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## The antsnake venom activities of *Parkia biglobosa* (Mimosaceae) stem bark extract

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### Abstract

Snake bites in rural Nigeria are commonly treated with plant extracts. We have studied the ability of one such traditionally used plant (*Parkia biglobosa*; [Jacq.] Benth., Mimosaceae) to reduce the effects of two snake venoms (*Naja nigricollis*, and *Echis ocellatus*) in several experimental models. A water-methanol extract of *P. biglobosa* stem bark significantly ( $p < 0.001$ ) protected the chick *biventer cervicis* (cbc) muscle preparation from *N. nigricollis* venom-induced inhibition of neurally evoked twitches when it was added to the bath 3–5 min before or after the venom. The extract also reduced the loss of responses to acetylcholine (Ach), carbachol and KCl, which are normally blocked by *N. nigricollis* venom, and significantly reduced the contractures of the preparation induced by venom. *P. biglobosa* extract (75, 150 and 300  $\mu\text{g/ml}$ ) significantly ( $p < 0.05$ ) protected C<sub>2</sub>C<sub>12</sub> murine muscle cells in culture against the cytotoxic effects of *N. nigricollis* and *E. ocellatus* venoms. The extract protected egg embryos exposed to lethal concentrations of *E. ocellatus* venom for more than 12 h and completely blocked the haemorrhagic activity of the venom at concentrations of 5 and 10  $\mu\text{g}/1.5 \mu\text{l}$ . *P. biglobosa* extract (400 mg/kg) did not protect mice injected i.p. with 5 and 2.5 mg/kg of *E. ocellatus* and *N. nigricollis* venoms, respectively. It, however, protected 40% of the mice from death caused by *E. ocellatus* venom after the extract and venom were pre-incubated for 30 min before injecting the mixture.

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### 1. Introduction

The use of plant remedies to treat snakebite victims in rural areas of Nigeria is a common practice. The natives who are predominantly rural farmers come in contact with snakes during their farming engagements. Due to high cost of hospital treatment and unavailability of antivenoms, most often the rural people find it more convenient to consult native doctors who are acclaimed for curing snakebite patients. Anecdotal evidence abounds to indicate that plant

remedies used by the native doctors are effective, and there appears to be a high rate of survival among snakebite patients treated in that way. There are claims of successful management of even cases at advanced clinical stages of venom toxicity.

Considering the high cost of conventional antivenoms and the significant percentage (8%) of patients who react adversely to them (Corrigan et al., 1978; Boyer et al., 2001), a systematic investigation of plant-based remedies for snakebite is justified. Plants used as remedy for snakebite abound in literature (Martz, 1992; Wang et al., 1997; Yang et al., 1998; Borges et al., 2000). However, many of the reported studies lack detailed scientific investigation, which is needed in the development of medicinal agents from plants (Martz, 1992; Talalay and Talalay, 2001). The present study investigates a local plant, *Parkia biglobosa*,

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used by traditional doctors in the south-east of Nigeria to treat snakebite patients. The hexane fraction of *P. biglobosa* showed analgesic and anti-inflammatory activities (Konadio et al., 2000), while an extract from the seed decreased arterial blood pressure in rats (Assane et al., 1993). It is used with other plants for the so called *cure salee* of cattle in the republic of Guinea; i.e. salted mixtures prepared from ground plant materials that are fed to cattle (Tringali et al., 2000). However, there have been no experimental studies on the reputed anti-venom effects. The present investigation used in vitro and in vivo methods to explore whether an extract of *P. biglobosa* could reduce the effects of venoms of typical West African snakes.

## 2. Material and methods

### 2.1. Preparation of plant material

The stem bark of *P. biglobosa* was collected with the assistance of a traditional healer. A botanist, Mr A. Ozioko, properly identified it as *P. biglobosa* (Jacq) Benth. (Mimosaceae) and a voucher sample was deposited in the herbarium of the Department of Botany, University of Nigeria Nsukka. One kilogram of the fresh bark was dried under mild sunshine. The dried sample was pulverized and stored in plastic bags.

### 2.2. Extraction of plant material

The pulverized stem bark of *P. biglobosa* was extracted with MeOH/H<sub>2</sub>O (1:1) by maceration for 72 h. The extract was detannified by passing it through polyvinylpyrrolidone (PVP) and lyophilised after the methanol content was removed by vacuum rotary evaporation.

### 2.3. Chick biventer cervicis (cbc) preparation

Preparations were obtained from 5 to 12 days old chicks following a standard method (Ginsborg and Warriner, 1960). The muscles were suspended in 3 ml baths containing modified Krebs solution [NaCl 118.4; KH<sub>2</sub>PO<sub>4</sub> 1.2; glucose 11.1; NaHCO<sub>3</sub> 25; CaCl<sub>2</sub> 2.5; MgSO<sub>4</sub> 1.4 and KCl 4.7 mM], bubbled with 95% O<sub>2</sub> + 5% CO<sub>2</sub> and maintained at 35 °C. Solutions of *P. biglobosa* extract in water were tested for activity on the cbc muscle preparation at various bath concentrations (50, 100, 200 and 300 µg/ml). The responses elicited by the extract or exogenously applied agonists (Ach 10<sup>-3</sup> M; carbachol 2 × 10<sup>-5</sup> M and KCl 4 × 10<sup>-2</sup> M) were recorded by a force displacement transducer (FT03) attached to a Grass Model 79 Polygraph. Muscle twitches were evoked by stimulating the motor nerve at 0.1 Hz with pulses of 0.2 ms duration and a voltage greater than that which produced a maximal response using ring electrodes and a Grass 88 B stimulator. The ability of

*P. biglobosa* extract to block post-synaptic or direct muscle effects of *Naja nigricollis* venom (10 µg/ml) was tested by introducing the extract 3–5 min after or before adding the venom to the bath.

### 2.4. Cytotoxicity

The effects of *P. biglobosa* extract and venoms (*N. nigricollis*, *E. ocellatus* and *B. arietans*) on cultured C<sub>2</sub>C<sub>12</sub> myoblast cells were investigated separately following the tetrazolium salt (MTT) assay method (Mossman, 1983). Cells were seeded in 96 well microtitre plates (10<sup>4</sup> cells per well in 100 µl medium) and were allowed to attach and reach log phase of growth (24 h). Various concentrations of *P. biglobosa* extract (75, 150 and 300 µg/ml) with or without venom (30 µg/ml of *N. nigricollis*, 30–45 µg/ml of *B. arietans* and 15 µg/ml of *E. ocellatus*) were added to each well in 100 µl medium. The plates were incubated with the cells at 37 °C for 18 h. Ten microliters of MTT (5 mg/ml) was added to each well and incubated for 4 h after which the medium was aspirated from the wells and 150 µl DMSO was added per well to solubilize the cells. The microplates were shaken for 2 min at 400 rpm on a microplate shaker. The optical density was measured at 570 nm using a Dynex MRX plate reader. The results were analysed using ANOVA and Student's *t*-test.

### 2.5. Antihemorrhagic test

Day-old fertile eggs obtained from a local hatchery were incubated till day 4 at 38 °C. The eggs were cracked on day 4 into Clingfilm hammocks following a standard method (Sells et al., 1997; Dunn and Boone, 1976) and incubated further till day 6. Discs of 2 mm diameter cut from filter paper (Whatman no. 2) were impregnated with a standard haemorrhagic dose (SHD) of *E. ocellatus* venom (3 µg/1.5 µl) alone or venom and various concentrations (2.5, 5.0, 7.5 and 10 µg/1.5 µl) of *P. biglobosa* extract. The discs were placed on the yolk sac membrane over a major bilateral vein and left for 3 h for haemorrhagic corona to form. The corona were measured with a ruler. Control experiments were performed with the buffered saline solution used to prepare the extract and venom solutions. Readings were taken in triplicate and analysed with Student's *t*-test. The minimum concentration required to abolish haemorrhage was recorded as the minimum effective neutralising dose (MEND).

### 2.6. Oral and intraperitoneal acute toxicity

Both oral and intraperitoneal (i.p.) acute toxicity tests were performed with *P. biglobosa* extracts in adult Wistar mice of both sexes (22–30 g). A total of 25 mice were grouped into five equal groups and dosed orally with increasing doses (250, 500, 750 and 1000 mg/kg) of *P. biglobosa* extract. The fifth group served as control and

received an equivalent volume of distilled water. The mice were watched for signs of acute toxicity and death over 24 h.

A similar experiment was performed using the i.p. route instead of the p.o. route. A total of 25 mice of both sexes distributed randomly into five groups were treated i.p. with increasing doses (200, 400, 600 and 700 mg/kg) of *P. biglobosa* extract. They were observed over 24 h for signs of acute toxicity and death. A control group was treated with an equivalent volume of distilled water following the same route.

### 2.7. Evaluation of *P. biglobosa* extract in vivo for antsnake venom activity

**Administration of extract 30 min prior to venom injection.** Twenty adult Wistar mice of both sexes (25–30 g) were divided into four equal groups of five mice each. Group 1 was injected with *N. nigricollis* venom (2.5 mg/kg, i.p.). Group 2 was injected with the same dose (2.5 mg/kg, i.p.) of *N. nigricollis* venom 30 min after *P. biglobosa* extract (400 mg/kg) was administered orally by gastric intubation. Group 3 was injected (i.p.) with *E. ocellatus* venom (5 mg/kg) while Group 4 was injected with the same dose (5 mg/kg, i.p.) of *E. ocellatus* venom 30 min after an oral intubation of the extract (400 mg/kg). All the mice were observed over the period of the experiment.

**Administration of both venom and extract after pre-incubation.** A similar experiment as described above was performed with the same number of mice per treatment group. Group 1 was injected with *N. nigricollis* venom (3.5 mg/kg i.p.). Group 2 was treated i.p. with a mixture of *N. nigricollis* venom and *P. biglobosa* extract (400 mg/kg) after both venom and extract were incubated in a test tube for 30 min. Group 3 was injected (i.p.) with *E. ocellatus* venom (i.p.) while Group 4 was injected (i.p.) with a mixture of both venom and extract after pre-incubation of both for 30 min in a test tube. The time it took for the mice to die was recorded and analysed using Student's *t*-test.

All the mice used for the experiments were subjected to minimal pain or distress. The regulations guiding the use of animals for experiments were strictly followed.

### 2.8. Source of venoms

The venoms were purchased from the Liverpool School of Tropical Medicine, Liverpool L3 5QA, UK.

## 3. Results

### 3.1. Chick biventer cervicis muscle (cbc) preparation

*P. biglobosa* extract when added alone at 100–300 µg/ml did not induce any effect on the cbc preparation. Preliminary experiments revealed that extract

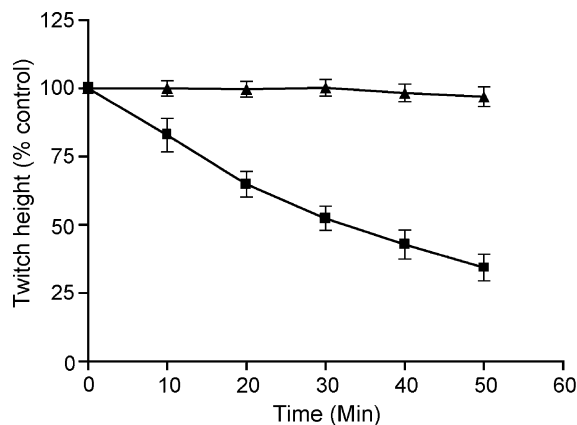


Fig. 1. The effect of *P. biglobosa* (Pb) extract on *Naja nigricollis* venom-induced changes in twitch height. Filled squares, venom (10 µg/ml) alone. Filled triangles, Pb (300 µg/ml) added 5 min before the venom (10 µg/ml). Points are means and bars are SEM ( $n = 4$ ).

at concentrations of 200 µg/ml and above reduced the toxic effects of *N. nigricollis* venom (data not shown). *P. biglobosa* extract (300 µg/ml) significantly ( $p < 0.001$ ) protected cbc preparations against the venom-induced block of neurally evoked twitches when the extract was added to the bath 5 min before the venom of *N. nigricollis* (10 µg/ml) (Fig. 1). It also significantly ( $p < 0.005$ ) blocked the muscle contracture induced by the venom (contractures were  $56 \pm 19\%$  of control twitch height with venom alone, but  $8 \pm 0.5\%$  when venom was added in the presence of extract). The responses to Ach, carbachol and KCl after exposure to *N. nigricollis* venom were maintained at 87, 82 and 72%, respectively of pre-venom controls (Fig. 2), whereas responses to agonists were abolished or reduced to less than 20% of control height following exposure to venom alone.

The addition of the extract 3 or 5 min after the venom resulted in a very significant ( $p < 0.001$ ) reduction of the inhibitory effect of the venom on muscle twitches (Fig. 3) and a significant ( $p < 0.001$ ) reduction of the characteristic contracture of the cbc muscle in response to the venom ( $20 \pm 4\%$  of control twitch height with venom followed by extract;  $40 \pm 9\%$  with venom alone). The responses to Ach, carbachol and KCl were maintained at values (85, 90 and 72%, respectively) similar to those obtained when the extract was added 5 min before the venom.

Lower concentrations (100 and 200 µg/ml) of *P. biglobosa* extract also significantly protected the cbc preparations from the toxic effects of *N. nigricollis* venom, though to a lesser extent than at 300 µg/ml.

Twitches of the cbc preparations exposed to venom and *P. biglobosa* extract were restored to control heights about 10 min after washing with Krebs solution. The effects of venom alone were not reversed by washing, even when washing was started 3 min after the addition of venom.

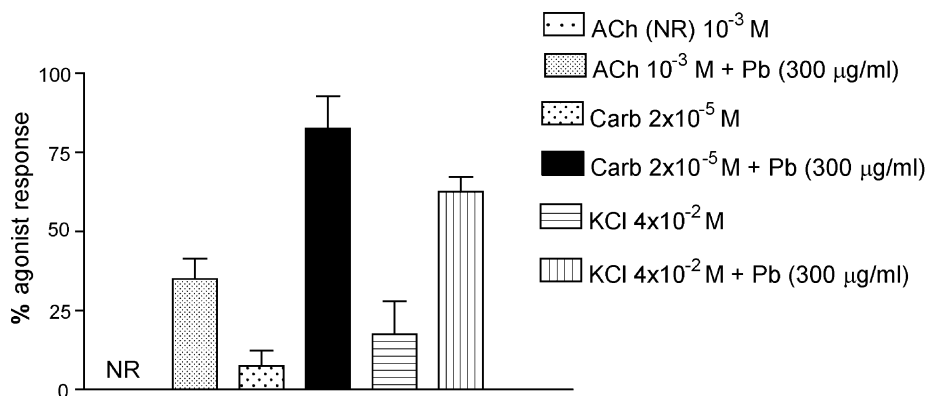


Fig. 2. The effect of *P. biglobosa* (Pb) extract on post-synaptic block induced by *Naja nigricollis* venom (10 µg/ml) venom. From the same preparations as in Fig. 1. Bars represent SEMs (n = 4). NR, no response.

### 3.2. Cytotoxicity test

*P. biglobosa* extract alone had no effect on the viability of  $C_2C_{12}$  muscle cells, but it significantly ( $p < 0.05$ ) protected muscle cells against the toxic effects of *N. nigricollis* venom (30 µg/ml) at all concentrations of the extract tested (75, 150 and 300 µg/ml) (Table 1). The maximum (57%) protective effect of the extract was exhibited with extract at 75 µg/ml (Fig. 4). The extract significantly ( $p < 0.001$ ) inhibited the cytotoxic effects of *E. ocellatus* venom only at 300 µg/ml (Table 1).

### 3.3. Antihemorrhagic test

*P. biglobosa* completely (100%) blocked the haemorrhagic activity of *E. ocellatus* at 5 µg/1.5 µl (3.3 mg/ml) of extract (Table 2). The MEND is 5 µg/1.5 µl.

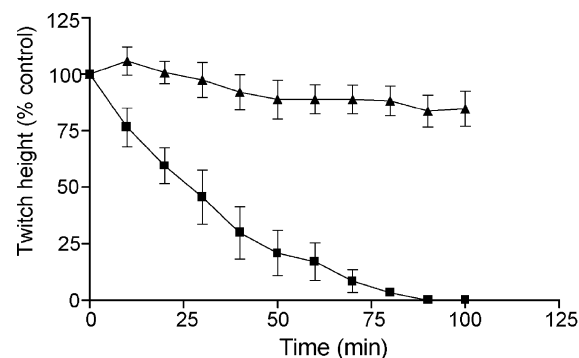


Fig. 3. The effect of *P. biglobosa* (Pb) extract on *Naja nigricollis* venom-induced changes in twitch height. Filled squares, venom (10 µg/ml) alone. Filled triangles, venom (10 µg/ml) added 3 min before addition of Pb (300 µg/ml). Points are means and bars are SEM (n = 4).

### 3.4. In vivo effects

**Acute toxicity.** Mice dosed orally with *P. biglobosa* extract were initially dull with significantly reduced movement for 20–30 min. No death was recorded even at the highest dose (1000 mg/kg) tested. After i.p. administration, however, some toxicity was observed: the LD<sub>50</sub> was estimated to be 457 mg/kg.

**Evaluation of *P. biglobosa* extract in vivo for antisnake venom activity.** All the mice treated with venoms alone (*N. nigricollis* 2.5 mg/kg and *E. ocellatus* 5 mg/kg) and venom with the plant extract (400 mg/kg) died. There was no significant difference between the time of death in the treated and control groups.

**Administration of venom and extract after pre-incubation.** None of the mice treated with *N. nigricollis* and *E. ocellatus* venoms alone survived. Although all mice treated with a mixture of *N. nigricollis* venom and the extract after pre-incubation died, the time of death was

Table 1

Effects of *P. biglobosa* extract against the cytotoxic actions of *E. ocellatus* venom on  $C_2C_{12}$  muscle cells

Treatment	Percentage cell survival ± SD	N	p Value <sup>a</sup>
Control cells (no treatment)	100.0 ± 21.8	6	
<i>Echis</i> (15 µg/ml) alone	27.6 ± 5.7	6	
Pb (75 µg/ml) + <i>Echis</i> (15 µg/ml)	39.4 ± 23.6	6	0.26
Pb (150 µg/ml) + <i>Echis</i> (15 µg/ml)	46.4 ± 29.3	6	0.15
Pb (300 µg/ml) + <i>Echis</i> (15 µg/ml)	68.0 ± 10.8	6	<0.0001
Pb (300 µg/ml) alone	100.0 ± 15.6	6	

Pb, extract of *P. biglobosa*.

<sup>a</sup> Tested against venom alone.

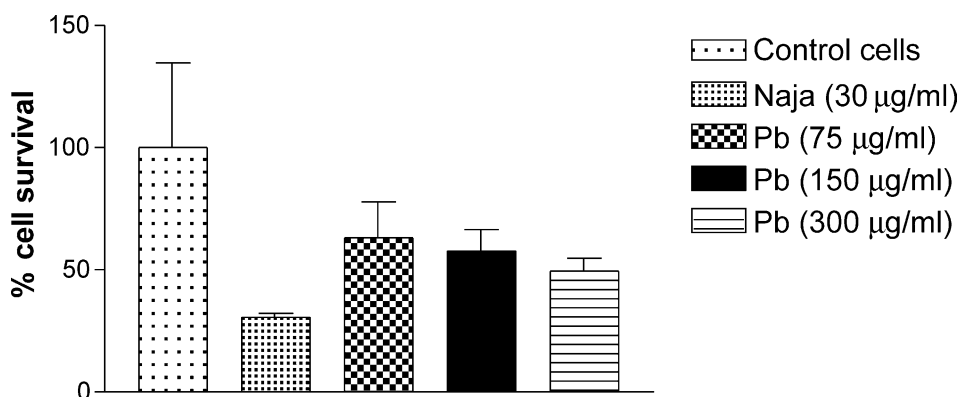


Fig. 4. The effects of *P. biglobosa* (Pb) extract (75, 150 and 300 µg/ml) on C<sub>2</sub>C<sub>12</sub> cells incubated with *N. nigricollis* venom (30 µg/ml). Bars are SD ( $N = 6$ ). Incubation time = 18 h.

significantly ( $p < 0.05$ ) increased from 0.95 h in the control to 5.47 h in the group treated with the mixture of extract and venom. Forty percent of mice treated with a mixture of *E. ocellatus* venom and the extract survived. The time of death for the other 60% was significantly ( $p < 0.05$ ) increased from 0.79 to 5.28 h.

#### 4. Discussion

*P. biglobosa* extract significantly blocked many of the toxic effects of *N. nigricollis*, and *E. ocellatus* venoms in vitro. These were exemplified by significant inhibitions of both the neurotoxic and myotoxic effects of *N. nigricollis* venom on chick *biventer cervicis* muscle preparations. Remarkably, the extract could block the neurotoxic activities of *N. nigricollis* venom on *cbc* muscle preparation whether the former was added three minutes before or 3–5 min after the venom when the venom's effects on the preparation are normally irreversible by washout of the venom. This observation is significant in two respects. The first is the possible clinical relevance of this action, since snakebite victims obviously present for treatment after a bite. The second is the possibility that the extract acts through a mechanistic intervention rather than a direct physical interaction with the venom in vitro, which is the likely mode of action of many polyphenolic compounds found in plant extracts, and which probably explains many of the 'protective' effects of plant extracts when they are

preincubated with venom before administration to the biological assay. Polyphenols (epi-gallocatechin, epi-catechin etc) and a long-chain ester of trans-ferulic acid have been isolated from the ethanol bark extract of *P. biglobosa* (Tringali et al., 2000), but the extract used in the present study was pretreated in an attempt to remove polyphenolic tannins (however, the complete absence of tannins, and particularly low molecular weight polyphenolics, could not be verified). Although the component of the plant extract responsible for the antivenom activity observed in the present study has not yet been identified, it is unlikely that the anti-venom activity is due to polyphenolic components.

Another positive attribute of the extract is its antihæmorrhagic activity against *E. ocellatus* venom. A MEND value as small as 5 µg/1.5 µl shows that the extract is very effective against the effect of *E. ocellatus* venom on the circulatory system.

Despite these protective effects of the bark extract of *P. biglobosa* in vitro, the results from the in vivo experiments in mice were less encouraging. The extract did not protect mice challenged with lethal doses of the two venoms when extract and venoms were administered independently. However, it significantly increased the survival time of the mice treated with a mixture of the extract and *N. nigricollis* venom after 30 min of preincubation. In addition, 40% of the mice treated with a mixture of *E. ocellatus* venom and the extract of *P. biglobosa* survived. Possibly the plant extract could be effective against snake venom activity in vivo if

Table 2

Effects of *P. biglobosa* extract on the hæmorrhage induced by *E. ocellatus* venom in the egg embryo

Extract/venom	Concentration of extract (µg/1.5 µl)	Hæmorrhagic zone (mm)	Reduction from control (%)	MEND (µg/1.5 µl)	State of embryo
<i>Echis</i> (3 µg/1.5 µl)	–	3.5	–		All died
<i>P. biglobosa</i> + <i>Echis</i> (3 µg/1.5 µl)	2.5	0.5	85.7		All alive
<i>P. biglobosa</i> + <i>Echis</i> (3 µg/1.5 µl)	5.0	0.0	100.0	5	All alive
<i>P. biglobosa</i> + <i>Echis</i> (3 µg/1.5 µl)	10.0	0.0	100.0		All alive

the experimental model is modified to simulate actual life experience. Two possible considerations are suggested for work in order to obtain more positive results. The dose of venom should be adjusted to achieve 75% instead of 100% mortality in the control mice to ensure that they are not challenged with very high dose of venom, beyond the dose any snake can inject into its victim. A very high dose will not give enough time for the extract to induce its effect. The other suggestion is to give repeated doses of the extract at various time intervals, which approximates to what obtains when humans are treated for snakebite. Treatment is normally continued until clinical signs of envenomation disappear (Vijeth et al., 2000).

In conclusion, the methanol extract of *P. biglobosa* has shown significant protection against the neurotoxic, haemotoxic and cytotoxic effects of venoms of poisonous snakes found in Nigeria. The use of the plant in ethnomedicine to treat snakebite appears to have a pharmacological basis.

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