Protective Effect of *Acanthus Montanus* in Carrageenan-Induced Models of Local Inflammation: Inhibitory Effect on Nitric Oxide (NO) Production

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Summary

Acanthus montanus (Nees) T. Anderson is a plant widely used in folk medicine in Central Africa for its antiinflammatory, antirheumatic, antiulcer, digestive and vasoprotective properties. In the present study, anti-inflammatory activity of the aqueous extract of *Acanthus montanus* was compared with that of diclofenac, a non-steroidal anti-inflammatory drug and L-Nitro arginine methyl ester (L-NAME), a nitric oxide inhibitor, using carrageenan-induced paw oedema in mice. The extract at the doses ranging from 100 to 400 mg/kg p.o, diclofenac (50 mg/kg, p.o.) and the NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; 100 mg/kg, s.c.) significantly inhibited the carrageenan-induced mouse paw. The highest anti-inflammatory effects of the extract (76.00%) and L-NAME (85.00%) were significantly antagonised by L-Arginine, a precursor of nitric oxide, at the dose of 300 mg/kg. L-Arginine has no effect on Diclofenac anti-inflammatory activity. These results suggest that, anti-inflammatory activities of *Acanthus montanus* aqueous extract could be due to nitric oxide (NO) inhibition. These findings give support to the ethnopharmacological use of the plant in the treatment of several inflammatory ailments.

Key words: *Acanthus montanus*; anti-inflammatory; paw oedema; nitric oxide inhibitor.

Introduction

Stretching from the forest of Benin in West Africa to Congo Basin and Angola in central Africa is *Acanthus montanus* (Nees) T. Anderson (Acanthaceae), one of the 50 species of Acanthus found in family Acanthaceae. In Nigeria, the leaves are used for cough, rheumatism, hypertension, skin infection, boil and witches [1] in Cameroon, it is use for cough, dysmenorrhoea, pain, epilepsy [2] and miscarriages, while in Gabon, it is used for cough, heart troubles, rheumatic pain and syphilis [3, 4] (Burkil, 1985: Adjanohoun et al, 1996).

Our laboratory previous studies showed that *Acanthus montanus* inhibited the uterine contraction induced histamine, serotonin and prostaglandin [5]. This plant is devoid of toxic effects in rats at the doses often used by the population, but its $MeOH/CH_2Cl_2$ extract is embryotoxic peri-natally at high doses [6, 7]. Aqueous extract of *Acanthus montanus* also possesses analgesic and anti-inflammatory properties through inhibition of the prostaglandins partway [8]. Nitric oxide (NO) is associated with inflammatory reaction and is produced by inducible nitric oxide synthase (iNOS) in certain cells activated by various proinflammotory agents such as lipopolysaccharide (LPS), tumor necrosis factor (TNF), interleukin-1 (IL-1) and interferon- g (IFN- g). In both in vitro and in vivo studies, NO activates COX-1 and COX-2, resulting in an enhanced production of Prostaglandins. It also reacts with $O2^-$ to form the cytotoxic radical peroxynitrite [9]. Thus, effective inhibition of NO accumulation represents a beneficial therapeutic strategy [10]. In the present study, we evaluated the anti-inflammatory activity of *Acanthus montanus* and its mechanism from the viewpoint of the NO system.

Methods

Plant material

Acanthus montanus (Nees) T. Anderson (Acanthaceae) plants were collected in Etoa area of Yaounde (Centre province, Cameroon) in Juin 2007. Botanical identification was performed at the National Herbarium, Yaounde, Cameroon where a voucher specimen was deposited under the number 1652SRF- 61CAM. 62

Extract preparation

The aqueous extract was prepared by maceration of 100 g of sun dried pulverised leaves of the plant in 1 L of boiled distilled water for 24 h. The extract was later filtered, and the solvent eliminated by concentration in a rotor evaporator and dried in an oven at 50°C to give 9.6 g (9.6 %) of aqueous extract.

Experimental animals

Adult males and females albino mice (23-26 g) were used for the study. They were raised in the animal house of the Faculty of Science where kept under constant conditions: light/dark cycle and a room temperature of 28±3 ◦C. The animals were fasted for 16h but allowed water *at libitum*. Authorization for the use of laboratory animals in this study was obtained from the Cameroon National Ethical Committee (Reg. no. FWA-IRB00001954).

Carrageenan-induced Mouse paws oedema

The method described by Koichi Tan-No et al.[11] was used. Following the overnight fast, 100, 200 and 400mg/kg of the aqueous extract of *Acanthus montanus* or 100 mg/kg of L-NAME were orally administered to the animals in different groups, using a cannula. At the same time, animals in the positive control groups received 50 mg/kg if diclofenac, orally while animals in the control group received saline, at the dose of 10 ml/kg. 30 minutes later, oedema was induced on the right hind paw of each mouse by intraplantar injection of 30µl of carrageenan (2 w/v % in physiological saline). Paw volume was measured before and at hourly intervals for 6 h after carrageenan injection, using a plethysmometer (Ugo Basile 7010). The paw oedema among the different group of animals was compared, the percentage inhibition of paw oedema was determined using the same methods as [12].

$$Pi\ (\%) = \frac{\left(\overline{Vt} - \overline{Vo}\right)c - \left(\overline{Vt} - \overline{Vo}\right)E}{\left(\overline{Vt} - \overline{Vo}\right)c} X\ 100$$

Vt : right hind paw volume at time t. C: control group. E: experimental group
Vo: right hind paw volume before sub-plantar injection of carrageenan

Effects of L-Arginine on the anti-inflammatory actions of *Acanthus montanus*, diclofenac and L-NAME

In order to determine the participation of nitric oxide system in the anti-inflammatory action of *Acanthus montanus* aqueous extract, diclofenac and L-NAME, the effect of L-arginine on the anti-inflammatory actions of theses compounds were examined. L-arginie was intraperitoneally administered 2h before the peak time of the anti-inflammatory effects. Namely, L-arginine was administered to animals treated with aqueous extract, diclofenac and L-NAME mice at 2 h, 3 h and 2 h respectively after carrageenan injection.

Statistical analysis

One way ANOVA followed by Dunnets multiple comparison tests were performed. Results are expressed as mean ± SEM. The differences between groups were considered significant when $P < 0.05$. The statistical package used for these analyses was Graphpad Instat.

Results

Anti-inflammatory effects of aqueous extract of *Acanthus montanus*, diclofenac and L-NAME

Intra-dermal injection of carrageenan into one of the hind paw of normal rats (control group treated orally with saline) caused a local inflammatory response, which reached a maximum intensity of oedema (87.72%) at hour 5 after application of the phlogistic agent (Table 1). The aqueous extract of *Acanthus montanus* at a dose of 100 mg/kg, did not alter the development of oedema of the foot compared to the control group. However, at a dose of 200 mg/kg it caused a statistically significant inhibition in the intensity of response, at hours 0.5, 1, 2, 3 and 4 after injection of the carrageenan (Table 1).
It was observed that L-NAME at the dose of 100 mg/kg produced 85.00 % percentage inhibition of paw oedema at the 4 th hour of drug administration while 70.73% was produced by diclofenac at the same time. These results are presented in table 2.

Effects of L-arginine on the Anti-inflammatory action of aqueous extract of Acanthus montanus, diclofenac and L-NAME

The administration of L-arginine prior to the injection of plogogistic agent does not significantly affect mice oedema development. The anti-inflammatory activities of *Acanthus montanus* (400mg/mg) were significantly inhibited by L-arginine (300 mg/kg). The same result was observed with L-NAME. The anti-inflammatory effect produced by diclofenac (50 mg/kg) was not inhibited by L-arginine (Table 3).

Table 1: Anti-inflammatory effects of aqueous extract of *Acanthus montanus*, diclofenac and L-NAME

Group	Dose (mg/kg)	n	Inflammation (%)						
			0,5 h	1 h	2 h	3 h	4 h	5 h	6 h
Control (Saline)	-	6	48,19±20,76	54,27±21,88	62,18±14,86	47,79±121,20	49,04±14,19	87,72±25,58	59,80±26,54
A. montanus	100	6	38,50±6,81	30,11±6,84*	40,49±9,67	30,69±5,83	22,02±7,09	32,68±7,73	22,55±7,89
A. montanus	200	5	13,10±2,72*	16,50±5,35**	17,61±5,68**	20,22±3,29*	8,75±4,39*	33,84±8,84*	26,04±8,13
A. montanus	400	6	19,46±4,27	41,97±7,04	29,32±6,22	21,86±5,47	15,70±8,22*	36,18±10,07*	31,78±13,15
L-NAME	100	5	29,04±7,59	39,48±7,29	28,44±4,72	16,62±6,23*	6,11±2,47**	23,30±7,07*	39,04±6,13
Diclofenac	50	5	31,41±5,00	32,06±3,07	25,35±4,90*	11,76±2,94**	7,71±4,48**	26,88±6,16*	24,35±4,19

Data are given as means ± S.E.M. Significant parameters were obtained statistically from one-way ANOVA and Dunnett's multicomparison test; *$p < 0.05$ is considered significant, compared to control.

Table 2: Anti-inflammatory activity of aqueous extract of *Acanthus montanus* in carrageenan-induced hind paw edema: expressed as a percentage of volume variation (ΔV in mL).

Traitement	Dose (mg/kg)	n	Volume variation in mL (% inhibition)						
			0,5 h	1 h	2 h	3 h	4 h	5 h	6 h
Control (saline)	-	6	0,08±0,02	0,10±0,02	0,09±0,01	0,07±0,02	0,07±0,01	0,12±0,01	0,08±0,03
A. montanus	100	6	0,07±0,01	0,05±0,01	0,07±0,02	0,05±0,01	0,04±0,01	0,06±0,01§	0,04±0,01
			(15,22)	(48,33)*	(21,15)	(24,39)	(45,00)	(53,52)*	(48,49)
A. montanus	200	5	0,03±0,00	0,03±0,01	0,03±0,01§	0,04±0,01	0,02±0,01	0,07±0,02	0,05±0,02
			(66,09)*	(68,00)**	(60,77)**	(41,46)*	(76,00)*	(42,54)*	(30,67)
A. montanus	400	6	0,03±0,01	0,07±0,01	0,05±0,01	0,04±0,01	0,03±0,01	0,06±0,02	0,05±0,02
			(58,70)	(30,00)	(44,23)	(46,34)	(62,50)*	(49,30)*	(33,33)
L-NAME	100	5	0,05±0,01	0,07±0,01	0,05±0,01	0,03±0,01	0,01±0,00	0,04±0,01	0,07±0,01
			(37,39)	(34,00)	(44,62)	(61,95)*	(85,00)**	(66,20)*	(12,00)
Diclofenac	50	5	0,05±0,01	0,06±0,00	0,04±0,01	0,02±0,01	0,01±0,01	0,05±0,01	0,04±0,01
			(29,57)	(44,00)	(49,23)*	(70,73)**	(79,00)**	(61,13)*	(44,00)

Data are given as means ± S.E.M. Significant parameters were obtained statistically from one-way ANOVA and Dunnett's multicomparison test; *$p < 0.05$ is considered significant, compared to control.

Table 3*:* Effect of L-arginine on the anti-inflammatory effect induced by the aqueous extract of *A. montanus*, diclofenac and L-NAME

Traitement	n	Volume variation in mL (% inhibition)						
		0,5 h	1 h	2 h	3 h	4 h	5 h	6 h
Control (saline)	6	0,08±0,02	0,10±0,02	0,09±0,01	0,07±0,02	0,07±0,01	0,12±0,02	0,07±0,03
L-Arg (300 mg/kg)	5	0,07±0,01 (14,28)	0,08±0,01 (20,00)	0,07±0,01 (22.22)	0,05±0,01 (28.57)	0,05±0,01 (28.57)	0,10±0,01 (16.66)	0,06±0,01 (14.28)
A.m (200 mg/kg) + L-Arg (300 mg/kg)	6	0,06±0,00 (25,00)	0,08±0,00 (20,00)	0,07±0,00 (22,22)	0,6±0,01 (14,28)	0,6±0,01 (14,28)	0,11±0,01 (8,33)	0,06±0,01 (14.28)
Diclo (50 mg/kg) + L-Arg (300 mg/kg)	6	0,03±0,00** (63,04)	0,05±0,01** (51,67)	0,02±0,01** (75,00)	0,03±0,01** (52,50)	0,04±0,00** (47,50)	0,02±0,00** (87,32)	0,03±0,00** (62,79)
L-NAME + L-Arg (300 mg/kg)	6	0,07±0,01 (12.50)	0,08±0,01 (20.00)	0,07±0,00 (22.22)	0,06±0,01 (14.28)	0,05±0,01 (28.57)	0,10±0,01 (16.66)	0,10±0,01 (-36,74)

Test groups were compared with the control group and significant difference were noted.
* $P<0.05$, **$P<0.01$.

Discussion

In rat carrageenan inflammation, the interaction between cyclooxygenase and the NO pathway may represent an important mechanism for the modulation of the inflammatory response [13]. Mouse paw oedema and pleurisy elicited by carrageenan is less explored than rat paw and rat pleurisy but it is a useful model for study of inflammation and the same mediators are involved in both models [14, 15, 11]. It is well known that different mechanisms may be involved in the genesis of inflammatory reactions. The development of the inflammatory response induced by carrageenan is characterized by an initial stage (1-2 h) which is dependent on the release of histamine, serotonin and bradykinin, followed by a later stage (3-4 h) which is maintained principally by the release of prostanoids [16]. It has also been shown that nitric oxide (NO) has an important role as much as in the regulation of vascular permeability as in cell migration induced by pro-inflammatory agents, including carrageenan [11, 16]. Several studies have shown that NO appears to play a crucial role in modulating the generation of prostaglandins at the inflammation site [13, 15].

In a previous study, we reported the anti-inflammatory activity of the aqueous extract of *Acanthus montanus* but we were not able to postulate if NO inhibition was implied in that anti-inflammatory effects [8]. In this study, significant ($P< 0.05$) anti-inflammatory activity exhibited by the aqueous extract of *Acanthus montanus* at the doses 200, and 400mg/kg against oedema induced by carrageenan in mouse compared to the control group was a confirmation that, the plant might serve as a useful source of anti-inflammatory agents. Diclofenac (a Non Steroidal Anti-Inflammatory drug) and L-NAME (an inhibitor of nitric oxide) also showed significant anti-inflammatory effects. NO is produced from L-arginine by NOS.

Three isoforms of NOS have been reported. Two of these, namely endothelial and neuronal NOS are calcium-dependant and constitutive. The third type, inductible NOS (i-NOS), is calcium-independent and can be induced in several cell types (macrophages, vascular smooth muscle) after activation by bacterial endotoxin and/or inflammatory cytokines. The quantity of NO in tissues increases during inflammatory responses while the inhibition of i-NOS activity and expression produced an anti-inflammatory effect on mouse paw oedema [11].

The anti-inflammatory effects of the aqueous extract of *Acanthus montanus* and L-NAME were significantly inhibited by L-arginine, a substrate of NOS. However, the anti-inflammatory action of the diclofenac (a cyclooxygenase inhibitor) has not been inhibited by the L-arginine. These results show that *Acanthus montanus* and the L-NAME would partially act at least by inhibition of the production of the nitric oxide in the inflammatory site. These are in agreement with previous report stating that NO appears to play an essential role in modulating the generation of prostaglandins at the inflammation site [17] and L-arginine completely reverses the inhibition of the rat paw oedema produced by the L-NAME [18].

On the other hand it has been indicated that the expression of i-NOS is controlled by NFkappaB (NFkB), a transcription factor that regulates gene expression [19, 20]. Our results indicated that the anti-inflammatory effects of the aqueous extract of *Acanthus montanus* may be due to the direct catalytic activities and/or the expression of the i-NOS through the suppression of NF- kB activation. This suppression of NF- kB activation can be attributed to the phenolic compounds since in our previous study the phytochemival screening of *A. montanus* reveal the presence of polyphenols [8] and it is well known that polyphenolic compounds prevent reactive oxygen species (ROS) formation and are NFkB inhibitor [21, 22].

Regardless of the mechanism action detailed, part of these anti-inflammatory effects may be related to an inhibition of the expression of the inducible NO synthases, while another part may be related to oxyradical and peroxynitrite scavenging since plants of the same genus demonstrated antioxidant activities [23].

Conclusion

The results obtained in this study provide strong evidence that the aqueous extracts of *Acanthus montanus* caused a significant reduction in the first and the second phase of inflammation and these anti-inflammatory effects in this model may be occurring via inhibition of NO generation.

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