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Journal of Ethnopharmacology 81 (2002) 23–29

Journal of ETHNO-PHARMACOLOGY

www.elsevier.com/locate/jethpharm

Antimalarial activity of medicinal plants used in traditional medicine in S. Tomé and Príncipe islands

Maria do Céu de Madureira [a,b,*], Ana Paula Martins [a,b], Milene Gomes [b], Jorge Paiva [a], António Proença da Cunha [a], Virgílio do Rosário [b]

[a] *Departamento de Farmacognosia, Faculdade de Farmácia, Universidade de Coimbra, R. Norte, 3000 Coimbra, Portugal*

[b] *Centro de Malária e outras Doenças Tropicais/Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Rua da Junqueira, 96, 1349-008 Lisboa, Portugal*

Received 10 August 2001; received in revised form 14 December 2001; accepted 17 December 2001

Abstract

The present study investigates the antimalarial activity of 13 medicinal plants used in traditional medicine in S. Tomé and Príncipe (STP) islands in the Gulf of Guinea, aiming at identifying the most effective plants for further research. Fieldwork was carried out with the collaboration of 37 traditional healers from both islands, during an ethnobotanical study, which was conducted from 1993 to 1999. Our results indicate that the traditional healers in STP use several medicinal plants against fever and/or 'malaria' which reveal strong antiparasitic activity in vitro: four of the plant extracts have evident antiplasmodial activity against chloroquine resistant *Plasmodium falciparum*, with IC_{50} values <10 μg/ml, and also revealed hepatic schizontocidal activity (<5–35 μg/ml). In vivo, the extracts caused partial reduction of *Plasmodium berghei* parasitaemia in mice.

Keywords: Antimalarial activity; Medicinal plants; *Struchium sparganophorum*; *Tithonia diversifolia*; *Pycnanthus angolensis*; *Morinda lucida*

1. Introduction

Malaria is the most important parasitic disease in tropical areas. In the last decades resistance to several antimalarials became widely disseminated, while the cost of effective treatment is prohibitive for the large majority of the populations in these areas. There is an urgent need for new chemotherapeutic compounds, which are easy to administer and store, and which are of low cost. One possible source for such affordable treatments lies in the use of traditional herbal remedies. The use of plants for the treatment of malaria extends over at least three continents, including several countries in Africa, in the Americas and in Asia (Phillipson et al., 1987). However, few data are available to assess the extent to which these plant remedies are used in the health care systems of tropical countries. The recognition and validation of traditional medical practices and the search for plant-derived drugs could lead to new strategies in malaria control. Since many modern drugs such as quinine and artemisinin originate from plants, it is essential that other medicinal plants which have folklore reputation for antimalarial properties are investigated, in order to establish their safety and efficacy, and to determine their potential as sources of new antimalarial drugs (Gessler et al., 1994).

To overcome some of the most common problems in this field, such as the identification of plant material and the variability of the chemical content of the locally used plant remedies, we evaluated, following normal procedures, some of the plants used by the traditional healers in the Gulf of Guinea islands of S. Tomé and Príncipe (STP), where two-thirds of the population live in regions where endemic malaria occurs (Loureiro et al., 1996).

Data on about 350 different species was collected by personal contact with local traditional healers during an ethnobotanical survey of medicinal plants, conducted from 1993 to 1999, in both islands. The present study investigates the antimalarial activity and toxicity of 13

* Corresponding author. Tel.: +351-39-859995; fax: +351-39-827126.

E-mail addresses: cmdt@esoterica.pt (M. do Céu de Madureira), martins.rubio@netcabo.pt (A. Paula Martins).

0378-8741/02/$ - see front matter
PII: S0378-8741(02)00005-3

medicinal plants that are used by traditional healers in STP for the treatment of malaria and fever.

2. Methodology

2.1. Ethnobotanical survey

In this paper, we present ethnobotanical data on medicinal plants that are used to treat malaria and other fevers, based on first-hand information collected by personal contact with traditional healers, ordinary villagers (old people), as well as through personal observations of applications of the herbal remedies administered by traditional healers.

Local traditional healers called 'curandeiros' were the main guides of the study. 'Fitchicélos', are another group, which uses witchcraft, and their treatments are based mainly on magical rites, sometimes combined with plant therapy; only a few 'fitchicélos' were consulted during the study. Old people, who had knowledge of the curative property of plants, were the second most important group with whom the research team has worked. It is also important to emphasize the importance of the 'mother of family' (mother, grand-mother), who is capable of treating the every day (less significant) ailments of her family using the medicinal plants grown in her own backyard.

The field team worked with 37 traditional healers on both islands, covering all the districts: Água Grande, Mézochi, Cantagalo, Caué, Lobata and Lembá (S. Tomé), and Pagué (Príncipe). Information was collected through informal interviews, which were recorded on tape and filmed during fieldwork with traditional healers. The gathering and recording of information were repeated several times, in different periods of the year. Each individual was asked about the plants used for medicinal purposes in their area, and for each plant the following descriptors were recorded: vernacular names, part of plant used, the mode of preparation, the form of administration of the remedies, the diseases and also the symptoms for which they were recommended.

2.2. Collection of plants

Thirteen plant species were selected based on ethnomedical use in the islands, on data from literature surveys (Makinde et al., 1994; Koshimizu, 1992) and on chemical and biological characteristics of related species.

These plants were collected under the guidance of the healers. Prior to collection the team always obtained permission from the healers, village chiefs, landowners and government (Ministry of Health). Voucher specimens of these medicinal plants, with specimen data including vernacular name, voucher number, collecting locality and date of collection are deposited at the Herbarium of the Instituto Botânico, Coimbra University (COI) (Table 1).

2.3. Extract preparation

Dried material was powdered and extracted with ethanol 70%, concentrated by rotary vacuum evaporation and lyophilized (CEE-crude ethanol extracts). Fresh material was only used for plants n° 7 and 13, which were initially homogenized, followed by same procedure. To obtain a preliminary separation of bioactive constituent groups, the CEE of each plant

Table 1
Medicinal plants traditionally used for malaria and fevers

Number	Botanical name/family	Local name	Local of collection	Voucher No. (COI)	Plant part used
1	*Struchium sparganophorum* (L.) Kuntze/Asteraceae	Libô d'aua	Água Crioula	MM 125	Leaves
2	*Vernonia amygdalina* Del.[a]/Asteraceae	Libô Mucambu	Born Jesus	MM 114	Leaves
3	*Vernonia amygdalina* Del.[a]/Asteraceae	Libô Qué	Morro Quinas	MM 21	Leaves
4	*Ageratum conyzoides* L./Asteraceae	Fiá Malé	Rio Ave	MM 28	Aerial P.
5	*Cinchona succirubra* Pav./Rubiaceae	Quina	Diogo Vaz	MM 25	Bark
6	*Aloe humilis* L./Liliaceae	Áliba Babosa	Blu-Blu	MM 354	Leaf sap
7	*Tithonia diversifolia* (Hmsl.) A. Gray/Asteraceae	Girassol	Monte Café	MM 625	Aerial P.
8	*Cedrela odorata* L./Meliaceae	Cidrela	Ubua Coconja	MM 321	Bark
9	*Premna angolensis* Curke/Verbenaceae	Pó Ama	Apaga-Foguinho	MM 619	Bark
10	*Pycnanthus angolensis* (Welw. Ward)/Myristicaceae	Pó Casson	Fraternidade	MM 426	Bark
11a	*Morinda lucida* Benth. (bark)/Rubiaceae	Gligô	Diogo Vaz	MM 26	Bark
11b	*Morinda lucida* Benth. (leaves)/Rubiaceae	Gligô	Diogo Vaz	MM 26	Leaves
12	*Cestrum laevigatum* Schlecht.var. *puberuleum* Sendtn./Solanaceae	Coedano	Guadalupe	MM 102	Leaves
13	*Canna bidentata* L./Canaceae	Salaconta	Morro Quinas	MM 14	Roots

[a] Different chemotypes of the same species, but locally distinguished by the traditional healers.

was dissolved in methanol and water 1:2 (MW) and subsequently partitioned between petroleum ether (PE), dichloromethane (DM) and ethyl acetate (EA); all the resulting fractions were lyophilized after concentration by rotary vacuum evaporation. Samples were frozen at −20 °C.

2.4. Determination of in vitro antiplasmodial activity in plant extracts

2.4.1. Blood stage assays

To test the antiplasmodial activity of plant extracts we used the susceptibility microassay technique (Desjardins et al., 1979; Carvalho and Krettli, 1991). Two strains of *Plasmodium falciparum:* 3D7–chloroquine sensitive clone of isolate NF54, and Dd2–chloroquine resistant clone from the W2-MEF line (Wellems et al., 1990) were continuously maintained in culture, by the method of Trager and Jensen (1976) and used in these assays.

Each CEE and fraction were dissolved in ethanol and diluted with RPMI 1640 culture medium, with 10% human serum, to prepare stock solutions (5 mg/ml with 1% ethanol). These solutions were sterilized through membrane filters of 0.45 μm and used to prepare a series of concentrations of 500, 250, 100, 50, 25,10, 5 and 1 μg/ml. Chloroquine was used as control and negative controls were prepared with the culture medium and 0.1% ethanol content.

For the microtest, 90 μl aliquots of the control and test solutions were added to all of the wells of the flat-bottomed microtitre plate, and aliquots of 10 μl of a 50% (v/v) suspension of parasitized erythrocytes in culture medium (0.5–1.0% parasitaemia) were added to all test wells. Three duplicate assays were carried out per parasite line; microtitre plates were placed in a candle jar with a gas mixture of 3% CO_2–5% O_2–92% N_2, and incubated at 37 °C for 24 h; thick films were prepared when control wells, stained with Giemsa, showed schizont formation, parasitaemias were established by optical microscopy. The results are expressed as percentage of growth inhibition. The concentration, which inhibited growth by 50% (IC_{50}) in comparison to control wells, was estimated by linear interpolation between drug concentrations values (Hills et al., 1986) (Table 2).

2.4.2. Hepatic stage assays

These assays evaluate the inhibition of development of the liver stage parasite, as well as the toxicity of the plant extracts to the hepatic host cells.

2.4.2.1. Viability assays (hepatotoxicity test). An in vitro culture of HepG2 A16 hepatic cells was mixed with William's E culture medium (25×10^3 cells/200 microlitre) and this volume was added to all of the 96 μl plate wells, and incubated at 37 °C in an enriched CO_2 environment for 18–24 h.

Plant extracts were dissolved with ethanol and diluted with William's E culture medium to obtain six concentrations: 500, 250, 100, 50, 25 and 10 μg/ml, with a maximum of 0.1% in ethanol content. Primaquine was used as positive control, and the negative controls were prepared with culture medium and 0.1% ethanol.

After the incubation period the HepG2 cells were adherent to the wells and the supernatant liquid was removed and substituted by 200 μl of the different extract concentrations and controls. The plate was incubated during 24 h more, in the same conditions as

Table 2
Antimalarial activity of plant extracts against *P. falciparum* (Dd2) in vitro

Number	Plant species	IC_{50}, median values (μg/ml)				
		CEE	PE	DM	EA	MW
1	*Struchium sparganophorum*	180	< 10	100	100	240
2	*Vernonia amygdalina*[a]	120	170	235	500	n.d.
3	*Vernonia amygdalina*[b]	340	200	80	10	n.d.
4	*Ageratum conyzoides*	150	110	55	220	n.d.
5	*Cinchona succirubra*	< 10	< 10	< 10	< 10	< 10
6	*Aloe humilis*	260	150	150	25	500
7	*Tithonia diversifolia*	15	< 10	< 10	140	500
8	*Cedrela odorata*	190	110	50	n.d.	n.d.
9	*Premna angolensis*	180	250	250	250	n.d.
10	*Pycnanthus angolensis*	< 5	100	100	100	n.d.
11a	*Morinda lucida* (*bark*)	< 10	50	50	100	500
11b	*Morinda lucida* (*leaves*)	10	130	60	500	125
12	*Cestrum laevigatum*	100	100	50	150	135
13	*Canna bidentata*	500	130	25	245	500

CEE, crude ethanolic extracts; PE, petroleum ether fraction, DM, dichloromethane fraction; EA, ethyl acetate fraction; MW, remanescent methanol and water fraction; n.d., not determined. IC_{50} (chloroquine), 0.094 μg/ml.

described before; after this period, the supernatant liquid was removed again and substituted by 200 μl more of the same extract or control solutions. At the end of the incubation period, 20 μl of MTT solution (5 mg/ml in RPMI 1640, without phenol red) was added to each well, after what the plate was incubated for more 3 h in the same conditions. The supernatant was then removed and 200 μl of acidified isopropanol added to the wells. These assays were performed in triplicate, to determine the maximum non-toxical concentration to the hepatic cells (Sinden et al., 1990).

Results were obtained by ELISA with a filter of 570 nm and a background of 630 nm and the minimum lethal dose (MLD) was determined for each extract (concentration that inhibits 30% of cell growth).

2.4.2.2. Antimalarial exoerythrocytic schizontocidal assay. Female Balb/C mice were infected with *Plasmodium berghei* ANKA malaria parasites; 5–8 days later, while exhibiting gametocytes, these mice were submitted to insectory maintained *Anopheles stephensi* females for an infectious blood meal, and 14–18 days later sporozoites were collected from dissected mosquito salivary glands and used to infect Hep G2 A16 cells (Trager and Jensen, 1976; Hills et al., 1986) in in-vitro cultures (Calvo-Calle et al., 1994; Hollingdale et al., 1983; Sinden, 1991; Denizot and Lang, 1986).

A cell suspension of 40×10^3 cells/300 μl in culture medium was added to each labtek microplate well and incubated, as described above, for 24 h. The supernatant liquid was replaced by a sporozoite suspension of 100×10^3/100 μl, and the microplate was incubated again for 3 h.

For each plant extract and fraction, 300 μl of the concentration which exhibited 70% of cell growth in the viability assay (MLD), as well as its immediate lower concentration, were now tested in triplicate in the in vitro culture of the exoerythrocytic stage of *P. berghei* in HepG2 cells (Hollingdale et al., 1983; Millet et al., 1986). Results were obtained through immunofluorescence assay (Danforth et al., 1978). The fixed cell cultures were incubated with 100 μl of a mixture of monoclonal antibody i72 (20 μl), stage-specific immune serum (20 μl) and phosphate-buffered-saline (PBS) (960 μl), for 30 min at 37 °C, in a humid chamber. The wells were washed with 400 μl PBS, and the cultures were reacted with 100 μl of a mixture of GAMIg/FITC (10 μl), blue Evans (50 μl) and PBS (940 μl), for another 30 min, in the same conditions. After washing with PBS the slides were prepared with a solution 1:1 glycerol/PBS and examined by immunofluorescence microscope. Tests were repeated in two separate experiments. The concentration, which inhibited the development of hepatic schizonts growth by 50% (IC_{50}), in comparison to control wells, was estimated by linear interpolation between drug concentrations values and negative controls (Table 3).

2.5. Determination of in vivo antimalarial activity in plant extracts

For in vivo tests the 'Four-day suppressive test' (O'Neill et al., 1987) was used, with female Balb/C mice (6-week old; ±20 g weigh) infected by intraperitoneal innoculations of 10^7 erythrocytes parasitised with *P. berghei* ANKA malaria parasite.

Plant extracts and fractions were solubilized or suspended in ethanol at the maximum proportion of 1% (v/v) in distilled water, tested in two concentrations (500 and 1000 mg/kg body weight), administered daily, orally, during 4 days, to infected mice (in groups of five animals). Blood films were taken on the fourth day (96 h after the first dose), Giemsa stained and examined microscopically and the level of parasitaemia was calculated. Extract activity was determinate by percent reduction of parasitaemia in treated groups compared with untreated infected mice. The statistical analysis was based on Student's *t*-test transformed in *P*-values ($P > 0.05$ is not significant; $P < 0.05$ is significant). Controls with local *Cinchona* extracts, at the same doses, were included. Survival studies were also carried out with these animals.

3. Results and discussion

From the group of studied plants only three species (*Cedrela odorata*, *Morinda lucida* and *Vernonia amygdalina*) have been previously investigated for their antimalarial activity (MacKinnon et al., 1997; Tona et al., 1999; Masaba, 2000), however, this is the first time that the hepatic schizontocidal activity of these plants was determined. To our knowledge, this is the first time that *Ageratum conyzoides*, *Aloe humilis*, *Canna bidentata*, *Cestrum laevigatum*, *Premna angolensis*, *Pycnanthus angolensis*, *Struchium sparganophorum* and *Tithonia diversifolia* are being assessed for their antiplasmodial properties.

The results of the in vitro tests with plant extracts against chloroquine resistant *P. falciparum* strain (Dd2) are presented in Table 2. Data on *P. falciparum* $3D_7$-culture line is not included due to similarity of results.

The extracts of *A. humilis* (EA), *C. odorata* (DM), *C. laevigatum* (DM) and *C. bidentata* (DM) showed moderate antiplasmodial activity with IC_{50} values ≤ 50 μg/ml. Six other medicinal plant extracts had evident antiplasmodial activity against chloroquine resistant *P. falciparum*, with IC_{50} values ≤10 μg/ml: *S. sparganophorum* (PE), *V. amygdalina*[b] (EA), *Cinchona succirubra* (CEE and all fractions), *T. diversifolia* (PE, DM), *P. angolensis* (CEE) and *M. lucida* (CEE). Comparing with

Table 3
Cytotoxicity and exoerythrocytic antimalarial activity of plant extracts against *Plasmodium berghei* in vitro (Hep G_2 cells)

Extract number	Plant species	Cytotoxicity MLD (μg/ml)	Tested doses (μg/ml)	Schizontocide activity IC_{50} (μg/ml)
1	*Struchium sparganophorum*	50	50/25	24
1PE	*Struchium sparganophorum*	100	100/50	n.d.
1DM	*Struchium sparganophorum*	25	25/10	35
1EA	*Struchium sparganophorum*	50	50/25	78
1MW	*Struchium sparganophorum*	500	500/250	482
2	*Vernonia amygdalina*	250	250/100	n.a.
3	*Vernonia amygdalina*[b]	250	250/100	305
4	*Ageratum conyzoides*	100	100/50	135
5	*Cinchona succirubra*	250	250/100	n.a.
6	*Aloe humilis*	500	500/250	n.a.
6PE	*Aloe humilis*	100	100/50	n.a.
6DM	*Aloe humilis*	10	10/1	19
6EA	*Aloe humilis*	500	500/250	354
6MW	*Aloe humilis*	100	100/50	344
7	*Tithonia diversifolia*	100	100/50	287
7PE	*Tithonia diversifolia*	50	50/25	18
7DM	*Tithonia diversifolia*	10	10/1	n.a.
7EA	*Tithonia diversifolia*	250	250/100	117
7MW	*Tithonia diversifolia*	500	500/250	n.a.
8	*Cedrela odorata*	250	250/100	158
9	*Premna angolensis*	500	500/250	n.a.
10	*Pycnanthus angolensis*	250	250/100	n.a.
10PE	*Pycnanthus angolensis*	100	100/50	34
10DM	*Pycnanthus angolensis*	500	500/250	n.a.
10EA	*Pycnanthus angolensis*	25	25/10	22
11a	*Morinda lucida* (*bark*)	500	500/250	415
11aPE	*Morinda lucida* (*bark*)	10	10/1	5
11aDM	*Morinda lucida* (*bark*)	10	10/1	n.d.
11aEA	*Morinda lucida* (*bark*)	50	50/25	137
11aMW	*Morinda lucida* (*bark*)	500	500/250	n.a.
11b	*Morinda lucida* (*leaves*)	50	50/25	76
11bPE	*Morinda lucida* (*leaves*)	250	250/100	103
11bDM	*Morinda lucida* (*leaves*)	100	100/50	167
11bEA	*Morinda lucida* (*leaves*)	500	500/250	n.a.
12	*Cestrum laevigatum*	250	250/100	n.a.
13	*Canna bidentata*	500	500/250	n.a.

MLD (minimal lethal dose)—dose that inhibits 30% of cell growth; CEE, crude ethanolic extracts; PE, petroleum ether fraction; DM, dichloromethane fraction; EA, ethyl acetate fraction; MW, remanescent methanol and water fraction; n.a., not active; n.d., not determined. IC_{50} (primaquine) = 0.003 μg/ml. MLD (primaquine) = 0.1 μg/ml.

the IC_{50} value obtained for *Artemisia annua* (3.9 μg/ml) and for *Azadirachta indica* (10 μg/ml) this group of plants has promising results (Phillipson et al., 1987). Nevertheless, plants that are frequently reported as used as antimalarials in various countries do not necessarily show high activity in the in vitro test. These findings can probably partly be explained because many of the plants used in the treatment of malaria could have other therapeutic activities either than antiparasitic effect, such as antipyretic or immunomodulatory.

In two of the studied medicinal plants (*P. angolensis* and *M. lucida*) the crude extracts showed marked antiplasmodial activities but the fractions were all much less active. A simple explanation could be due to possible synergisms between the complex and heterogeneous mixture of different compounds in the crude extracts that are lost during fractionation. Other possibility is the instability of the fractionated and/or purified molecules, which are otherwise protected by chlorophyll and/or other components (Zani et al., 1997).

In the cytotoxicity and exoerythrocytic antimalarial activity of plant extracts against *P. berghei* in vitro (Hep G2 cells) (Table 3), eleven of the tested extracts and fractions revealed hepatic schizontocidal activity, in concentrations below the respective MLD values (*S. sparganophorum* crude extract and methanol/water fraction, *A. humilis* EA fraction, *T. diversifolia* PE and EA fractions, *C. odorata* crude extract, *P. angolensis* PE and EA fractions, *M. lucida* bark crude extract and PE fraction and *M. lucida* leaves PE fraction). From these,

four plants revealed evident schizontocidal activity with IC_{50} values from 5 to 34 μg/ml (*S. sparganophorum*, *T. diversifolia*, *P. angolensis* and *M. lucida* bark).

The plant extracts and fractions that showed significant activity in in vitro tests have been selected for in vivo assays for antimalarial activity (*S. sparganophorum*, *C. succirubra*, *T. diversifolia*, *C. odorata* and *P. angolensis*). Such preliminary screening does not preclude the possibility that the other extracts, not assayed, may be active antimalarial agents by the oral route. In general, the tested plant extracts were partly active against the rodent malaria, except for *Cinchona* extracts that showed a total chemosuppression of parasitaemia in mice infected with *P. berghei*. However, two of them, *S. sparganophorum* and *C. odorata* crude extracts, exhibited a significant inhibition ($P < 0.05$) of the parasite multiplication (85 and 73%, respectively), at oral doses of 1000 mg/kg per day. The parasitaemia (mean ± standard deviation) of the infected control group at the end of the 4-day test was 10.34 ± 2.11%; for the mice treated with *S. sparganophorum* the parasitaemia was 1.54 ± 1.38% and for the mice treated with *C. odorata* the parasitaemia was 2.84 ± 0.99%.

The median time of mice survival of the infected treated animals versus the control group (23 ± 3 days) was calculated and one plant extract increased this time by 35% (*C. odorata*).

4. Conclusions

The resistance of *P. falciparum* to chloroquine is now a major health problem in STP, as in many parts of Africa, and these data suggest a potential role for medicinal plant extracts in the treatment of chloroquine-resistant falciparum malaria.

Our study indicates that traditional healers in STP use several medicinal plants against fever and/or malaria which have demonstrated varying activity against blood stage parasites of chloroquine resistant *P. falciparum*, as well as against the hepatic development of *P. berghei* in Hep G2 liver cells. The effectiveness against liver stage development suggests that they could also be used as preventive medicines. The development of phytopharmaceutical products with these capacities could be of relevance for the protection of risk groups, in areas with such high levels of resistance.

Phytochemical studies of the four most active plants (*S. sparganophorum*, *T. diversifolia*, *P. angolensis* and *M. lucida*) are being performed, in order to identify the most effective fraction or compound, bearing in mind that the optimal product may not always correspond to single compounds.

In order to develop more appropriate formulations for local production and administration of these products, in collaboration with local traditional healers, further studies (non-clinical toxicological studies, as well as local clinical observations to confirm safety and efficacy) are required.

This will have an obvious economic advantage for the local populations, as the developed medicinal plant products will be the cheapest therapeutic alternative for malaria.

Acknowledgements

We would like to thank to traditional healers of S. Tomé and Príncipe, in particular to Sum Gino and Sum Pontes, with whom we learnt and worked, and to the Ministry of Health of RDSTP. We also express our gratitude to Professor Laurent Rénia for providing the biological material for the hepatic stage tests, F. Nogueira for the performance of hepatic schizontocidal activity tests and to Ana Neto for extracts preparation. A special thanks to Nina Etkin for the helpful suggestions on this paper. This study was financed by Fundação para a Ciência e Tecnologia (PRAXIS/PSAU/P/SAU/38/96) and M. Gomes supported by PRAXIS XXI (BM/12880/97).

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