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COMPOSITION AND ANTIMALARIAL ACTIVITY *IN VITRO* OF VOLATILE COMPONENTS OF *LIPPIA MULTIFLORA*

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Key Word Index—*Lippia multiflora*; Verbenaceae; volatile components; antimalarial; *Plasmodium falciparum*; *in vitro*.

Abstract—The essential oil of *Lippia multiflora* was prepared by hydrodistillation of leaves and stalks and characterized by GC and mass spectroscopy. The oil was tested for antimalarial activity on *in vitro* cultures of *Plasmodium falciparum* (FcB1-Columbia chloroquine-resistant strain and F32-Tanzania chloroquine-sensitive strain). The dilutions inhibiting the *in vitro* growth of the parasite by 50% 24 and 72 hr after administration of the essential oil to the parasite culture were 1/12 000 and 1/21 000, respectively. When tested on a highly synchronized culture, the essential oil inhibited growth mostly at the trophozoite–schizont step, indicating a potential effect on the first nuclear division of the parasite.

INTRODUCTION

Malaria is of worldwide interest because of its high morbidity and mortality rates [1, 2]. The increasing spread of *Plasmodium falciparum* strains which are resistant to standard treatments has initiated numerous studies aimed at identifying new antimalarials. Research on and identification of active plant constituents are important in the search for new antimalarial compounds. Traditional medicine can be a very important source of active plants [3, 4]. One of the recent achievements in the treatment of malaria is the use of artemisinin and its derivatives obtained from *Artemisia anmua*, a plant traditionally used in Chinese medicine [5]. African traditional medicine uses numerous plants, the efficiency of which against malaria is being tested [6–8]. In the course of an extensive study on Ivory Coast (RCI) antimalarial medicinal plants, our interest was focused on *Lippia multiflora* Mold [9]. This plant is of common use in West Africa. Its antiviral and antifungal activity has already been reported elsewhere [10]. The present work describes the composition of the essential oil of *L. multiflora* and its antimalarial activity *in vitro* on *Plasmodium falciparum*.

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RESULTS AND DISCUSSION

Hydrodistillation of the dried plant material gave a light yellow–green essential oil with a mean yield of 0.8% (by volume).

The oil was analysed by GC and GC-mass spectrum (Table 1). Eighteen components were identified and quantified in this way [11, 12]. *L. multiflora* shows rather great chemical variability with regard to its essential oil composition [10, 12, 13]. The sample described in the present report was characterized by a high content in linear terpenes: nerolidol (45.2%), linalool (20.2%) and β-farnesene (10.5%); germacrene D, β-caryophyllene and 1-8-cineol were important minor components.

Serial dilutions of the essential oil of *L. multiflora* were tested *in vitro* on two strains of *P. falciparum* (Fig. 1). No differences were observed between the dilutions that inhibited by 50% the growth (ID_{50}) of the chloroquine-resistant (FcB1) and chloroquine-sensitive (F32) strains of the parasite (1/12 000 and 1/21 000 after 24 and 72 hr incubation, respectively, for both strains). This equivalent activity on the two strains tested is an encouraging result since it might help in circumventing the problem of chloroquine resistance [6]. Furthermore, the differences (about three dilutions) observed in the ID_{50} at the two incubation times tested (24 and 72 hr) might indicate a potential cumulative effect of the essential oil on *P. falciparum* growth.

When tested on 4 hr synchronized cultures, it appeared that at the highest dose tested (1/6000) the oil interfered mostly with the second part of the the erythrocytic cycle (16–48 hr) (Fig. 2). More interesting were the results obtained with the dilutions of 1/8000 and 1/12000, for which maximal inhibition was observed between 24 and 32 hr, corresponding to the trophozoite–schizont stage (S-phase of the cycle [14]). A lower inhibitory effect was also observed at these doses on the last part of the cycle (Fig. 2). Higher dilutions (1/16000 and 1/18000) of the essential oil had either a very weak or no detectable effect.

Table 1. Volatile components of *L. multiflora*

R_t (min)	Identification	Percentage of total oil
09.8	Sabinene	0.3
10.3	Myrcene	0.2
10.7	α-Phellandrene	1.8
11.3	*para*-Cymene	0.2
11.5	1,8-Cineole	3.1
11.5	Limonene	0.2
13.6	Linalool	20.2
16.4	α-Terpineol	0.3
17.3	Nerol	0.4
22.2	α-copaene	0.3
22.4	β-Bourbonene	0.3
23.5	β-Carophyllene	3.8
24.2	(*Z*)b-Farnesene	10.5
24.4	α-Humulene	0.4
24.6	$C_{15}H_{24}$	0.2
25.2	Germacrene D	9.8
25.5	$C_{15}H_{24}$	0.6
25.8	M = 222	0.2
26.2	δ-Cadinene	0.6
26.9	M = 222	0.2
27.4	(*Z*)-Nerolidol	45.2
27.9	Caryophyllene oxide	0.4

We have recently initiated an extensive study of Ivory Coast antimalarial medicinal plants. Our interest was focussed on *L. multiflora* because of the activity of its aqueous extracts (IC_{50} = 2–4 μg ml^{-1}) [9]. The antimalarial activity of the aqueous extracts was also observed after cryoconservation. However, the essential oil seems to be responsible for the antimalarial activity.

The direct application of an essential oil in the treatment of malaria has so far not be considered. However, the presence of potential antimalarial compounds in *L. multiflora* essential oil as well as the low toxicity of the oil at rather low doses [10] might open possibilities for further investigations in the field of antimalarial compounds.

EXPERIMENTAL

Plant. The plant material was harvested in Bouake (République de Côte d'Ivoire) in November 1994. The botanical identification was made by comparison with an authentic sample. A voucher specimen is deposited at the Faculté de Pharmacie of Abidjan (République de Côte d'Ivoire).

Essential oil preparation and analysis. The essential oil was prepared by hydrodistillation using a Clevenger type apparatus. The distillation was performed with 100 g dried plant material in 2.5 l H_2O for 4 hr. The qualitative analysis by GC-MS was performed under the following conditions: fused silica capillary polydimethylsilicone DB1 column (30 m × 0.253 mm, film thickness 0.25 μm) in melted silica. Carrier gas was He (0.9 ml min^{-1}); injector temp. 200°; oven temp. 50° for 2 min, then raised to 200° at 4° min^{-1}; detector temp. 220°. MS: a selective quadrapole type detector of 5970 A class; ionization voltage 70 eV.

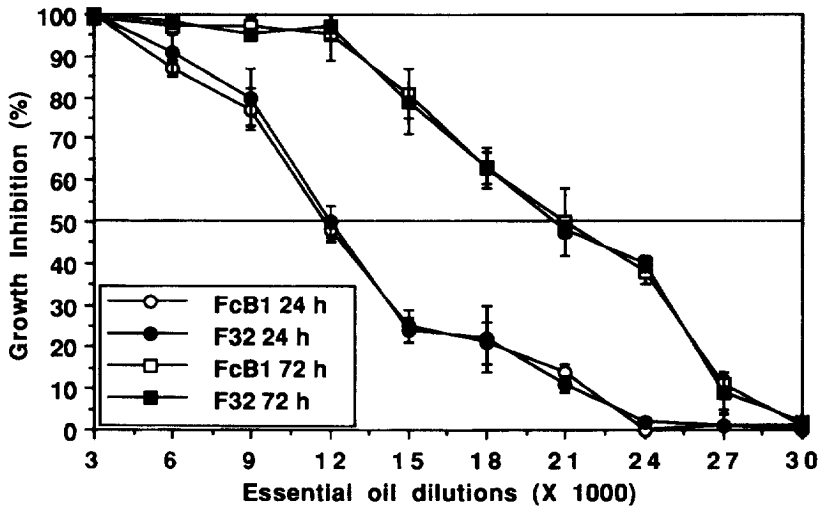

Fig. 1. The antimalarial activity of the essential oil of *L. multiflora* determined by [^{3}H]hypoxanthine incorporation. Similar values were obtained for *P. falciparum* FcB1 chloroquine-resistant and F32 chloroquine-sensitive strains. Incubation times are indicated in the box. Values are the mean of three independant experiments, vertical bars represent 2 SD.

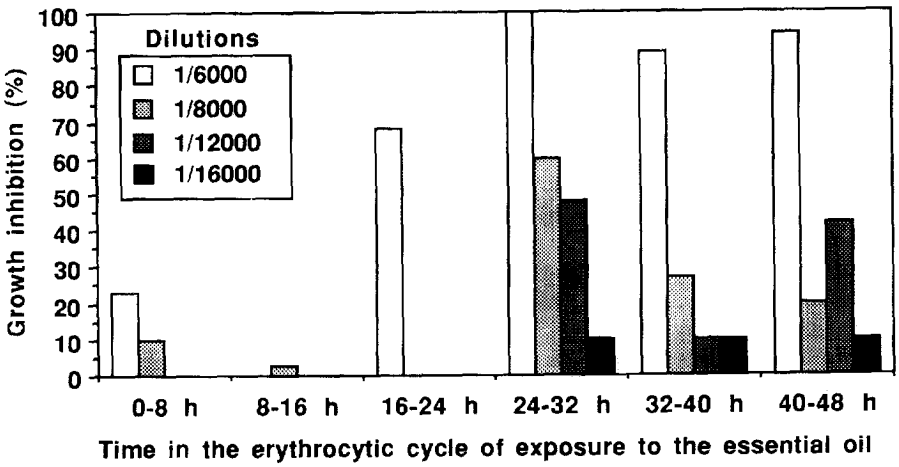

Fig. 2. Sensitivity of the different stages of *P. falciparum* (FcB1 strain) to *L. multiflora* essential oil. The different stages of a culture synchronized on a 4 hr period were subjected to 8 hr contacts (corresponding to one sixth of the erythrocytic cycle time) with *L. multiflora* essential oil at various dilutions. After contact, the cells were washed and returned to normal culture conditions until the beginning of the second erythrocytic cycle and then parasitaemia was determined. At a dilution of 1/20000, no effect was observed. Each bar corresponds to the mean of two independent experiments.

The quantitative chromatographic analyses were conducted on polydimethylsilicone B1 capillary column (25 m × 0.25 mm) with a FI detector; carrier gas: N_2 working temps were identical to those mentioned above.

Parasite strains. Two strains of *P. falciparum* were used. One was F32-Tanzania, chloroquine-sensitive (IC_{50} for chloroquine: 15 ± 1 ng ml^{-1}) and the other one FcB1-Columbia, chloroquine-resistant (IC_{50} for chloroquine 200 ± 10 ng ml^{-1}) [8].

The parasites were cultured according to ref. [15] with modifications [8]. They were maintained *in vitro* in human red blood cells (O^+), diluted to 1–2% haematocrit in RPMI 1640 medium (Gibco, Paisley, U.K.) supplemented with 25 mM Hepes and 30 mM $NaHCO_3$ and 5% human AB^+ serum. The cultures were synchronized by gelatine (Plasmagel, Roger Bellon, Paris, France) flotation and 5% D-sorbitol (Merck, Darmstadt, Germany) lysis [16, 17].

In vitro *antimalarial activity testing.* The method used was adapted from the radioactive micromethod of ref. [18]. The essential oil was tested 3 times in triplicate in 96-well plates (TPP, Switzerland) with cultures at a ring stage (synchronization interval: 16 hr) at 0.5–1% parasitaemia (haematocrit: 1%). For each test, the parasite cultures were incubated with essential oil of increasing dilutions for 24 and 72 hr. The first dilution (1/100) was performed with DMSO Merck), and the following with RPMI 1640. Parasite growth was estimated by [^{3}H]hypoxanthine (Amersham-France, Les Ulis, France) incorporation. The [^{3}H]hypoxanthine incorporated by the parasite in the presence of the essential oil was compared with that of control cultures without essential oil (referred to as 100%). Dilutions inhibiting parasite [^{3}H]hypoxanthine incorporation by 50% (ID_{50}) were determined graphically by means of vs percentage inhibition plots.

The sensitivity of the different stages of *P. falciparum* to the *L. multiflora* oil was determined on *in vitro* cultures synchronized on a 4 hr period. Sorbitol lysis (t_0) was performed 4 hr after gelatine flotation and the culture (parasitaemia, 2%, haematocrit, 1%) was distributed in 24-well plates (TPP, Switzerlzand). The culture in 6 wells was subjected to 8-hr periods of exposure to the essential oil (various dilutions) from t_0 to $t_{0+8\ hr}$ (1st contact corresponding to the 1st sixth of the erythrocytic cycle time), then in 6 other wells to $t_{0+8\ hr}$ to $t_{0+16\ hr}$ (2nd-contact) *et seq.* over the 48 hr of the erythrocytic cycle. After exposure, the culture in the wells was washed (× 3) with 5% serum supplemented RPMI and returned to normal culture conditions (without essential oil) until the next cycle. At $t_{0+62\ hr}$ (ring stage of the next erythrocytic cycle) parasitaemia was calculated by visual numeration on 2000 erythrocytes on thin Giemsa stained smears [19].

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