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In vivo antiplasmodial activity of ZS-2A: a fraction from chloroform extract of *Zizyphus spina-christi* root bark against *Plasmodium berghei berghei* in mice

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ABSTRACT

Zizyphus spina-christi is used in ethnomedical practice for the treatment of fever. Bio-assay guided investigation of the plant's root bark was initiated and ZS-2A, a fraction from the chloroform extract of the material, eluted with hexane-ethylacetate (50:50) using flash column chromatography, was evaluated for *in vivo* antiplasmodial activity against *Plasmodium berghei* in mice. Four-day suppressive, curative effect against established infection and prophylactic models of antiplasmodial studies were used. The fraction (25, 50 and 100 mg/kg, p.o.) showed a potent activity against the parasite in the suppressive and curative tests. The result suggests that ZS-2A may be a promising agent for malaria treatment.

Keywords: Fraction ZS-2A, *Zizyphus spina-christi*, *Plasmodium berghei berghei.*

INTRODUCTION

Despite the increase threat of malaria to lives especially in Africa, success in controlling the disease is possible (White et al., 2004). Different approaches are currently being advocated to achieve this, which includes: exploring evidences of immunity, social science input, revisiting the abandoned vector control methods and investigation into traditionally used herbal medicines (Wright, 2005).

Zizyphus spina-christi Willd (Rhamnaceae) is a wild plant that can be domesticated. It grows in tropical Africa and Asia and has versed reported medicinal values (Burkill, 1997). Among the plant's popular ethnomedical uses is the treatment of fever (Al-Said, 1993). To the best of our knowledge, scientific basis for such usage has not been documented. As part of our continued evaluation of *Zizyphus spina-christi* root bark, we tested fraction (numbered ZS-2A) from chloroform extract of the plant for its antiplasmodial activity against rodent plasmodia (*Plasmodium berghei berghei*) *in vivo* in mice for possible chemosuppressive effect against the parasite. The root bark of the plant was first sequentially extracted with four solvents (hexane, chloroform, ethylacetate and methanol) along their polarity using soxhlet extractor (Quickfit, England) and the extracts preliminarily tested for antiplasmodial activity. The chloroform extract that gave the highest activity was fractionated using flash column and evaluated for suppressive, curative and prophylactic activities against *P.*

berghei berghei. The phytochemical composition of the chloroform extract was determined and its LD_{50} established.

MATERIALS AND METHODS

Plant material

The plant material was collected at Midlu, Adamawa State, Nigeria between April and May, 2004. Its authentication, voucher specimen (NIPRD #4108) preservation and extraction were as earlier reported (Adzu et al., 2007). Briefly, the root bark of the plant was carefully removed, cleaned and dried under shade. The dried root bark was ground into powder and 1.29kg of the powdered material sequentially extracted to obtain hexane (ZS-1), chloroform (ZS-2), ethylacetate (ZS-3) and methanol (ZS-4) extracts. The combined extract of each solvent was concentrated and evaporated to dryness. The total yield of the extracts was 21.86% w/w of crude starting material.

Phytochemical test

The phytochemical composition of the chloroform extract was determined using standard procedures (Harborne, 1998). TLC was also carried out to determine spot zones which were viewed under UV light (254/365nm Eagle Scientific Ltd, UK).

Fractionation of chloroform extract

Ten grams of the chloroform extract (ZS-2) were mixed with 15 g of silica gel (Aldrich Chemical; 230-400 mesh) and loaded onto a flash column containing another 100 g of silica gel. It was then flushed with hexane, and eluted with gradient of ethylacetate in hexane, then methanol in multiples of 100 ml, using pump (ABM, Germany). Each of the fractions consisting of 100 ml was individually collected. A total of 34 fractions (100 mL each) were collected and combined into six main groups (ZS-2A…ZS-2F) on the basis of their TLC profiles. These combined fractions (ZS-2A to ZS-2F) were concentrated over water bath and allowed to evaporate to dryness at room temperature. The fractions were preliminarily tested for antiplasmodial potency and ZS-2A which gave the most potent effect was fully evaluated.

Animals

Four weeks old albino mice obtained from Animal Facility Centre, NIPRD, Abuja, were used for the study. They were housed in plastic cages with saw dust as beddings and given food and water *ad libitum*. The mice were used in accordance with NIH Guide for the Care and Use of Laboratory Animals; NIH Publication (No 83-23) revised (1985) and NIPRD's Standard Operation Procedures (NIPRD-SOPs).

Acute toxicity test

The LD_{50} of the fraction was tested in order to determine the safety of the agent using Lorke's (1983) method. Briefly, ZS-2A was administered in geometric doses of 10, 100 and 1000 mg/kg i.p. to four groups of mice (n = 3). Another mouse was given normal saline to serve as the control and all the mice were kept under same conditions and observed for toxic signs and mortality for 24h.

Rodent parasite (*Plasmodium berghei berghei*)

The rodent parasite was sourced from National Institute for Medical Research (NIMR), Lagos, Nigeria by a co-author (U.A. Katsayal) and kept at Department of Veterinary Parasitology and Entomology, Ahmadu Bello University, Zaria, Nigeria. The parasites were kept alive by continuous reinfestation (i.p.) in mice (Calvalho et al., 1991) every 4 days. The reinfected mice were moved to the Department of Pharmacology and Toxicology, NIPRD, Abuja where the study was carried out. Prior to the commencement of the study, one of the infected mice was kept and observed to reproduce diseases similar to human infection (English et al., 1996).

Chemicals and test agents

All the chemicals used for the extraction, fractionation and chromatography were of analar grade and were purchased from Sigma-Aldrich representative in Nigeria (Zayo International Ltd, Jos, Nigeria). ZS-2A was prepared as aqueous tragacanth (a biologically inert surfactant) and were freshly prepared prior to each experiment.

Antiplasmodial studies

Suppressive test

A modification of the classical 4-day earlier infection method (Peters et al., 1993; Okokon et al., 2006) was used for the test. Twenty five mice were inoculated as follows:

A donor mouse with parasitaemic level of (++) = 11 - 100 parasites per 100 thick film fields (WHO, 1985) was anaesthetised with chloroform. Blood (1 ml) was collected through cardiac puncture with needle and syringe and made up to 20ml with saline. All the mice received 0.2 ml of the diluted inoculum i.p. and were then group into groups 1 - 5 (n = 5) keeping the groups mean weight as near as possible. Group 1 was not treated and served as negative control. Groups 2 – 4 received ZS-2A (25, 50 and 100 mg/kg, p.o.) while group 5 was treated with chloroquine (5 mg/kg, s.c.), all on the first day (D_0). Treatment then continued daily ($D_1 – D_3$) until D_4 when blood was collected from tail of each mouse and smeared onto a microscopic slide to make a film (Saidu et al., 2000). The films were fixed with methanol, stained with 4% Giemsa at pH 7.2 for 45 min (Saidu et al., 2000) and parasitaemia (WHO, 1994) examined microscopically (Kirby et al., 1993).

Rane (curative) test

The inoculation and treatment protocols used for the Rane test (Ryley and Peters, 1970) were similar to the suppressive test described above, except that in this test, treatment started on D_3 after infection was established. On D_3, a pretreatment blood smear of each mouse was collected and the mice were treated as described above. Treatment then continued daily ($D_4 – D_6$) until D_7 when post treatment blood smears were collected and examined for parasite suppression. The mice were observed for 30 days and mean survival time of each grouped recorded (Adzu et al., 2003).

Repository test

The ability of ZS-2A to exhibit prophylactic activity was tested using the residual infection procedure (Peters, 1965). In this test, treatment (as described above) was initiated on D_0 and continued till D_4 when the mice were all infected with the parasite as earlier described. Smears were then made from each mouse 72 h after treatment (Abatan and Makinde, 1986) and increase/decrease in parasitaemia determined. The pre- and post-treatment body weights were taken.

Data analysis

Results were expressed as mean ± SEM. Student t-test was used to analyse the data between groups and Analysis of Variance (ANOVA) among groups. $P < 0.05$ was considered significant in all cases.

RESULTS

Phytochemical test

The chloroform extract was found to contain carbohydrates (+++), resins (++), saponins (++), terpenes (+++) and traces of alkaloids (+).

Acute toxicity tests

There was no mortality recorded in the mice, even in the ones that were 1000 mg/kg, p.o. This indicates that the experimental doses used are relatively safe.

Suppressive activity

Percent inhibition of parasitaemia by ZS-2A was calculated as:

$$\frac{PC - PT}{PC} \times 100$$ (Hilou et al., 2006),

where PC=parasitaemia in control, and PT=parasitaemia in treated group.

The result is presented in Table 1. The fraction exhibited potent dose dependent activity in the test.

Curative effect

The fraction was able to reduce the parasitaemia observed in the pretreatment data, but didn't totally clear the parasite after the termination of dosing. The mice survival was improved from the average of less than 10 days in the control group to over 3 weeks in the group that received 100 mg/kg. However, except in the chloroquine (CQ) group, mortality was not totally abolished at the end of 30 days duration of observation (Table 2).

Prophylactic effect

ZS-2A failed to exhibit significant prophylactic effect since the parasitaemia remained high. It showed some improvement, but didn't totally prevent loss in body weight (Table 3). The plausible cause of this is discussed.

Table 1: Effect of ZS-2A against *P. bergei* infection in mice on suppressive test.

Treatment	Dose (mg/kg, p.o.)	Parasitaemia count D_4	% Inhibition
Control	-	19.2 ± 4	-
ZS-2A	25	11.4 ± 2	40.63*
	50	8 ± 2	58.33*
	100	6.6± 2	65.63*
CQ	5	3 ± 1	84.38*

* indicates significant difference ($p < 0.05$); D_4 = Day 4 after inoculation. CQ = chloroquine

Table 2: Effect of ZS-2A against *P. bergei* infection in mice during established infection (Curative test).

Treatment	Dose (mg/kg, p.o.)	Parasitaemia count		Survival time (Days)
		D_3	D_7	
Control	-	16.2 ± 0	23.4 ± 4	9.6 ± 2
ZS-2A	25	18.4 ± 5	11.6 ± 2	12.4 ± 3
	50	17.6 ± 5	8.6 ± 1*	20.4 ± 3
	100	18.2 ± 3	6 ± 2*	25.4 ± 2
CQ	5	20.2 ± 2	2.6 ± 1*	30

* indicates significant difference ($p < 0.05$); D_3 = Day 3 and D_7 = Day 7 after inoculation. CQ = chloroquine.

Table 3: Prophlactic effect of ZS-2A against *P. bergei* infection in mice (Repository test).

Treatment	Dose (mg/kg, p.o.)	Parasitaemia count	Body Weights (g)	
		D_7	D_0	D_7
Control	-	26.3 ± 4	23.15 ± 2	19.2 ± 2
ZS-2A	25	24.7 ± 5	22.92 ± 2	20 ± 1
	50	17.4 ± 3	23.17 ± 3	21.67 ± 2
	100	9.4 ± 3*	25.83 ± 2	23 ± 0
CQ	5	7.6 ± 4 *	23.12 ± 2	22.6 ± 2

* indicates significant difference ($p < 0.05$); D_0 = Day of inoculation and D_7 = Day 7 after inoculation. CQ = chloroquine.

DISCUSSION

The rodent parasite *(P. berghei*) discovered by Vinckey and Lips in 1948 have been used in studying the activity of potential antimalarials in mice (Thomas et al., 1998) and of recent, in rats (Pedroni et al., 2006). On the other hand, plants have proved to be sources of antimalarial agents, especially with the success of quinine isolated from the Peruvian *Cinchona* bark and artemisinin from *Artemisia annua*.

We chosed 4-week old mice for the study so as to exclude the effect of anaemia in the old mice and the possible changes it may induce (Pierrot et al., 2003). We opted for this *in vivo* model because it takes into account any prodrug effect and the likelihood of immune system in controlling infection (Waako et al., 2005). Oral dosing of ZS-2A was used, to replicate the ethnomedical method of administration and the likely route during clinical evaluation.

From the results, the fraction (ZS-2A) gave significant effect on both suppressive and curative test. Agents with suppressive activity against *P. berghei* were known for antimalarial activity (Calvalho et al., 1991). The fraction however failed to exhibit significant repository effect in the groups of mice that received low doses. This can be attributed to short duration of action of ZS-2A, perhaps limited by rapid metabolism. It can also be due to the *in vivo* model used, which lacks the insect vector, and the manner of inoculation and the doses used that result in rapid infection of the erythrocytes (Ager, 1984) without the parasite going through the liver stages. Another possibility was that ZS-2A might have acted through metabolic activation of the immune system (Waako et

al., 2005) and so parasite clearance could not be total. There is also the fact that not all antimalarials are completely active in *P. berghei* model (Dow et al., 1998).

However, *Zizyphus spina-christi* is used in areas where malaria is endemic and individuals might possess at least some degree of immunity in which relief may in addition be symptomatic. The fraction might have probably acted like antimalarial plants in their crude form, acting mainly by causing elevation of red blood cell oxidation (Etkin, 1997) and/or by inhibiting protein synthesis (Kirby et al., 1989).

It is evident based on these findings that ZS-2A is a potential antiplasmodial agent, justifying its folkloric usage as an antimalarial (Al-Sad, 1993), indicative of its potential as a chemotherapeutic antimalarial. The activity may be attributed to either the terpenes of traces of alkaloids detected in the parent chloroform extract. Previous antiplasmodial screenings of plant substances have implicated terpenes, alkaloids and flavonoids (Phillipson and Wright, 1990; Christensen and Kharazmi, 2001). In addition, our earlier studies have shown that crude aqueous extract of *Zizyphus spina-christi* root bark has central analgesic and sedative effects (Adzu and Haruna, 2007). Agents with such activity were reported to provide relief to malaria patients (Addae-Kyeremu et al., 2001).

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