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In-vivo antimalarial activity of *Cassia occidentalis*, *Morinda morindoides* and *Phyllanthus niruri*

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The ethanolic, dichloromethane and lyophilized aqueous extracts of *Cassia occidentalis* root bark, *Morinda morindoides* leaves and whole plants of *Phyllanthus niruri* were evaluated for their antimalarial activity *in vivo*, in 4-day, suppressive assays against *Plasmodium berghei* ANKA in mice. No toxic effect or mortality was observed in mice treated, orally, with any of the extracts as a single dose, of 500 mg/kg body weight, or as the same dose given twice weekly for 4 weeks (to give a total dose of 4 g/kg). No significant lesions were observed, by eye or during histopathological examinations, in the hearts, lungs, spleens, kidneys, livers, large intestines or brains of any mouse.

At doses of 200 mg/kg, all the ethanolic and dichloromethane extracts produced significant chemosuppressions of parasitaemia (of $>60\%$ for *C. occidentalis* root bark and *Ph. niruri* whole plant, and of 30% for *M. morindoides* leaves) when administered orally. The most active ethanolic extract, that of *Ph. niruri*, reduced parasitaemia by 73%. The dichloromethane extracts of *M. morindoides* and *Ph. niruri* produced similar reductions (74% and 72% chemosuppression, respectively), whereas that of *C. occidentalis* was slightly less active (60% chemosuppression). Each lyophilized aqueous extract was less active than the corresponding ethanolic extract.

The development in *Plasmodium falciparum* of resistance to the effects of antimalarial drugs and the emergence of multi-resistant strains of the parasite, mainly in tropical and subtropical countries, have given impetus to research on the development of new antimalarial drugs from medicinal plants (Björkman and Phillips-Howard, 1990; Wernsdorfer, 1991, 1994; Tracy and Webster, 1996). The selection of plants on the basis of ethnopharmacological data has proven to be a fruitful way to the discovery of new drugs (Muñoz *et al.*, 2000). Several medicinal plants belonging to various families from the African flora are known to

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be (or are, at least, used as) analgesics and febrifuges. Traditional healers claim that they find other plant species to be effective against malaria in their daily practises, and such plants are being selected and screened for their putative antimalarial properties *in vitro* and/or *in vivo* (Weenen *et al*., 1990; Jurg *et al*., 1991; Gessler *et al*., 1994, 1995; Benoït *et al*., 1996; Omulokoli *et al*., 1997; El Tahir *et al*., 1999*a*, *b*, *c*; Rasoanaivo *et al*., 1999; Tona *et al*., 1999; Muñoz *et al*., 2000). The toxicity of the plants, as complete crude extracts or isolated constituents of such extracts, must also be investigated (Ratsimamanga-Uverg *et al*., 1990, 1991; Marshall *et al*., 1994; Gessler *et al*., 1995).

An evaluation of the antimalarial activity of some medicinal plants used in the Democratic Republic of Congo (DRC; Tona *et al*., 1999) led to nine species being selected and tested *in vitro*. In the present study, extracts of the three plant species found to have the highest antimalarial activity *in vitro* (Tona *et al*., 1999) were tested *in vivo*, in a *Plasmodium-berghei*–mouse model.

MATERIALS AND METHODS

Plant Material

Extracts were prepared from the root bark of *Cassia occidentalis*, the leaves of *Morinda morindoides* and whole plants of *Phyllanthus niruri*. All the plants used were collected in Kinshasa, capital city of the DRC, in March 1999. They were identified by M. M. Nlandu of the Institut National d'Etudes et de Recherches en Agronomie (INERA) of the University of Kinshasa, where a voucher specimen of each plant species has been deposited. All plant materials were dried at room temperature and then reduced to a powder.

Preparation of Crude Extracts (Table 1)

Fifty g of each powdered plant material were macerated with 300 ml ethanol or dichloromethane (three macerations, each with 100 ml solvent and for 24 h). Each mixture was then filtered and the filtrate evaporated to dryness *in vacuo*, yielding dried, ethanolic or dichloromethane extracts.

A further 100 g of each powder were mixed with 1500 ml distilled water, heated at 100°C for 30 min, cooled and filtered. The filtrate was lyophilized at < 42°C for 24 h, yielding a lyophilised aqueous extract of each plant material.

Prior to use, each extract was dissolved in water (aqueous extracts) or a 9:1 (v/v) water:ethanol mix (ethanolic and dichloromethane extracts) so that the dose required was contained in 0.5 ml of the solution.

Toxicology and Histology

ACUTE TOXICOLOGY

Thirty-three, white, adult, Swiss mice, with a mean (S.D.) body weight of 20 (2) g, from the Institut National de Recherches Biomédicales (INRB) in Kinshasa, were acclimatized to laboratory conditions and randomly divided into 10 groups each of three mice (one group for each extract plus one control group given water and another given the 9:1 water:ethanol mix). Each mouse was starved for 24 h prior to treatment by gavage with a single dose of dissolved extract (500 mg/kg bodyweight) or diluent. The mice were given food 30 min after the gavage. Each mouse was weighed and checked for signs of toxicity (including death) daily for 7 days.

SUBACUTE TOXICITY

The procedure for testing subacute oral toxicity was similar to that used to test acute toxicology, but the mice were treated twice a week for 4 weeks (each of those given an extract receiving eight doses, each of 500 mg/kg). Each mouse was again starved for 24 h prior to each treatment and offered food 30 min later. Each mouse was weighed and checked for signs of toxicity daily until 30 days after the first treatment.

Histology

On day 30 after first treatment, the animals used in the toxicology tests were all killed by

TABLE 1
Characteristics of the plant extracts investigated

Plant	*Voucher specimen*	*Part extracted*	*Dry weight of extract (%)*		
			Ethanolic	*Dichloromethane*	*Lyophilized aqueous*
Cassia occidentalis L. (Caesalpiniaceae)	P03998ML	Root bark	6.34	4.00	12.36
Morinda morindoides (Baker) Milne–Redhead (Rubiaceae)	P03995ML	Leaves	6.02	4.57	14.21
Phyllanthus niruri L. (Euphorbiaceae)	P03999ML	Whole plant	9.12	4.8	16.53

decapitation. The hearts, lungs, livers, kidneys, spleens, large intestines and brains were carefully dissected out and fixed in Bouin's liquid, embedded in paraffin wax, cut into 3–5-μm sections and triple-stained with haematoxylin, eosin and safranin. The histology so revealed in the organs of mice treated with the extracts was compared with that seen in the organs of the control mice which had only been given diluent.

In-vivo Antimalarial Testing

The activities of the ethanolic, dichloromethane and lyophilized aqueous extracts against *Plasmodium berghei* ANKA (kindly provided by Professor N. P. Ngimbi of the Institute of Tropical Medicine, Faculty of Medicine, University of Kinshasa) were evaluated *in vivo*, in the classical, 4-day, suppressive test (Peters and Robinson, 1992). White, adult, Swiss (OF1) mice, with a mean (S.D.) body weight of 21 (2) g, were each inoculated intraperitoneally with 5×10^6 erythrocytes parasitised with *Pl. berghei* ANKA, in 0.9% saline, on day 0. Each extract was dissolved in the same diluents as for the toxicology tests but to a concentration such that each dose required was contained in 0.2 ml of solution. Twenty-four mice were divided into groups of three (three mice for each dose of each extract and for each of the two controls). The test mice were treated daily from day 0 (immediately after infection) to day 3, with an oral dose of 200 or 800 mg/kg.day. On the same days, mice in the positive-control group were given quinine dihydrochloride (dissolved in water to give a 0.2-ml dose volume) at 10 mg/kg.day and those in the negative-control group were only given water (0.2 ml/day) in the same way.

Each day from day 0 to day 4, a thin film was made from a tail-blood sample from each mouse and stained with Giemsa so that the level of parasitaemia (%) could be evaluated (as half the number of schizonts with at least three nuclei each, counted in 200 erythrocytes). On day 4, the mean parasitaemia in each group of mice was determined so that the percentage chemosuppression for each dose of each extract could be calculated as:

$$[(A - B)/A] \times 100$$

where A was the mean parasitaemia in the negative-control group and B the parasitaemia in the test group.

Statistical Analysis

The statistical significance of differences in parasitaemias between control and test groups was assessed using Student's t-tests. P-values of 0.05 or less were considered significant.

RESULTS

Toxic Effects of the Crude Extracts

Results from the toxicological tests indicate that all the mice treated orally with the aqueous extracts (Figs 1 and 2) or any other of the extracts, either as a single dose of 500 mg/kg or as eight such doses over 4 weeks, continued to gain bodyweight at a similar rate to that seen in the untreated controls ($P > 0.05$ for each comparison). As no external toxic effects or mortality were observed within 30 days of treatment, the median lethal dose of each extract is presumably greater than the higher total dose tested (i.e. 4 g/kg, given over 4 weeks). None of the vital organs examined had major lesions detectable by routine histology, and the minor lesions that were seen in the treated mice (cytonuclear pleomorphism, nuclear vacuolization, cellular necrosis and binucleation in the livers of the mice given *M. morindoides* or *Ph. niruri* extracts, and interstitial haemorrhage and intratubular materials in the kidneys of the same mice) were seen equally frequently in the control mice.

Antimalarial Activities

The in-vivo antimalarial activities of the ethanolic and dichloromethane extracts of the three selected medicinal plants are illustrated in Figures 3 and 4 and Table 2. Although the level of parasitaemia in the untreated control mice increased throughout the period of observation, that in the positive-control mice given quinine dihydrochloride gradually decreased to 0% on day 4. Parasitaemias in all the mice given any of the plant extracts except

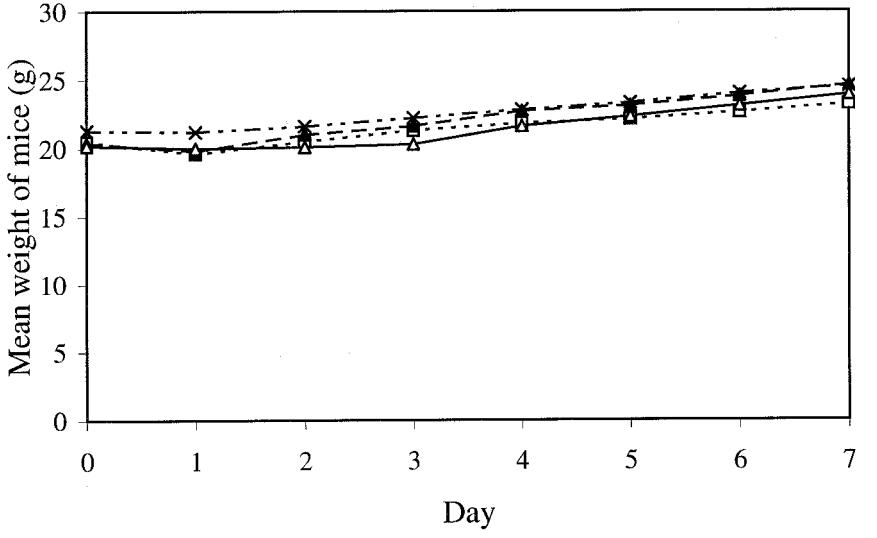

Fig. 1. Acute oral toxicity: effect of lyophilized, aqueous extracts of *Cassia occidentalis* (▲), *Morinda morindoides* (×) or *Phyllanthus niruri* (△), each given as a single, oral dose of 500 mg/kg on day 0, on the mean bodyweight of treated mice, compared with the bodyweights of untreated, control mice (□).

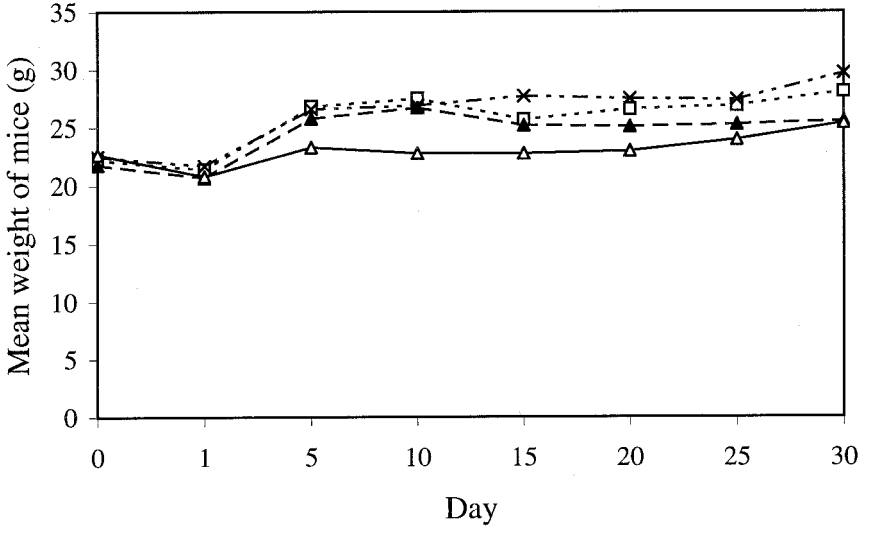

Fig. 2. Subacute oral toxicity: effect of lyophilized, aqueous extracts of *Cassia occidentalis* (▲), *Morinda morindoides* (×) or *Phyllanthus niruri* (△), each given as eight, oral doses of 500 mg/kg (on day 0 and then twice weekly for 4 weeks), on the mean bodyweight of treated mice, compared with the bodyweights of untreated, control mice (□).

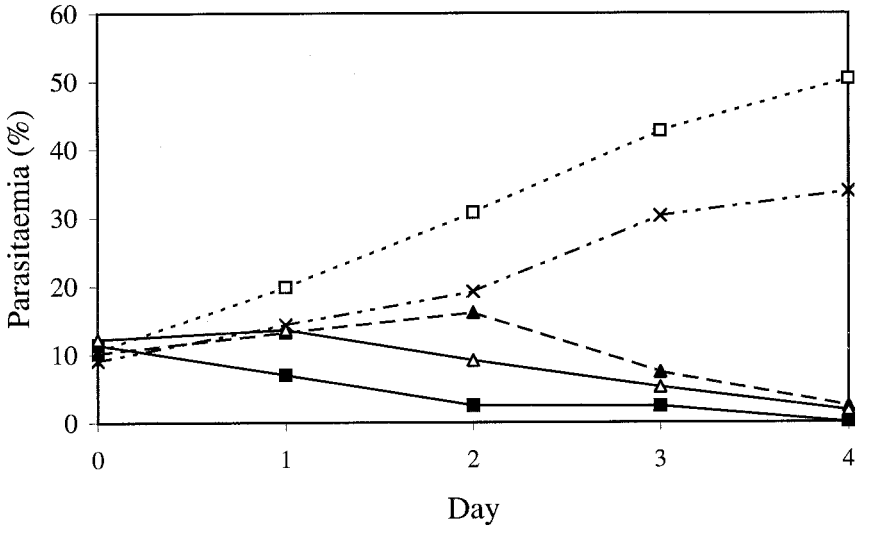

Fig. 3. Changes in parasitaemia in mice infected with *Plasmodium berghei* ANKA on day 0, following daily, oral treatment on days 0–3 with an ethanolic extract of *Cassia occidentalis* (▲), *Morinda morindoides* (×) or *Phyllanthus niruri* (△), each at 200 mg/kg.day, or with quinine dihydrochloride (10 mg/kg.day, as a positive control; ■), or with water (0.2 ml/day, as a negative control; □).

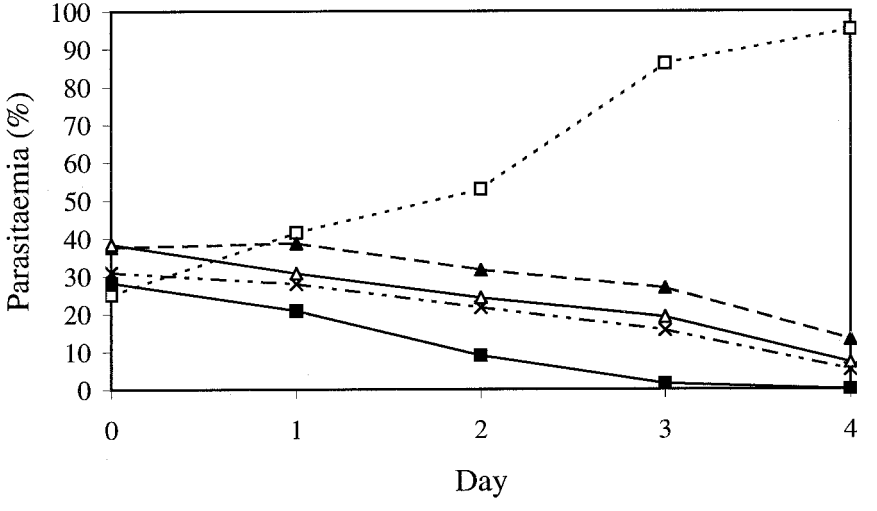

Fig. 4. Changes in parasitaemia in mice infected with *Plasmodium berghei* ANKA on day 0, following daily, oral treatment on days 0–3 with a dichloromethane extract of *Cassia occidentalis* (▲), *Morinda morindoides* (×) or *Phyllanthus niruri* (△), each at 200 mg/kg.day, or with quinine dihydrochloride (10 mg/kg.day, as a positive control; ■), or with water (0.2 ml/day, as a negative control; □).

TABLE 2
In–vivo antimalarial activity of the plant extracts

Treatment	*Extract*	*Dose (mg/kg.day)*	*Mean (S.D.) value (%)*	
			Parasitaemia	*Chemosuppression**
Cassia occidentalis	Ethanolic	200	10.0 (4.0)	68.0 (3.3)
		800	6.5 (4.1)	79.0 (3.2)
	Dichloromethane	200	12.0 (4.0)	61.3 (2.7)
		800	11.0 (2.6)	64.5 (2.2)
	Lyophilized aqueous	200	14.3 (4.7)	54.0 (4.4)
Morinda morindoides	Ethanolic	200	21.3 (3.6)	31.3 (2.8)
		800	19.5 (5.0)	37.1 (5.2)
	Dichloromethane	200	8.1 (1.0)	74.0 (3.3)
		800	5.0 (2.7)	84.0 (4.5)
	Lyophilized aqueous	200	24.0 (4.0)	22.6 (3.9)
Phyllanthus niruri	Ethanolic	200	8.4 (2.0)	73.0 (2.6)
		800	6.2 (2.1)	80.0 (4.0)
	Dichloromethane	200	8.5 (4.0)	72.6 (2.6)
		800	5.5 (3.2)	82.2 (2.4)
	Lyophilized aqueous	200	11.3 (4.6)	63.5 (3.7)
None		—	31.0 (3.7)	—
Quinine dihydrochloride		10	0	100.0 (0.0)

* All chemosuppressions were statistically significant ($P < 0.05$ for each). With the dichloromethane extracts, the chemosuppression produced by the higher dose of *M. morindoides* or *P. niruri* (but not *C. occidentalis*) was significantly greater than that produced by the lower.

the ethanolic extract of *M. morindoides* were lower on day 4 than on day 0. However, on day 4, even the mean parasitaemia in the mice given the ethanolic extract of *M. morindoides* (which was higher than in any other group of treated mice) was significantly lower than that in the untreated controls ($P < 0.001$). Mice given the most active extract, the ethanolic extract of *Ph. niruri*, had parasitaemias on days 1, 2, 3 and 4 which were significantly lower than the concurrent parasitaemias in the untreated controls ($P < 0.001$ for each comparison). Parasitaemias in all the mice given dichloromethane extracts fell with time, always to a value of $< 20\%$ by day 4 (Fig. 4). Although each lyophilized aqueous extract was less active than the corresponding ethanolic or dichloromethane extract, the chemosuppression observed with each aqueous extract was significant (Table 2). When tested at a dose of 200 mg/kg.day, the dichloromethane extracts of *M. morindoides* and *Ph. niruri* produced similar levels of chemosuppression ($P > 0.05$).

DISCUSSION

Cassia occidentalis

Various parts of *Cassia occidentalis* L. (Caeasalpiniaceae) plants are used in traditional medicine, for several therapeutic purposes. The leaves, for example, are used as a febrifuge (Kerharo and Adam, 1974; Kambu, 1990). The toxicity of the seeds, which has been recognized since at least 1913 (Kerharo and Adam, 1974), has been investigated extensively (Martin *et al.*, 1981; Colvin *et al.*, 1986; Flory *et al.*, 1992; Calore *et al.*, 1998; Haraguchi *et al.*, 1998) but the present study appears to be the first to investigate the toxicity of this species' root bark. Although their appeared to be no indications in the published literature that *C. occidentalis* might be useful in the treatment of malaria, Tona *et al.* (1999) discovered that traditional healers in Kinshasa use the root bark in the treatment of this disease. Ethanolic and dichloromethane extracts of the root bark have subsequently

been found to have marked antimalarial activity, both *in vitro* (Tona *et al.*, 1999) and *in vivo* (present study). Phytochemical investigation of the root bark revealed the presence of steroids, terpenes and anthracene derivatives, and these compounds (extracted from other plants) are known to inhibit *Pl. falciparum in vitro* (Koumaglo *et al.*, 1992; Cimanga, 1997; Sittie *et al.*, 1999).

Morinda morindoides

Morinda morindoides (Baker) Milne-Readh. (Rubiaceae), commonly called *nkongabululu* or *nkongobololo* in the DRC, has been used as a medicinal plant for many years. An aqueous decoction of fresh leaves—the most typical of the traditional preparations—is used for the treatment of fever, rheumatism and various diseases, including malaria (Kambu, 1990). It may well be effective as an antirheumatic, since flavonoids from an 80% methanol extract of the leaves had anticomplementary and radical-scavenging activity and inhibited xanthine oxidase (Cimanga *et al.*, 1995, 1997, 1999). Again, despite the frequent use of *M. morindoides* in traditional medicine, the present toxicological study on this plant appears to be the first. A methanol extract of the root was reported to be inactive against the chloroquine-resistant, K1 strain of *Pl. falciparum* and did not show any toxicity to brine shrimps at a concentration of 100 μg/ml (Addae-Kyereme and Wright, 1997). A chloroform extract of the leaves did inhibit the growth of the chloroquine-sensitive, NF 54 strain of *Pl. falciparum in vitro*, this activity being attributed to the chryzarin and alizarin in the extract (Cimanga, 1997). In the present study, treatment of infected mice with ethanolic or dichloromethane extracts of the leaves, at an oral dose of 200 mg/kg.day, led to significant reductions in parasitaemia (31% and 74%, respectively; $P < 0.001$ for each) compared with those seen in the untreated controls. Curiously, the chemosuppression observed in the present study when mice were treated with a dichloromethane extract of leaves collected in March (74%) was markedly higher than that seen when the extract used had been prepared in exactly the same manner from leaves collected in August (33%; Tona *et al.*, 1999). The timing of plant collection (and possibly the locality) may therefore influence the concentration of the component(s) with antimalarial activity (Capasso, 1985). Further studies *in vivo* are required to determine if, as suspected (Cimanga, 1997), the antimalarial activity of *M. morindoides* is attributable to the flavonoids and anthracene derivatives present in this species.

Phyllanthus niruri

Phyllanthus niruri L. (Euphorbiaceae) is widely used as a medicinal plant in various regions of the world. Calixto *et al.* (1998) reported that an aqueous infusion of the whole plant—a typical preparation in traditional medicine—was clinically tested in human volunters with hepatitis B or severe renal disturbances attributed to calculi, and in healthy subjects. At a daily, oral dose of 20 g, the aqueous extract was well tolerated and apparently non-toxic. Both aqueous and ethanolic extracts of the leaves helped reduce hepatic injury in rats and mice given lead nitrate and aluminium sulphate (Dhir *et al.*, 1990) or carbon tetrachloride (Prakash *et al.*, 1995) *per os*. Although *Ph. niruri* is known to be toxic to fish and frogs (Kerharo and Adam, 1974), an aqueous extract did not have any apparent effect on the kidney cells of dogs (Calixto *et al.*, 1998) and none of the present extracts of *Ph. niruri* appeared to effect the livers or kidneys of mice treated with them. At an oral dose of 200 mg/kg.day, both the ethanolic and dichloromethane extracts of the whole plant produced a significant ($> 70\%$) chemosuppression of parasitaemia ($P < 0.001$) in the present study. Curiously, a 50% ethanolic extract from *Ph. niruri* failed to inhibit *Pl. berghei* growth in infected mice (Kerharo and Adam, 1974; Oliver-Bever, 1986). Many compounds of medicinal interest, including alkaloids, benzene derivatives, flavonoids, coumarins, terpenes and steroids, have been isolated from *Ph. niruri* (Calixto *et al.*, 1998). Alkaloids, flavonoids and terpenes (from other plant species) have been found to inhibit *Pl. falciparum*, in both *in vitro* and *in vivo* tests (Bray *et al.*, 1990; Nkunya *et al.*, 1991; Phillip

son *et al*., 1993; François *et al*., 1994; Marshall *et al*., 1994; Bickii *et al*., 2000).

Comparisons and Conclusions

The results of the present toxicological experiments indicate that all of the extracts tested were well tolerated in mice. The ethanolic and dichloromethane extracts of *C. occidentalis* root bark and of *Ph. niruri* whole plant and the dichloromethane extract of *M. morindoides* leaves each reduced parasitaemia by $>50\%$ when tested at an oral dose of 200 mg/kg.day, indicating that the median effective dose (ED_{50}) of each of them was <200 mg/kg.day. Not surprisingly, therefore, a four-fold increase in dose, to 800 mg/kg.day, generally produced little benefit. The present results correlate well with those of the earlier studies of the activities of similar plant extracts against *Pl. falciparum in vitro* (Tona *et al*., 1999).

In conclusion, the results of the present study appear to justify the use of *Ph. niruri*, *C. occidentalis* and *M. morindoides* as traditional medicines for the treatment of malaria. The ethanolic extracts of *C. occidentalis* root bark and *Ph. niruri* whole plant, and all the dichloromethane extracts tested had the best activities *in vivo* activity. These extracts will be investigated further, in an attempt to isolate and identify their active constituents.

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