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Full Length Research Paper

The importance of including toxicity assays when screening plant extracts for antimalarial activity

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Identifying plant extracts as sources of antimalarial compounds needs to be addressed as numerous studies screen extracts without the means of eliminating extracts that are merely cytotoxic. Fifty-nine organic solvent extracts from South African plants were screened for antiplasmodial activity using the [3H]-hypoxanthine incorporation assay against the chloroquine-resistant *Plasmodium falciparum*. Variable antiplasmodial activity and toxicity was observed. Extracts form *Combretum erythrophyllum* and *Crinum bulbispermum*, had IC50 values ≤ 1 µg/ml with the ethyl acetate extracts of *C. bulbispermum* roots and bulbs having values comparable to chloroquine (0.04 µg/ml). Nine extracts had toxicity indexes ≥ 100. Lycorine, isolated from *C. bulbispermum* was as active as chloroquine (IC50 of 0.03 µg/ml) and had a favourable security index.

Key words: Antiplasmodial, *Combretum erythrophyllum*, *Crinum bulbispermum*, *Maytenus heterophylla*, *Pavetta gardeniifolia*, lycorine.

INTRODUCTION

It is estimated that 80% of people living in developing countries are almost completely dependent on traditional medicinal practices for their primary health care needs. Higher plants are also known to be the main source of drug therapy in traditional medicine. While the scientific world races to find a new cure for the mosquito-borne disease, a child dies every 3 to 5 minutes of malaria, attributing to the 300 to 500 million clinical cases each year and a staggering 1.5 to 2.7 million deaths (Breman, 2001). Malaria is a protozoal disease caused by four *Plasmodium* species. *Plasmodium falciparum* is one of the agents of malaria. Resistance to the most common antimalarial drugs has spread to almost every part of the world contributing to the urgency for the development of new compounds for malaria therapy (Winstanley, 2000). Efforts are now being directed towards obtaining drugs with different structural features, along with new strategies in malaria control and the recognition and validation of traditional medical practices.

A large number of reports on the *in vitro* antiplasmodial activities of plant extracts without reference to toxicity have been published lately (Clarkson et al., 2004; Saxena et al., 2003). In these circumstances it is not always clear if the reported activity could be related to a general cytotoxicity. A publication by van Zyl and Viljoen (2002) reported on both the antiplasmodial activity and toxicity of *Aloe* species; while Atindehou et al. (2004) and Prozesky et al. (2001) reported the antiplasmodial activity of a substantial list of plant extracts, but the toxicity of only a selected few of the list of extracts.

In this study fifty-nine different extracts from six plant species selected from literature were screened for antiplasmodial activity as well as toxicity. This was done to confirm antiplasmodial activity as opposed to general cytotoxicity and to improve the selection process for promising extracts.

MATERIALS AND METHODS

Selection and collection of plant material

Six South African plants were selected based on reported antiplasmodial activity in related species (Clarkson et al., 2004) and their

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Table 1. Medicinal plants used in this study.

Family	Species	Part used	Traditional uses of this genus
Amaryllidaceae	*C. bulbispermum* (Burm. F.) Milne-Redh. and Schweick	Leave Roots Bulbs	To treat colds, rheumatism, earache, septic sores, haemorrhoids and rheumatic fever. Also used as a rat poison (Watt and Breyer-Brandwijk, 1962)
Anacaridaeceae	*R. pyroides* Burch.	Leaves Seeds Stems	Antimicrobial concoctions (Saxena et al., 1994), gastritis, stomach cancer and arteriosclerosis (Lee et al., 2004)
Combretaceae	*C. erythrophyllum* (Burch.) Sond.	Leaves Stems	Malaria (Benoit-Vical et al., 1999), bilharziasis, hookworm, antimicrobial infections, fever, headaches, (Masoko et al., 2007)
Celasteraceae	*M. heterophylla* Eckl. and Zeyh.	Leaves Seeds Stems	Treat infectious diseases and the recurrent fever typical of malaria (El Tahir et al., 1999)
Liliaceae	*A. greatheadii* Schonl. *var davjana* (Schonl.) Glen and Hardy	Leaves	Used for antibacterial, antifungal, antiviral, antimalarial properties. Against trypanosomiasis and for cathartic effects (van Zyl and Viljoen, 2002).
Rubiaceae	*P. gardeniifolia* A.Rich.	Leaves Seeds Stems	Used for antimalarial activity (Sanon et al., 2003), respiratory and abdominal disorders, fevers (Grosvenor et al., 1995).

availability (Table 1). The plants were collected from the Botanical Garden of the North-West University (Potchefstroom Campus) and identified by the curator of the garden, Mr Peter Mortimer. Voucher specimens are kept at the A.P. Goossens Herbarium (PUC), North-West University, Potchefstroom.

Preparation of extracts

Plant samples were separated into different morphological parts. Leaves, stems and seeds were dried at room temperature for five days, while roots and bulbs were frozen. Dried plant parts were grounded to a coarse powder, while the frozen parts were cut into smaller pieces. Extracts were prepared by sequential Soxhlet extraction of 5-20 g of plant material with petroleum ether, dichloromethane, ethyl acetate and ethanol. Solvents were removed using rotary vacuum evaporation.

***In vitro* antiplasmodial screening**

A chloroquine-resistant strain (FCR-3) of *P. falciparum* was continuously cultured according to the method of Trager and Jensen (1976) which entailed the daily replacement of culture medium and the addition of washed erythrocytes every second day when parasites were in the trophozoite-schizont stage. Washed erythrocytes were obtained by centrifuging whole blood at 400 x g for 5 minutes and then removing the plasma portion and leukocyte buffy coat. The [^{3}H]-hypoxanthine incorporation assay of Desjardins et al. (1979) was used to determine antiplasmodial activity on synchronised ring stage parasites (Lambros and Vandenberg, 1979). Synchronisation of the ring stage was achieved by adding sorbitol to the medium every second day when the parasites were in the ring stage. The parasite culture was adjusted to 0.5% parasiteamia and 1% heamatocrit. Parasiteamia was determined with Giemsa staining. 96-Well microplates were prepared by filling each well with standardised parasite culture and adding solubilised plant extracts in various dilutions. Plant extracts were reconstituted in DMSO and then diluted to render solutions with a maximum concentration of 1% DMSO. A negative control (uninfected erythrocytes) and an untreated parasite control were included. After incubation of 24 hours at 37°C under anaerobic conditions [^{3}H]-hypoxanthine was added to all wells and the plates were again incubated for 24 hours at 37°C under anaerobic conditions. Incorporation of [^{3}H]-hypoxanthine into DNA was measured by liquid scintillation counting. The results were expressed as a percentage of parasite growth and the IC_{50} values of the plant extracts and standard antimalarials were calculated from log sigmoid dose response curves by the Enzfitter® programme. Chloroquine diphosphate and quinine sulphate were used as reference compounds. All assays were done in triplicate.

Cytotoxicity screening

To determine the toxicity of the plant extracts on human kidney epithelial (Graham 293) cells, the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] toxicity assay was used (Mosmann, 1983). Graham cells were cultured in Ham F10 medium containing heat inactivated foetal calf serum and 0.1% gentamicin. The medium was replaced every second day, trypsinised once weekly and then allowed to reach confluency. Dilutions and appropriate DMSO controls were prepared and plated out in quadruplicate in 96-well plates to a cell suspension of 0.5 million cells/ml. A concentration of 1% DMSO (v/v) was not toxic to the epithelial cells. Each plate contained an untreated cell control and a blank control (containing no cells). Prepared plates were incubated at 37°C for 44 hours in a 5% CO_2 environment. After incubation sterile MTT solution was added to each well and plates incubated for another 4 hours. Absorbance of the formed formazan product was measured at a test wavelength of 540 nm and a reference wavelength of 690 nm. Results were expressed as percentage cellular viability of the extract and cell-free controls. All assays were done in quadruplicate. Security indexes (Benoit-Vical et al., 1999) were calculated as an indication of the toxicity of the prepared plant extracts in relation to its antimalarial activity.

Security index = Cytotoxicity (IC_{50} µg/ml)/Antiplasmodial activity(IC_{50} µg/ml)

Isolation of lycorine

Isolation of pure lycorine from *C. bulbispermum* was achieved by column chromatography on silica gel (size 0.063-0.2 mm, Macherey-Nagel) with petroleum ether-dichloromethane-ethyl acetate (4:8:1) as mobile phase. ^{13}C and ^{1}H nuclear magnetic resonance (NMR) spectra were recorded on a Varian Gemini 300 in DMSO-d_6. ^{13}C spectra were recorded at a frequency of 75,462 MHz and ^{1}H spectra at a frequency of 300,075 MHz. All chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane ($\delta = 0$).

RESULTS

Results obtained from the [^{3}H]-hypoxanthine incorporation assay, the MTT-assay and the calculated security indexes of the tested plant extracts and reference compounds (chloroquine and quinine) are shown in Table 2.

Eleven extracts had IC50 values ≤ 1 µg/ml with two extracts comparable to chloroquine (0.04 µg/ml). Nine extracts had toxicity indexes ≥ 100. The extracts of *C. bulbispermum* were the most promising candidates for further study.

The comparison of spectral data with that found in the literature to confirm the structure of lycorine (Figure 1) is depicted in Table 3.

DISCUSSION

Most extracts inhibited parasite proliferation by more than 50% at a concentration of 50 µg/ml. Only nine of the fifty-nine extracts had security indexes of greater than 100 and were considered to have antiplasmodial activity as opposed to general cytotoxicity (highlighted in Table 2). The most promising extract was the ethyl acetate bulb extract of *C. bulbispermum* with an IC_{50} value of 0.08 µg/ml and a security index of 2203.13.

A. greatheadii showed very little antiplasmodial activity and appeared to be toxic against the human kidney epithelial cell line. The weak antiplasmodial activity of *Aloe* species in general is confirmed by van Zyl and Viljoen (2002), as well as Clarkson et al. (2004) with IC_{50} values mostly being greater than10 µg/ml.

M. heterophylla extracts showed moderate but variable antiplasmodial activity, with IC_{50} values ranging between 1.20 and 89.47 µg/ml, with the best activity being displayed by the dichloromethane stem extract (IC_{50} value: 1.20 ± 0.93 µg/ml). The high security index (224.82) of the ethanol extract of the seeds of this species indicated that it possessed more antiprotozoal activity as opposed to being cytotoxic. Extracts of *M senegalensis* and *M undata* were also reported by Clarkson et al. (2004) to have little antiplasmodial activity with IC_{50} values greater than 15 µg/ml against the chloroquine-sensitive D10 strain. Atindehou et al. (2004) also reported IC_{50} values of greater than 5 µg/ml for *M senegalensis*; while Prozesky et al. (2001) reported values of greater than 50 µg/ml against the chloroquine-resistant PfUP1 strain.

Both *R. pyroides* and *P. gardeniifolia* showed moderate to good activity in the tritiated hypoxanthine incorporation assay, but the extracts were considered to be more cytotoxic rather than antiplasmodial. Sanon et al. (2003) and Weninger et al. (2004) also reported weak antiplasmodial activity for *Pavetta* species.

C. erythrophyllum extracts displayed potent antiplasmodial activity (IC_{50} values ranging from 0.70 and 10.68 µg/ml), especially the leaves with an IC_{50} value of 0.87 µg/ml. Clarkson et al. (2004) reported weaker activity for the twigs of *C. zeyheri* (IC_{50} value of 15 µg/ml) and Atindehou et al. (2004) also reported IC_{50} values of more than 5 µg/ml for *C. molle*, *C. racemosum* and *C. smeathnannii* extracts.

C. bulbispermum root and bulb extracts showed remarkably potent antiplasmodial activity with varying toxicity, although Clarkson et al. (2004) reported poor activity for *C. macowanii* bulb extracts. The ethyl acetate extract of *C. bulbispermum* bulbs had the best profile with an IC_{50} value of 0.08 µg/ml for antiplasmodial activity and a security index of 2203. Due to these promising properties, further purification of this extract was performed. On comparison of the physical data (Table 3) to that found in the literature (Likhitwitayawuid et al.,1993), the compound was identified as lycorine (Figure 1, Table 3), which has been isolated from *C. amabile* and *Brunsvigia radulosa* (Campbell et al., 2000). Lycorine was identified as a potent antiplasmodial compound with an IC_{50} value of 0.03 µg/ml against the chloroquine-resistant strain (FCR-3) of *P. falciparum*, which is comparable to the activity of the crude extract and chloroquine. Likhitwitaywuid et al. (1993) reported an IC_{50} value of 0.3 µg/ml for lycorine isolated from *C. amabile* against the chloroquine-resistant strain (W-2). Campbell et al. (2000) reported IC_{50} values of 0.6 and 0.7 µg/ml for lycorine isolated from *B. radulosa* against the strains D-10 and FAC8, respectively. Reports on the cytotoxicity of lycorine vary considerably and this study reports an IC_{50} value of 445.47 µg/ml for lycorine against human kidney epithelial cells. Likhitwitaywuid et al. (1993) reported IC_{50} values for ranging from 0.3 to 1.6 µg/ml against a series of human cancer cells. The anti-cancer activity of lycorine was also reported by Li et al. (2007) as an effect due to the apoptosis inducing effect of lycorine. Lycorine further has been found to inhibit protein synthesis (Jimenenz et al., 1976).

Although different species and extracts of the plants investigated in this study have been reported on in literature, the findings of this study seem consistent with the fact that when the results of an antiplasmodial screening are combined with toxicity studies, very few plants can be considered as good candidates for further investigation. Bearing in mind that natural phytomolecules such as quinine and artemisinin have acted as templates for the development of antiplasmodial agents, it is encouraging to find that the antiplasmodial activity of

Table 2. IC_{50} values for antiplasmodial and toxicity assays and calculated security indexes for South African plant extracts.

Plant extract	Antiplasmodial activity IC_{50} (µg/ml) ± S.D. (n = 3)	Toxicity IC_{50} (µg/ml) ± S.D. (n = 4)	Security index
A. greatheadii* var *davyana			
leaves:			
petroleum ether	4.90 ± 2.98	4.7 ± 0.78	0.97
dichloromethane	32.45 ± 6.58	392.96 ± 83.89	12.11
ethyl acetate	43.18 ± 21.84	144.51 ± 60.37	3.35
ethanol	27.04 ± 12.89	94.30 ± 38.90	3.49
C. erythrophyllum			
leaves:			
petroleum ether	1.18 ± 0.49	66.04 ± 7.58	55.97
dichloromethane	0.70 ± 0.25	42.78 ± 7.24	61.11
ethyl acetate	1.04 ± 0.36	32.92 ± 2.65	31.65
ethanol	**0.87 ± 0.28**	**925.72 ± 20.38**	**1064.05**
stems:			
petroleum ether	1.47 ± 0.38	2.74 ± 0.23	1.86
dichloromethane	**0.70 ± 0.21**	**70.51 ± 6.59**	**100.73**
ethyl acetate	10.68 ± 0.03	20.85 ± 7.74	1.95
ethanol	4.41 ± 1.65	66.97 ± 4.38	15.19
C. bulbispermum			
leaves:			
petroleum ether	0.89 ± 0.19	45.17 ± 6.67	50.75
dichloromethane	1.96 ± 1.10	11.08 ± 1.96	5.65
ethyl acetate	15.16 ± 3.21	20.56 ± 1.72	1.35
ethanol	62.29 ± 16.86	11.69 ± 1.73	0.19
roots:			
petroleum ether	3.54 ± 1.48	16.08 ± 9.99	4.54
dichloromethane	1.03 ± 0.08	2.36 ± 0.65	2.29
ethyl acetate	**0.02 ± 0.01**	**9.60 ± 5.02**	**480.00**
ethanol	18.23 ± 0.56	171.08 ± 56.73	9.38
bulbs:			
petroleum ether	**0.40 ± 0.09**	**177.57 ± 45.57**	**443.93**
dichloromethane	0.38 ± 0.03	9.71 ± 8.94	25.55
ethyl acetate	0.08 ± 0.01	176.25 ± 12.58	2203.13
ethanol	**0.32 ± 0.13**	**8.71 ± 0.31**	**27.22**
M. heterophylla			
leaves:			
petroleum ether	12.58 ± 4.45	36.67 ± 3.61	2.91
dichloromethane	2.10 ± 0.57	7.37 ± 1.57	3.51
ethyl acetate	3.84 ± 0.96	26.17 ± 0.11	6.82
ethanol	89.47 ± 46.65	64.41 ± 11.52	0.72
seeds:			
petroleum ether	23.09 ± 7.58	5.44 ± 0.29	0.23
dichloromethane	20.07 ± 2.42	90.19 ± 41.71	4.49
ethyl acetate	3.00 ± 0.46	47.01 ± 4.37	15.72
ethanol	27.84 ± 6.50	6259.30 ± 45.57	224.82
stems:			
petroleum ether	6.36 ± 0.32	50.61 ± 5.18	7.96
dichloromethane	1.20 ± 0.93	34.21 ± 5.25	28.51

Table 2. contd.

ethyl acetate	1.36 ± 0.54	58.84 ± 2.41	43.26
ethanol	10.56 ± 0.53	230.82 ± 5.40	21.86
P. gardeniifolia			
leaves:			
petroleum ether	5.50 ± 0.58	25.93 ± 2.37	4.71
dichloromethane	12.91 ± 1.24	238.95 ± 8.14	18.51
ethyl acetate	7.28 ± 4.14	236.86 ± 143.28	32.54
ethanol	21.15 ± 1.35	729.71 ± 245.39	34.50
seeds:			
petroleum ether	3.39 ± 1.43	225.47 ± 37.37	66.51
dichloromethane	**1.30 ± 0.49**	**153.43 ± 61.08**	**118.02**
ethyl acetate	35.42 ± 14.64	495.94 ± 161.32	14.00
ethanol	4.30 ± 0.61	384.31 ± 171.10	89.37
stems:			
petroleum ether	1.83 ± 0.20	50.75 ± 14.73	27.73
dichloromethane	**1.47 ± 0.39**	**1065.90 ± 1610.6**	**725.10**
ethyl acetate	3.32 ± 0.54	87.22 ± 8.26	26.27
ethanol	19.92 ± 0.36	540.15 ± 219.48	27.12
R. pyroides			
leaves:			
petroleum ether	1.68 ± 0.11	50.32 ± 4.17	29.95
dichloromethane	1.00 ± 0.41	7.17 ± 0.93	7.17
ethyl acetate	1.32 ± 0.31	84.11 ± 1.85	63.72
ethanol	9.30 ± 1.84	942.53 ± 800.00	101.34
seeds:			
petroleum ether	9.67 ± 0.43	82.85 ± 17.13	8.57
dichloromethane	1.60 ± 0.95	22.91 ± 6.67	14.32
ethyl acetate	21.10 ± 0.24	230.19 ± 60.56	10.91
stems:			
petroleum ether	1.11 ± 0.50	10.64 ± 0.97	9.59
dichloromethane	3.05 ± 0.60	5.33 ± 1.59	1.75
ethyl acetate	1.35 ± 0.30	34.37 ± 12.76	25.46
ethanol	9.30 ± 0.46	112.79 ± 19.23	12.13
Chloroquine	0.04 ± 0.006	125.56 ± 5.04	3139.00
Quinine	0.13 ± 0.01	136.06 ± 4.06	1046.62
Lycorine	0.03 ± 0.01	445.47 ± 18.08	14849.00

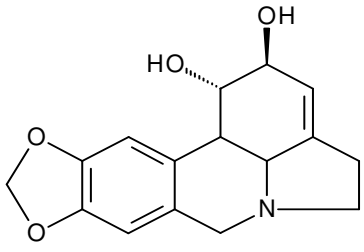

Figure 1. Lycorine.

lycorine, a compound structurally unrelated to both quinine and artemisinin, is consistently reported. This compound or structural derivatives thereof could inhibit parasite growth via the induction of apoptosis as already described for artemisinin derivatives (Jones et al., 2009).

The authors recommend the inclusion of toxicity testing with all *in vitro* antiplasmodial screening of plant extracts.

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Table 3. ^{1}H and ^{13}C NMR spectral assignments for isolated compound and lycorine (Likhitwitayawuid et al., 1993).

Position	$^{1}H^{a}$	$^{1}H^{b}$	$^{13}C^{a}$	$^{13}C^{b}$
1	4.28 (s)	4.27 (s)	71.66	70.21
2	3.98 (s)	3.97 (s)	70.14	71.72
3	5.35 (s)	5.37 (s)		118.48
4			141.54	141.68
4a	2.61 (d)	2.60 (d)	60.72	60.83
6α	3.34 (d)	3.32 (d)	56.59	56.73
β	4.05 (d)	4.02 (d)		
6a			129.56	129.75
7	6.65 (s)	6.68 (s)	106.96	107.01
8			145.16	145.20
9			145.52	145.65
10	6.80 (s)	6.81 (s)	105.01	105.06
10a			129.60	129.57
10b	2.48 (m)	2.50 (m)	42.0	40.18
11α,β	2.44 (m)	2.44 (m)	d28.10	28.13
12α	2.20 (ddd)	2.19 (ddd)	5.24	53.31
β	3.20 (dd)	3.19 (dd)		
OCH_2O	5.92 (s)	5.94 (s)	100.51	100.57

[a]Chemical shifts are reported in ppm (δ) in DMSO-d_6 for ^{1}H and ^{13}C.
[b]Data obtained form Likhitwitaywuid et al. (1993).

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