Anti-inflammatory Activity and Mechanism of Action of *Vitex negundo* Linn

1Vishal Ramprakash Tandon and 2Rajesh Kumar Gupta
1Post Graduate Department of Pharmacology and Therapeutics, Government Medical College Jammu (J and K) India-180001
2Department of Pharmacology, Mahatma Gandhi Institute of Medical Sciences (Sevagram), Wardha, Maharashtra, India

Abstract: To evaluate the anti-inflammatory activity of *Vitex negundo* (VN) leaf extract and its mechanism of action in experimental animals. Carrageenin induced hind paw edema and cotton pellet granuloma test in albino rats were employed to study the anti-inflammatory activity of *Vitex negundo* (VN) leaf extract. The mechanism of anti-inflammatory action was explored by observing its effect on oxytocin induced contractions in rat uterus and oxidative stress. VN extract was administered orally in graded doses (100, 250 and 500 mg kg\(^{-1}\)) as single dose therapy and twice daily for 7 days in respective inflammatory experimental models. The effects were compared with phenylbutazone (100 mg kg\(^{-1}\)) orally in carrageenin induced hind paw edema method and ibuprofen (200 mg kg\(^{-1}\).B.DX 7 days) orally in cotton pellet granuloma test as standard controls, respectively. The test drug showed significant anti-inflammatory activity in dose dependant manner in both experimental models. VN inhibited oxytocin induced contractions of rat uterus and plasma MDA (malondialdehyde) levels significantly. These observations suggest that VN possesses anti-inflammatory activity against acute as well as sub-acute inflammation, which appear to be due to prostaglandin inhibition and reduction of oxidative stress respectively, which needs to be substantiated by further study.

Key words: *Vitex negundo*, anti-inflammatory, oxidative stress, prostaglandin carrageenin, cotton granuloma, malondialdehyde

INTRODUCTION

*Vitex negundo* (VN) Linn (verbenaceae), a large aromatic shrub with typical five foliate leave pattern, found throughout the greater part of India at warmer zones and ascending to an altitude of 1500 m in outer, Western Himalayas. It has been claimed to possess many medicinal properties (Tandon, 2005). Although, leaves of VN have been investigated for its anti-inflammatory activity in past (Telang et al., 1999; Juna et al., 1999; Singh, 1978; Sharma and Singh, 1980; Dharmasiri et al., 2003). But it was only Telang et al. (1999) who first noticed NSAID’s like activity of VN. Similarly, fresh leaves of VN have been suggested to possess anti-inflammatory and pain suppressing activities possibly mediated via PG synthesis inhibition, antihistamine, membrane stabilising and antioxidant activities (Dharmasiri et al., 2003). On the contrary, Sahni et al., have shown VN to possess anti-ulcer activity against piroxicam (NSAID’s) induced ulcers, probably by increasing PG levels. As, there are conflicting reports for the mechanism of anti-inflammatory activity of VN and it yet remain to be clearly explored against acute and sub-acute inflammation. Moreover very few reports (Dharmasiri et al., 2003) are available who have tried to explore the mechanism of action of VN anti-inflammatory activity in past.

Therefore, the present study was undertaken to investigate anti-inflammatory activity and mechanism of action of ethanolic leaf extract of VN Linn., per orally.

MATERIALS AND METHODS

The plant material and preparation of extract: The plant was collected from local area of Sevagram in the month of June, 2002. It was identified and authenticated by an expert (botanist) from J.B College of Science, Sevagram (Wardha) Maharashtra, India. The fresh leaves of VN were cleaned of extraneous matter, shade dried and powdered. The powder was macerated for 24 h in 70% V/V ethanol. Then it was subjected to percolation by using 70% V/V ethanol as solvent. The menstrum collected was again shade dried and viscous extract suspended in 1% gum acacia for the present study. Total yield of extract was 11.5%.

Corresponding Author: Dr. Vishal Ramprakash Tandon, Plot No. S/B, Near Arya Samaj, Bakshinagar, Jammu-J and K-180001
Tel: 91-0191-2543546 Mobile: 09419195126

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Animals: Albino rats of either sex (weight 125-180 g) of wistar strain, procured from National Institute of Nutrition, Hyderabad, India were used for the present study. The clearance for the use of animals for experimental purpose was obtained from institutional ethical committee constituted for the purpose at M.O.I.M.S, Sevagram, (Wardha). Animals were housed in poly- propylene cages (4 per cage) with dust free rice husk as a bedding material under laboratory conditions with control environment of temperature 22±3°C, humidity (60±10%) and 12 h light/dark cycle (6.00-18.00). They were fed ad libitum with rodents chow and free access to drinking water. Before subjecting them to experimentation, the animals were given a week’s time to get acclimatized with laboratory conditions. The animals were fasted overnight before the experiment.

Drugs
Test drug: Ethanolic leaf extract of VN in the form of suspension was fed orally in a volume of 10 mL kg⁻¹ wt. in doses of 100, 250 and 500 mg kg⁻¹ of body weight in both the experimental models. For mechanism of anti-inflammatory action, VN in the dose (500 mg kg⁻¹, p.o), which produced maximum anti-inflammatory action was used to study effect of test drug on oxidative stress whereas, it was used in dose concentration ranging from (0.5-2.5 mg mL⁻¹) to study its interaction with oxytocin in rat uterus preparation.

Dose selection of test drug was based on previously reported studies (Telang et al., 1999; Jana et al., 1999; Tandon and Gupta, 2005), preliminary trial carried out in our laboratory over a dosage range of VN varying from (5-500 mg kg⁻¹, p.o) and oral LD₅₀ dose of VN leaf extract (7.8 g kg₁ b.w) in one of our previously reported study (Tandon and Gupta, 2004).

Phenylobutazone: (Ciba Geigy), was used as a 2% suspension in gum acacia in doses of 100 mg kg⁻¹ orally as standard drug.

Ibuprofen: (Boots Company, India), was used as a 2% suspension in gum acacia in doses of 200 mg kg⁻¹ B.D.X 7 days, orally as standard drug.

Oxytocin (Sandoz) and oestradiol valerate (Sigma organon) were also used for the present study. Oxytocin was dissolved in distilled water to get a desired concentration.

Chemicals: Carrageenin (Analar, BDH), for rat hind paw edema method, Malondialdehyde (Sigma Chemicals), Trichloroacetic acid 20% solution, Thiobarbituric acid (Loba chemia India), Sulphuric acid 0.05 M, Sodium Sulphate Solution: 2 M, N-butyl alcohol were used for MDA estimation and Dejalon’s physiological salt solution (Dejalon’s et al., 1945) was used for rat uterus preparation.

Experiment design and drug treatment
For anti-inflammatory activity: The animals were divided into five groups with each group consisting of ten animals. Group I received distilled water and served as control. Group II, III and IV were administered three graded doses of test drug i.e., 100, 250 and 500 mg kg⁻¹ orally as single dose therapy in carrageenin induced rat hind paw edema method and twice daily for seven consecutive days in cotton pellet granuloma test. Group V received phenylbutazone (100 mg kg⁻¹ orally) and Ibuprofen (200 mg kg⁻¹ B.D. X 7 days, p.o) as standard drugs in respective experimental models for comparison.

For mechanism of anti-inflammatory activity: The effects of VN in dose concentration ranging from (0.5-2.5 mg mL⁻¹) were studied on oxytocin induced contractions in rat uterus. Whereas, to study the effect of test drug on oxidative stress by MDA level estimation. The animals from cotton granuloma test were used in four groups with ten animals in each group. Group I of cotton granuloma test receiving distilled water served as diseased control. Groups of cotton granuloma test receiving maximal dose of test drug (500 mg kg⁻¹ B.D. X 7 days orally) and Ibuprofen (200 mg kg⁻¹ B.D. X 7 days p.o) served as Group II and III, respectively. Whereas, Group IV consisted of healthy normal control for baseline MDA levels. The results were compared with Group I.

Two methods were employed to evaluate the anti-inflammatory activity as well as potentiation effect of phenylbutazone and Ibuprofen anti-inflammatory action by VN.

Carrageenin induced rat hind paw edema method: Used by Winter et al. (1962) was used to study the effect of test drug on acute phase of inflammation. The paw volume was measured first at half an hour and then at 1, 3, 5 and 6 h after administration of drugs. The reduction in the volume displacement of hind foot in comparison to control was taken as anti-inflammatory effect.

Cotton Pellet granuloma test: Used by Meir et al. given in Ghosh (1984). Fundamentals of Experimental Pharmacology was followed to screen the effect of drugs on exudative and proliferative phases of inflammation by forming the granuloma pouch. The difference in wet and dry weights of granuloma from control group to that of treated group indicates the anti-inflammatory activity.

For mechanism of action: Effect of test drug on oxytocin induced rat uterus contractions described by Dejalon et al. (1954) and Perry (1970) was carried out in the present study.
The response of uterine tissue to oxytocin (0.01 IU mL⁻¹) before and after the incubation of tissues with the extract of VN (0.5-2.5 mg mL⁻¹) recorded with contact period of 5 min on a smoked drum. Each response was recorded for 30 sec. Effects of test drugs on oxytocin induced rat uterus contractions were compared to original response of oxytocin in term of height in cms, which served as control. The experiment was repeated for ten times.

Effect of VN on oxidative stress in sub-acute inflammatory model.

**Plasma MDA (Malondialdehyde) estimation:** After 7 days drug treatment in cotton pellet granuloma method, 3-5 mL of blood was collected from the anterior part of eye from each animal using capillary tube, in a vial containing EDTA as an anticoagulant. Plasma was separated by centrifugation at 3000 rpm for 10 min. It was stored at -20°C and used to estimate MDA levels. The method of Sotoli, 1978 using thiobarbituric acid reaction was used for it. The reduced level of MDA was taken as indicator of anti-lipoperoxidative activity, which can be taken as index of reduced oxidative stress.

**Statistical Analysis:** All data were expressed as mean±SEM. The results were analyzed by one-way analysis of variance (ANOVA) followed by post hoc Dunnett’s multiple comparison test, using spss software (version 2.0, jandel scientific Inc. USA). Differences between means were considered to be significant at p<0.05.

**RESULTS**

In carrageenin induced hind paw edema method, the oral administration of VN in graded doses (100, 250 and 500 mg kg⁻¹) produced significant reduction in paw volume in dose dependent manner in comparison to control. The maximum effect was seen in the dose of 500 mg kg⁻¹ orally which showed significant (p<0.001) reduction as 39% in paw volume in comparison to control. The anti-inflammatory activity in this dose of the test drug was comparable to phenylbutazone (100 mg kg⁻¹ P.O). The maximum anti-inflammatory action was observed in 3 h in all the doses of test drug. The action started at half an hour except with 100 mg kg⁻¹ dose of the test drug, where it was delayed to 1 h. The anti-inflammatory effect persisted for varied time as 3, 5 and 6 h in different doses (100, 250 and 500 mg kg⁻¹), respectively (Table 1).

In cotton pellet granuloma test VN extract in graded doses (100, 250 and 500 mg kg⁻¹, B. DX 7 days, orally) showed significant reduction of both wet as well as dry weights of granuloma in dose dependent manner, when compared to control with maximum effect in 500 mg kg⁻¹ dose of the test drug showing 55.7 and 60% reduction of wet and dry weights of granuloma respectively. The anti-inflammatory activity in this dose was also comparable with Ibuprofen (250 mg kg⁻¹ B. DX 7 days, orally) as shown in Table 2.

In the method studied for mechanism of anti-inflammatory activity, against acute inflammation, the VN extract in different dose concentration (0.5, 1, 1.5, 2 and 2.5 mg mL⁻¹) showed significant inhibition of height of rat uterus contraction induced by oxytocin (0.01 IU mL⁻¹) in dose dependent manner. The maximum inhibition with p<0.001 was observed in 2 mg mL⁻¹ dose concentration of the test drug (Fig. 1 and 2).

Whereas, in oxidative stress model, VN extract (500 mg kg⁻¹ B. DX 7 days, orally) produced significant (p<0.01) reduction in plasma MDA levels which was 34.20% in comparison to diseased control. However, standard drug recorded greater reduction of MDA levels as shown in Fig. 3.

**DISCUSSION**

The present study indicated VN to possess significant anti-inflammatory activity in dose dependent manner against acute as well as sub-acute inflammatory experimental models. Present findings are in agreement with the studies of Jana et al. (1995), Singh (1978), Sharma and Singh (1998) which have shown anti-inflammatory activity against carrageenin induced paw edema in rats as well as with the findings of Telang et al. (1999) and Jana et al. (1999) that have shown anti-inflammatory activity of VN in cotton granuloma test.

In this study, VN extract showed inhibiting effect on oxytocin induced rat uterus contractions in a dose dependent manner. These findings were in agreement with the findings of Telang et al. (1999). It is well established that oxytocin induced uterine contractions are partly mediated through prostaglandins (Rall, 1978). The prostaglandins are known to play important role in genesis of sign and symptoms of acute inflammation (Mocada et al., 1978). It is also known that NSAID’s act by inhibiting the cyclooxygenase enzyme thereby inhibiting the synthesis of prostaglandin (Vane, 1971; Vane et al., 1998). Therefore, these findings suggest that VN has probably produced anti-inflammatory action against acute inflammation by inhibiting prostaglandin synthesis, although the result is not conclusive, as other mediators in addition to PG’s like SHT and calcium have a role to play in contractions of rat uterus, which were not studied in the present study. Moreover a direct estimation
Table 1: Anti-inflammatory effect of *Vitex negundo* extract in albino rats (By carrageenan induced rat hind paw edema method)

<table>
<thead>
<tr>
<th>Group</th>
<th>Drug</th>
<th>Dose (mg/mL kg(^{-1}))</th>
<th>ADA</th>
<th>1 h</th>
<th>3 h</th>
<th>5 h</th>
<th>6 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>DW</td>
<td>10</td>
<td>2.2±0.06</td>
<td>3.8±0.12</td>
<td>4.1±0.14</td>
<td>5.0±0.10</td>
<td>3.9±0.11</td>
</tr>
<tr>
<td>II</td>
<td>VNE</td>
<td>100</td>
<td>2.2±0.07</td>
<td>3.6±0.07</td>
<td>3.7±0.07</td>
<td>4.3±0.20</td>
<td>3.9±0.31</td>
</tr>
<tr>
<td>III</td>
<td>VNE</td>
<td>250</td>
<td>2.2±0.08</td>
<td>3.1±0.15</td>
<td>3.2±0.19</td>
<td>3.2±0.14</td>
<td>2.8±0.22</td>
</tr>
<tr>
<td>IV</td>
<td>VNE</td>
<td>500</td>
<td>2.1±0.10</td>
<td>2.7±0.10</td>
<td>3.0±0.05</td>
<td>3.5±0.09</td>
<td>2.7±0.22</td>
</tr>
<tr>
<td>V</td>
<td>FEZ</td>
<td>10</td>
<td>2.2±0.10</td>
<td>2.8±0.09</td>
<td>2.6±0.07</td>
<td>2.6±0.06</td>
<td>2.5±0.06</td>
</tr>
</tbody>
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One-way ANOVA:
- p<0.001
- p=0.001
- p=0.001
- p=0.001
- p=0.001

n=No. of animals; DW=Distilled water; SEM=Standard Error Mean; p.o.=Per Orally; B.D.A.=Before Drug Administration; ADA=After Drug Administration; VNE=Vitex negundo extract; FEZ=Fenugreek seed; One-way ANOVA followed by Dunnett’s test. *p<0.05, **p<0.01, ***p<0.001 in comparison to control (Group I)

Table 2: Anti-inflammatory effect of *Vitex negundo* extract in albino rats (By cotton pellet granuloma method)

<table>
<thead>
<tr>
<th>Each</th>
<th>Dose (mg/mL kg(^{-1}))</th>
<th>ADA</th>
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<tr>
<td>10</td>
<td></td>
<td></td>
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<tr>
<td>II</td>
<td>10</td>
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</tr>
<tr>
<td>III</td>
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</tr>
<tr>
<td>IV</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>0.01 IU mL(^{-1})</td>
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<tr>
<td>VNE</td>
<td>0.05 IU mL(^{-1})</td>
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<tr>
<td>VNE</td>
<td>1.0 IU mL(^{-1})</td>
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<tr>
<td>VNE</td>
<td>1.5 IU mL(^{-1})</td>
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<tr>
<td>VNE</td>
<td>2.0 IU mL(^{-1})</td>
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<tr>
<td>VNE</td>
<td>2.5 IU mL(^{-1})</td>
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<tr>
<td>OXY</td>
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<td>OXY</td>
<td>0.01 IU mL(^{-1})</td>
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Note: Inhibitory effect of *Vitex negundo* extract on oxytocin induced contractions in rat uterus in a dose dependant manner

OXY → OXYTOCIN, R → RECOVERY; VNE+OXY → *VITEX NEGUNDO* EXTRACT + OXYTOCIN

Fig. 1: Effect of *Vitex negundo* extract on oxytocin induced contractions in rat uterus

of PG’s can throw more light to indicate its involvement, which remain to be substantiated in future studies. The finding of present study strengthens the finding of Dharmasiri et al. (2003) who suggested anti-inflammatory and pain suppressing activities of VN possibly mediated via PG synthesis. Where as, the results are contrary to the findings of Sahni et al. (2001).

The present study also showed significant reduction in MDA (Malondialdehyde) levels by VN. The oxidative stress is the condition where Reactive Oxygen Species
(ROS) generation exceeds endogenous anti-oxidant defense (Sen, 1995) and it is well known that in chronic and sub-acute inflammation reactive oxygen species play an important role in modulating the extent of inflammatory response and consequent tissue and cell injury (Robbin et al., 1999). MDA is a metabolic product of lipid peroxidation, the level of which is increased in oxidative stress. Therefore, reduction of oxidative stress by anti-lipoperoxidative activity might possibly be the mechanism of anti-inflammatory action of VN in model of sub-acute inflammation. Moreover, leaves of VN are known to possess various anti-oxidant chemical constituents like flavonoids (Banerji et al., 1998) vitamin-c and carotene (Basu et al., 1947) which are known to reduce oxidative stress (Fraga et al., 1987; Frei, 1991; Krinsky, 1989). Hence, reduction of oxidative stress produced by VN might possibly be due to these chemical constituents. These findings are in agreement to findings of Dharmasiri et al. (2003) suggested to possess anti-inflammatory and pain suppressing activities of VN possibly mediated via membrane stabilising and antioxidant activities in addition to PG inhibiting
properties. More over anti-ulcer activity of VN reported by Sahni et al. (2001) might possibly be mediated by the same mechanism.

CONCLUSION

These observations suggest VN to possess anti-inflammatory activity against acute as well as sub-acute inflammation which appears probably to be due to prostaglandin inhibition and reduction of oxidative stress respectively.

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