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Research Article

Anti-arthritic effect of ethanolic extract of leaves of *Vitex negundo* Linn., (Verbenaceae) in male albino Wistar rats

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ABSTRACT

To determine the anti-arthritic effect of ethanolic extract of leaves of *Vitex negundo* Linn., (Verbenaceae) in male albino Wistar rats using Freund's complete adjuvant model. The plant was collected in agricultural college, Madurai and preliminary phytoconstituents were identified by chemical analysis. The fatty materials from the leaves were extracted with petroleum ether by hot percolation method. The dried leaves were extracted with ethanol and used for the pharmacological experiments. The male Wistar rats were used for the chronopharmacological and anti-arthritic study. The arthritis was induced by Freund's complete adjuvant (FCA) and anti-arthritic effect of ethanolic extract of leaves of *Vitex negundo* Linn (VNE), was studied at 100, 250 and 500 mg/kg dose levels. At the end of the study, the liver function test and radiological examination were carried out to assess the anti-arthritic effect of the VNE. VNE at 250 and 500 mg/kg showed significant inhibition of FCA-induced arthritis in evening time drug administered group. The animals which received VNE at evening time showed significant anti-arthritic effect than morning treated animals. VNE-treated animals showed significant ($p<0.05$) increase in body weight. Present study concluded that the leaves extracts of *Vitex negundo* showed significant anti-arthritic activity against Freund's complete adjuvant-induced arthritis in male Wistar rats.

Keywords: *Vitex negundo;* Freund's complete adjuvant; arthritis; anti-arthritic activity.

INTRODUCTION

Arthritis is one of the most common diseases in old geriatric people and occurs in different forms. The most common form is osteoarthritis (degenerative joint disease) which results in trauma and infection in the joints. Arthritis is a major problem in oldage people and it needs chronic treatment with analgesics. The chronic treatment may cause adverse effects and that may increase further complications. Hence we need some alternative system of natural medicine of to treat arthritis. *Vitex negundo* Linn. (Verbenaceae) is widely distributed in the region of south Asia and it has anti-inflammatory, antibacterial, antifungal and analgesic properties. This plant is used in the treatment of superficial bruises, injuries, sores, and skin infections. The present study was planned to assess the chronopharmacological and anti-arthritic activities of leaves extract of *Vitex negundo* Linn. (Verbenaceae) compared to indomethacin.

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MATERIALS AND METHODS

Taxonomically identified leaves of *Vitex negundo* Linn. (Verbenaceae) were collected from Agricultural college, Madurai during August 2009. The plant was certified by Botanist, American college, Madurai. The plant was identified as aromatic large shrub or small tree of about 3m in height with quadrangular branches, leaves opposite, extipulate, long petioled and digitately 3-5 folioate, all leaflets with petiolules, the middle one longer, flower bluish purple in panicles upto 30cm long, fruits globose or ovoid of obovoid four seeded drupe, black when ripe.

Animals: Male Wistar rats were housed under 22 ± 2° C temperature, 40-60 % humidity and 12-12 ± 1 h light dark cycle. The animals were fed with standard rodent chow (Hindustan liver limited, Bangalore, India) and water *ad libitum*. The animals were maintained as per the norms of CPCSEA approved guidelines and the experimental protocol were approved by IAEC (UCP/IAEC/2009/042).

Extraction of the leaves of *Vitex negundo*: The collected leaves were washed with running water and allowed for natural drying under the shade. The leaves were powdered and packed in the soxhlet apparatus, extracted with petroleum ether about 50°C for 72 h using a hot percolation method. The petroleum ether extract was filtered, concentrated and the solid marc

was again extracted with 95% ethanol at 70°C using hot percolation method for 72h.

Phytochemical evaluation: One gram of the petroleum ether and ethanolic extract of *Vitex negundo* leaves (VNE) was dissolved in 100ml of its own mother solvent to obtain a 1% w/v stock solution. The stock solution was analysed for presence of the carbohydrates, proteins, sterols, alkaloids, tannins, glycosides, flavonoids, phenolic compounds and saponins.

Anti-arthritic activity of ethanolic extract of *Vitex negundo* leaves (VNE): The chronopharmacological (morning administration Vs evening administration) and anti-arthritic effect of leaves extract of *Vitex negundo* was studied using rat model. Male Wistar rats were divided in to six groups, each containing six animals. In each group, half of the animals received test drug in morning hours and remaining animals received in evening hours. Finally, the chronopharmacological effect of ethanolic extract of *Vitex negundo* leaves was compared within the group and control animals. The animals were divided as follows,

Group 1: Vehicle control (0.5ml 1% w/v Tween 80)

Group 2: Arthritis control

Group 3: Indomethacin 10mg/kg

Group 4: VNE 100mg/kg

Group 5: VNE 250mg/kg

Group 6: VNE 500mg/kg

The extract was suspended in 1% w/v Tween 80 solution on the day of the experiment and administered orally. The dose of the VNE was selected based on previous study reports (Tandon *et al.,* 2006; Tandon *et al.,* 2008).

Arthritis was induced by Frenaud's complete adjuvant (FCA) (Difco labs, USA) and this method was described by Newbould. Adjuvant arthritis was induced by subcutaneous injection of FCA (0.1ml) into subplantar tissue of the right hind paw of each rat. The test group consisted of FCA injected rats challenged with the respective doses of the test drugs administered orally 24h before FCA injection, while, the control rats were injected with 0.1ml of liquid paraffin (incomplete freund's adjuvant) only. The drug treatment was continued daily in the same time after the challenge for 20 more days. The swelling in the injected and contralat-

Table 1: Effect of VNE on FCA induced arthritis male albino Wistar rat

Drug administration time	Treatment / Days	Swelling volume(ml) ±SEM on injected paw							
		Post insult time of assay in days							
		7	9	11	13	15	17	19	21
Morning administration	Control	0.10 ±0.00	0.10 ±0.00	0.10 ±0.00	0.10 ±0.00	0.10 ±0.00	0.10 ±0.00	0.10 ±0.00	0.10 ±0.00
	Arthritis	1.19 ±0.00	0.93 ±0.00	0.85 ±0.00	0.85 ±0.00	0.86 ±0.00	0.86 ±0.00	0.88 ±0.00	0.91 ±0.00
	Indomethacin 10mg/kg	0.79 ±0.00	0.79 ±0.00	0.67 ±0.00	0.73 ±0.00	0.74 ±0.00	0.63 ±0.00	0.62 ±0.00	0.57 ±0.00
	VNE 100mg/kg	1.13 ±0.01	0.90 ±0.00	0.81 ±0.01	0.83 ±0.00	0.83 ±0.01	0.84 ±0.00	0.87 ±0.00	0.89 ±0.00
	VNE 250 mg/kg	0.88 ±0.00	0.89 ±0.00	0.7 ±0.00	0.66 ±0.01	0.69 ±0.01	0.73 ±0.01	0.67 ±0.00	0.75 ±0.01
	VNE 500 mg/kg	0.86 ±0.00	0.83 ±0.00	0.80 ±0.00	0.68 ±0.00	0.74 ±0.00	0.53 ±0.00	0.51 ±0.00	0.70 ±0.00
Evening administration	Control	0.10 ±0.00	0.10 ± 0.00	0.10 ±0.00	0.10 ±0.00	0.10 ±0.00	0.10 ±0.00	0.10 ±0.00	0.10 ±0.00
	Arthritis	1.18 ±0.00	0.92 ±0.00	0.84 ±0.01	0.85 ±0.00	0.85 ±0.00	0.86 ±0.01	0.88 ±0.00	0.91 ±0.01
	Indomethacin 10mg/kg	0.73 ±0.00**	0.74 ±0.01**	0.74 ±0.01**	0.61 ±0.02**	0.67 ±0.01**	0.56 ±0.01**	0.54 ±0.01**	0.50 ±0.00**
	VNE 100 mg/kg	1.16 ±0.00	0.91 ±0.00	0.83 ±0.00	0.83 ±0.00	0.84 ±0.00	0.86 ±0.00	0.88 ±0.00	0.91 ±0.00
	VNE 250 mg/kg	0.93 ±0.00*	0.81 ±0.02*	0.68 ±0.00*	0.69 ±0.00*	0.65 ±0.00*	0.77 ±0.010*	0.69 ±0.0*	0.70 ±0.00*
	VNE 500 mg/kg	0.78 ±0.00**	0.74 ±0.01**	0.71 ±0.01**	0.74 ±0.01**	0.7 ±0.00**	0.58 ±0.00**	0.56 ±0.00**	0.55 ±0.01**

Values are expressed as Mean ± SEM, n =3 rats in each group.

*P < 0.01, **P < 0.001, ns means not significant compared to morning administration

eral hind paw were monitored daily using mercury displacement plethysmometer. Increase in the extent of erythema and edema of the tissues shows the severity of the inflammation. The difference between the experimental groups and arthritis control group were analysed. The changes in body weight were recorded daily (Castell et al., 1988).

Biochemical analysis: End of the experiment, blood was withdrawn from all the groups of the animals through retro-orbital plexus puncture. Haemoglobin (Hb), red blood cells (RBC), differential count (DC) and white blood cells (WBC) count, erythrocyte sedimentation rate (ESR) were estimated immediately after blood collection. The plasma SGOT, SGPT, ALP and total protein levels were analysed by Auto analyser using auto pack kids (Mumbai). The C-reactive protein (CRP) levels were estimated using the ELISA kit obtained from Alpha diagnostics Intl., USA and copper levels was estimated using colorimetric Bathocuproin disulfonate method (Gao *et al.,* 2008; Kaneria *et al.,* 2007).

Radiographic analysis: On the 20th day of the experiment animals were anesthetized with diethyl ether and placed in digital x-ray machine for the radiographic analysis of the knee joints. X- ray was taken at the knee joints for the confirmation and evaluation of the severity of arthritis in FCA induced rats (Yu et al., 2002; Woode et al., 2008).

Statistical analysis: The data was expressed as mean ± SEM. Statistical comparisons were performed by Student's t-test comparison with morning administration and P <0.05 was considered as significant.

RESULTS

The yields of the petroleum ether and ethanol extracts were 6% w/v and 7.5% w/v, respectively. The petroleum ether extract showed the presence of sterols, flavanoids and ethanolic extract showed presence of reducing sugars, proteins, amino acids, sterols, alkaloids, tannins, glycosides, flavanoids, saponins, phenolic compounds.

The latency of secondary response occurred after few days. The response was characterised by joint swelling and nodule which was evident on the 7th day. The administration of VNE (250mg/kg) significantly (p<0.01) protected against joint swelling in arthritis-induced paw when compared with arthritis control group and morning administration of VNE. But the significant reduction was observed from day 11 to 13 in the VNE

Table 2: Effect of VNE on secondary response of FCA induced arthritis rat

Drug administration time	Treatment Days	Swelling volume(ml) ±SEM on Non injected paw							
		Post insult time of assay in days							
		7	9	11	13	15	17	19	21
Morning administration	Normal	0.12 ±0.00	0.10 ±0.00	0.11 ±0.00	0.10 ±0.00	0.10 ±0.00	0.10 ±0.00	0.10 ±0.00	0.10 ±0.00
	Arthritis	0.34 ±0.00	0.43 ±0.00	0.51 ±0.00	0.74 ±0.01	0.91 ±0.00	0.89 ±0.01	0.95 ±0.00	1.64 ±0.00
	Indomethacin 10mg/kg	0.27 ±0.00	0.27 ±0.00	0.33 ±0.00	0.49 ±0.01	0.53 ±0.01	0.63 ±0.01	0.58 ±0.01	0.65 ±0.00
	VNE 100 mg/kg	0.31 ±0.00	0.42 ±0.00	0.47 ±0.01	0.68 ±0.00	0.80 ±0.00	0.82 ±0.00	0.90 ±0.00	0.96 ±0.00
	VNE 250 mg/kg	0.27 ±0.00	0.40 ±0.00	0.47 ±0.00	0.65 ±0.01	0.71 ±0.01	0.73 ±0.01	0.82 ±0.01	0.93 ±0.00
	VNE 500 mg/kg	0.31 ±0.00	0.36 ±0.00	0.42 ±0.01	0.62 ±0.00	0.68 ±0.00	0.68 ±0.00	0.71 ±0.00	0.78 ±0.00
Evening administration	Normal	0.12 ±0.00	0.10 ±0.00	0.11 ±0.00	0.11 ±0.00	0.11 ±0.00	0.11 ±0.00	0.11 ±0.00	0.10 ±0.00
	Arthritis	0.34 ±0.00	0.42 ±0.00	0.50 ±0.00	0.74 ±0.00	0.90 ±0.00	0.88 ±0.00	0.95 ±0.00	1.60 ±0.00
	Indomethacin 10 mg/ kg	0.22 ±0.00**	0.23 ±0.00**	0.27 ±0.00**	0.44 ±0.00**	0.48 ±0.00**	0.58 ±0.00**	0.67 ±0.00**	0.61 ±0.00**
	VNE 100 mg/kg	0.31 ±0.00	0.41 ±0.00	0.48 ±0.00	0.69 ±0.00	0.78 ±0.01	0.83 ±0.01	0.91 ±0.00	0.96 ±0.01
	VNE 250 mg/kg	0.31 ±0.01*	0.37 ±0.00*	0.44 ±0.00*	0.60 ±0.01*	0.68 ±0.00*	0.68 ±0.01*	0.78 ±0.00*	0.90 ±0.00*
	VNE 500 mg/kg	0.22 ±0.01**	0.3 ±0.00**	0.37 ±0.01**	0.53 ±0.01**	0.61 ±0.00**	0.60 ±0.00**	0.66 ±0.00**	0.73 ±0.00**

Values are expressed as Mean ± SEM, n =3 rats in each group.

*P < 0.01, **P < 0.001, ns means not significant compared to morning administration

Table 3: Biochemical changes in VNE on FCA induced arthritis male albino Wistar rat biochemical estimation

Group		Normal control	Arthritis control	Indomethacin 10mg/kg	VNE 100mg/kg	VNE 250mg/kg	VNE 500mg/kg
Morning administration	SGOT (U/L)	105.40 ±0.26	236.56 ±1.01	127.76 ±08	230.60 ±0.65	185.50 ±2.94	146.53 ±0.73
	SGPT (U/L)	55.56 ±0.89	156.16 ±0.41	90.86 ±0.22	153.60 ±0.25	131.96 ±1.15	111.83 ±1.18
	ALT (U/L)	176.80 ±2.52	285.93 ±2.71	233.70 ±1.06	279.43 ±0.35	259.20 ±0.55	247.06 ±0.79
	Total protein (U/L)	7.91 ±0.021	11.33 ±0.03	8.60 ±0.05	11.13 ±0.09	10.40 ±0.06	9.90 ±0.057
	Serum copper (µg/dL)	105.76 ±1.91	188.46 ±7.39	121.30 ±1.06	181.33 ±0.35	142.23 ±0.99	133.56 ±0.84
Evening administration	SGOT (U/L)	105.86 ±1.28	236.23 ±20.70	121.10 ±0.63**	230.83 ±0.78	169.30 ±4.40*	141.26 ±0.52**
	SGPT (U/L)	55.66 ±1.45	156.26 ±0.68	83.53 ±0.87**	153.30 ±0.47	126.66 ±1.52*	105.33 ±0.75**
	ALT (U/L)	176.33 ±0.57	286.33 ±2.48	226.00 ±1.15**	279.10 ±1.39	252.86 ±1.47*	240.46 ±0.47**
	Total protein (U/L)	7.96 ±0.033	11.46 ±0.03	8.00 ±0.10**	11.10 ±0.10	10.10 ±0.05*	8.60 ±0.25**
	Serum copper (µg/dL)	106.66±1.85	188.3 ±8.21	115.70 ±0.40**	181.00 ±1.53	136.80 ±0.80*	128.43 ±0.33**

Values are expressed as Mean ± SEM, n =3 rats in each group.

*P < 0.01, **P < 0.001, ns means not significant compared to morning administration

(250mg/kg) treated group. However the effect of 500mg/kg treatment was found to be significant (p<0.001) from the initial stage of secondary response and maintained throughout the experiment and shows P<0.01 level of significance during 15 to 19 days after FCA injection as that of the group treated with the reference standard indomethacin. The summary of the anti-arthritic effect of VNE at 100, 250 and 500 mg/kg was summarised in table 1 and 2. The influence of the biochemical and haematological parameters were shown in table 3 and 4, respectively.

Treatment with VNE extract showed significant (p<0.05) increase in body weight as that of vehicle treated group (control) and morning administered groups of indomethacin and VNE treated groups. Biochemical parameters including SGOT, SGPT, ALP, CRP, copper and total protein levels were significantly inhibited while administration of *Vitex negundo* and indomethacin in evening administration compared to morning administration. Arthritic rats exhibited a reduced RBC count, reduced Hb level and an increased ESR and VNE treated group improved the RBC count, Hb level and the ESR. The WBC count was increased in arthritic group and the migration of leukocytes is significant decrease in the WBC count. Haematological parameters like Hb, RBC, WBC, lymphocytes, neutrophils, ESR did not shown any significant difference between evening and morning administration of the indomethacin as wells as *Vitex negundo* treated groups.

The clinical analysis (radiological analysis) of rheumatoid arthritis allows therapeutic monitoring which remains the standard method for evaluating the disease progress. The loss of articular cartilage leads to diminished joint space, which may be brought by a variety of pathological mechanism were analysed by radiological examination. The degree of bone resorption, diminished joint space and tissue swelling were markedly reduced in VNE (250 mg/kg and 500 mg/kg) treated groups.

DISCUSSION

VNE 250 mg/kg and 500mg/kg, (evening administration) showed significant (p<0.001) anti-arthritic effect against FCA-induced arthritis. FCA injection on rat hind paw showed a pronounced swelling, and hyperalgesia appeared without the involvement of contralateral paw. This response is usually considered as a primary reaction. There is also a delayed hypersensitivity response which is considered as latent secondary systemic response known to induce arthritis which occurs after few days on the contralateral paw and characterized by tibiotarsal joint swelling and nodule formation in the experiment. The secondary response could be due to the liberation and overproduction of bradykinin, prostaglandins and kinins in paw tissue, which accompanies leukocyte migration (Winder *et al.,* 1969). *Vitex negundo* extract showed significant (p<0.05) increase in body weight and adjuvant arthritis is characterized by reduced weight loss and body weight loss is associated with increased production of pro-inflammatory

Table 4: Haematological changes in VNE on FCA induced arthritis male albino Wistar rat biochemical estimation

Group		Normal control	Arthritis control	Indomethacin 10mg/kg	VNE 100mg/kg	VNE 250mg/kg	VNE 500mg/kg
Morning administration	WBC cells/cu.mm	7.34 ±0.00	7.87 ±0.00	7.02 ±0.01	7.84 ±0.00	7.26 ±0.00	7.15 ±0.00
	RBC cells/cu.mm	4.81 ±0.05	3.73 ±0.10	4.64 ±0.03	3.75 ±0.07	4.58 ±0.03	4.60 ±0.01
	ESR mm/h	3.32 ±0.00	7.05 ±0.00	4.06 ±0.03	7.0 ±0.03	5.1 ±0.05	4.56 ±0.03
	Hb gm/dl	12.65 ±0.08	8.91 ±0.00	11.70 ±0.05	8.6 ±0.30	9.5 ±0.05	10.16 ±0.03
	Lymphocytes %	50.0 ±0.57	56.66 ±2.18	34.2 ±0.24	34.56 ±0.26	30.3 ±0.32	53.6 ±0.34
Evening administration	WBC cells/cu.mm	7.33 ±0.00	7.87 ±0.00	7.03 ±0.01	7.85 ±0.00	7.26 ±0.00	7.16 ±0.00
	RBC cells/cu.mm	4.76 ±0.04	3.73 ±0.08	4.64 ±0.01	3.85 ±0.02	4.61 ±0.00	4.63 ±0.01
	ESR mm/hr	3.31 ±0.00	7.05 ±0.00	4.13 ±0.06	7.0 ±0.003	5.16 ±0.03	4.53 ±0.03
	Hb gm/dl	12.62 ±0.06	8.91 ±0.01	11.73 ±0.08	8.63 ±0.26	9.43 ±0.03	10.13 ±0.01
	Lymphocytes %	49.33 ±0.66	55.0 ±1.73	35.5 ±0.40	35.46 ±0.26	31.46 ±0.13	55.2 ±0.25

Values are expressed as Mean ± SEM, n =3 rats in each group.

cytokines such as TNF-α and interleukin (Roubenoff and Freeman 1997; Campo and Avenoso 2003).

In the present study, the arthritic rats exhibited a decreased RBC count, reduced Hb level and an increased ESR. ESR is an estimate of the suspension stability of RBC's in plasma. It is related to the number and size of the red blood cells and to the relative concentration of plasma proteins, especially fibrinogen and β globulins. Increase in the ESR rate is an indication of active but obscure disease processes. The treatment with the VNE improved the RBC count, Hb level and ESR to near normal level indicating the significant recovery from the anemic condition and arthritic progress and thus confirms it has potential role in the recovery of arthritic conditions. In arthritic conditions, there is a mild to moderate rise in WBC count due to release of IL-IB. IL-IB increases the production of both granulocyte and macrophages colony stimulating factor (Mowat 1971)

Ceruloplasmin, an enzyme synthesised in the liver contains eight atoms of copper in its structure. Free copper ions are powerful catalysts of free radical damage. By binding copper, ceruloplasmin prevents free copper ions from catalyzing oxidative damage. An increase copper ions concentration indicates the exact of inflammatory conditions. The arthritic rats exhibited significantly elevated copper levels, which were suppressed in VNE and indomethacin treated rats.

The radiographic analysis of the knee joint in arthritic and drug treated animals further supported and confirms the potent anti-arthritic effect of VNE in a dose dependent manner which suppress the pathological changes, such as pannus formation and bone destruction.

FCA-induced arthritis used to study pathogenesis of rheumatoid arthritis for testing therapeutics (Mizushima *et al.,* 1972). One of the reasons for the wide utilization of this model is due to the strong correlation between the efficacy of therapeutic agents in this model and in rheumatoid arthritis in humans and it is characterized by very rapid erosive disease. In adjuvant arthritis, bacterial peptidoglycans and muramyl dipeptide are responsible for arthritis induction (Hunneyball *et al.,* 1986; Crofford *et al.,* 1982)

Paw swelling is one of the major factors in assessing the degree of inflammation and curative efficacy of drugs. It occurs through cell mediated-autoimmunity by structural mimicry between mycobacteria and cartilage proteoglycons in rats (Vijayalaksmi *et al.,* 1997). In the present study, rats were selected to induce arthritis because rats develop a chronic swelling in multiple joints with influence of inflammatory cells, erosion of joint cartilage and bone destruction (Singh and Majummdar 1996). There are also reports on the flavonoid vitexin as a potent anti-inflammatory agent; vitexin may exert its anti-inflammatory activity by inhibiting the 5- lipoxygenase pathway, which together with the COX-2 pathway, is very important in producing and maintaining inflammation (Gautam *et al.,* 2008; Achari *et al.,* 1984; Bhargava *et al.,* 1989).

CONCLUSION

The present study was concluded that the *Vitex negundo* exert its potent anti-arthritic activity by signifi-

cantly altering the pathogenesis during arthritic without exerting any side effect in Freund's complete adjuvant induced arthritis in male Wistar rats.

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