirta*, *Phyllanthus niruroides* (Euphorbiaceae), *Tetrapleura tetraptera* (Mimosaceae), *Mitragyna inermis*, and *M. stipulosa* (Rubiaceae) are widespread in the Greater Accra Region of Ghana and are used against malaria or fever by herbalists (Bruce, 1998 and 2000). Based on our screening results (Table I) we chose *Microglossa pyrifolia* (Lam.) Kuntze (*syn.*: *Conyza pyrifolia* Lam., Asteraceae) for further investigation. *M. pyrifolia* is a shrub widespread in tropical Asia and Africa (Dokosi, 1998). In Ghana an aqueous leaf decoction is used against fever and malaria. In other West African countries the leaf extract is taken against abdominal pains, rheumatism, diarrhoea and many other diseases (Neuwinger, 1994). Subsequent fractionation of the lipophilic extract coupled with an antiplasmodial bioassay (Desjardins *et al.*, 1979) was performed to detect the active principles responsible for the antiprotozoal activity of *M. pyrifolia.*

Experimental

General experimental procedures

For fractionation, silica gel 60 (63–200 μm) and reversed phase material (LiChroprep® RP-18, 40–63 μm) were used. Preparative high performance liquid chromatography (HPLC) was performed on a Knauer Eurochrom 2000 with Knauer pumping system and a Knauer WellChrom DAD K-2700 UV-detector 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

[§] Part 7 in the series 'Herbal remedies traditionally used against malaria', for part 6 see Onegi *et al.* (2002).

F_{254} were used. Mass spectra were determined with a Finnigan MAT CH7A (220 °C, 70 eV) and FAB-MS were recorded on a VARIAN MAT CH5DF (Xenone, 7 kV). ^{1}H-NMR spectra, ^{1}H-^{1}H-COSY, and HMBC experiments were obtained using acetone-d_6 and $CDCl_3$ as solvents with a Bruker AVANCE DPX 400 (400 MHz, TMS as internal standard). Cultures of *P. falciparum* were harvested with an Inotech cell harvester and IC_{50} values determined by a liquid scintillation counting.

Plant material

The plant species were collected in the Greater Accra Region from February to March 2000 and April 2001 by scientists of the Department of Botany, Legon-University Accra in Ghana. Voucher specimens of the following plants have been deposited in the herbarium of the Department of Botany, Legon-University Accra: *Gomphrena celosioides* (GC 39071), *Picralima nitida* (GC 39064), *Pergularia daemia* (GC 39070), *Emilia sonchifolia* (39072), *Microglossa pyrifolia* (GC 47681), *Euphorbia hirta* (GC 39068), *Phyllanthus niruroides* (GC 39069), *Tetrapleura tetraptera* (39065), *Mitragyna inermis* (GC 39066), *Mitragyna stipulosa* (GC 39067). *Adansonia digitata* fruits were collected in April 2001 in Navrongo (Upper East Region).

Extraction and isolation

For the screening program, the air dried plant material (20 g) was crushed and extracted three times for 2 h with 150 ml petrol ether-EtOAc (1:1, v/v) at room temperature to gain the lipophilic extracts. Afterwards the plant material was air dried and treated three times with 150 ml MeOH to afford the hydrophilic extracts. The preparation of the aqueous extracts was carried out according to (Bruce, 2000): Stem barks and roots were boiled for one hour in an aqueous solution of $NaHCO_3$ (1 teaspoon/300 ml). Vegetative aerial parts or fruits were extracted by an infusion with such a solution.

For further investigations of *M. pyrifolia*, vegetative aerial parts (500 g) were ground and extracted for 24 h at room temperature with petrol ether-EtOAc 1:1 (2 l). This procedure was repeated three times. Solvents were evaporated at 40 °C under reduced pressure; the residue was subjected to the antiplasmodial test. The oily residue (30 g) was purified by column chromatography on RP-18 material and sequentially eluted with MeOH-H_2O mixtures (4:6; 5:5; 6:4; 7:3; 8:2; 9:1, v/v), MeOH, and $CHCl_3$ (800 ml, each). Fractions 6–10 eluting with MeOH-H_2O (8:2; 9:1, v/v, 300 ml each) and MeOH (500 ml), respectively, proved to be the most active ones in the antiplasmodial assay. Fraction 6 (residue 120 mg) was chromatographed on silica gel (7 g) with CH_2Cl_2, CH_2Cl_2-MeOH (9:1, v/v) (200 ml each) to yield compound **13** (3 mg). Further purification of the remaining fraction 6A by HPLC with MeOH-H_2O (30:70, v/v) to MeOH within 40 min lead to **11** (60 mg, R_T = 29.64 min). Fraction 7 (250 mg) was separated on silica gel (12 g) with petrol ether-EtOAc (3:1 and 1:1, v/v respectively; 170 ml each) and CH_2Cl_2-MeOH (10:1, v/v, 200 ml) and finally by preparative TLC (CH_2Cl_2-MeOH 10:1, v/v) to yield acacetin **1** (3 mg, R_f = 0.42), compound **4** (5 mg, R_f = 0.52) and **5** (10 mg, R_f = 0.48). Fraction 8 (1 g) was purified on silica gel (40 g) with petrol ether-EtOAc gradient (10:1; 3:1; 1:1, v/v, respectively; 200 ml each) and yielded the active fractions 8A and 8B. Compound **6** (12 mg, R_f = 0.49) was isolated from 8A by preparative TLC (petrol ether-EtOAc 2:1, v/v). Purification of 8B by preparative TLC (CH_2Cl_2-MeOH 10:1, v/v) lead to **7** (30 mg, R_f = 0.57). Fraction 9 (6 g) was purified on silica (60 g) with petrol ether-EtOAc and CH_2Cl_2-MeOH gradients, respectively (10:1, 5:1 and 40:1, 20:1, v/v, respectively; 250 ml each) and lead to the active fractions 9A and 9B. Compounds **8** (3 mg, R_f = 0.60), **9**, and **10** (6 mg, R_f = 0.62) were isolated from 9A by preparative TLC (CH_2Cl_2-MeOH 10:1, v/v). From fraction 9B, using preparative TLC (CH_2Cl_2-MeOH 10:1 and 20:1, v/v, respectively), we isolated compound **12** (12 mg, R_f = 0.62). Fractionation of 10 (1.5 g) on silica (20 g) lead to linoleic acid **2** (15 mg); fraction 10A was purified by preparative TLC ($CHCl_3$-MeOH 9:1, v/v) and yielded **3** (2 mg, R_f = 0.47). Fractionation of the inactive aqueous extract by column chromatography on RP-18 material with MeOH-H_2O mixtures of decreasing polarity (up to 90% MeOH) lead to active fractions, which contained compounds **7** and **11**.

1-Acetyl-6E-geranylgeraniol-19-oic acid (**12**): ^{1}HNMR (400 MHz), acetone-D_6: δ 6.77 (1H, t, J = 7 Hz, H-6); 5.39 (1H, tq, J = 7 Hz, 1 Hz, H-2); 5.19 (1H, tq, J = 7 Hz, 1 Hz, H-10); 5.10 (1H, tq, J = 7 Hz, 1 Hz, H-14); 4.59 (2H, d, J = 7 Hz, H-1);

2.41–2.33 (4H, m, H-8, H-9); 2.25–2.12 (4H, m, H-4, H-5); 2.05 (3H, s, OAc); 1.98 (4H, obscured by solvent, H-12, H-13); 1.72 (3H, s, H-20); 1.66 (3H, s, H-18); 1.60 (3H, s, H-16 or H-17); 1.59 (3H, s, H-17 or H-16) ppm; (-)-FAB-MS: 361 $[M-H]^-$.

Sinapyl diangelate (**13**): ^{1}HNMR (400 MHz), acetone-D_6: δ 6.90 (2 H, s, H-2, H-6), 6.73 (1 H, brd, J = 16 Hz, H-7), 6.47 (1H, td, J = 6 Hz, 16 Hz, H-8), 6.23 (1 H, m, H-3′ or H-3″), 6.12 (1H, qq, J = 1 Hz, 7 Hz, H-3″ or H-3′), 4.81 (2 H, dd, J = 1 Hz, 6 Hz, H-9), 3.84 (6 H, s, $OCH_3$3 and $OCH_3$5); 2.20 (3 H, m, H-4″ or H-5″), 2.01 (3 H, obscured by solvent, H-5″ or H-4″), 1.97 (3 H, dq, J = 7 Hz, 1.5 Hz, H-4′), 1.90 (3 H, quint, J = 1.5 Hz, H-5′) ppm; EI-MS: $[M]^+$ 374 (5), 292 (12), 83 (100), 55 (46); HR-MS: m/z = 374.17273 (calculated 374.17294 for $C_{21}H_{26}O_6$), 292.13099 (calculated 292.13108 for $C_{16}H_{20}O_5$), 83.04966 (calculated 83.04969 for C_5H_7O).

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

Lipophilic extracts of all tested plant species exhibited a moderate antiplasmodial activity with *M. pyrifolia* aerial parts as the most active ones [IC_{50}: 13.1 μg/ml (Dd2), 10.5 μg/ml (PoW)]. Bioassay-guided fractionation of the aerial parts of *M. pyrifolia* revealed the fractions eluting from a RP-18

Table I. Activity of lipophilic[#] plant extracts against *Plasmodium falciparum*.

Plant family	Species	Plant part	Traditional uses	Mean IC_{50} values [μg/ml[a]]	
				PoW	Dd2
Amaranthaceae	*Gomphrena celosioides* Mart.	whole plant	antimalarial (Bruce, 1998)	36.9	31.5
Apocynaceae	*Picralima nitida* (Stapf) Th. & H. Durand	stem bark	substitute for quinine (Neuwinger, 1994), antimalarial (Bruce, 1998), (François *et al.*, 1996)	> 50	14.5
Asclepiadaceae	*Pergularia daemia* (Forssk) Chiov.	stem bark	remedy for cold (Dokosi, 1998), and fever (Bruce, 2000)	> 50	> 50
Asteraceae	*Emilia sonchifolia* (L.) DC.	aerial parts	fever remedy (Abbiw, 1990), antimalarial (Azuine, 1998)	36.9	43.6
	Microglossa pyrifolia (Lam.) Kuntze	aerial parts, root	fever remedy (Abbiw, 1990), antimalarial (Bruce, 1998)	10.5	13.1
Bombacaceae	*Adansonia digitata* L.	fruit pulp[b]	fever remedy (Abbiw, 1990), antimalarial (Azuine, 1998)	> 50	> 50
Euphorbiaceae	*Euphorbia hirta* L.	whole plant	antimalarial (Dokosi, 1998)	> 50	> 50
	Phyllanthus niruroides Müll. Arg.	aerial parts	antimalarial (Bruce, 1998)	11.5	27.3
Mimosaceae	*Tetrapleura tetraptera* (Schuhmach. & Thonn.) Taub.	fruits	antimalarial (Neuwinger, 1994)	> 50	> 50
Rubiaceae	*Mitragyna inermis* (Willd.) Kuntze	leaves,	analgesic properties	> 50	34.6
		stem bark,	(Abbiw, 1990),	39.6	14.6
		root	antimalarial (Bruce, 1998), (Mustofa *et al.*, 2000)	> 50	28.2
	Mitragyna stipulosa (DC.) Kuntze	leaves,	antimalarial (Bruce, 1998),	32.6	20.4
		stem bark,	antidote to poison, e.g.	> 50	36.1
		root	(Abbiw, 1990)	> 50	48.7

[#]: Petrol ether-EtOAc (1:1, v/v).
[a]: Performed in duplicate.
[b]: Tested as aqueous extract.

column with 80% to 100% MeOH to be most active with IC_{50} values ranging from 2.5 to 18.7 μg/ml against a chloroquine-sensitive strain (PoW) and a chloroquine-resistant clone (Dd2) of *Plasmodium falciparum.* Further separation by several chromatographic methods yielded 13 compounds. We isolated acacetin (**1**), linoleic acid (**2**), *E*-phytol (**3**), benzyl 2,6-dimethoxybenzoate (**4**) (Lu *et al.*, 1993), 13-hydroxy-octadeca-9*Z*,11*E*,15*Z*-trien-oic acid (**5**) (Reddy *et al.*, 1994), and 1-hydroxy-calamenene as a mixture of two isomers (**6**) (El-Seedi *et al.*, 1994). Furthermore, we obtained the following furanoditerpenes: strictic acid (**7**) (Tandon and Rastogi, 1979), hardwickiic acid (**8**) (Heymann *et al.*, 1994), 10α-nidoresedic acid (**9**) and 10β-nidoresedic acid (**10**). The spectral data of **9** and **10** were quite similar to those of the already known methyl esters (Singh *et al.*, 1988, Bohlmann and Fritz, 1978). Several active fractions yielded 6*E*-geranylgeraniol-19-oic acid (**11**) (Zdero *et al.*, 1990). The ^{1}H NMR of **12** was very similar to that of **11**. An additional singlet at 2.06 ppm (3H, s, OAc) pointed to an acetate group. Due to the presence of the acetate group, the signal for the two protons at C-1 displayed a chemical shift of 4.59 ppm (2H, d, J = 7 Hz, H-1) versus the signal for H-1 of **11** at 4.09 ppm (2H, d, J = 7 Hz, H-1). The (−) FAB MS gave a parent ion $[M\text{-}H]^-$ at *m/z* 361 thus confirming the structure and lead to the identification of **12** as 1-acetyl-6*E*-geranylgeraniol-19-oic acid. The isolation of the 6*E*-isomer has not yet been reported, in contrast to its 6*Z*-isomer (Herz and Kulanthaivel, 1985). The EIMS spectrum of **13** showed a molecular ion peak at *m/z* 374. The ion fragment peak at *m/z* 292 indicated the loss of an angelate group. In the ^{1}H NMR compound **13** displayed a singlet at 6.90 ppm (2H, s, H-2, H-6) and two singlets for two methoxyl groups at 3.84 ppm (6H, s, OMe-3 and OMe-5), belonging to a methoxylated aromatic system. Additionally a doublet at 6.73 ppm (1H, d, J = 16 Hz, H-7) and a triplet doublet at 6.47 ppm (1H, td, J = 6 Hz, 16 Hz, H-8) pointed to a *trans*-configurated double bond in conjugation with the aromatic system. The chemical shift of the allylic methylene group at 4.81 ppm (2 H, dd, J = 1 Hz, 6 Hz, H-9) indicated an esterification at this position. Additionally typical signals for two angelate moieties could be observed: 6.23 ppm (1 H, m, H-3′ or H-3″), 6.12 ppm (1H, qq, J = 1 Hz, 6 Hz, H-3″ or H-3′), 2.20 ppm (3 H, m, H-4″ or H-5″), 2.01 ppm (3 H, obscured by solvent, H-5″ or H-4″), 1.97 ppm (3 H, dq, J = 7 Hz, 1.5 Hz, H-4′), 1.90 ppm (3 H, quint, J = 1.5 Hz, H-5′). Comparison with literature data of known sinapyl alcohol derivatives (Bohlmann *et al.*, 1969) lead to the identification of **13** as sinapyl diangelate, which represents a new natural compound. This is the first report on the occurrence of **1**-**10**, **12**, and **13** from *M. pyrifolia*.

In our antiplasmodial test system the compounds linoleic acid (**2**), *E*-phytol (**3**), 13-hydroxy-octadeca-9*Z*,11*E*,15*Z*-trien-oic acid (**5**), and 6*E*-geranylgeraniol-19-oic acid (**11**) exhibited an activity against *P. falciparum* with IC_{50} values between 2.5 μg/ml and 13.7 μg/ml (Table II). From several active fractions we isolated the diterpene 6*E*-geranylgeraniol-19-oic acid (**11**). Thus, we assume that the aliphatic unsaturated compounds of *M. pyrifolia* are representing the antiprotozoal principle of this species. In our test system the methanolic and aqueous extracts of all tested species did not show any activity. However, fractionation of the aqueous extract of *M. pyrifolia* lead to the detection (^{1}H NMR and EIMS spectra) of **7** and **11** in active fractions. Thus, **11** is detectable in the aqueous extract despite of its rather lipo-

Table II. Antiplasmodial activity of compounds isolated from *Microglossa pyrifolia* against *Plasmodium falciparum in vitro.*

	Mean IC_{50} values*			
	PoW		Dd2	
Compound	[μg/ml]	[μM]	[μg/ml]	[μM]
Linoleic acid (octadeca-9,12-dienoic acid)# (**2**)	6.1	21.8	8.7	31.1
E-Phytol (**3**)	2.5	8.5	3.4	11.5
Benzyl 2,6-dimethoxybenzoate (**4**)	9.0	33.1	> 25	> 91.9
13-Hydroxy-octadeca-9*Z*,11*E*,15*Z*-trienoic acid (**5**)	6.7	22.8	13.7	46.6
6*E*-Geranylgeraniol-19-oic-acid (*11*)	4.3	12.9	5.2	15.6
Chloroquine × 2 H_3PO_4	0.008	0.015	0.073	0.14

* Performed in triplicate.

Previously tested (Köhler *et al.*, 2002).

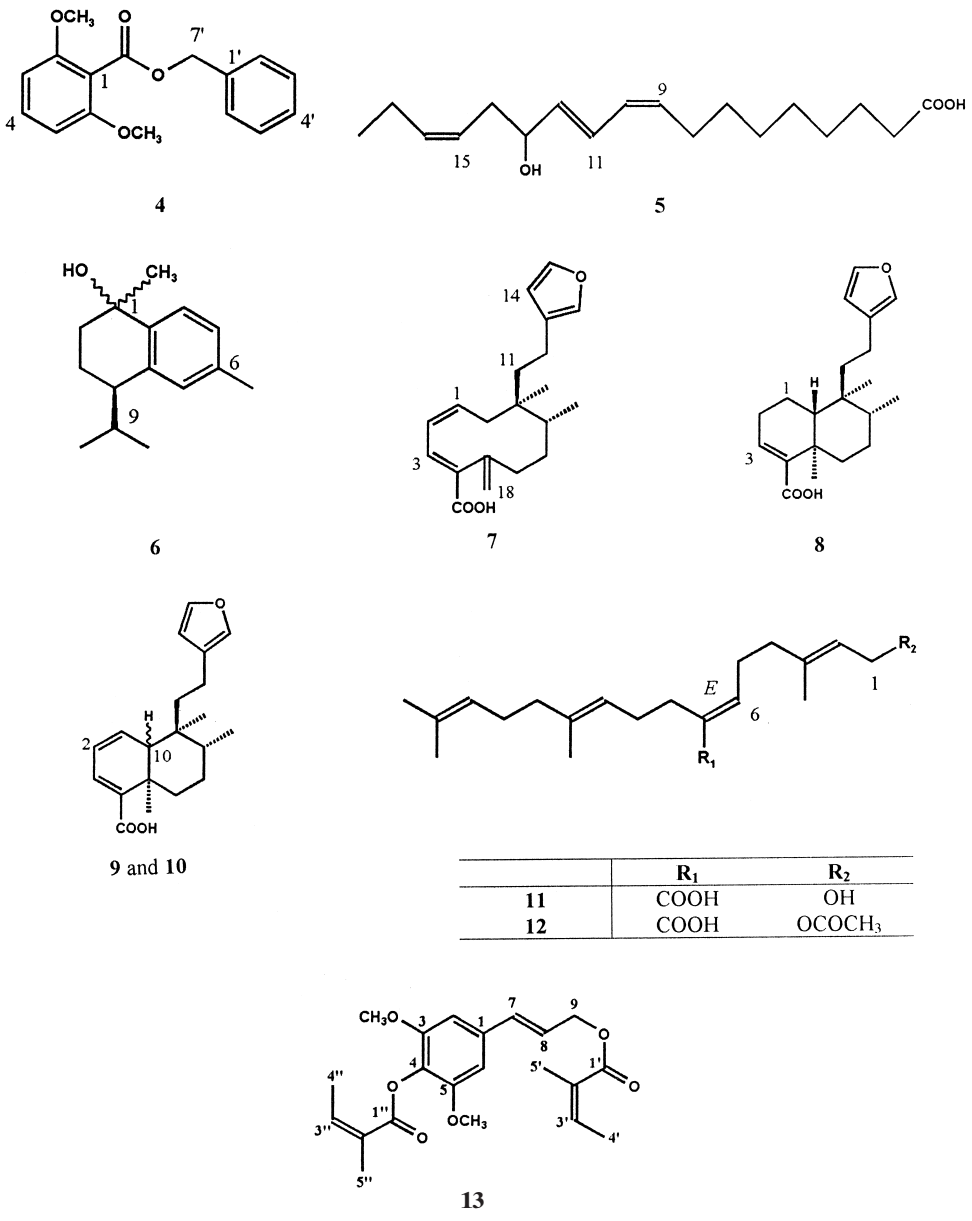

Fig. 1. Structures of compounds isolated from *Microglossa pyrifolia.*

philic character. Therefore, we conclude that 6*E*-geranylgeraniol-19-oic acid (**11**) is mainly responsible for a moderate antiplasmodial activity of a traditional plant preparation from *M. pyrifolia.*

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