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Tanzanian Medicinal Plants used Traditionally for the Treatment of Malaria: *in vivo* Antimalarial and *in vitro* Cytotoxic Activities

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Seventeen fractions of extracts obtained from 11 Tanzanian medicinal plants, which had previously been shown to possess a high antimalarial activity *in vitro* were submitted to the 4-day suppressive test in *Plasmodium berghei*-infected mice, and were investigated for cytotoxic activity in human carcinoma cell lines *in vitro*. Several fractions administered orally to the mice (500 mg/kg body weight/day) produced a significant reduction of parasitaemia. The most effective plant fractions investigated were those of the root and stem bark of *Maytenus senegalensis* (90% and 63% suppression of parasitaemia, respectively) and of the roots of *Cissampelos mucronata* (59% suppression). Highest cytotoxic activities were found with all fractions of *Maytenus senegalensis* (IC_{50} < 1 µg/mL) and with the PE fraction of the roots of *Salacia madagascariensis* (median IC_{50} = 1.2 µg/mL for HT 29 and 2.3 µg/mL for KB).

Keywords: in vivo antimalarial activity; *Plasmodium berghei*; *in vitro* cytotoxicity; human cell lines; plant extracts; traditional medicine; Tanzania.

INTRODUCTION

Attempts to control the spread of malaria in African countries south of the Sahara with insecticides or synthetical antimalarials have so far failed (Grammiccia and Beales, 1988). Besides the problem of resistance to the chemicals there are also other problems; the ethnomedical perceptions and practices relating to the illness 'malaria' are very often different from the biomedical ones on which malaria control measures are based. In western medicine, malaria is connected with mosquitos and *Plasmodium*. In tropical countries one may find completely different explanations of the causative agent of the illness, such as excessive external heat or eating the wrong kind of food (Agyepong, 1992). Plants have always proved to be a rich source for remedies of diseases, and also of new drugs. Many of the antimalarial drugs in use at present were either obtained from plants—for example quinine and artemisinin—or have been developed using the chemical structure of quinine or more recently the peroxide moiety of artemisinin as a template (Wright and Phillipson, 1990; Warrell, 1993). There are several reviews of the pharmacological and chemical work already completed in the study of antimalarial drugs from plants (Phillipson *et al.*, 1993; Phillipson and Wright, 1991; Vasanth *et al.*, 1990).

To screen different plant extracts for antimalarial activity, *in vitro* microdilution tests are convenient and rapid to perform, but they give no information about the selectivity of the toxicity. For this reason it is also important to investigate antimalarial efficacy in the rodent test using *Plasmodium berghei* in mice (since we have no practicable *in vivo* model with *Plasmodium falciparum*) and to determine the cytotoxic activity against human cells *in vitro*.

These tests are well established but are still associated with problems or phenomena that are not easy to interpret or to overcome. For example, there are plant extracts or compounds such as some quassinoids isolated from plant species belonging to the family of the Simaroubaceae which have an enormous antimalarial activity but are also toxic in laboratory tests (O'Neill *et al.*, 1988). Others which show activity *in vitro* but are not active in the *in vivo* mouse model, and there are cases in which crude extracts are much more active than any individual isolated compound (Kirby *et al.*, 1993a).

In the framework of research projects on phytochemical studies of Tanzanian medicinal plants, 43 plant species, belonging to 26 families, were collected in Tanzania in 1991 and 1992 on the basis of ethnobotanical information, and subsequently screened for their antimalarial activity *in vitro* (Gessler *et al.*, 1994).

Motivated by the promising results it was decided to investigate the antimalarial potential *in vivo* and the cytotoxic activity against human cell lines *in vitro* of 17 fractions of 11 different plant species which proved to be highly active in the antimalarial *in vitro* screening against *Plasmodium falciparum*.

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Table 1. Antimalarial activity of plant fractions against *Plasmodium berghei* in mice

	Plant species	Plant part	Fraction[a]	*In vitro* IC_{50}[b] (μg/mL)	Number of mice	*In vivo* suppression of parasitaemia (%) (SE)[c]	Survival[d] (days) (SE)
A[e]	*Maytenus senegalensis*	Stem bark	EtAc	0.16	3	62.8 (±9.7)	5.3 (±0.3)
	(Kilombero District)	Root bark	EtAc	0.62	6	89.9 (±7.5)	9.2 (±1.2)
	(Kagera District)	Stem bark	EtAc	3.0	6	23.3 (±17.0)	6.5 (±0.3)
	Cissampelos mucronata	Roots	EtAc	0.38	6	58.5 (±14.5)	8.5 (±1.1)
	Zanthoxylum chalybeum	Root bark	H_2O	0.43	6	4.1 (±12.3)	6.2 (±0.2)
	Salacia madagascariensis	Roots	PE	0.80	6	19.0 (±5.8)	4.3 (±0.9)
B[e]	*Achyranthes aspera*	Root bark	EtAc	3.0	6	8.3 (±11.4)	6.0 (±0.3)
	Cussonia zimmermannii	Root bark	PE	3.3	6	5.9 (±12.3)	6.0 (±0.3)
	Erythrina sacleuxii	Root bark	EtAc	3.0	6	9.2 (±14.5)	5.8 (±0.3)
	Harungana madagascariensis	Roots	EtAc	4.0	6	9.4 (±11.2)	6.2 (±0.2)
	Harungana madagascariensis	Roots	PE	6.0	6	13.2 (±11.8)	6.0 (±0.3)
	Harungana madagascariensis	Stem bark	EtAc	10	6	11.2 (±11.7)	6.2 (±0.2)
	Harungana madagascariensis	Stem bark	PE	10	6	29.3 (±8.0)	6.5 (±0.2)
	Keetia zanzibarica	Roots	EtAc	4.0	3	9.6 (±7.2)	6.0 (±0.0)
C[e]	*Vepris lanceolata*	Root bark	EtAc	7.0	3	15.2 (±12.3)	6.0 (±0.4)
	Vepris lanceolata	leaves	H_2O	9.5	6	19.3 (±17.0)	6.8 (±0.5)
	Abutilon grandiflorum	Roots	EtAc	10	6	21.7 (±9.2)	6.2 (±0.2)
Chloroquine tested at 4 mg/kg body weight/day.					5	100.0 (±5.1)	17.6 (±2.0)

[a] EtAc, ethyl acetate; PE, petroleum ether; H_2O, water.
[b] Median values of *in vitro* antimalarial tests published in detail in Gessler *et al.* (1994) are given for comparison.
[c] Mean suppression of parasitaemia in % at a dose of 500 mg/kg/day.
[d] Mean survival in days; mean survival of total of 30 control mice, 5.7 day (SD, 0.5).
[e] Plant species grouped by the *in vitro* antimalarial activity of the most active fraction: A<1 μg/mL; B 1–5 μg/mL; C 6–10 μg/mL (Gessler *et al.*, 1994).

MATERIALS AND METHODS

Plant materials and extractions. The plant materials were collected in the Morogoro, Dar es Salaam and Kagera regions in Tanzania in 1991 (May–August) and 1992 (August–November). The selection of the plants was based on ethnobotanical information, either from the literature or directly from personal communication with traditional healers. The different plant parts were extracted with ethanol (EtOH) and fractionated with petroleum ether (PE), ethyl acetate (EtAc) and water (H_2O) as described in detail elsewhere (Gessler *et al.*, 1994). Voucher herbarium specimens are deposited in the herbarium of the Department of Botany at the University of Dar es Salaam (Tanzania) and of the 'Institut für pharmazeutische Biologie' at the University of Freiburg (Germany).

Selection of plant fractions for *in vivo* antimalarial and *in vitro* cytotoxic tests. The plants used were among 43 plant species which had previously been screened for *in vitro* antimalarial activity (Gessler *et al.*, 1994). The fractions selected were those which had shown an *in vitro* IC_{50} of ≤ 10 μg/mL against *Plasmodium falciparum* strains K1 and NF54.

The plants selected were:

Amaranthaceae	*Achyranthes aspera* L.
Araliaceae	*Cussonia zimmermannii* Harms
Celastraceae	*Maytenus senegalensis* (Lam.) Excell; *Salacia madagascariensis* (Lam.) DC.
Fabaceae	*Erythrina sacleuxii* Hua
Guttiferae	*Harungana madagascariensis* (Lam.ex) Poir.
Malvaceae	*Abutilon grandiflorum* G.Don
Menispermaceae	*Cissampelos mucronata* A. Rich.
Rubiaceae	*Keetia zanzibarica* (Klotzsch) Bridson
Rutaceae	*Vepris lanceolata* (Lam.) G.Don; *Zanthoxylum chalybeum* Engl.

***In vivo* antimalarial tests with the rodent malaria species *Plasmodium berghei*.** Male Moro mice (SPF line), weighing ±25 g were infected by injecting 2×10^7 erythrocytes parasitized with *Plasmodium berghei* strain ANKA S intravenously via a tail vein. The volume of inoculum was 0.2 mL. All test mice were infected on day 0. The number of mice treated with each plant fraction was 6 (exceptions are shown in Table 1). A total of 30 infected but untreated mice served as controls. The plant fractions were dissolved in 20% Tween 80 with the aid of ultrasonication and were further diluted with water to achieve an end concentration of 500 mg/kg body weight. The animals were treated orally (p.o.) once on days 0, 1, 2 and 3 with a volume of 0.01 mL/g body weight (Peters *et al.*, 1975). Chloroquine was included in the tests at concentrations of 4, 2 and 1 mg/kg with a total of 5 mice in each group. The mice received as diet NAFAG pellets (9009 PAB-45) and were held at room temperature. The survival of the mice was checked twice a day. Parasitized erythrocytes were counted in Giemsa stained thin films from tail blood on day 4. The percentage suppression of parasitaemia for each plant fraction was calculated as:

$$100 - 100 \times (\text{mean \% parasitaemia in treated mice} / \text{mean \% parasitaemia in control mice}).$$

The average survival time of the animals in each group was recorded. The standard error of the percentage suppression was computed using the formula for the variance of a ratio given by Armitage and Berry (1991).

***In vitro* microtest for assessing cytotoxicity against two human cell lines.** The cytotoxicity was assessed using two different procedures for the same test plates: first, the minimum inhibitory concentration (MIC) was determined

by comparing microscopically the cells of the different drug concentrations with the cells of the control. Second, the colorimetric cytotoxicity assay described by Skehan *et al.* (1990) and Likhitwitayawuid *et al.* (1993), and slightly modified by Kaminsky (personal communication), which uses sulforhodamine B to stain cellular protein, was applied. The estimation of cellular protein can be used as a measure of cell growth. The optical density can be read at 540 nm by using an enzyme-linked immunosorbent assay (ELISA) reader.

The human cell lines used were KB, which originated from an epidermoid carcinoma, and HT 29, which originated from a colon carcinoma. The cells were maintained in Eagle's modified minimum essential medium (MEM) with 10% fetal bovine serum (FBS). The test fractions were dissolved in dimethyl sulfoxide (DMSO) with a maximum concentration of 0.1% and diluted with MEM medium to 1000 μg/mL. Each fraction was evaluated in duplicate at 7 threefold dilutions, beginning at 1000, 100 or 10 μg/mL to obtain the optimum concentration range. Each test was carried out three times. Chloroquine was included as a control. After 72 h of incubation at 37 °C the minimum inhibitory concentration (MIC) was microscopically determined and 0.4% sulforhodamine B dissolved in 1% acetic acid (HOAc) was added. The concentrations at which growth was inhibited by 50% (IC_{50}) were estimated by linear interpolation between the two adjacent drug concentrations above and below the 50% incorporation line (Hills *et al.*, 1986; Huber and Koella, 1993).

RESULTS

Antimalarial activity *in vivo*

As summarized in Table 1, the antimalarial potential in mice of each of the plant fractions was investigated with *Plasmodium berghei* ANKA S. To measure the potential, two criteria were considered; the suppression of the parasitaemia and the survival of the mice. Appreciable activity, measured as suppression of the parasitaemia was demonstrated at a concentration of 500 mg/kg body weight/day with several fractions compared with the untreated controls (mean parasitaemia of 30 control mice 79.5%, SD, 5.6). The highest antimalarial activities were observed with the EtAc fractions of the root bark (mean 89.9% suppression of the parasitaemia) and the stem bark (mean 62.8% suppression) of *Maytenus senegalensis* (collected in Kilombero District), and with the EtAc fraction of the roots of *Cissampelos mucronata* (mean 58.5% suppression).

Although *Salacia madagascariensis* (roots, PE fraction) had been shown to possess a very high *in vitro* antimalarial activity, the reduction of parasitaemia *in vivo* was rather moderate (mean 19.0%). Among other plant fractions showing suppression ≥20% were those of *Harungana madagascariensis* (stem bark, PE) and *Abutilon grandiflorum* (roots, EtAc). All other investigated fractions showed activities less than 20%.

The EtAc fraction of the root bark of *Maytenus senegalensis* prolonged the survival of the mice compared with the control by 3.5 days, and the EtAc fraction of the roots of *Cissampelos mucronata*, prolonged survival by 2.8 days.

Chloroquine caused 100% suppression of parasitaemia in mice at a concentration of 4 mg/kg body weight/day, 91% suppression at 2 mg/kg body weight/day and 11% at 1 mg/kg body weight/day. The mean survival time was 17.6, 10.4 and 6.2 days, respectively.

In general, no toxic signs—survival for a shorter time than the untreated control mice, or changes in appearance and habits—were observed. Exceptions were mice treated with the EtAc fraction of the stem bark of *Maytenus senegalensis* and with the PE fraction of *Salacia madagascariensis*, which died (day 5.3 and day 4.3, respectively) before the untreated control mice (day 5.7).

Cytotoxic activity against human cell lines

The *in vitro* cytotoxic results we obtained with the cell lines HT 29 and KB are summarized in Table 2. The values represent the median values of three experiments. The minimum inhibitory concentrations (MIC) were higher in all experiments than the IC_{50} values determined by assessment of the cellular protein. Most of the tested fractions were found to demonstrate a general cytotoxic response with only little discernible cell-type selectivity. However, the EtAc fraction of the stem bark of *Maytenus senegalensis* was ten-fold more toxic for HT 29 cells than for KB cells. The highest activities were found with all tested fractions of *Maytenus senegalensis* ($IC_{50} < 1$ μg/mL) and with the PE fraction of the roots of *Salacia madagascariensis* (median $IC_{50} = 1.2$ μg/mL for HT 29 and 2.3 μg/mL for KB). The lowest cytotoxic activities were expressed by the tested fractions of *Vepris lanceolata* (median $IC_{50} = 205$ μg/mL for HT 29 and 589 μg/mL for KB) and of *Keetia zanzibarica* (median $IC_{50} = 205$ μg/mL for HT 29 and 241 μg/mL for KB). Chloroquine was used as a reference drug (median $IC_{50} = 58$ μg/mL for KB).

DISCUSSION

Some of the investigated plant fractions, like those of *Maytenus senegalensis* and *Cissampelos mucronata*, are able to reduce the level of parasitaemia in rodents by a high degree. This may support the consistent reports by traditional healers that these plants are effective in treating malaria in humans. The roots of *Maytenus senegalensis* are not only used as an antimalarial drug in Tanzania (Gessler *et al.*, 1994), but also in Senegal (Von Sengbusch, 1980). The antimalarial use of *Cissampelos mucronata* has not yet been reported outside of Tanzania.

Nevertheless, the results for *in vivo* antimalarial activity do not necessarily correlate with those for *in vitro* antimalarial activity which were described previously (Gessler *et al.*, 1994). The water fraction of *Zanthoxylum chalybeum*, which had a high antimalarial activity *in vitro* (median $IC_{50} = 0.43$ μg/mL), was inactive in the mouse model.

Also *Cussonia zimmermanii* and *Erythrina sacleuxii* (median IC_{50} *in vitro* = 3.0 μg/mL, 3.3 μg/mL respectively) showed no antimalarial potential *in vivo*. The lack of activity of certain extracts or compounds *in vivo*, which are active or cytotoxic *in vitro*, might be

due to low absorption or because structures necessary for activity may be altered by metabolic processes. This phenomenon has already been described for other extracts or compounds, like cryptolepine, which was isolated from *Cryptolepis sanguinolenta* and berberine isolated from *Enantia chlorantha* (Phillipson *et al.*, 1993), or for *Azadirachta indica* and its terpenoid lactone nimbolide (Rochanakij, 1985). All exhibit a high activity against *P. falciparum in vitro* but are inactive in *P. berghei*-infected mice. Drugs or herbal medicines may also act by more than one mechanism, for example they may have an indirect effect on the immune system (e.g. biological response modifier) or other pathways that are not yet understood (Rasoanaivo *et al.* 1992). In mice, treated with fractions which have a good *in vivo* antimalarial activity, we observed a decrease of segmented neutrophile granulocytes and an increase of monocytes in the blood. This cannot be easily explained by the parasitaemia itself because the amount of those specific cells in infected control mice was not different from those in uninfected control mice (Gessler *et al.* unpublished).

Some fractions from different plant parts of one plant species showed a different antimalarial activity pattern *in vivo* from that found *in vitro*. The stem bark of *Maytenus senegalensis in vitro* was five times more active than the root bark, whereas *in vivo* it was about 31% less active than the root bark. Similar results were found for different plant parts of *Vepris lanceolata* and *Harungana madagascariensis* (see Table 1). With *Harungana madagascariensis* it was interesting to note that the apolar fractions (petroleum ether) of the two plant parts investigated always exhibited a better result *in vivo* than the more polar fractions (ethyl acetate). This could be due to better resorption processes for more lipophilic mixtures.

The ratios of cytotoxicity to *in vitro* antiplasmodial activity (i.e. median IC_{50} in cultured human cells/ median IC_{50} in cultured *P. falciparum* strains) were in the range of 0.18–100 for most of the tested fractions, so they have a poor selectivity index. Ratios of <1, i.e. the cytotoxic activity *in vitro* was higher than the antimalarial activity *in vitro*, were found in HT 29 and KB cells with all tested fractions of *Maytenus senegalensis*. The best ratios were obtained with fractions of *Zanthoxylum chalybeum* (100 for HT 29), leaves of *Vepris lanceolata* (62 for KB), *Keetia zanzibarica* (60 for KB) and of *Cissampelos mucronata* (50 for KB). For chloroquine the ratio is calculated in our tests with KB cells and *Plasmodium falciparum* strain K1 as 483. It has been proposed that the ratio for a good therapeutic remedy should be ≥1000, as it is for example for quinine (Likhitwitayawuid *et al.*, 1993).

The cytotoxic activity of the chloroform extract of the stem bark of *Maytenus senegalensis* collected in Ghana has already been described by Tin-Wa *et al.* (1971). They found the extract to be active against CA-9KB cells with an $IC_{50} = 0.01$ μg/mL. Our extract (EtAc) of the stem bark tested against KB was 70 times less active.

With the root bark of *Maytenus senegalensis* we obtained an extract with an excellent antimalarial activity *in vivo*, with no obvious toxic signs in mice. However, this same extract showed a serious cytotoxic action on cell lines *in vitro*. *In vitro* cytotoxicity does not necessarily mean an extract cannot be used in humans; this plant has been used in humans for years in traditional medicine in several countries, not only for the treatment of malaria (Chhabra *et al.*, 1989; Haerdi, 1964; Von Sengbusch, 1980). Similarly, Kirby *et al.* (1993b) describe the extreme toxicity *in vitro* and *in vivo* of highly active antimalarial quassinoids isolated from *Brucea* species, although human clinical studies using crude preparations of *Brucea* fruits have shown antimalarial efficacy without toxicity. Not much is known about the pharmacological activities of *Cissampelos mucronata*, except that it contains several alkaloids with curare like activity (Correia da Silva and Quiteria Paiva, 1964). However, several other plants of the family Menispermaceae have been shown to con-

Table 2. *In vitro* cytotoxic activity of plant fractions tested against HT 29 and KB cell lines

	Plant species	Plant part	Fraction[a]	MIC (μg/mL)[b] HT 29	MIC (μg/mL)[b] KB	Sulforhodamin[c] IC_{50} (μg/mL) HT 29	Sulforhodamin[c] IC_{50} (μg/mL) KB
A[d]	*Maytenus senegalensis*	Stem bark	EtAc	0.12	1.11	0.06	0.77
	(Kilombero District)	Root bark	EtAc	0.37	0.37	0.29	0.19
	(Kagera District)	Stem bark	EtAc	1.11	1.11	0.83	0.54
	Cissampelos mucronata	Roots	EtAc	12.4	37	8.5	19
	Zanthoxylum chalybeum	Root bark	H_2O	111	37	43	13
	Salacia madagascariensis	Roots	PE	1.4	4.1	1.2	2.3
B[d]	*Achyranthes aspera*	Root bark	EtAc	111	111	62	67
	Cussonia zimmermannii	Root bark	PE	37	37	6.3	16
	Erythrina sacleuxii	Root bark	EtAc	37	37	23	19
	Harungana madagascariensis	Roots	EtAc	37	37	25	9.7
	Harungana madagascariensis	Roots	PE	37	37	34	9.1
	Harungana madagascariensis	Stem bark	EtAc	37	37	28	17
	Harungana madagascariensis	Stem bark	PE	37	37	22	10
	Keetia zanzibarica	Roots	EtAc	333	333	205	241
C[d]	*Vepris lanceolata*	Root bark	EtAc	37	100	30	45
	Vepris lanceolata	Leaves	H_2O	333	1000	205	589
	Abutilon grandiflorum	Roots	EtAc	111	333	79	90
Chloroquine				n.d.	111	n.d.	58

[a] EtAc, ethyl acetate; PE, petroleum ether; H_2O, water.
[b] Minimum inhibitory concentration (MIC); median value of 3 experiments.
[c] Assessment of the cellular protein with sulforhodamin B staining; median value of 3 experiments.
[d] Plant species grouped by the *in vitro* antimalarial activity of the most active fraction: A <1 μg/mL; B 1–5 μg/mL; C 6–10 μg/mL.

tain antimalarial active bis-benzylisoquinoline alkaloids (Ratsimamanga-Urveg *et al.*, 1992).

Taking the high cytotoxicity *in vitro* into account, further work is needed to clarify the effective potential of the tested plants in clinical use. It is also very important to investigate the concentration and dosage of the active principles when the plants are prepared and used in traditional remedies. Furthermore, herbal remedies frequently contain other ingredients which might affect the activity and toxicity of the active principle.

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