International Journal of Pharmacognosy and Pharmaceutical Research

ISSN Print: 2664-7168
ISSN Online: 2664-7176
Impact Factor: RJIF 8
IJPPR 2023; 5(1): 01-05
www.pharmacognosyjournals.com
Received: 02-11-2022
Accepted: 08-12-2022

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Phytochemical investigation and pharmacological evaluation of Anti-inflammatory activity of *Vitex negundo linn bark* extracts

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DOI: https://doi.org/10.33545/26647168.2023.v5.i1a.44

Abstract
Asthma is a disease characterized by acute exacerbation of coughing dysponea, wheezing and chest tightness. Patients usually have reduced forced expiration volume in one sec as well as reduced airflow The current study aimed to evaluate the phytochemical investigation and pharmacological evaluation of anti-inflammatory activity of *Vitex negundo linn bark* extracts. The results when compared with 0 Hr reading of same group at specific dose levels tested (200 mg/kg, 400mg/kg and 600 mg/kg), it was observed that methanolic extract of *Vitex negundo linn bark* started showing the protection after 2 hrs at 200 mg/kg and 600mg/kg whereas 400 mg/kg showed at the end of 1 hr itself and continued till the end of 5 hrs and quite comparable with that of Ibuprofen. These results are encouraging enough to pursue bioactivity-guided fractionation of this extract and structure elucidation of the active phyto constituents.

Keywords: Anti-inflammatory, bark extracts, *Vitex negundo linn*, asthama

Introduction
Asthma is a disease characterized by acute exacerbation of coughing dysponea, wheezing and chest tightness. Patients usually have reduced forced expiration volume in one sec as well as reduced airflow. Other features airway inflammation bronchial hyper responsiveness, which are not unique to the other diseases. Morbidity and mortality rates have recognized the growing seriousness of asthma in the general population in the past 20 years from 1980 to 1987 the prevalence rate of asthma in the United States increased by 29%18. Asthma is also increased in severity out the world Salter is a London physician who defined asthma in 1860 with remarkable insight as “paroxysmal dysponea of a peculiar character with intervals of healthy respiration between attacks” and recognized that these episodes were caused by contraction of smooth muscle Asthma is one of the most common disorder, encountered in clinical medicine in both children and adults characterized by inflammation of the airway that is central to airway dysfunction One of the common disease that effect mankind is a allergy, in its diverse manifestation. The Prevalence of allergy and asthma has risen in recent years despite the general health improvement in the population. The pathophysiological hallmark of asthma is the infiltration of inflammatory cells, including eosinophils, lymphocytes and macrophages. These cells release various inflammatory mediators, including histamine and cytokines (Numerous studies have also found elevated levels of histamine in the plasma of patients with asthma similar effects have been noted in the lung tissues of guinea pigs. Elevated levels of tumor necrosis factor (TNF) - α, interleukin (IL-t and IL-6 have been noted in broncho alveolar lavaged fluid from asthmatic patients after allergen challenge.

Materials and methods
Plant collection and authentication
The leaves of *Vitex negundo linn bark* were collected from the foot hills of Tirumala, Chittoor Dist. Andhra Pradesh, India. The plant species were identified and authenticated by the Botanist Dr. K. Madhava Chetty, Sri Venkateswara University, Titupathi, A.P., India. (Lr. No. BSI/SC/5/23/2006-07/Tech-2117. Dt. 05/02/2016) The collected plant material was washed, dried, powdered and stored in labelled airtight containers in a cool and dry place till

further use.

Microscopy and anatomical Studies

The required samples of different organs were cut and removed from the plant and fixed in FAA (Farmalin-5ml + Acetic acid -5 ml + 70% Ethyl alcohol-90 ml). After 24 hrs of fixing, the specimens were dehydrated with graded series of tertiary-butyl alcohol as per the schedule given by Sass, 1940. Infiltration of the specimens was carried by gradual addition of paraffin wax (Melting point 58-60oC) until TBA solution attained super saturation. The specimens were cast into paraffin blocks. The powdered plant material was subjected for cold maceration using 50% hydro alcohol for 15 days intermittent shaking. The extracts were then filtered and the filtrate was concentrated to dryness under reduced pressure and controlled temperature to yield semisolid extract and concentrated under vacuum. 5 gm of the air-dried entire plant (coarsely powder) was macerated with 100 ml of 50% v/v ethanol in a closed flask for 24 hrs. Shaking frequently during the first 6 hrs. and allowed to stand for 18 hrs. There after filtered rapidly taking precaution against the loss of ethanol. The filtrate was evaporated to dryness in a tarred flat bottom shallow dish at 105oC and weighed. The percentage of ethanol solvent extractive value with reference to air dried drug had been calculated.

Acute Anti-inflammatory studies

(a) Carrageenan-induced paw oedema in rats

Oedema was induced by injecting 0.1ml of carrageenan (1% w/v) in normal saline into the sub planter region of the left hind paw, after 1 hr of extract / standard drug administration orally. The volume of paw was measured with pleythesmometer after 1 hr, 2hr, 3hr, and 5hr of carrageenan injection. Results were determined as the percent inhibition of oedema and were compared with the control.

Acetic acid-induced vascular permeability in mice

The mice were dosed orally with the test substances suspended in 0.3% carboxy methyl cellulose solution (CMC) 30 min before the injection of 0.7% acetic acid-saline solution (i.p., 0.1 ml/10 g b.w). 4% pontamine sky blue (10 ml/kg) was also injected by tail vein after 30 minutes of acetic acid injection. After 20 min, the mice were sacrificed and then the pigment exuded to abdominal cavity was washed with 10 ml of distilled water and centrifuged (3000rpm for 10 min). The absorbance of supernatants was measured at 580 nm using UV–vis spectrophotometer. The vascular permeability effects were expressed in terms of dye amount per 30 g weight of mouse, which leaked into the peritoneal cavity. The results were compared with positive control aspirin.

Results& Discussions

Pharacognostical studies

Pharacognostical studies play an important role in the standardization of plant material. In the present study, plant *Vitex negundo linn bark* was selected based on their ethno medical uses and were authenticated. A detailed study of microscopic characters of the plant along with the photographs serves in identifying the special characters of the plant. Its extractive values, ash values and microscopical characters were determined. The various extracts of *Vitex negundo linn bark* were subjected to HPTLC studies. The various extracts of both *Vitex negundo linn bark* were subjected to HPTLC studies in order to identify the various chemical compounds present in them.

Table 1: Extractive values of *Vitex negundo linn bark*

S. No	Plant	Parts used	Solvent used	Extractive value (% W/W)
1.	*Vitex negundo linn bark*	Leaves	50% v/v ethanol	27.94

Table 2: Preliminary phytochemical analysis of *Vitex negundo linn bark*

Preliminary Phyto constituents	*Vitex negundo linn bark*
Carbohydrates	+
Alkaloids	+
Proteins & Amino acids	+
Tannins & Phenolics	+
Flavonoids	+
Triterpenoids	+
Sterols	+
Glycosides	-
Cyano lipids	+
Fixed oils	+
Gums	-
Mucilages & Saponins	+

Pharmacological studies

Acute Anti-inflammatory studies

(a) Effect of *Vitex negundo linn bark* on Carrageenan induced paw edema in rats

The effects of 50% methanolic extract of *Vitex negundo linn bark* at three dose levels (200 mg/kg, 400mg/kg and 600 mg/kg) in carrageenan induced rat paw edema was studied. The results revealed that all extracts tested at three dose levels (200 mg/kg, 400mg/kg and 600 mg/kg) showed significant protection in the acute inflammation induced by carrageenan. In particular, at 400 mg/kg both the extracts were capable of producing 83-88% protection. The onset of action in all the extracts tested was observed more than 50% at the end of 2hrs itself.

Further, the results when compared with 0 Hr reading of same group at specific dose levels tested (200 mg/kg, 400mg/kg and 600 mg/kg), it was observed that methanolic extract of *Vitex negundo linn bark* started showing the protection after 2 hrs at 200 mg/kg and 600mg/kg whereas 400 mg/kg showed at the end of 1 hr itself and continued till the end of 5 hrs and quite comparable with that of Ibuprofen.

(b) Effect of *Calotropics gigantea* on acetic acid induced vascular permeability in mice

The intraperitoneal injection of acetic acid caused squirming and increased the capillary permeability that was measured by direct estimation of plasma-bound dye (Pontamine Sky Blue) which has leaked into the peritoneal cavity. The study revealed that all extract of *Vitex negundo linn bark* (200 mg/kg, 400mg/kg and 600 mg/kg) significantly decreased the vascular permeability. Moreover, aqueous extract 600 significantly (p<0.001) decreased the vascular permeability when compared to aqueous extract 200 and 400.

Table 3: Effect of *Vitex negundo linn bark* on acetic acid induced vascular permeability in mice

Treatment	Paw Volume (ml) and % inhibition of paw edema								
	0 Hr	1 Hr		2 Hr		3 Hr		5 Hr	
Control (CMC)	0.11 ± 0.008	0.66 ±0.03###	-	0.95 ± 0.04###	-	1.18 ± 0.02###	-	1.51 ± 0.07###	-
Ibuprofen (25 mg/kg)	0.11 ± 0.010	0.21 ±0.08	66	0.17 ±0.006	80	0.15 ±0.006	85	0.13 ±0.02	93
VNB methanol extract (200 mg/kg)	0.12 ± 0.017	0.41 ±0.03#	38	0.37 ±0.03#	61	0.31 ± 0.04	74	0.23 ±0.04	85
VNB methanol extract (400 mg/kg)	0.13 ± 0.007	0.36 ±0.04#	46	0.32 ±0.03	67	0.27 ±0.02	77	0.18 ±0.02	88
VNB methanol extract (600 mg/kg)	0.13 ±0.008	0.22 ±0.02	77	0.19 ±0.006	85	0.18 ±0.02	88	0.14 ±0.02	92
VNB Aq extract (200 mg/kg)	0.19 ± 0.007	0.53 ±0.04##	21	0.47 ±0.03##	50	0.4 ±0.03#	66	0.31 ±0.03	80
VNB Aq extract (400 mg/kg)	0.13 ± 0.01	0.47 ±0.03##	30	0.41 ±0.03#	56	0.34 ±0.02	72	0.25 ±0.02	83
VNB Aq extract (600 mg/kg)	0.14 ±0.02	0.33 ±0.02	48	0.28 ±0.02	75	0.21 ±0.02	81	0.16 ±0.02	89

Table 4: Effect of *Vitex negundo linn bark* on Carrageenan induced paw edema in rats

Treatment	Paw Volume (ml) and % inhibition of paw edema								
	0 Hr	1 Hr		2 Hr		3 Hr		5 Hr	
Control (CMC)	0.11±0.008	0.66±0.03###	-	0.95±0.04###	-	1.18± 0.02###	-	1.51±0.07###	-
Ibuprofen (25 mg/kg)	0.11±0.010	0.21±0.08	66	0.17±0.006	80	0.15±0.006	85	0.13±0.02	93
VNB methanol extract (200 mg/kg)	0.12±0.017	0.41±0.03#	38	0.37±0.03#	61	0.31±0.04	74	0.225±0.04	85
VNB methanol extract (400 mg/kg)	0.13±0.007	0.36±0.04#	46	0.32±0.03	67	0.27±0.02	77	0.18±0.02	88
VNB methanol extract (600 mg/kg)	0.13±0.008	0.22±0.02	77	0.19±0.006	85	0.18±0.02	88	0.14±0.02	92
VNB Aq extract (200 mg/kg)	0.19±0.007	0.53±0.04##	21	0.47±0.03##	50	0.4±0.03#	66	0.31±0.03	80
VNB Aq extract (400 mg/kg)	0.13±0.01	0.47±0.03##	30	0.41±0.03#	56	0.34±0.02	72	0.25±0.02	83
VNB Aq extract (600 mg/kg)	0.14±0.02	0.33±0.02	48	0.28±0.02	75	0.21±0.02	81	0.16±0.02	89

Values are expressed as mean ± S.D; n=6,
#p<0.05, ##p<0.01, ###p<0.001 when compared to 0 Hr reading
One way ANOVA was applied followed by Dunnet's test

Table 5: Effect of *Vitex negundo linn bark* on acetic acid induced vascular permeability in mice

S. No	Group	Amount of P.S.B (µg/30g b.wt)
1	Normal	97.96±4.72
2	Control	247.11±12.14
3	Asprin (100 mg/kg)	114.06±6.63***
4	VNB methanol extract (200 mg/kg)	152.23±6.01***
5	VNB methanol extract (400 mg/kg)	132.85±4.66***
6	VNB methanol extract (600 mg/kg)	121.35±6.23***€€€
6	VNB AQ extract (200 mg/kg)	217.33± 8.43**
7	VNBAQ extract (400 mg/kg)	188.25± 5.78***†††
8	VNB AQ extract (600 mg/kg)	162.33±3.78***†††

Values are expressed as mean ± S.D; n=6
***p<0.001 when compared to control group
€€€p<0.001when compared to CG methanol extract 200 and 400
†††p<0.001 when compared to CG AQ extract 200 and One way ANOVA was applied followed by Turkey's multiple comparison test

Discussions

The present study demonstrates, for the first time, the immunostimulant potential of the aqueous and methanolic extract of *Vitex negundo linn bark*. The results of the *in vitro* PMN function test showed a significant increase in the percentage phagocytosis and phagocytic index for methanol and water extracts. This indicates that these extracts enhance the phagocytic efficacy of the PMN cells by causing more engulfment of the Candida cells versus control, thereby stimulating a non-specific immune response. As the methanolic extract showed promising immunostimulant activity in the *in vitro* test, it was taken up for *in vivo* animal studies. The results of *in vivo* animal studies showed an increase in the early and delayed hypersensitivity reaction to SRBC at doses of 100 mg/kg and 200 mg/kg. This indicated the stimulatory effect of these extract on chemotaxis dependent leucocyte migration. In the early hypersensitivity reaction, the antigen antibody formed immune complexes, which are known to induce local inflammation with increased vascular permeability, edema and infiltration of PMN leucocytes. The early increase in vascular permeability as well as neutrophil influx has been ascribed to the complement C 5a fragment which is activated by this immune complex. Antibody molecules which are secreted by plasma cells mediate the humoral immune response. The extracts showed an increase in the hemagglutination titer at doses of 100 mg/kg and 200 mg/kg in animal studies. This augmentation of the humoral response to SRBC indicated an enhanced responsiveness of the macrophages and T and B lymphocyte subsets involved in antibody synthesis.

The extracts probably stimulate lymphocyte proliferation, which in turn leads to production of cytokines that activate other immune cells such as B cells, antigen-presenting cells and other T cells. Studies such as the lymphocyte transformation test and cytokine studies are currently underway to understand the exact mechanism for the observed immunostimulant.

Conclusions

The methanolic extract of *Vitex negundo linn bark* was found to have a significant immunostimulant activity on

both the specific and non-specific immune mechanisms. These results are encouraging enough to pursue bioactivity-guided fractionation of this extract and structure elucidation of the active phytoconstituents.

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