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Anti-inflammatory and Anti-snake Venom Activity of *Andrographis stenophylla* Leaf

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In the present study, the anti-inflammaotry and anti-snake venom properties of methanol extract of *Andrographis stenophylla* leaf was investigated. The anti-inflammatory activity was checked using carrageenan-induced acute pedal paw edema model and freund's complete adjuvant induced chronic inflammatory moel. In both acute and chronic inflammatory models, the extract exhibited significant anti-inflammatory activity at two doses of 50 and 100 mg/kg. The methanol extract of *Andrographis stenophylla* leaf, also effectively neutralized Naja najanaja venom induced lethal, cardiotoxic, neurotoxic and hemorrhagic effects. The leaf extract was found to potentiate the polyvalent snake venom antiserum action in experimental animals. The extract provides a 3.5 fold protection against the lethal effect of venom and 350 fold potentiation of anti serum action. The plant possesses significant anti-inflammatory and anti-snake venom activity and the work provides pharmacological evidence for the folklore claim of anti-snake venom activity.

Key Words: *Andrographis stenophylla*, Anti-inflammatory, Anti-snake venom.

INTRODUCTION

Andrographis stenophylla (Fam: Acanthaceae) is an erect glabrous under shurb with very narrow leaves and stems from a stout root stock, distributed mainly in hill areas of Tamilnadu, India[1-3]. The leaves of the plant are used as folklore medicine for the treatment of snake venom poisoning and diabetes. There is so far no report available on this plant from NAPRALERT or other sources. The authors observed that several plants used popularly as anti-snake venom show anti-inflammatory activity. Hence the present investigation was under taken to evaluate the anti-inflammatory and anti-snake venom activity of *Andrographis stenophylla* leaf.

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EXPERIMENTAL

The plant was collected during the month of July, from Marudhamalai hills of Coimbatore district, Tamilnadu. The plant was authenticated by Mr. P. Daniel, Deputy Director, Botanical Survey of India, Southern circle, Coimbatore, India and a voucher specimen (No. BSI/SC/5/21/2000-358) was deposited in the Botanical Survey of India, Coimbatore. The leaves of the plant were removed and dried under shade and powdered in a mechanical grinder. The powdered leaf was extracted with petroleum ether (40-60ºC), benzene, dichloroethane, methanol and water successively in a Soxhelet apparatus[4]. The solvents were then evaporated under reduced pressure to obtain dry extracts. Yields of the extracts were found petroleum ether (40-60°)-5.286 %, benzene-7.456 %, dichloroethane-2.280 %, methanol-8.606 %, water-3.180 % (w/w), respectively.

Naja najanaja venom was obtained from Department of Zoology, Government Arts College, Coimbatore and preserved in a refrigerator till further use. The venom was dissolved in tris buffer, (SD fine Ltd.) and the venom concentration was expressed as mL of venom/mL of buffer. Lyophilized polyvalent snake venom antiserum (Bharath serums and vaccine Ltd, Thane, India) was obtained from PSG Hospitals, Coimbatore. Before use antiserum was weighed and dissolved in Tris buffer. The antiserum concentration was expressed in terms of mg/mL (20 mg/mL). Methanol extract was also dissolved in Tris buffer. Animals were purchased from Agricultural University, Trissur, Kerala. Swiss albino mice weighing 25-30 g, Male Wistar rats weighing 150-200 g, Wistar rabbits weighing 2-2.5 Kg and Guinea pigs weighing 750-800 g were housed under standard environmental conditions of temperature, humidity and light and provided with standard rodent food (Ashirwad Industries, Chandigarh, lndia) and water *ad libitum*. Institutional animal ethics committee has approved the experimental protocol. The extracts as well as standard drugs were suspended in 2% Tween 80 for screening anti-inflammatory activity.

Evaluation of anti-inflammatory activity:

Carrageenan-induced acute paw edema method: Wistar rats selected by random sampling technique were used for the study. Hind paw edema was induced by injecting 0.1 mL of 1 % carrageenan in normal saline on the sub-plantar region. The plant extract was administered orally, 1 h prior to carrageenan administration[5]. Paw volume was measured at 0 to 4 h after the administration of carrageenan. 1 mL of 2 % Tween 80 served as control and diclofenac sodium 5 mg/Kg was used as standard.

Freund's adjuvant-induced chronic inflammatory method: Wistar rats selected by random sampling technique were used for the study. Chronic inflammation was induced by sub-cutaneous injection of 0.1 mL of freund's

complete adjuvant into the right hind plantar region[6]. The methanol extract was fed orally at two dose levels, one week pre-treatment, at 12 h intervals. The paw volume was measured plethismographically on day 0, 1, 2, 4, 7, 10, 15, 21 and 30, after injection of freund's complete adjuvant.

Evaluation of anti-snake venom activity

Inhibition of venom lethal effect: The LD_{50} of *Naja najanaja* venom was determined by injection of different concentration of venom intra peritoneal to male albino mice. Various doses of venom were mixed with fixed amount of extract, incubated at 37ºC for 45 min, cooled and injected intra peritoneal to mice. Median lethal dose was calculated from the number of deaths occurring within 24 h of injection of the incubate. Effective dose (ED) was calculated as the minimum dose of the plant extract that neutralized the median lethal dose of venom[7].

Anti hemorrhagic assay: In order to determine the ability of the plant extract to neutralize the hemorrhagic effect of venom, an anti hemorrhagic assay was performed[8]. The minimum hemorrhagic dose (MHD) was determined, which is defined as the number of micrograms of venom that causes 7.5 mm hemorrhagic diameter when injected intradermally into the back of depilated rabbit. Equal amounts of 20 MHD/mL solution of crude venom (2 μL/mL) were mixed with serial dilutions of methanol extract and incubated for 1 h at 23ºC cooled and 0.1 mL of the venom/extract mixture was injected intradermally. The hemorrhagic diameter was measured after 24 h and the result is expressed as anti hemorrhagic dose (AHD), which is defined as micrograms of extract used to neutralize 1 MHD of venom.

Anti cardioc toxic assay: Minimum cardio toxic dose (MCTD) was determined, which is the least amount of venom which stopped auricular contraction within 15 min. Isolated guinea pig auricle was prepared[9] and suspended in oxygenated (95% O_2 and 5% CO_2) Ringers solution at 29-30ºC. The spontaneous contraction of auricle was recorded on a smoked drum through lightly sprung heart lever. The result is expressed as anti cardio toxic dose (ACTD) which is the micrograms of extract used to neutralize 1 MCTD of venom which was determined by adding the incubate of 1 MCTD of venom and different dilutions of extract incubated at 37ºC for 1 h. The mixture of venom/extract was added to a glass bath (2 mL) containing the auricle and the nature of contraction were recorded.

Anti neuro toxic assay: Minimum neurotoxic dose (MNTD) was determined and defined as the least amount of venom, which blocked rat phrenic nerve action within 30 min. Isolated rat phrenic nerve diaphragm was prepared by the method of Bulbring[10]. The preparation was suspended in oxygenated (95% O_2 and 5% CO_2) Tyrode's solution at 29-30°C in a glass bath (5 mL) and stimulated with a square wave electronic stimulator (Grass, USA). Contractions were recorded with a lightly sprung lever on a

smoked drum. Anti neurotoxic dose (ANTD) was estimated as venom/ extract incubated at 37ºC for 1 h, cooled and added to glass bath containing the phrenic nerve preparation. The result was expressed as anti neurotoxic dose defined as the microgram of extract, which effectively neutralized one minimum neurotoxic dose.

Anti-serum action potentiation: Fixed dose of commercial anti-serum was mixed with various doses of venom, incubated at 37ºC for 1 h, centrifuged at 2000 rpm for 10 min and the supernatant was injected, in male albino mice, anti-serum action potentiation by the plant extract was estimated by injecting venom, anti-serum and extract incubate in male, albino mice and lethality was recorded up to 24 h, anti-serum action potentiation is expressed in terms of LD_{50} value[11].

Statistical analysis: The mean ± S.E.M values are calculated for each group for determining the significance of intergroup difference. Each parameter was analyzed separately and one way analysis of variance (Anova) was carried out. Dunnet's test[12] was used to determine the effect of drug against control group, $P < 0.001$ was considered significant

RESULTS AND DISCUSSION

The results demonstrated significant anti-inflammatory activity of methanol extract of leaves of *Andrographis stenophylla*. It was noted that the plant extract was able to reduce the paw edema volume in carrageenan induced acute paw edema model (Table-1). Similarly in chronic inflammatory model, the plant extract produced significant reduction in inflammation of paw (Table-2). The paw edema volume was reduced in a dose dependent manner ($P < 0.001$).

TABLE-1
EFFECT OF METHANOL EXTRACT OF *Andrographis stenophylla* LEAF ON CARRAGEENAN-INDUCED EDEMA

Group	Mean paw volume (mL) ± S.E.M.				
	0 h	1 h	2 h	3 h	4 h
MEAS 50 mg/kg	0.36 ± 0.02	0.38 ± 0.02	0.40 ± 0.08*	0.48 ± 0.09*	0.53 ± 0.06*
MEAS 100 mg/kg	0.36 ± 1.01	0.37 ± 0.02	0.38 ± 0.01*	0.42 ± 0.08*	0.46 ± 0.08*
Diclofenac 5 mg/kg	0.35 ± 0.08	0.37 ± 1.01	0.38 ± 0.09*	0.38 ± 0.14*	0.39 ± 0.02*
Control	0.34 ± 0.02	0.58 ± 0.01	0.79 ± 0.02	0.85 ± 0.03	0.95 ± 0.02

MEAS = Methanol extract of *Andrographis stenophylla*; N = 6; *P<0.001

TABLE-2
EFFECT OF METHANOL EXTRACT OF *Andrographis stenophylla* LEAF ON FRUND'S ADJUVANT-INDUCED CHRONIC INFLAMMATION

No. of days	Groups			Control
	MEAS 50 mg/kg	MEAS 100 mg/kg	Diclofenac 5 mg/kg	
0	0.28 ± 1.48	0.26 ± 0.40	0.28 ± 0.48	0.25 ± 1.04
1	0.42 ± 0.72*	0.39 ± 0.11*	0.49 ± 1.38	0.58 ± 1.32
2	0.52 ± 0.96*	0.42 ± 1.42*	0.38 ± 1.22*	0.64 ± 1.52
4	0.64 ± 1.40*	0.53 ± 1.02*	0.38 ± 1.42*	0.98 ± 1.64
7	0.58 ± 1.01*	0.49 ± 1.12*	0.44 ± 1.28*	0.71 ± 1.21
10	0.52 ± 1.12*	0.41 ± 1.22*	0.46 ± 1.22*	0.74 ± 1.64
15	0.48 ± 1.24*	0.37 ± 1.22*	0.42 ± 1.52*	0.68 ± 1.52
21	0.44 ± 1.12*	0.35 ± 1.05*	0.38 ± 1.02*	0.61 ± 1.02
30	0.38 ± 1.21*	0.31 ± 1.08*	0.32 ± 1.05*	0.52 ± 1.33

MEAS = Methanol extract of *Andrographis stenophylla*; N = 6; *P < 0.001

The LD_{50} of Naja najanaja venom in male albino mice was found to be 8 μg. The minimum hemorrhagic dose (MHD) of Naja najanaja venom in rabbits was found to be 0.1 μg. Naja najanaja venom induced, minimum cardio toxic dose, (MCTD) was 32 μg in isolated guinea pig auricle and minimum neurotoxic dose (MNTD) was 68 μg in isolated rat phrenic nerve diaphragm preparation. The methanol extract of *Andrographis stenophylla* was found to neutralize 3.5 fold of LD_{50} dose of venom (Table-3). The effective dose of methanol extract which neutralized 28 mcg of venom was found to be 45 ± 1:46 mg/kg. In *in-vitro* studies the anti hemorrhagic dose was 500 μg, anti cardio toxic dose was 320 μg and anti neurotoxic dose was 295 μg. The methanol extract inhibited the lethal effect in mice, which was comparable with the antiserum as positive control.

TABLE-3
INHIBITION OF NAJA NAJANAJA VENOM LETHAL EFFECTS BY METHANOL EXTRACT OF *Andrographis stenophylla* LEAF

Venom concentration (μg)	No. of animals / No. of animals taken	Fold of neutralization	ED_{50} mg/kg
8	0/6	1.0	
12	0/6	1.5	
16	0/6	2.0	
20	0/6	2.5	45 ± 1.46*
24	0/6	3.0	
28	0/6	3.5	
32	6/6	No protection	
32	0/6	4.0	2 ± 0.76**

*P < 0.05, for the extract when compared with anti-serum, student's t-test was used; **ED_{50} of anti-serum

The cobra venom is cardio toxic, neurotoxic in nature. Hence the cobra venom neutralization studies were focused on lethal, cardio toxic

and neuro toxic activities. Since haemolysis may be a common envenomation symptom hemorrhagic assay was also performed. It was observed that the methanol extract of *Andrographis stenophylla* effectively neutralized the cobra venom induced patho physiological effects in different *in vivo* and *in vitro* test models. The extract also produced 350 fold potentiation of the action of poly valent anti serum (Table-4). Preliminary phytochemical studies[13] indicate the presence of terpenes and phenolic compounds in methanol extract and the so called secondary metabolites are proved to be effective in snake venom neutralization[14]. The present study revealed for the first time the anti inflammatory and anti-snake venom activity of the leaf extract of *Andrographis stenophylla* and the folklore claim is thus justified.

TABLE-4
ANTI-SERUM ACTION POTENTIATION BY METHANOL EXTRACT OF *Andrographis stenophylla* LEAF

Venom + anti-serum	Venom + ME	Venom + ME + anti-serum	% Potentiation
16 mcg + 1.5 mg	28 mcg + 45 mg	56 mcg + 45 mg + 2 mg	350

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