

EVALUATION OF THE ANTI-ASTHMATIC ACTIVITY OF LEAVES OF *VITEX NEGUNDO*

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Objective: To evaluate the antiasthmatic activity of leaves of *Vitex negundo*.

Materials and Methods: Ethanolic extract (AE) and various fractions like petroleum ether (PF), aqueous (AF) and ethyl acetate (EAF) of leaves of *Vitex negundo* were prepared. The antiasthmatic activity of AE, PF, AF and EAF was evaluated by various experimental models like mast cell degranulation by compound 48/80, passive cutaneous anaphylaxis, and egg-albumin induced asthma. Dexamethasone (5mg/kg) was used as a reference standard.

Results: AE, EAF, and AF showed significant protection of rat mesenteric mast cells from disruption caused by compound 48/80. Animals treated with AE, EAF, and AF showed significantly lesser the amount of dye leakage as compared to control animals at the site of heterologous antibody injection. Animals treated with AE, EAF, and AF showed significantly lesser the amount of eosinophils, serum bicarbonate level and lung body weight ratio and significantly higher tidal volume as compared to untreated, egg-albumin sensitized animals.

Conclusion: Present study concluded that AE, EAF, and AF of leaves of *Vitex negundo* are found to be effective in various experimental models of asthma. Stabilization of mast cells, inhibitory effects on immediate hypersensitivity reactions and antieosinophilic activity appear to be involved in its mode of action.

Keywords : Asthma, Mast cells, Anaphylaxis, BAL Fluid.

INTRODUCTION

Asthma literally means 'panting'. It is a broad term used to refer to a disorder of the respiratory system that leads to episodic difficulty in breathing. Currently, asthma is recognized as a chronic lung disease with airway obstruction and inflammation and bronchial hyper-responsiveness to variety of stimuli. These symptoms may be due to liberation of endogenous and intrinsic mediators like histamine, leukotrienes (LTs), bradykinin, prostaglandins (PGs), nitric oxide, platelet activating factors (PAF), chemokines and endothelin from mast cells during the allergic reactions and inflammation of the air passages in the lungs.¹

Large number of drugs belonging to β_2 agonist, corticosteroids, mast cell stabilizers, methylxanthins, leukotriene antagonists and others are in use for treating asthma. However none of them seems to be an ideal drug. The search for new drug is still the need of the day. There is high prevalence of usage of alternative traditional system of medicines for the treatment of asthma. Ayurveda offers a unique insight into comprehensive approach to asthma management through proper care of the respiratory tract. More than 400 medicinal plant species have been used ethnopharmacologically and traditionally to treat the symptoms of asthmatic and allergic disorders worldwide. The world health organization (WHO) has recognized herbal medicine as an essential building block for primary health care of vast countries like India and China. Herbal

medicines are a treasure house of the information, from which we may derive leads to fill many blank spots in the modern medicine.²

Vitex negundo (VN) seems to be a promising plant for treatment of bronchial asthma because of its reported immunomodulatory and anti-inflammatory activity.³ Ethanolic extract of VN leaves showed inhibitory effect on degranulation of rat peritoneal mast cells induced by compound 48/80 and egg albumin.⁴ Aqueous extract of mature leaves of VN showed anti-inflammatory effect.⁵ However, direct studies on asthma models have not been conducted till date.

In the light of above mentioned facts, the objective of our present investigation was to evaluate the anti-asthmatic activity of leaves of VN using various experimental models.

MATERIALS AND METHODS

Animals

Rats (150- 200g), mice (20 to 30g) and G. pigs (300-600g) of either sex were used during whole period of experiments. IAEC- Institute Animal Ethics Committee permission of KBIPER as per CPCSEA guidelines permission was obtained KBIPER/0667.

Preparation of plant extract and fractions

The leaves of plant VN were collected from medicinal plant garden of K.B. Institute of Pharmaceutical Education and Research (KBIPER). Its botanical identification was confirmed by the department of Pharmacognosy, KBIPER. The ethanolic extract (AE) was prepared using Soxhlet

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extractor using ethanol as a solvent. Petroleum ether (PF), aqueous (AF) and ethyl acetate (EAF) fractions were prepared using Soxhlet extractor using the below mentioned procedure. Leaves of VN was extracted using petroleum ether as a solvent at 50°-60° C, filter and solvent was concentrated under controlled temperature below 50° C (known as PEF). Residue was dried for 1 day and extracted with Ethyl acetate as a solvent at 50°- 60° C, filter and solvent was concentrated under controlled temperature below 50° C (known as EAF). Again Residue was dried for 1 day and extracted with distilled water as a solvent at 50°- 60° C, filter and solvent was concentrated under controlled temperature at 100° C (known as AF).

Preparation of the drug:

The extract was weighted and dissolved in sufficient amount of water containing 0.5% CMC to prepare required concentration.

Study on rat mesenteric mast cell degranulation by compound 48/80

The pieces of rat mesentery were collected in petri dish containing Ringer Locke solution and then subjected to the following treatment schedules. Petri dish no.1- Ringer Locke solution (vehicle control), Petri dish no.2- Ringer Locke solution (positive control), Petri dish no.3- Dexamethasone (10µg/ml), Petri dish no.4- AE of VN leaves (500µg/ml), Petri dish no.5- EAF of VN leaves (500µg/ml), Petri dish no. - PEF of VN leaves (500µg/ml); Petri dish no.7- AF of VN leaves (500µg/ml). Each petri dish was incubated for 10 min at 37°C. Later 0.2 ml

of compound 48/80 having concentration 10µg/ml was added and again incubated for 10 min at 37°C. After that all pieces were transferred to 4% formaldehyde solution containing 0.1% touidine blue and kept a side for 20 to 25min. After staining and fixation of mast cells, mesentery pieces were transferred through acetone and xylene two times and mounted on slides. All the pieces were examined under light microscope with 450x magnification. A minimum of 100 cells were counted for intact and disrupted mast cells and from that % protection form degranulation was calculated.⁶

Heterologous passive cutaneous anaphylaxis model

The rats were injected with 0.1ml of egg albumin and 0.1 ml of Bordetella Pertusis vaccine i.p. on 1st, 3rd and 5th day. After 21 days from the first day of the immunization, blood was collected by cardiac puncture under light ether anesthesia. Serum was separated by centrifugation at 3000 rpm for 15 min. and stored below -20°C before use. Mice used for receiving rat antiovalbumin antisera were randomly divided into following groups: Group 1. Control group, Group 2. Dexamethasone (5 mg \kg), Group 3. AE of VN leaves (300 mg/kg), Group 4. EAF of VN leaves (300 mg/kg), Group 5. PEF of VN leaves (300 mg/kg), Group 6. AF of VN leaves (300 mg/kg). To all the mice, the anti-ovalbumin antisera were injected i.d. on the clipped dorsal skin. On the very same day, the drug extracts or dexamethasone or vehical was administered and was repeated once daily for 3 day. After 48 hrs of passive sensitisation, 1 ml of 0.5% Evans blue solution containing

TABLE- 1

		Serum bicarbonate level m.eq/litre	Tidal volume (ml)	Lung/bodyweight ratio
Group 1		30.33 ± 1.08b	4.54 ± 0.05b	0.0090 ± 0.0001 b
Group 2		56.83 ± 2.16b	2.01 ± 0.03c	0.0113 ± 0.001 c
Group 3		31.83 ± 0.94 b	4.45 ± 0.04b	0.0084 ± 0.0006 b
Group 4		34.16 ± 1.01b	4.37 ± 0.02b	0.0089 ± 0.003 b
Group 5		33.50 ± 1.23 b	4.34 ± 0.04b	0.0095 ± 0.001 b
Group 6		36.50 ± 1.60	4.19 ± 0.03b	0.0093 ± 0.0009 b
Group 7		56.66 ± 2.14	2.13 ± 0.06b	0.0136 ± 0.006
One Way	F	57.35	625.18	378.156
ANOVA	df	6,35	6,35	6,35
	p	0.001	0.001	0.001

Values are mean ± SEM; n=6 in each group. Group-1: Control group (vehicle control), Group-2: Egg albumin sensitized, Group-3: Dexa. (5 mg \kg), Group-4: AE of VN leaves (300 mg/kg), Group-5: EAF of VN leaves (300 mg/kg), Group-6: PEF of VN leaves (300 mg/kg), Group-7: AF of VN leaves (300 mg/kg). Values in a column with different superscripts differ, P<0.001.

20mg of egg albumin was injected intravenously through tail vein. 24 hrs after the injection of Evans blue dye solution, mice were sacrificed and definite area of skin were removed and transferred to the solution of 70% acetone for 24 hrs to extract out the Evans blue dye. The Evans blue content was measured in this 70% acetone colorimetrically at 612 nm.⁷

Dexamethasone (5 mg/kg), Group 4. AE of VN leaves (300 mg/kg), Group 5. EAF of VN leaves (300 mg/kg), Group 6. AF of VN leaves (300 mg/kg), Group 7. PEF of VN leaves (300 mg/kg). The G. pigs, with the exception of the group 1 were sensitized with 1 ml of 10% egg albumin i.p. After a week of sensitization with egg albumin, group 3, 4, 5, 6 and 7 were treated with respective

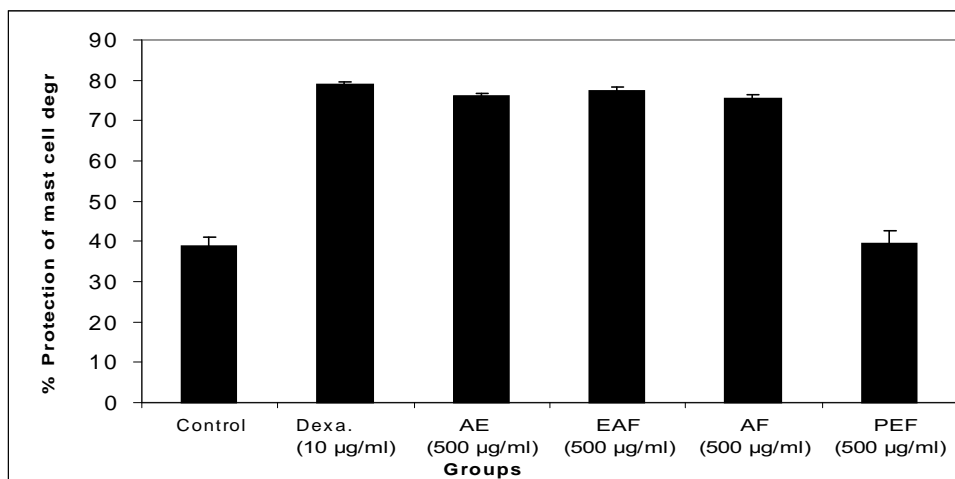


FIGURE 1. Effect of Various fractions of VN leaves on mast cell degranulation induced by compound 48/80.

* Significantly different from control at $p < 0.001$.

Each bar in the graph represents mean \pm SEM of six observations.

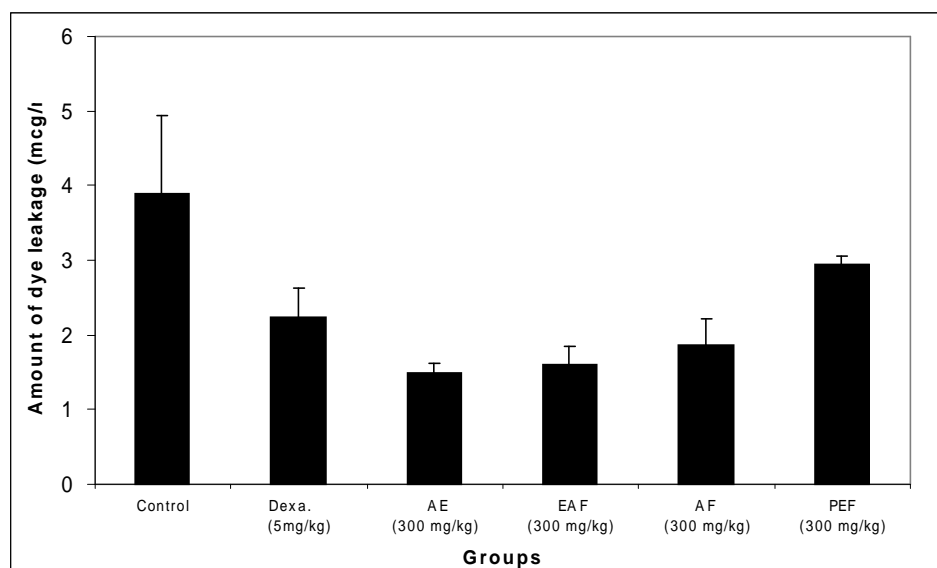


FIGURE 2. Effect of Various fractions of VN leaves on passive cutaneous anaphylaxis in mice.

* Significantly different from control at $p < 0.001$.

Each bar in the graph represents mean \pm SEM of six observations.

Egg-albumin induced asthma in guinea pigs⁸

The effect of various fractions of VN leaves in bronchoalveolar lavage was carried by dividing G. pigs in to following groups. Group 1. Control group (vehicle control), Group 2. Egg albumin sensitized, Group 3.

treatments orally for a week. Two hrs after the last dose of treatment (i.e. 15th day of sensitization) the animals were challenged with 0.5 ml of 2% egg albumin i.v. through saphenous vein. After 1 hour of challenge with egg albumin, animals of all groups were examined for serum

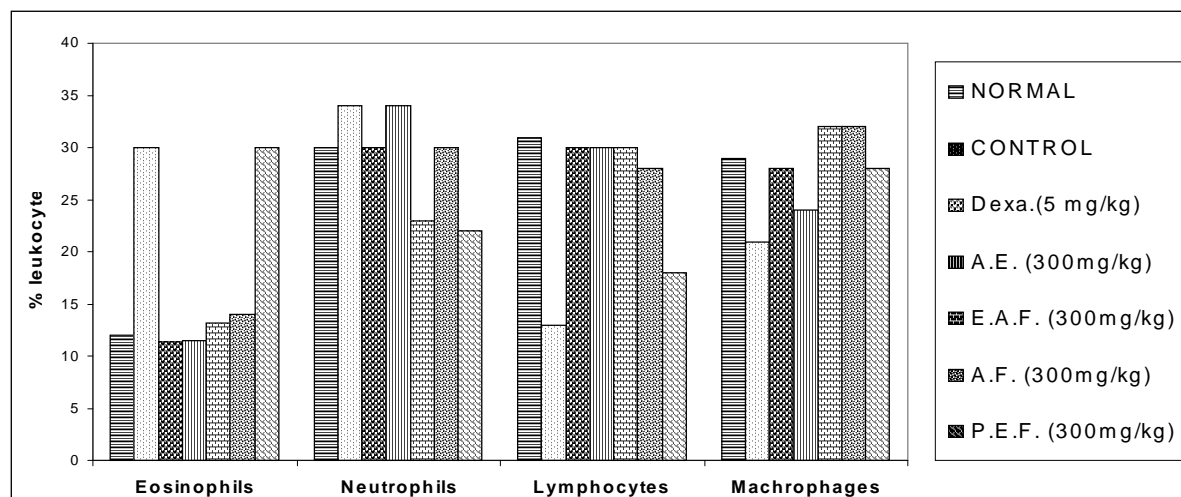


FIGURE 3. Effect of various fractions of VN leaves on differential leukocytes count of bronchial fluid in egg-albumin
* Significantly different from control at $p < 0.001$.

Each bar in the graph represents mean \pm SEM of six observations.

bicarbonate level (method described by Godkar, 1996), tidal volume (method described by Khandpur, 1996) and lung/bodyweight ratio.⁹⁻¹⁰

Three hrs post challenge, tracheo-bronchial tree was lavaged with saline to collect the bronchoalveolar lavage (BAL) by the method described in section 3.1.

BAL fluid study-differential leucocytes count.

After 3 hours of challenge with egg albumin or just prior to death of animal, the trachea-bronchial tree was lavaged with 10ml of saline by inserting cannula and the bronchoalveolar lavage fluid was collected. Centrifugation was done at 2000 rpm for 5 min. and the pellet was resuspended in 0.5 ml saline. 0.2ml of geimsa stain in buffered saline (6.8 pH) was added to it. After 5 min of adding geimsa stain the numbers of each type of leucocytes in 50 μ l BAL fluid was determined under the light microscope at 450x magnification. The result obtained was compared with unsensitized (Group 1) and untreated egg albumin sensitized (Group 2) animals.

RESULTS

Effect of Various fractions of VN leaves on mast cell degranulation induced by compound-48/80

Compound 48/80 produced significant disruption of mast cells. Ethanolic extract (AE) and ethyl acetate fraction (EAF), aqueous fraction (AF) produced lesser degranulation of mast cell (Fig: 1). AE, EAF and AF (500 μ g/ml) showed significant protection [(76.167 \pm 0.477; $p < 0.001$), (72.13 \pm 0.764; $p < 0.001$), (70.267 \pm 0.922; $p < 0.001$) respectively], when compared with positive control group [(38.833 \pm 1.212; $p < 0.001$)] (Figure 1).

Dexamethasone (Dexa.) produced similar results.

Effect of Various fractions of VN leaves on passive cutaneous anaphylaxis in mice

The anti-ovalbumin antiserum treatment showed immediate hypersensitivity reactions on the clipped dorsal skin. Pretreatment with AE, EAF and AF showed significant protection against the immediate hypersensitivity induced by egg albumin. AE, EAF and AF produced significantly lesser amount of dye leakage at 300 mg/kg [(1.4991 \pm 0.1149; $p < 0.001$), (1.5996 \pm 0.2529; $p < 0.001$) and (1.8651 \pm 0.3546; $p < 0.001$) respectively], when compared with the control group (3.8880 \pm 1.0440; $p < 0.001$). Dexamethasone produced similar results. (Figure 2).

Differential leukocytes count in BAL fluid

Significantly higher eosinophils count was observed in untreated sensitized animals (30 \pm 1.065; $p < 0.05$), when compared with normal control group (12 \pm 0.447; $p < 0.05$). Animals pretreated with AE, EAF and AF and dexamethasone showed significant lesser eosinophils count [(11.5 \pm 0.764; $p < 0.05$), (13.167 \pm 0.73; $p < 0.05$), (14 \pm 0.96; $p < 0.05$) and (11.333 \pm 0.803; $p < 0.05$) respectively], when compared with untreated sensitized animals (30 \pm 1.065; $p < 0.05$). See figure 3.

DISCUSSION

The present study was undertaken to evaluate the antiasthmatic activity of various fractions of VN leaves. VN seems to be a promising plant for treatment of bronchial asthma because of its reported immuno - modulatory and anti-inflammatory activity.³ Earlier studies

from our own laboratory have shown the effectiveness of ethanolic extract (AE) in animal models of bronchial asthma. In present study, we evaluated the petroleum ether (PEF), ethyl acetate (EAF), and aqueous (AF) fractions for their antiasthmatic activity.

Intravenous administration of egg-albumin in guinea pigs showed significantly higher serum bicarbonate level. This is mainly due to the higher carbon dioxide tension in blood which is transported as bicarbonates. AE, EAF and AF treated animals showed significantly lesser serum bicarbonate level. Due to airway obstruction, there may be low volume of air inspired or expired per breath which indicates there was sudden decreased in tidal volume. AE, EAF and AF treated animals showed significantly higher tidal volume.

The above results indicate antiasthmatic activity of AE, EAF and AF of leaves of VN. In an attempt to determine possible mechanism, we studied mast cell stability activity, effect against PCA and cellular content of BAL fluid.

A significant protection of rat mesenteric mast cells from disruption caused by compound 48/80 was observed in animals treated with AE, EAF and AF of VN leaves in our study.

It is now well known that mast cells are extensively involved in the pathophysiology of bronchial asthma. Mast cell disruption is mediated by activation of IgE antibodies. Stabilization of mast cell membrane could be one of the possible mechanisms of various fractions of leaves of VN. In mice anaphylaxis induced mortality or anaphylactic hypotension and passive cutaneous anaphylaxis are used as indicators of the level of anaphylaxis. Pretreatment with antiovalbumin antisera is reported to produce inflammation and wheals. Mediators like LTs, prostaglandins, platelet activating factors and cytokines are reported to be responsible for such inflammatory response. In our study, penetration of the dye in to the skin area of mice, where antiovalbumin antisera were injected, is an indicator of amount of inflammatory response produced. The amount of dye penetration in control animals was significantly higher, suggesting the significant extent of inflammation development due to antigen antibody reaction. The leakage of dye was significantly less in the animals treated with AE, EAF and AF of VN leaves. This can partly be due to inhibition of LTs synthesis. There was no effect of PEF on leakage of dye.

Intravenous administration of egg-albumin to guinea pigs after 14 days of antigen challenge results in selective pulmonary eosinophilia, a response that has been associated with airway hyper reactivity.^{11,12} Eosinophils

cause epithelial damage by releasing major basic protein, which may lead to increased airway reactivity, either by exposing sensory nerve endings or by removing the protective effects of an epithelial derived relaxant factor. In our study, we have used sensitized guinea pigs to demonstrate the effect of various fractions of VN leaves on eosinophils accumulation following antigen challenge. The guinea pig is well suited for such studies since airway hyper reactivity and eosinophilia can be readily demonstrated in this species. As compared with unsensitized animals, significantly higher level of eosinophils was observed in untreated sensitized animals challenged with egg albumin. Animals treated with AE, EAF and AF of VN leaves showed significantly lower level of eosinophils as compared to untreated sensitized animals. The result of this study shows that the AE, EAF and AF of leaves of VN are found to be effective in various experimental models of asthma. Stabilization of mast cells, inhibitory effects on immediate hypersensitivity reactions and antieosinophilic activity appear to be involved in its mode of action. However, isolation, characterization and pharmacological studies of active principles from active fractions of drug are needed to be done.

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