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Antimalarial Activity of Alkaloids from *Pogonopus tubulosus*

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The antimalarial activity of the Bolivian medicinal plant *Pogonopus tubulosus* (D.C.) Schumann was evaluated by *in vitro* testing on trophozoite stages of resistant and sensitive strains of *Plasmodium falciparum* and by *in vivo* tests on *P. berghei* and *P. vinckei petteri* in mice. The bark of this medicinal plant yielded three alkaloids: tubulosine, psychotrine, cephaeline. Tubulosine showed an interesting activity *in vitro* with an IC_{50} of 0.006 μg/mL against the sensitive strain of *P. falciparum* and an IC_{50} of 0.011 μg/mL against the resistant strain of *P. falciparum*. This compound had good *in vivo* antimalarial activity with an ED_{50} of 0.05 mg/kg/day on *P. vinckei petteri* strain and an ED_{50} of 0.45 mg/kg/day on *P. berghei*.

Keywords: antimalarial; *Plasmodium falciparum*; *P. berghei*; *P. vinckei*; tubulosine; psychotrine; cephaeline; *Pogonopus tubulosus*.

INTRODUCTION

Pogonopus tubulosus (D.C.) Schumann (Rubiaceae), a tree that grows in the southern South American subtropical rain forest, provides one of the numerous drugs called 'falsa quina' in South America, used against malaria (Killeen *et al.*, 1993). In our attempt to find new antimalarial products, extracts from the stem bark of *Pogonopus tubulosus* have been assayed for their antimalarial activities. We report here on the isolation and chemical identification of the three active alkaloids tubulosine **1**, psychotrine **2** and cephaeline **3** and on their *in vitro* and *in vivo* antimalarial activities.

MATERIALS AND METHODS

Chemistry. Plant materials were collected during ethnobotanical field work in the Chaco Province of Bolivia, near the city of Monte Agudo in November 1991. A voucher specimen (Moretti 1509) has been deposited in the National Herbarium of La Paz, Bolivia. A decoction of stem bark of this species named 'tumpa ropea' or 'falsa quina' by the Chiriguano is specifically used in this region as an antimalarial.

The crude alkaloids (1.58%) were obtained from the defatted powdered bark (165 g) following a classical procedure (Muñoz *et al.*, 1994). The alkaloids mixture (2.6 g) was fractionated by flash chromatography (silica gel, 230–400 mesh), eluted with $CHCl_3$ and $CHCl_3$/MeOH mixtures of increasing polarity (0–20%). Final purification was performed by successive column chromatographies (silica gel 60), and preparative thin layer chromatographies eluted with the same solvent and led to the isolation of alkaloid compounds **1** (0.157 g), **2** (0.024 g) and **3** (0.018 g) in a pure state. ^1H and ^{13}C NMR spectra were recorded at 300 and 75 MHz respectively. 2D experiments were performed using standard Bruker microprograms. More details of the isolation procedure and copies of the original spectra are available from the authors.

***In vitro* testing against *Plasmodium falciparum*.** This was carried out using a method based on that of Desjardins *et al.* (1979). Cultures of *P. falciparum*, (chloroquine sensitive strain 2087 and chloroquine resistant strain INDO) were maintained in human erythrocytes using a method developed by Trager and Jensen (1976). Dimethyl sulphoxide (50 μL) was added to samples of extracts or pure alkaloids which were then dissolved in RPMI 1640 medium with the aid of mild sonication in a sonicleaner bath (Branson Ltd), and further diluted as required in medium. The DMSO concentration for tested dilutions was no greater than 0.1%. 150 μL of total culture medium with the diluted extract and the suspension of human red blood cells in medium (0^+, 5% haematocrit) with 1% parasitaemia, were placed into the wells of 96-well microtitre plates. All tests were performed in triplicate. After 24 h of incubation at 37 °C using the candle jar method, the medium was replaced daily by fresh medium and incubation was continued for a further 48 h. On the third day of the test, a blood smear was taken from each well and parasitaemia counted. Each test included an untreated control, control with solvent and chloroquine as an internal standard. The parasitaemia for each well was obtained and the % inhibition of parasitaemia for each concentration of extract was calculated in relation to the control. Linear regression analysis was used to determine the best fitting straight line from which IC_{50} values were determined.

***In vivo* testing against *P. vinckei* and *P. berghei*.** The 4-day suppressive test against *P. vinckei* and *P. berghei* infection

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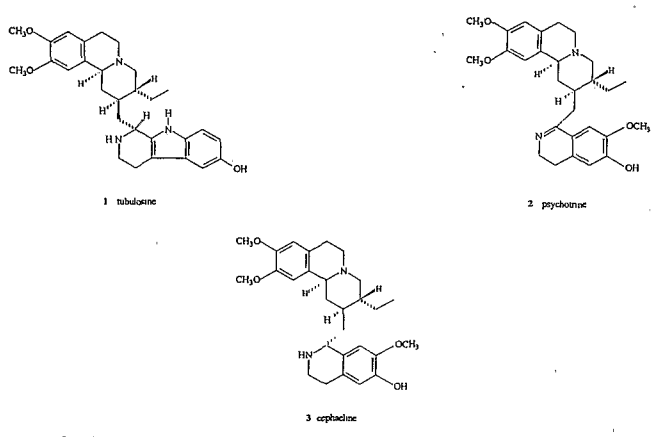
in mice described by Peters (1980) was used. This work was carried out as previously described (Deharo *et al.*, 1993). Mice were inoculated with *P. berghei* NK 65 or *P. vinckei petteri* 279BY on day 1 of the experiment and inoculated daily for 4 consecutive days with the extract or drug under test. On day 5 of the test, a blood smear was taken. ED_{50} values were computed by comparing the parasitaemias present in infected controls with those of test animals.

RESULTS AND DISCUSSION

A preliminary *in vivo* antimalarial bioassay showed that the activities were concentrated in the crude alkaloid mixture extracted from stem bark, and to a lesser extent in the petroleum extracts. Chemical study of the latter extract is in progress.

Identifications of **1**, **2** and **3** were carried out by comparing their physical and spectral data ($[\alpha]_D$, U.V., S.M., ^{1}H-NMR); with literature data for **1** and **2** (Ma *et al.*, 1990; Budzikiewicz *et al.*, 1954), and for **3**, with an authentic sample available in our laboratory. ^{13}C-NMR spectrum and 2 D ^{1}H-NMR experiments provided further confirmation for their structure.



Tubulosine **1** was previously isolated from the same species (Brauchli *et al.*, 1964); psychotrine **2** has already been detected in the ornamental species *Pogonopus speciosus* (Ma *et al.*, 1990); however this is the first reported occurrence of cephaeline **3** in this genus, which, having only 2 to 3 species seems chemically homogeneous.

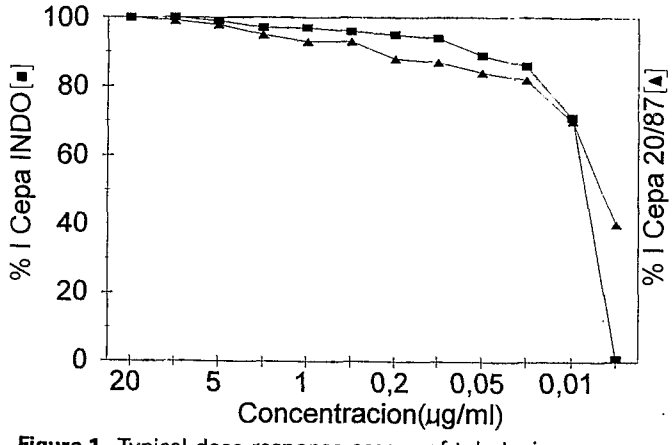


Figure 1. Typical dose-response assays of tubulosine on asynchronous cultures of *P. falciparum*. Each point is the mean of triplicate values. 20/87 chloroquine-sensitive strain (▲) and INDO resistant strain (■) after a 72 hr incubation.

Table 1. 50% inhibitory concentrations (µg/mL) of alkaloids from *Pogonopus tubulosus* against two isolates of *Plasmodium falciparum in vitro*

Drug	Isolate of *P. falciparum* 2087	Isolate of *P. falciparum* INDO
Chloroquine	0.02	0.08
Tubulosine **1**	0.006	0.011
Psychotrine **2**	0.14	0.39
Cephaeline **3**	0.027	0.011

Alkaloid **1** showed a good *in vitro* antiplasmodial activity with an IC_{50} of 0.006 µg/mL against the chloroquine-sensitive strain, and with an IC_{50} of 0.011 µg/mL against the resistant strains (Fig. 1 and Table 1). These activities are similar to those already published for this alkaloid against other strains, with an IC_{50} of 0.02 µg/mL with chloroquine resistant strain K-1 (Wright *et al.*, 1991). The *in vitro* activities of cephaeline (**3**) (Fig. 2 and Table 1) with an IC_{50} of 0.027 µg/mL against the chloroquine-sensitive strain, and with an IC_{50} of 0.011 µg/mL on the resistant strain were similar to tubulosine (**1**). Psychotrine (**2**) was less active (Fig. 3 and Table 1). Tubulosine (**1**) has been shown to have cytotoxicities against tumour cell lines. Its IC_{50} value on 9KB cells was 0.01 µg/mL (Ma *et al.*, 1990). These results suggest that compound **1** exhibits better selective toxicity against *Plasmodium* than that against mammalian cells; however its selectivity seems lower than that of chloroquine.

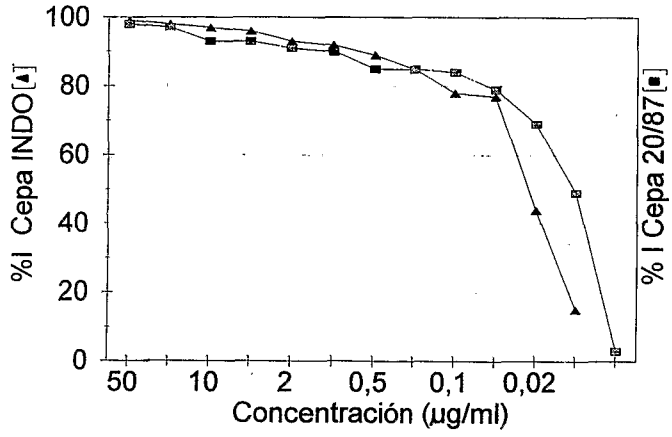


Figure 2. Typical dose-response assays of cephaeline on asynchronous cultures of *P. falciparum*. Each point is the mean of triplicate values. 20/87 chloroquine-sensitive strain (■) and INDO resistant strain (▲) after a 72 hr incubation.

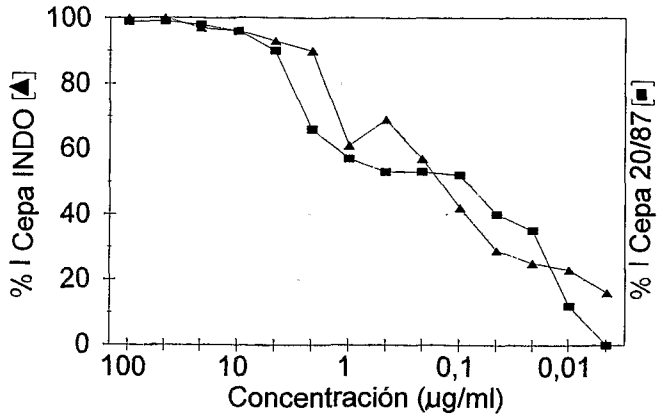


Figure 3. Typical dose-response assays of psychotrine on asynchronous cultures of *P. falciparum*. Each point is the mean of triplicate values. 20/87 chloroquine-sensitive strain (■) and INDO resistant strain (▲) after a 72 hr incubation.

Table 2. Antimalarial activity *in vivo* of tubulosine (1), psychotrine (2) and cephaeline (3)

Strain	Concentration	Parasitaemia	SEM	% Inhibition	Toxic deaths out of 10 mice at each dose
P. berghei NK 65	2.00	19	4	68	0
	1.00	22	5	63	0
	0.50	25	3	58	0
	0.10	34	1	42	0
	0.05	36	2	39	0
	control	59	4		
	IC_{50} of 1	=0.45 mg/kg/day			
P. vinckei petteri 279 BY	2.00	4	3	91	6
	1.00	5	2	89	0
	0.50	6	4	88	0
	0.10	12	3	74	0
	0.05	20	4	57	0
	control	45	4		
	IC_{50} of 1	≈0.05 mg/kg/day			
P. berghei NK 65	1.83	37	3	33	0
	0.1	39	3	28	0
	control	54	5		
	IC_{50} of 2	>2 mg/kg/day			
P. berghei NK 65	6.53	25	3	55	7
	3.13	43	4	21	0
	1.13	47	3	14	0
	control	54	5		
	IC_{50} of 3	=6 mg/kg/day			

Tubulosine (**1**) was also active *in vivo* with an ED_{50} value of 0.05 mg/kg/day on the *P. vinckei petteri* strain, at a lower concentration than its lethal dose (Table 2). All the mice were alive at the dose of 1 mg/kg/day. Mortality appeared at 2 mg/kg/day with 6/10 mice dead. This activity was confirmed on the *P. berghei* strain with an ED_{50} of 0.45 mg/kg/day but without toxicity at the doses tested (Table 2). This difference observed between the two *Plasmodium* species is reminiscent of the innate sensitivity of the same parasite species to chloroquine. Beauté-Lafitte *et al.* (1994), classified the different species of rodent malaria according to their CQ sensitivity. They found that *P. berghei* was less sensitive than *P. vinckei petteri*, and attributed these differences to a respective increase in the synchronicity of the infection, because the pharmacokinetics of chloroquine in the mouse are rapid and only the trophozoite stage is sensitive to the drug (Cambie *et al.*, 1991). It is therefore possible that the antimalarial effect of tubulosine is also stage-dependent. The two other alkaloids **2** and **3** are less active *in vivo* (Table 2).

The structure of tubulosine (**1**) is related to the strychnos dimer-alkaloids as well as to the ipeca alkaloids of the emetine group. It may be regarded as a mixed isoquinoline-indol analogue of emetine. Recent studies on molecular modelling (Quetin-Leclercq *et al.*, 1991) showed that this type of alkaloid cannot take the planar conformation as proposed previously (Ma *et al.*, 1990). A common (non planar) conformation exists where the angles between the two planes containing an aromatic ring are nearly the same, conserving the electronic environment of the nitrogen atom with its free electron at a certain distance from the aromatic rings. Emetine like alkaloids (**2, 3**) are more cytotoxic while the introduction of an indol moiety enhances the affinity for protozoan receptor. The relative *in vitro* inactivity of **2** in comparison with **3** can be explained by its double bond in ring C which enhances the coplanar conformation and electron environment. Structure/activity relationship studies indicate that minor changes in strychnos alkaloid structures may significantly affect their antiparasitic activities (Wright *et al.*, 1991, 1994). Our results show that tubulosine is more active than alkaloids of the usambarensine group which are relatively active *in vitro* and inactive *in vivo* (Wright *et al.*, 1991).

The results presented here lend support to the traditional common use of this plant as an antimalarial. However tubulosine, the principal antimalarial compound isolated from this species unfortunately presents little interest in the curative treatment of malaria because of its toxicity.

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