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Effect of *Annona senegalensis* rootbark extracts on *Naja nigricotlis nigricotlis* venom in rats

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Abstract

Annona senegalensis Pers (family: Annonaceae) is used traditionally in Nigeria to treat victims of snakebite. The potency of the methanol extract of the root bark of the plant was tested against cobra (*Naja nigricotlis nigricotlis* Wetch) venom in rats. The extract was also tested on brine shrimp (*Artemia saline* Leach). The activity of the extract against the venom induced mortality, occurrence of toxic signs, activity on liver enzymes as well as its ability to reverse experimentally induced increase in body temperature were evaluated. Results indicated that the extract caused reduction in the induced hyperthermia and directly detoxified the snake venom used by 16–33%. It, however, failed to restore the biochemical functions (sGOT and sGPT) of the liver. The extract exhibited an LC₅₀ of 232.7 µg/ml in the brine shrimp test.

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Keywords: *Annona senegalensis*; *Naja nigricotlis nigricotlis*; Antisnake venom; Brine shrimp

1. Introduction

In Africa, majority of the rural population use traditional medicines for their healthcare needs. This usage is attributed to its relatively easy access and affordability to the people who live mostly in poor rural communities. Such traditional medicines often contain herbs and other plant materials whose efficacy and safety have not been determined. Interest in evaluating snake venom antidotes from plant sources has increased recently due to certain drawbacks in the applications of the current remedies that consist almost entirely of serum therapy, coupled with poor availability, storage and allergic reaction problems (Pereira et al., 1995). Some of these plants have been reputed to be active and have been used against snake venom (Rizzini et al., 1988). Their ac-

tive components bind to the venom proteins, in the process detoxifying the venom (Otero et al., 2000). These plants include: *Diospyros kaki* (Okonogi et al., 1979), *Hemidesmus indicus* (Alam et al., 1994), *Eclipta prostrata* (Melo et al., 1994; Pithayanuku et al., 2004), *Brownea rosademonte* (Otero et al., 2000), *Bauhinia cumansensis*, *Cecropia peltata*, *Aristolochia rugosa*, *Pithocellobium unguis-cati*, *Cola nitida*, *Renealmia alpine* (Lans et al., 2001), *Alocasia cucullata* (Wang, 1986), *Cissus assamica*, *Aristolochia fordiana* (Wang et al., 1997), *Marsypianthes chamaedrys* (Castro et al., 2003), *Guiera senegalensis* (Abubakar et al., 2000) and *Harpalyce brasiliana* (Silva et al., 1997).

Annona senegalensis Pers (Annonaceae), which grows wild in tropical Africa, is the plant of interest in this study. It has aromatic flowers, which are used to flavour food. The fruit, yellow in colour when ripe, has a pleasant smell with sweet edible jelly. The plant is reputed to be of great medicinal value and is used in native medicine (Dalziel, 1937) for

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chest pain, coughs, anaemia, urinary tract infection (Burkill, 1985; Muanza et al., 1994), cancer treatment (Durodola, 1975; Graham et al., 2000), diarrhoea, dysentery (Muanza et al., 1994; Ekpendu et al., 1998; Kudi and Myint, 1999), antritis and rheumatism (Audu, 1989). Among its several other documented uses are: intestinal and guinea worms (Watt and Breyer-Brandwick, 1962; Alawa et al., 2003), venereal diseases (Durodola, 1975; Bhat et al., 1990; Tabuti et al., 2003), head and body ache (Arnold and Gulumian, 1984; Chhabra et al., 1987), against leishmaniasis (Akendengue et al., 1999) and typanosomiasis (Atawodi et al., 2003), lice treatment (Hirschmann and Rojas De Arias, 1990), eyelid swelling (Klaus and Adala, 1994), bloody stool (Hedberg et al., 1983), and treatment of snakebites (Durodola, 1975; Kela, 1990; Selvanayahgam et al., 1994). *Annona senegalensis* is widely used in both western and northern Nigeria. Despite the widespread use of this plant, little is known of the scientific basis for its use in treating victims of snakebites.

Earlier scientific investigation of the plant has revealed its activity against malaria (Balansard and Timon-David, 1985). The plant has also been shown to be effective as an antiulcer/antacid, smooth muscle relaxant (Langason et al., 1994), antibacterial (Muanza et al., 1994), antiprotozoan (Igweii and Onabanjo, 1989), molluscicidal (Sofowora and Adewunmi, 1980), antitumor agent (Fatope et al., 1993; Sahpaz et al., 1994) and hormonal activities (Jacobson et al., 1975). More recently, our own studies on the plant (Adzu et al., 2003a) revealed that the methanol extract of the root bark of this plant has analgesic and anti-inflammatory activities that might be exerted through peripheral mechanisms and phytochemical tests indicating the presence of saponins, tannins and resins. The plant has been reported to contain in addition, wax, alkaloids (Philipov et al., 1995; You et al., 1995), proteins, amino acids, antraquinones (Bamba et al., 1984; Burkill, 1985; Ekpendu et al., 1998), sterols, glycosides, flavonal terpenoids (Mackie and Misra, 1956; Mackie and Ghatce, 1958; Adesogan and Durodola, 1976; Fatope et al., 1996; Sahpaz et al., 1996) and terpenes (Ekundayo and Oguntimein, 1986).

The traditional healers often administer the medicinal preparation by pounding the fresh roots into a paste, and applying the mashed product over incisions made at the point of bite holding it into place with a bandage. Some herbalists prefer to administer the prepared extracts orally. We, therefore, investigated activities of the methanol extract of the root bark against cobra (*Naja nigricollis nigricollis* Wetch) venom. We decided on cobra venom because it (cobra) was reported to be the major cause of snakebite injury in Nigeria (Houghton and Harvey, 1989). The detoxifying effects of the extract were investigated and activities against liver enzymes assessed by looking at the serum glutamate-oxalate-transaminase (sGOT) and serum-pyruvate-transaminase (sGPT) both of which indicate the condition of liver function (Hsu et al., 1998). The ability of the extract to reverse yeast-induced pyrexia as well as the toxic activity (cytotoxic) on zoologic system was also tested using brine shrimp lethality test.

2. Materials and methods

2.1. Plant sample

Fresh roots of *Annona senegalensis* were collected from Midlu, Adamawa State in April 2003. The plant material was authenticated at the Department of Medicinal Plant Research and Traditional Medicine, National Institute for Pharmaceutical Research and Development (NIPRD), Abuja, Nigeria. A voucher specimen (no. 5481) was deposited at the herbarium of the institute for future reference.

The root bark was removed, cleaned and air-dried. The dried material (500 g) was added to 1 l of methanol (M&B, England) in a flask followed by occasional shaking using flask shaker (GFL 3017, Germany) and extracted using soxhlet extractor (Quickeit, England) for 24 h. Thereafter, the solvent was evaporated under reduced pressure using rotary evaporator. This gave a yield of 26.4 g (5.28%, w/w).

2.2. Venom sample

The venom sample was obtained by M.S. Abubakar using the milking method (Markfarlane, 1967) from locally caught cobra (*Naja nigricollis nigricollis* Wetch), kept at the Herpetarium Unit, Department of Pharmacognosy and Drug Development, Ahmadu Bello University, Zaria, Nigeria. The pooled venom was lyophilized and stored. The LD₉₉ of the venom was established to be 9.55 mg/kg, i.p., in mice (Abubakar et al., 2000).

2.3. Animals

Adult Wistar rats (*Mou norvegicus albinus*) obtained from the Animal Facility Centre, NIPRD, were used for the study. They were kept in plastic cages with sawdust as bedding under conditions of 12:12 h light and dark cycle and fed with standard diet. Equal numbers of male and female rats were used in each experimental group, keeping their mean weight as near as possible. Their usage was according to the standard protocol of 'principles of laboratory animals' care' NIH publication NO. 85-23 revised (1978).

2.4. Brine shrimp lethality test

The brine shrimp lethality test was used to test the activity of the extract and to estimate its toxicity against zoologic systems (Meyer et al., 1982). The test was performed according to the method described by Mackean et al. (2000) but slightly modified to suit our local laboratory settings. Briefly, 50 mg of brine shrimp (*Artemia saline* Leach) eggs (HOBBY®, Germany) were sprinkled into a 50 ml beaker containing natural seawater (collected at Bar Beach, Victoria Island, Lagos, Nigeria) and placed in a secure place for 48 h to hatch. The phototropic nauplii of the hatched shrimps were harvested with plastic pipette by covering three-fourths of the beaker with black carbon paper, as they move towards a torchlight

directed at the uncovered portion. Stock of the extract (10 mg/ml) was prepared using doubly distilled water. From this stock, 1000, 500, 250 and 125 µg/ml of the extract were prepared and 1 ml of each preparation transferred into a 1 ml, 8 × 12 vial (Falcon) in triplicate. Ten–fifteen shrimp nauplii were added to each vial. The number of survivors against total over 24 h was recorded and LC₅₀ calculated using Finner (1971) method-based computer program.

2.5. Animal experiments

2.5.1. Inhibition of lethality

A total of 24 rats (180–260 g) separated into four groups of six (three male and three female) each were used. Group 1 was treated with saline (10 ml/kg, i.p.) while groups 2–4 received graded doses of the extract (50, 100 and 200 mg/kg, i.p., respectively). The animals were then injected 0.2 ml of the reconstituted venom (10 mg/ml), prepared in physiological saline through the tail vein (Theakston and Reid, 1983) 30 min after administration of the extracts. The animals were observed for signs of toxicity and mortality within 24 h was recorded.

2.5.2. In vitro detoxification effect

The test was performed using the method earlier described by Abubakar et al. (2000) and Otero et al. (2000). Briefly, the reconstituted venom was incubated separately with various concentrations of the extract at 37 °C for 10 min in such a way that the separate concentrations of the extract contained an equivalent of 10 mg/ml of the venom. The mixtures were then administered to the groups as follows: group 1 received only the venom (0.2 ml; 10 mg/ml), while groups 2–4 received 10 mg/ml venom and equivalent of 50, 100 and 200 mg/kg of the extracts, respectively. Onset and signs of toxicity after the administration of mixture were noted and recorded. The toxic signs were recorded as descriptive scores, using the following predetermined scoring indices: (3) for severe dyspnea and/or coma, and includes all spontaneous neurological abnormalities (e.g. convulsion and tremors); (2) for respiratory or movement impairment; (1) for only mild difficulty in breathing or movement, and (0) for the absence of any sign. Mean score of each group were taken and activity expressed as percentage inhibition of the toxic signs.

2.5.3. Suppression of damage to enzyme function

Twenty-four rats grouped into four ($n=6$) were used. One group was treated with saline (10 ml/kg, i.p.), which served as the control group. The remaining groups were administered the extract (50, 100 and 200 mg/kg i.p., respectively). All the groups received the venom (0.2 ml; 2 mg/ml) 24 h later. The animals were anaesthetized with chloroform 6 h after the venom injection, and blood immediately collected from the neck vein using a syringe was carefully transferred into a centrifuge tube and allowed to clot. The clotted blood was then centrifuged (Beckman CS-15, Germany) at 2500 rpm for 5 min to obtain the serum (Hsu

et al., 1998), which was kept frozen until analysis. The serum glutamate-oxalate-transaminase (sGOT) and serum-pyruvate-transaminase (sGPT) values were estimated using Randox kits (Randox Lab Ltd., UK) and measured with a spectrophotometer (Spectrum Lab 21 A Lensguana Tech, China).

2.5.4. Effect on body temperature

This test was performed using the yeast-induced-pyrexia procedure (Al-Ghamdi, 2001) as previously adopted (Adzu et al., 2002). In the procedure, the initial rectal temperatures of the rats were taken using clinical thermometer (Hartmann, Germany). They were injected with 10 ml/kg s.c. of 15% aqueous solution of yeast (Vahine professionnel, France). The temperature readings were repeated 24 h later, and animals that did not show a minimum increase of 0.5 °C in temperature were discarded. Selected animals were grouped into four groups of six rats each and treated with either saline or extract (50, 100 and 200 mg/ml i.p., respectively) and temperature readings taking at predetermined time intervals of 0.5, 1, 1.5, 2 and 6 h. Activity was assessed based on the ability of the extract to reverse the induced pyrexia (Adzu et al., 2003b).

2.6. Data analysis

The results of the study were expressed as mean ± standard error of mean (S.E.M.). Analysis of variance (ANOVA) was used to analyze the results followed by Dunnett's test for multiple comparisons.

3. Results

3.1. Brine shrimp test

The LC₅₀ of the extract was established to be 232.7 µg/ml.

3.2. Inhibition of lethality

The extract showed an improvement in the survival of rats in the test group dose dependently but did not totally abolish mortality. Whereas all the animals in the saline control group died, five, four and three died in the extract-treated groups (50, 100, and 200 mg/kg i.p., respectively).

3.3. In vitro detoxification

The extract inhibited the toxic signs induced by the venom dose dependently (Table 1). The onset of toxic signs was prolonged while the severity was reduced compared to saline group.

3.4. Inhibition of damage to liver enzyme function

The extract failed to restore the liver function to normal as measured by the sGOT and sGPT values. Rather, the extract

Table 1

In vitro detoxifying effects of the rootbark extract of *Annona senegalensis* against snake venom (10 mg/kg) in rats

Treatment	Doses (mg/kg, i.p.)	Mean onset time of toxic signs (min)	Mean toxicity score	Percentage inhibition
Saline (10 ml/kg, i.p.)	–	5.5 ± 0.6	3.0 + 0	0
Extract	50	7.4 ± 0.5	2.5 + 0.35	16.67
	100	18.6 ± 2.9	2.17 + 0.31	17.70
	200	28.1 ± 3.6	2.0 + 0.37	33.33

Table 2

Effects of *Annona senegalensis* rootbark extracts on the liver function of venomized rats

Treatment	Doses (mg/kg, i.p.)	Values (µg/l) ^a	
		sGOT	sGPT
Saline (10 ml/kg, i.p.)	–	38.5 ± 2.51	25.5 ± 8.53
Extract	50	84.67 ± 4.34	40.00 ± 6.01
	100	86.33 ± 2.67	43.00 ± 7.10
	200	95.00 ± 3.00	70.67 ± 7.54

^a Standard values are 12 µg/l.

Table 3

Effect of *Annona senegalensis* rootbark extracts against yeast induced pyrexia in rats

Treatment	Doses (mg/kg, i.p.)	Time (h)					
		–24	0	0.5	1	1.5	2
Saline (10 ml/kg)	–	37.11 ± 0.08	37.64 ± 0.02	37.83 ± 0.13	37.99 ± 0.08	38.00 ± 0.11	37.65 ± 0.17
Extract	50	37.05 ± 0.08	37.74 ± 0.04	37.84 ± 0.02	38.02 ± 0.06	38.06 ± 0.04	38.09 ± 0.04
	100	37.03 ± 0.11	37.55 ± 0.15	37.34 ± 0.14*	37.34 ± 0.25*	37.35 ± 0.14*	37.61 ± 0.09*
	200	37.14 ± 0.11	37.65 ± 0.26	37.55 ± 0.23*	37.44 ± 0.21*	37.43 ± 0.21*	37.22 ± 0.16*

Values in parenthesis are the rectal temperature ± S.E.M.

* Significant difference, F (3, 23) = 3.27; *p* < 0.05.

seemed to potentiate the activity levels of sGOT and sGPT further above the venom-induced levels, thereby worsening the situation (Table 2).

3.5. Effect on body temperature

Result (Table 3) showed that the extract exhibited significant (*p* < 0.05) activity in the group that received 100 and 200 mg/kg, i.p. of the extract when compared to saline control group.

4. Discussion

This study was carried out to establish the scientific basis for the traditional application of *Annona senegalensis* in the treatment of victims of snakebite among the indigenous people of Nigeria (Durodola, 1975; Kela, 1990). The methanol extract of the plant contains an active compound that can be subjected to bioassays as shown by the brine shrimp test. The test is used in screening agents that can be toxic (cytotoxic) to zoologic systems and to predict potential biological activity in unknown pharmaceutical samples. For instance, agents that elicit LC₅₀ ≤ 30 µg/ml are considered to have significant

bioactive component (Meyer et al., 1982) while LC₅₀ of about 650 µg/ml towards brine shrimp nauplii was considered to be only mildly toxic (Mackean et al., 2000). The results of the extract of *Annona senegalensis* (LC₅₀ = 232.7 µg/ml) against the nauplii is, therefore, indicative of the presence of components from which potentially active and relatively safe phytopharmaceutical may be developed (Anderson et al., 1991; McLaughlin et al., 1991).

The methanol extract of the plant reduced the mortality and significantly inhibited the onset, and severity of neurotoxic signs induced by the venom. Since the traditional healers sometimes administered these agents by making it into paste and tying them over incisions made at the point of bites, it is possible that the toxic enzymes might be neutralized through the process. Some plant constituents have the ability to bind venom proteins (Otero et al., 2000). Toxicological properties of snakebite are thought to be associated with enzymes (Stocker, 1990) especially phospholipase A₂ (PLA₂), which is believed to be its most toxic component (Mahanta and Mukherjee, 2001) implicated in haemorrhage (Melo and Ownby, 1999). The effects of the extract on the venom might thus be linked to its activity against the toxic enzymes (Haslam, 1989). Mahanta and Mukherjee (2001) postulated that neutralization of these enzymes might lead

to inhibition of lethality of venom at the site of application. The results of the in vitro detoxification test suggest that the extract might act by neutralising the activity of the venom at the site of bite, thereby reducing the severity of the toxic effects.

The sudden change associated with venom poisoning may be linked to breakdown of biochemical functions of the liver. Results from our study, however, did not indicate this, since the activities of the sGOT and sGPT were rather enhanced. Opinion here is that the liver may be involved directly but perhaps on a cumulative basis. Another possibility may be a symptomatic or physiological antagonism by the extract in which the toxic features, such as convulsion which lead to eventual suffocation and death could be attenuated, thereby reducing the fatality of the venom—a symptomatic relief. Watt (1967) as well as Eshiet and associates (1971) has provided evidence for the anticonvulsant activity of this plant. It is, therefore, possible that the anticonvulsant properties of the plant extract could have attributed to the observed effects against the venom.

Some traditional healers have observed resolution of fever among some of their patients who were placed on *Annona senegalensis*. The extract exhibited antipyretic effects by antagonising the effects of yeast on the rat temperature. Yeast induces pyrexia by increasing synthesis of prostaglandin and is used to screen agents with antipyretic effects (Al-Ghamdi, 2001). The resolution of fever by the extract may be related to its mechanism, thereby contributing to its usefulness because fever and hyperthermia are often associated with snakebites.

These results as well as our previous findings, especially the anti-nociceptive and anti-inflammatory effects (Adzu et al., 2003a) of *Annona senegalensis* might explain the basis for the success claimed by traditional healers in the use of the plant to treat victims of snakebite. Some compounds from plants used for inflammation were known to inhibit enzymes from snake and scorpion venoms (Houghton and Osibogan, 1993; Hutt and Houghton, 1998) by complexing with the venom constituents, thereby rendering them unable to act on receptors (Hutt and Houghton, 1998). Although the mechanism by which the extract achieves its effect remains unclear, a plausible speculation at this point is likelihood of the existence of an active component, which might be responsible for the observed effects. The various classes of compounds identified in the phytochemical study of the extract (Adzu et al., 2003a) could be studied closely. Akunyili and Akubue (1986) had earlier related antivenom activity to alkaloidal glycosides they traced in the plant they studied. Tannins are also known to unspecifically inactivate proteins (Okonogi et al., 1979; Abubakar et al., 2000). The activity of the extract may, therefore, be linked to its tannin contents. Furthermore, phytochemical constituents of plants used for snakebites tend to have similar (isoflavone skeleton, acidic nature and deoxygenated) functions (Reyes-Chilpa et al., 1994).

In conclusion, our study on the methanol extract of *Annona senegalensis* has demonstrated some useful activities that support its traditional use against snakebite.

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