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RESEARCH ARTICLE

SYNERGISTIC ANTI-INFLAMMATORY ACTIVITY OF ETHANOLIC LEAVES EXTRACT OF *CALOTROPIS GIGANTEAN* ns *ACHYRANTHES ASPERA*

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ABSTRACT

Achyranthes aspera & *Calotropis gigantean* plant are used in folklore medicines for a number of ailments. The freshly collected leaves of above plants were screened. Shade dried & firstly defatted with petroleum ether & then ethanol respectively and the leaf extracts were subjected to various physicochemical studies. The percentage yield of both plant of extract was (5.1%) for *Calotropis gigantean* & (5.25%) for *Achyranthes aspera* obtained with ethanol. The obtained extracts separately & in combination were used to carry out anti-inflammatory action. *Achyranthes aspera* leaf extract also exhibited anti-inflammatory activity. The obtained result showed that the EE at dose of 300 mg/kg showed 28.74% inhibition in carrageenan induced paw oedema, 14.64% inhibition of granuloma dry weight & 11.11% inhibition against formalin induced paw edema. *Calotropis gigantean* leaf extract also exhibited anti-inflammatory activity. The obtained result showed that the EE at dose of 300 mg/kg showed 34.97% inhibition in carrageenan induced paw edema, 18.82% inhibition of granuloma dry weight & 15.77% inhibition against formalin induced paw oedema. *Calotropis gigantean* & *Achyranthes aspera* leaf extract in combination also exhibited anti-inflammatory activity. The obtained result showed that the EE at dose (300+100) mg/kg showed 43.62% inhibition in carrageenan induced paw oedema, 25.81% inhibition of granuloma dry weight & 20.77% inhibition in formalin induced paw oedema.

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INTRODUCTION

Inflammation is the part of the complex biological response of body tissue to harmful stimuli such as pathogens, damaged cells or irritants [Guyton, 2006; Ross and Wilson, 2004]

Types of inflammation

There are two types of inflammation according to the duration of action of the response and depending on the host & initiating agent i.e. acute inflammation and chronic inflammation.

Acute inflammation

Acute inflammation is defined as a rapid and short onset (days to a few weeks) of action with increased formation of tissue fluid, blood flow including migration of leukocytes. Different mediators released during acute phase of inflammation are shown in the table below:

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Chronic inflammation

The chronic inflammation processes are very similar to acute inflammation but this process for longer duration and destroy more tissue. The characteristic feature of chronic inflammation is the presence of inflammatory cell eg. Lymphocytes (neutrophils), Fibroblasts are activated leading to collagen, fibrosis & granulomas (collection of defensive cells) example of mycobacterium tuberculosis [Ross and Wilson, 2004]

Plant profile

Classification of *Achyranthes aspera* [Srivastav et al., 2011**]:**

Kingdom	Plantae
Subkingdom	Tracheobinota
Super Division	Spermatophyta
Division	Mangoliophyta
Class	Mangoliophsida
Subclass	Caryophyllidae
Order	Caryophyllales
Family	Amaranthaceae

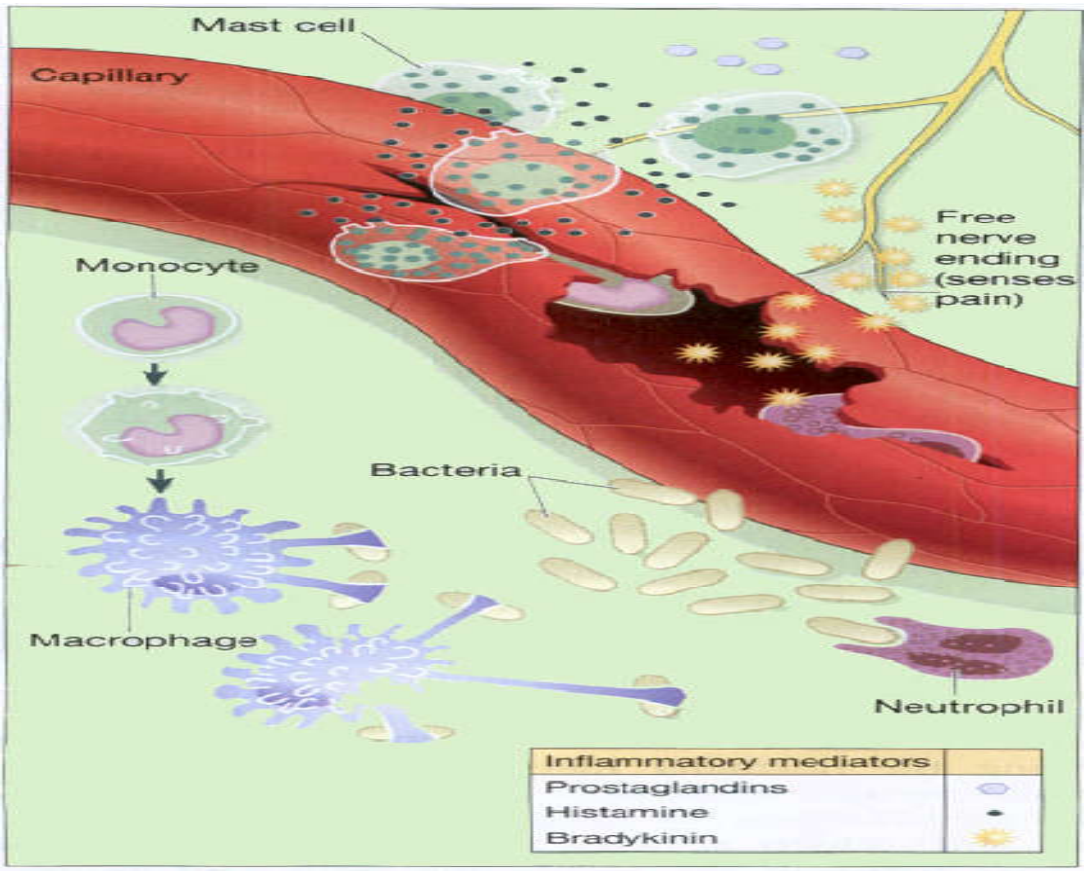

Fig.1. Mechanism of inflammation

Table 1: Cardinal signs of inflammation and physiological change [Srivastav et al., 2011]

Cardinal Sings	Physiological Change
Rubor (redness)	Increased vascularity
Tumor (swelling)	Exudation of fluid
Calor (heat)	Increased blood flow in artery
Dolor (pain)	The pain receptors & nerves by the inflammatory exudates.

Fig. 2. Plant of *Achyranthes aspera*

Fig. 3. Plant of *Calotropis gigantea*

Genus *Achyranthes*
Species *Aspera*
Common name Latjira, Chirchira

Geographical Source

The plant is found on road sides, field boundaries & waste places as a weed throughout India up to an altitude of 2100 m including in South Andaman Islands.

The plant is also widespread in Baluchistan, Ceylon, Tropical Asia, Africa, Australia & America [Benton et al., 2011].

Chemical constituents

The main constituents are ecdysterone achyranthine, betaine, vanillic acid, syringic acid, *p*-coumaric acid saponin [Priya et al., 2012]

Uses [Dey, 2011]

The plant is used as anti-asthmatic, diuretic, immunostimulant, hypolipidemic, antifertility, anti-inflamamtory, anti-dandruff, anti snake venom & in renal disorders.

Calotropis gigantean

Classification of *Calotropis gigantea*

Kingdom	Planatae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Dicotyledones
Sub class	Asteridae
Series	Bicarpellatae
Order	Gentianales
Family	Apocynaceae
Subfamily	Asclepidiaceae
Genus	*Calotropis*
Species	*Gigantea*
Common name	Madar

Geographical source

The plant is a native of India, Bangladesh, Burma, China, Indonesia, Malaysia, Pakistan, Philippines, Thailand & Sri Lanka **[**Sarkar et al., 2014**]**

Chemical constituents [Kumar et al., 2013]

Plant are contains flavonoids, tannins, sterol, saponins, cardiac glycosides. amyrin, amyrin acetate, ß-sitosterol, urosolic acid and cardenolides.

Uses

The plant is widely used as an anti-asthmatic, anti-inflammatory, antihelmintic, anti cancer, antiviral, anti-diarrhea

MATERIAL AND METHODS

Collection of plant leaves

The leaves of *Achyranthes aspera* & *Calotropis gigantean* were collected from "Mohabbatpur" village of Barabanki, Utter Pradesh, India. **Authentication:** the leaves of plant *Achyranthes aspera & Calotropis gigantean* were authenticated by a Senior Botanist Dr. D.C kasana; head of deportment of botany, I.P College of science, Bulandshahr (U.P), India.

Preparation of extract

Leaves of plant *Achyranthes aspera & Calotropis gigantean* were collected, reduced to small size after drying in shade for ten days and crushed to form coarse powder.

The powdered drug (500 gm) was subjected to continuous hot extraction with the help of soxhlet apparatus using petroleum ether & ethanol successively. Each time before extracting with the next solvent the plant material was dried in hot air oven at 50^0C for an hour. After the effective extraction, the solvent were distilled off, the extract were then concentrated on water bath to become dried. The obtained extract with each solvent was weighed and stored in an air tight container.

The percentage yield was determined using the formula:

$$\text{Percentage yield (W/V)} = \frac{\text{wt of extract obtained}}{\text{weight of crude drug taken}} \text{ x } 10$$

Pharmacological evaluations

In-Vivo study

Experimental Animals

Swiss albino rats (125gm) of either sex were used in the present study and obtained from the central Animal House facility of TMCOP, Moradabad (U.P). All animal protocols were approved by Institutional Animal Ethical committee (IAEC) of the organiztion (Reg. No. 1205/PO/C/21/04/08/CPCSEA). All animals were maintained under standard conditions of humidity (50±10 %), temperature (22 ± 2^0c) & light (12 hours light & 12 hours dark). Animals were fed with standard food (Lipton India Ltd. Pellets) & water *ad libitum.* They were acclimatized for 1 week before examination which was performed in accordance with CPCSEA (committee for the purpose of control and supervision of experimentation on animals) guidelines.

Acute toxicity study [http://iccvam.niehs.nih.gov/ suppdocs/oecd/oecd/ G1423. Pdf**]**

Procedure

Young male Swiss albino rats were selected for toxicity studies. Weight of each rat was recorded. Rats were divided into two groups (each containing three) and labelled. Solution of plant leaf extract was prepared & administered orally at different doses i.e. 5, 50, 500, and 2000 mg/kg. Any type of adverse effect and death of rat were observed upto 24 hours.
The acute toxicity test was performed according to the OECD 423 guidelines.

Dose selection

On the basis of acute toxicity study, two test doses were selected for pharmacological screening. As there was no lethality observed upto 2000 mg/kg two doses i.e. 300 mg/kg & 500 mg/kg were chosen for further studies.

Anti-inflammatory activity

Carrageenan induced paw edema [Vetriselvan et al., 2013; Neha Mohan et al., 2013]

The experiment model was based on various inflammatory mediator released by carrageenan. Edema formation in the rat paw is a biphasic event. In the first phase is attributed to the release of histamine & serotonin while second phase is the release of prostaglandin, protease & lysosome.

First phase begins after the injection (s.c) of carrageenan and gets diminished in two hours while second phase continues up to five hours. The experiment method was performing by winter 1962.

Procedure

An anti-inflammatory evaluation of *Achyranthes aspera, Calotropis gigantean* leaf extracts & their combination were used against carrageenan induced paw edema model. Experimental animals were divided into following five groups: All the groups were pretreated according to their treatments, 1 hour before the administration of 0.1 ml of 1% carrageenan (suspended in sterile 0.9% normal sterilized saline) in subplanter region of right hind paw of rat. The initial paw volume (IPV) and final paw volume (FPV) was measured after 60,120,180,240 & 300 minutes of carrageenan administration using plethysmometer. The difference initial and final paw volume was used to calculate the percentage inhibition using following equation:-

$$\text{Percentage inhibition} = \frac{(X-Y)}{X} \times 100$$

Where,

X= increase in paw volume of rats in the control group
Y= increase in paw volume of rat in the drug treated group

Cotton pellet induced granuloma [Jian-Yu et al., 2011]

The exudative & proliferative phase of inflammation was measured against cotton pellet induced granuloma method.

Procedure

The experimental animals were divided in to five groups according to sex and weight approximately 180 to 200 gm:

Experiment animal were fasted over night and feed was removed. Only tap water was provided. Animal were anaesthetized with ketamine (50 mg/km; i.m.). The back skin was saved using 70% ethanol as disinfecting agent. An incision was made in the lumber region and subcutaneous tunnels were formed by a bulunted forceps. Then after previously sterilized pre-weighed cotton pellets of (10 mg) was placed on both side in the scapular region. Each animal was kept in single cage and drug treatment was started two hour after cotton pellet implantation & continued to 7 consecutive days. On the 8^{th} day, animal were sacrificed and granulomas were removed. They were dried 24 hours in an oven at 60^0C until the weight was stabilized. The weight of dry cotton pellet was determined. The weight of granulomas tissue formed were calculated by subtracting initial weight from the final dry weight of cotton pellet and percentage protection by the drug was calculated using following formula.

$$\text{Percentage Inhibition} = \frac{(X-Y)}{X} \times 100$$

Where,

X = increase in weight of cotton pellet in the control group
Y = increase in weight of cotton pellet of rat in drug treated group

Formalin induced paw oedema model [Dimo et al., 2005; Gupta1 et al., 2010**].** Formalin induced paw oedema was measured using the method of T.Dalmo et al., (2005). Percentage inhibition of formalin induced paw oedema was calculated. Formalin was administered by S.C. injection in sublanter region of right hind paw of rat. Formalin produced biphasic response in which the first phase was the release of histamine while and second phase 5-HT was released.

Preparation of 1% formalin solution

1% formalin solution (v/v) was prepared by mixing 1ml of formaldehyde solution mixed with 98 ml of 0.9% saline volume was mad up to 100 ml.

Procedure

Animals were divided into five groups and each group contains six animals as follows:

Animal were fasted overnight before experiment and feed was removed water ad libitam. 0.1 ml of prepared formalin solution was injected subcutaneously in the subplanter region of rat right hind paw on the first day, one hour after drug, extract or vehicle administration. The administration of the extracts and diclofenac sodium was continued once per day for 9 consecutive days. Paw volume was measured using plethysmometer before 3and 6 hours daily for 9 consecutive days after formalin injection. Percentage inhibition was calculated using following formula:-

$$\text{Percentage anti-inflammatory} = \frac{C-D}{C} \times 100$$

Where,

D = represents the percentage difference in paw volume after extracts/fractions were administered to the rats
C = represents the percentage difference of volume in the control group.

RESULT AND DISCUSSION

Anti-Inflammatory Activity

Carrageenan induced paw oedema

The ethanolic extracts of leaves of *Calotropis gigantean* & *Achyranthes aspera* were evaluated for anti-inflammatory activities using the carrageenan induced paw oedema model. At the dose levels of *C.gigantean* (300 mg/kg) & *A.aspera* (300 mg/kg) combination (300 + 100 mg/kg) body weight where as diclofenac sodium (50 mg/kg) was used as positive reference standard and the result were as shown below: Carrageenan induced inflammation is biphasic phenomena. The first phase of edema is attributed to the release of histamine & 5-HT like substance and second acceleration phase of swelling is attributed to PG like substance. The results (Table 16 & Table 17) of acute inflammation model indicated that the ethanolic combined extract of leaves of *C. gigantean & A.aspera* at dose level of 300 + 100 mg/kg showed reduction in paw edema volume when compared with control and *C. gigantean & A.aspera* (ethanolic extract) treated groups at 3, 4 & 5 hours observation i.e. 15.33%, 31.86% & 43.62% respectively.

Table 2. Mediators released in acute inflammation

Substance	Released from	Trigger for release	Pro -inflammatory actions
Histamine	Mast cells, basophils; stored in cytoplasmic granules	Binding of antibody to mast cells & basophils	Vasodilatation, itching, vascular permeability, degranulation, smooth muscle contraction like broncho constriction
Serotonin (5-HT)	Platelets, mast cells & basophils, CNS (acts as neurotransmitter)	uprun activation of platelets and degranulation of mast cells, basophils degranulate	Vasoconstriction, increased vascular Permeability
Prostaglandins (PGs)	Nearly all cells present; not stored, but released from cell membrane phospholipids when required	various stimuli like drugs, toxins, other inflammatory mediators, hormones, trauma	Diverse, sometimes opposing, e.g. fever, pain, vasodilatation
Heparin	Liver, mast cells, basophils	Released when cells degranulate	Anticoagulant, which maintains blood supply (nutrients,O2) to injured tissue & washes away microbes & wastes
Bradykinin	Tissues & blood	When blood clots, in trauma & inflammation	Pain, Vasodilatation

Groups	Treatments
Group 1 (control)	received vehicle
Group 2 (standard)	received Diclofenac sodium dose of (50 mg/kg)
Group 3 (test-1)	received *Calotropis gigantean* leaf extract dose of (300 mg/kg)
Group 4 (test-2)	received *Achyranthes aspera* leaf extract dose of (300 mg/kg)
Group 5 (test-3)	received combination (*Calotropis gigantean, Achyranthes aspera* dose of 300+100 mg/kg)

Groups	Treatments
Group 1 (control)	received vehicle
Group 2 (standard)	received Diclofenac sodium dose of (50 mg/kg)
Group 3 (test-1)	received *Calotropis gigantean* leaf extract dose of (300mg/kg)
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Group 4 (test-2)	received *Achyranthes aspera* leaf extract dose of (300 mg/kg)
Group 5 (test-3)	received combination (*Calotropis gigantean, Achyranthes aspera* dose of 300+100 mg/kg)

Table 16 Effects of ethanolic extracts of leaves of *Achyranthes aspera* & *Calotropis gigantean* and there combination on carrageenan induced paw volume (in cm)

	Paw oedema volume (cm) measured					
	Mean± SEM					
Groups	0 hr	1 hr	2 hr	3 hr	4 hr	5 hr
Control	1.205± .004	1.816± .026	2.231± .010	2.210± .007	2.423± .010	2.453± .016
Standard 50 mg/kg	1.055±. 014	1.196±. 014***	1.320± .024***	1.40± .022***	1.34± .023***	1.28± .020***
Test 1 *C.giganten* 300mg/kg	1.153± .003*	1.391± .011*	1.476± .016*	1.883± .044*	1.861± .039*	1.595± .037*
Test 2 *A.Aspera* 300mg/kg	1.121± .009*	1.420± .022*	1.548± .035*	1.945± .029*	1.906± .020*	1.748± .022*
Test 3 C.G.& A.A.(300+100) mg/kg	1.116± .010**	1.416± .011**	1.545± .019**	1.871± .020**	1.651± .027**	1.383± .026**

All valves were shown as mean ± SEM n=6 by one way ANOVA, *P<0.05, **P<0.01, ***P<0.001 vs control.

Table 17: inhibition of edema by ethanolic extracts of leaves of *Achyranthes aspera* & *Calotropis gigantean* and there combination at deferent time intervals

	Percentage (%) Inhibition				
Groups	1 hr	2 hr	3 hr	4 hr	5 hr
Standard 50 mg/kg	34.14 %	35.00 %	36.65 %	44.69 %	47.81 %
Test 1 *C.gigantean* 300mg/kg	23.84 %	27.32 %	14.79 %	23.19 %	34.97 %
Test 2 *A.Aspera* 300mg/kg	21.8 %	23.78 %	11.99 %	21.33 %	28.74 %
Test 3 C.G.& A.A(300+100)mg/kg	22.02 %	23.92 %	15.33 %	31.86 %	43.62 %

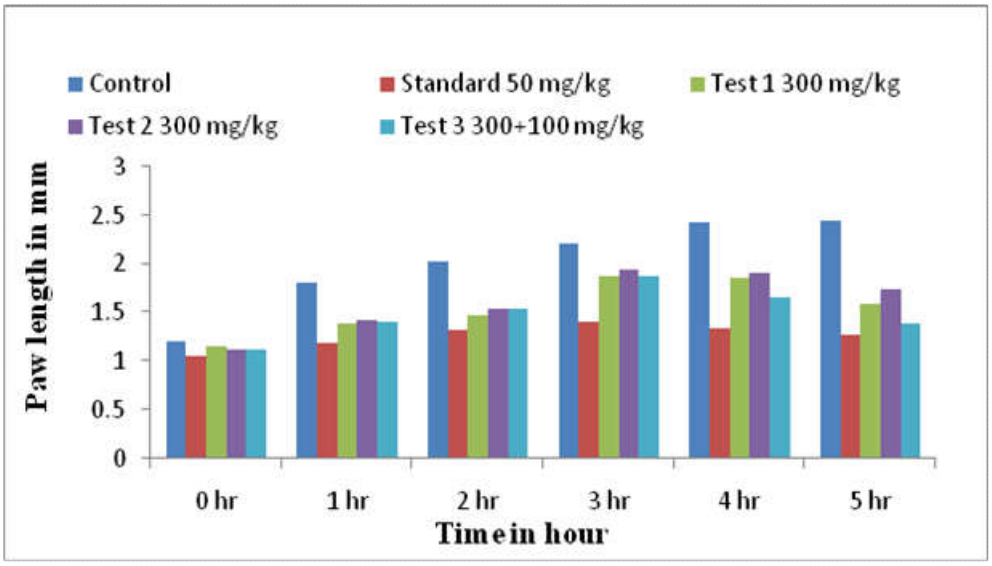

Fig. 9. Graph representing mean increase in paw volume in mm at different time intervals

Table 17: inhibition of edema by ethanolic extracts of leaves of *Achyranthes aspera* & *Calotropis gigantean* and there combination at deferent time intervals

	Percentage (%) Inhibition				
Groups	1 hr	2 hr	3 hr	4 hr	5 hr
Standard 50 mg/kg	34.14 %	35.00 %	36.65 %	44.69 %	47.81 %
Test 1 *C.gigantean* 300mg/kg	23.84 %	27.32 %	14.79 %	23.19 %	34.97 %
Test 2 *A.Aspera* 300mg/kg	21.8 %	23.78 %	11.99 %	21.33 %	28.74 %
Test 3 C.G.& A.A(300+100)mg/kg	22.02 %	23.92 %	15.33 %	31.86 %	43.62 %

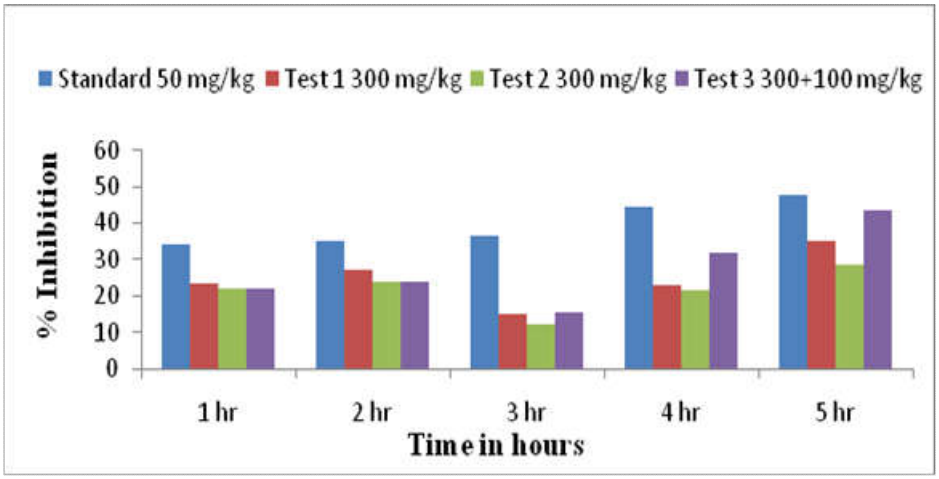

Fig. 10. Graph showing the % Inhibition in paw edema at deferent time intervals

Table 18: Mean of weight cotton pellet induced granuloma

Treatment	Dose	Granuloma weight in mg	
		Weight of first cotton pellet	Weight of second cotton pellet
Control		107.83±.708	92.16±.654
Standard	50mg/kg	51.66±1.475***	62.83±.792***
Test 1(C.G.)	300mg/kg	61.50±1.231*	74.83±1.077*
Test 2 (A.A.)	300mg/kg	65.50±1.565*	78.66±1.782*
Test 3(C.G+A.A)	300+100mg/kg	52.50±.428**	65.33±1.452**

All valves were shown as mean ± SEM, n=6 by one way ANOVA, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs control

Effect on cotton pellet induced granuloma

Ethanolic extracts of leaves of *Achyranthes Aspera* (300mg/kg) & *Calotropis gigantean* (300 mg/kg) and there combination (300 + 100 mg/kg) were evaluated by comparing granuloma dry weight cotton & percentage granuloma inhibition in different groups of animals as show in table 18 & 19 fig 11 & 12. Cotton pellet granuloma is well established test for the assessment of chronic anti-inflammatory process. Chronic condition predominantly consists of a transudative and proliferative test the dry weight of the implanted cotton pellet

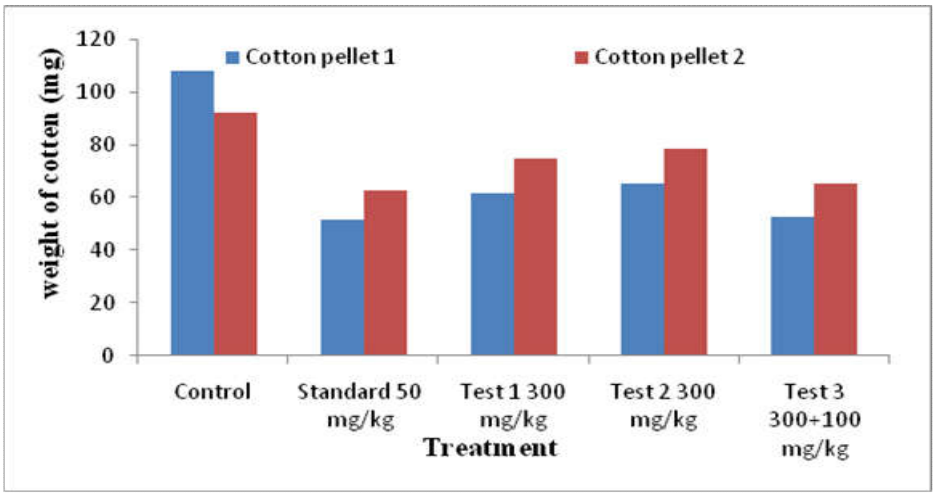

Fig 11: Graph representing weight variation in two cotton pellet from deferent treatment group

Table 19: Result of inhibition in cotton pellet induced granuloma

Treatment	Dose	Percentage inhibition	
		First pellet	Second pellet
Control			
Standard	50mg/kg	51.32%	31.82%
Test 1(C.G.)	300mg/kg	42.96%	18.82%
Test 2 (A.A.)	300mg/kg	39.25%	14.64%
Test 3(C.G+A.A)	300+100 mg/kg	49.53%	25.81%

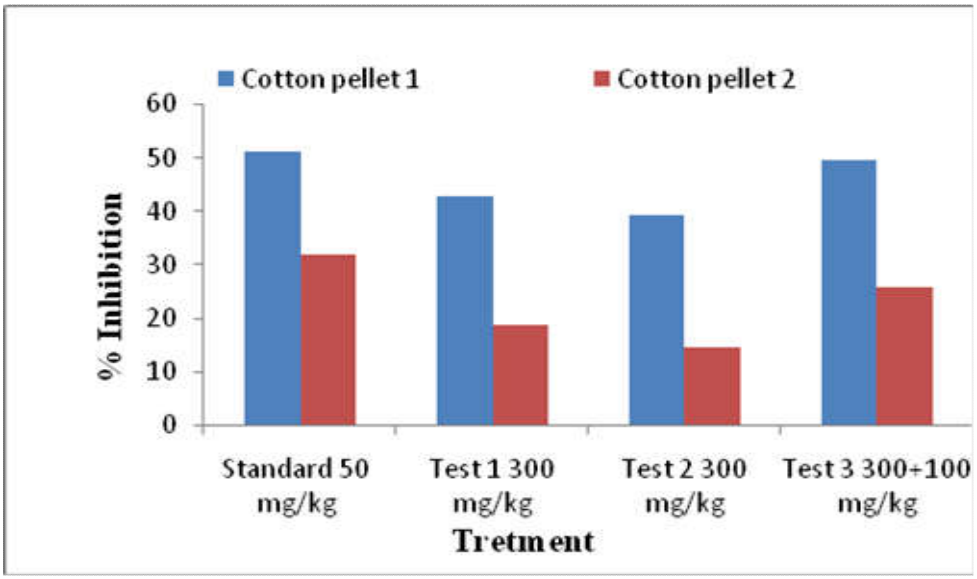

Fig 12: Graph of cotton pellet induced granuloma studies indicating the % inhibition of granuloma in various treatment groups

Table 20: Effects of ethanolic extracts of leaves of *Achyranthes Aspera* & *Calotropis gigantean* and there combination on carrageenan induced paw volume

Treatment (Groups)	Paw edema volume (mm) measured				
	Base line 0 hours	Increase after 3hours	Increase after 6hours	Increase On 3th day	Increase On 7th day
Control	1.133±.021	2.1±.170	2.05±.220	2.0±.227	1.8±.233
Standard 50 mg/kg	1.133±.021***	1.700±.093***	1.6±.101***	1.5±.042***	1.2±.025***
Test1 *C.gigantean* 300mg/kg	1.133±.021*	1.900±.044*	1.800±.205*	1.700±.077*	1.516±.060*
Test 2 *A.Aspera* 300mg/kg	1.116±.016*	1.97±.033*	1.900±.044*	1.833± .055*	1.6±.055*
Test 3 C.G.& A.A. (300+100) mg/kg	1.116±.016**	1.800±.036**	1.733±.114**	1.633±.021**	1.426±.030**

All valves were shown as mean ± SEM n=6 by one way ANOVA, *P<0.05, **P<0.01, *** P<0.001 vs control.

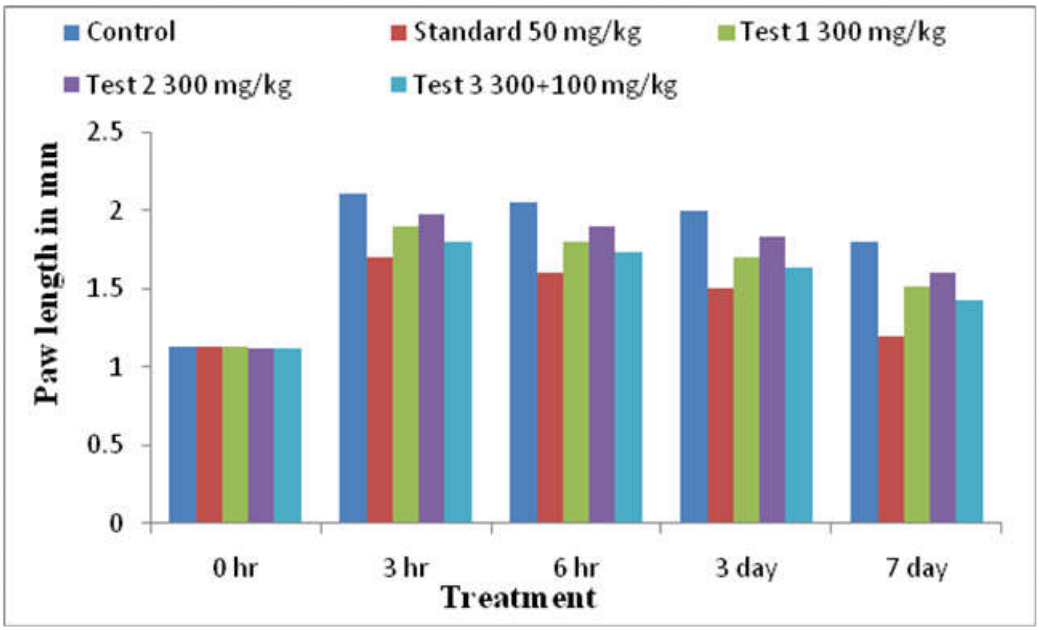

Fig 13. Graph representing mean increase in paw volume in mm at deferent time intervals

Table 21: % inhibition of edema by ethanolic extracts of leaves of *Achyranthes aspera* & *Calotropis gigantean* and there combination at deferent time intervals

Groups	Increase after 3 hours	Increase after 6 hours	Increase On 3th day	Increase On 7th day
Control	-	-	-	-
Standard 50 mg/kg	19.04%	21.95%	25.5%	33.3%
Test1 *C.giganten* 300 mg/kg	9.52%	12.19%	15%	15.77%
Test 2 *A.Aspera* 300 mg/kg	6.19%	7.31%	8.35%	11.11%
Test 3 C.G.& A.A.(300+100)mg/kg	14.28%	15.46%	18.35%	20.77%

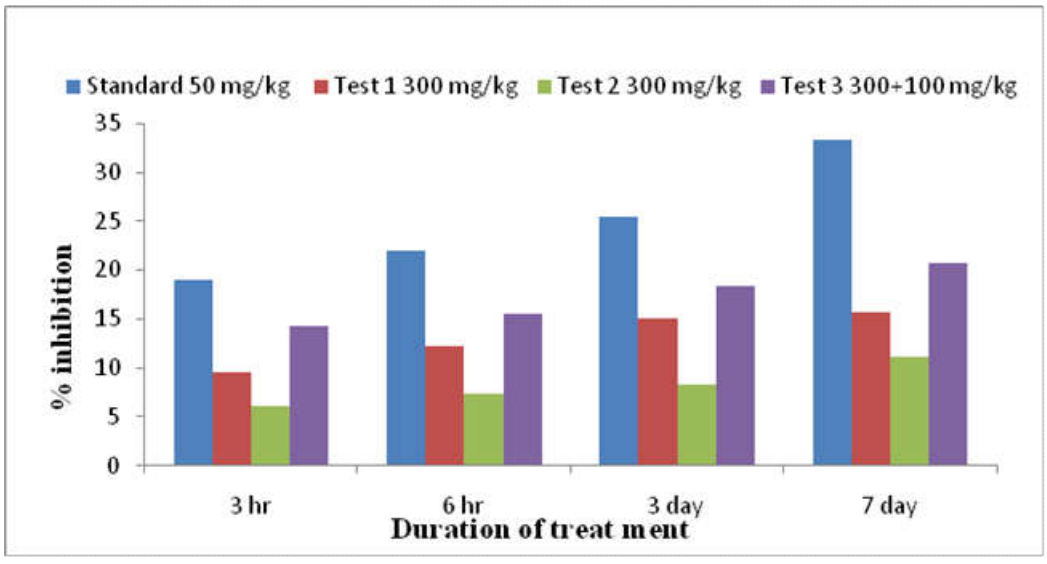

Fig 14: Graph showing the % inhibition in paw edema at deferent time intervals

correlates with the amount of granulomatous tissue formation. The mean dry weight of cotton pellet were as presented in table 18 the standard drug diclofenac sodium showed significant anti-inflammatory activity by redusing dry weight of the cotton pellet when compared with normal control. The ethanolic leaves extract combination of *C.gigantean & A.aspera* at dose level of 300 + 100 mg/kg showed significant decrease in drug weight of cotton pellets & inhibition was noted comparable to the control group. Hence decrease in the weight of granuloma indicated that the proliferative phase was less suppressed by the combination of *C. gigantean & A.aspera.*

Formalin induced paw oedema

Ethanolic extracts of leaves of *Achyranthes Aspera* & *Calotropis gigantean* and there combination were evaluated

for anti-inflammatory activities using the Formalin induced paw oedema model at dose of 300 mg/kg (test 1) & 300 mg/kg (test 2) and their combination 300 + 100 mg/kg (test 3) body weight respectively where as diclofenac sodium as standard and the result were shown in table 20 & 21, fig 19 & 14. The anti-inflammatory effect might be attributed to inhibition action of 5-HT, histamine & PEG. After formalin injection the paw length increased which were inhibition *C. gigantean & A.aspera* and diclofenac sodium the percentage of inhibition of paw edema in case of (*C. gigantean + A.aspera*) extract after 3hours, 6 hours, 3 days & 7 days. After formalin injection were 14.28%, 15.46%, 18.35%, 20.77% respectively.

Conclusion

The obtained plant extract were subjected to pharmacological studies.

Calotropis gigantean & Achyranthes aspera ethanolic leaves extract indiviusally and in combination exhibited better anti-inflammatory activities using carrageenan induced paw edema ,cotton pellet induced granuloma & formalin indused paw edema comparable to standard diclofenac. Hence it was concluded that the combined extract revealed more significant synergist effect for anti-inflammatory rather than individual ethanolic leaf extract when compared to the standard.
Therefore it seems worthy to develop the formulation containing the combination of both extracts optimized affects in chronic & acute inflammation condition.

Aknowlegement

Aurthor are beholden to Director Dr. K.K JHA & Mr. Neenalchal Trivedi, Teerthanker Mahaveer College of pharmacy to be providing laboratory facilities for this research work. This paper forms the parts of M. Pharm thesis. Thesis submitted by author to TMU, Moradabad, U.P.

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