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Anti-inflammatory and analgesic activities of mature fresh leaves of *Vitex negundo*

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Abstract

This study confirmed the oral anti-inflammatory, analgesic and antihistamine properties of mature fresh leaves (MFL) of *Vitex negundo* L. (Verbenaceae) claimed in the Ayurveda medicine by orally treating a water extract of the leaves to rats. The early phase (2 h) of carrageenan-induced rat paw oedema was significantly ($P < 0.01$) suppressed in an inversely does-dependent ($r^2 = 1$, $P < 0.01$) manner by MFL. The EC_{50} was 2 g/kg of MFL. In the formaldehyde-induced rat paw oedema test, the 2.5 and 5 g/kg leaves significantly ($P < 0.05$) suppressed the inflammation on days 4–6 of the test. In the hot plate test, 2.5 and 5 g/kg of MFL showed a significant ($P < 0.05$) and directly dose-dependent analgesic activity at 1 h of treatment while the activity was absent in the tail flick test in rats. The EC_{50} for the analgesic activity was 4.1 g/kg. In the formalin test, 1.25, 2.5 and 5 g/kg of MFL significantly ($P < 0.05$) suppressed the pain in both the phases of the test like aspirin. The leaves showed an inversely dose-dependent in vivo antihistamine and in vitro prostaglandin (PG) synthesis inhibition, membrane stabilising and antioxidant activities. Naloxone did not abolish the analgesic activity in the hot plate test. A 5 g/kg of MFL did not impair muscle strength and co-ordination and did not induce sedation. The treatment of 5 g/kg of MFL did not show signs of acute toxicity or stress. Fourteen-day oral treatment of 5 g/kg of MFL significantly increased the serum activity of AST. Flowering of the tree did not abolish the analgesic and anti-inflammatory activities of the leaves. These observations revealed that the fresh leaves of *Vitex negundo* have anti-inflammatory and pain suppressing activities possibly mediated via PG synthesis inhibition, antihistamine, membrane stabilising and antioxidant activities. The antihistamine activity can produce the anti-itching effect claimed in Ayurveda medicine.

Keywords: *Vitex negundo* fresh leaves; Anti-inflammatory activity; Analgesic activity; Antihyperalgesic activity; Antihistamine activity; Prostaglandin synthesis inhibition activity; Antioxidant activity; Membrane stabilisation

1. Introduction

As a result of adverse side effects, like gastric lesions, caused by NSAIDs and tolerance and dependence induced by opiates, the use of these drugs as anti-inflammatory and analgesic agents have not been successful in all the cases. Therefore, new anti-inflammatory and analgesic drugs lacking those effects are being searched all over the world as alternatives to NSAIDs and opiates. During this process, the investigation of the efficacy of plant-based drugs used in the traditional medicine have been paid great attention because they are cheap, have little side effects and according to WHO still about 80% of the world population rely mainly on plant-based drugs (Kumara, 2001).

Vitex negundo L. (Verbenaceae), Nika in Sinhala, is a small tree of which water extract of fresh mature leaves are used in Ayurveda medicine as anti-inflammatory, analgesic and anti-itching agents internally and externally (Gunatillake, 1994). The tree is distributed in Sri Lanka, India, Malaya, The Philippine Islands and East Africa. Flowers occur throughout the year (Jayaweera, 1981). However, the claimed activities of the leaves have not been investigated using controlled experiments in detail. The purpose of this study was to investigate in rats (a) the effectiveness of the leaves as anti-inflammatory, analgesic and antihistaminic agents, (b) whether flowering of the tree abolishes the anti-inflammatory and analgesic activities and (c) any possible toxic effect caused after short-term use of the leaves.

2. Materials and methods

2.1. Chemicals

Carrageenan (Sigma Chemical Co., St. Louis, MO, USA), aspirin, indomethacin, chlorpheniramine (State Pharmaceutical Corporation, Colombo, Sri Lanka), naloxone

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hydrochloride, formaldehyde (Fluka, Buchs, Switzerland), assay kits (Randox Laboratories Ltd., Co., Antrim, UK).

2.2. Collection of plants and preparation of extracts

Mature fresh leaves (MFL) of *Vitex negundo* at 1–3 weeks before flowering and 1–2 weeks after flowering were collected from a tree in the campus garden of the University of Colombo, Colombo, Sri Lanka, between March and July 2002 and authenticated by Prof. A.S. Seneviratne, Department of Botany, University of Colombo. Voucher specimen (23-VN) has been deposited at the museum of the Department of Zoology, University of Colombo. Fifty grams of MFL was macerated with 100 ml of distilled water (Jayasinghe, 1975) using an electric blender for 3 min, squeezed through muslin cloth and 50% (w/v) extract was obtained. Doses were determined according to the information provided by an Ayurvedic physician, Dr. S. Ediriweera, and animals were orally treated with the extract so that they received MFL 1.25, 2.5 and 5 g/kg of body weight (5 g/kg dose per rat is approximately equal to the human dose when extrapolated). The extract was prepared every day for the experiments.

2.3. Animals

Wistar male and female rats (150–200 g) housed under standardised animal house conditions were used in all the experiments. They had access to pelleted food (Vet House Ltd., Colombo, Sri Lanka) and water ad libitum. The animals were assigned to different groups to be treated in experiments.

2.4. Anti-inflammatory activity

2.4.1. Carrageenan-induced paw oedema

Male rats ($n = 9$/group) were treated with 1.25, 2.5 and 5 g/kg of MFL at preflowering stage of the tree, 5 g/kg of MFL at flowering stage, 5 ml/kg water and 5 mg/kg indomethacin, respectively (Forestieri et al., 1996). After 1 h, these rats were injected with 0.05 ml of 1% carrageenan suspension into the foot pad of left hind paw (Winter et al., 1962). The paw volumes of these rats were measured using a Plethysmometer (Letica Scientific Instruments, Barcelona, Spain) at 1 h before, and 2 and 4 h after the carrageenan injection and the paw oedema was calculated.

2.4.2. Formaldehyde-induced paw oedema

Male rats ($n = 9$/group) were treated with 1.25, 2.5 and 5 g/kg/day of MFL and 5 ml/kg/day of water for 7 consecutive days. After 1 h on days 1 and 3 of treatment, these rats were injected with 0.1 ml of 2% formaldehyde into the foot pad of left hind paw (Selye, 1949). Paw oedema was measured 1 h before formaldehyde injection and at 4 h after the injection on day 1 and everyday at 1 h after the treatment for 7 consecutive days.

2.5. Analgesic activity

2.5.1. Hot plate and tail flick tests

Female rats ($n = 6$/group) were treated with 1.25, 2.5 and 5 g/kg of MFL at preflowering stage, 5 g/kg of MFL at flowering stage, 5 ml/kg of water and 100 mg/kg aspirin. The reaction times of these rats were measured 1 h prior to the treatment, 1 and 3 h after the treatment using hot plate and tail flick techniques as described by Langerman et al. (1995). In the hot plate test, the rat was placed in a hot plate analgesia meter (Model MK 35 A, Muromachi Kikai Co. Ltd., Tokyo, Japan) at 50 °C and the time taken to lick the hind paw or to jump was recorded. In the tail flick test, the tail of the rat 4–5 cm from its tip was immersed in a water bath at 55 °C and the time taken to flick the tail was recorded. Rats showing a pre-treatment reaction time greater than 15 s in the hot plate test and 5 s in the tail flick test were not used in the experiment. A cut off time of 25 s was set to avoid tissue damage. The hot plate test was repeated with naloxone (5 mg/kg s.c.) and 5 g/kg dose.

2.5.2. Formalin test

Rats of either sex ($n = 10$/group) were treated respectively with 1.25, 2.5 and 5 g/kg of MFL, 5 ml/kg of water and 100 mg/kg of aspirin. One hour later, these rats were injected with 0.05 ml of 2.5% formaldehyde in normal saline into the foot pad of one of the hind paw (Dubuisson and Dennis, 1977), immediately placed in a transparent plastic cage separately and the licking time and frequency of the injected paw were recorded from the first 0 to 5 and 20 to 25 min (Hunskaar et al., 1985).

2.6. Mechanisms of anti-inflammatory and analgesic activities

2.6.1. Prostaglandin (PG) synthesis inhibition

The experiment was carried out according to Lindsey et al. (1999) and Dharmasiri et al. (2003). One centimetre portions of isolated dioestrous rat uteri were suspended in a 50 ml organ bath containing Krebs–Henseleit solution (pH 7.4), maintained at 37 °C and aerated with 5% CO_2 and 95% O_2 gas mixture. The spontaneous contractions of the uteri were recorded using an isometric sensor (Star Medicals, Tokyo, Japan) for 10 min and then, the organ bath was treated in triplicate with the extracts so that the final concentrations of MFL in the organ bath became 100, 200, 300 and 400 μg/ml and the contractions were recorded for further 10–15 min. Aspirin was used as the positive reference drug. The latent period for the initiation of contractions and the percent reduction of the amplitude and frequency of contractions with respect to the normal contractions was calculated.

2.6.2. Membrane stabilising activity

The experiment was done using heat-induced haemolysis of rat erythrocytes in vitro as described by Perez et al. (1995)

and Dharmasiri et al. (2003). Vials containing 20 μl fresh rat blood in 1 ml of phosphate-buffered saline were treated in triplicate with the extract so that the final concentration of the MFL in the vials became 2.5, 5, 7.5 and 10 mg/ml. Fifteen microlitres of saline was used as the control while aspirin was used as the positive reference drug. The vials were then incubated for 15 min at 37 °C followed by 54 °C for 25 min, centrifuged and the absorbance of the supernatant was measured at 540 nm spectrophotometrically. The percent inhibition of haemolysis with respect to the control was calculated.

2.6.3. Antioxidant activity

The experiment was carried out using thiobarbituric acid reactive substances assay as described by Dorman et al. (1995). The vials containing the reagents were treated in triplicate with the extract so that the final concentrations of the MFL in the vials became 1.25, 1.875, 2.5 and 3.125 mg/ml. A 100 μg/ml of butylated hydroxytoluene (BHT) was used as the positive reference and distilled water was used in the control. The vials were mixed well and incubated at 95 °C for 60 min, allowed to cool, 5 ml of butanol was added, mixed well and centrifuged at 1500 × *g*. The absorbance of the butanol layer was measured at 532 nm and the antioxidant index was calculated as follows:

$$\text{Antioxidant index} = \left(1 - \frac{T}{C}\right) \times 100$$

where *T* is the absorbance of test and *C* absorbance of control.

2.6.4. Antihistamine activity

Rats of either sex ($n = 9$/group) of which fur on posterior lateral side have been shaved 24 h earlier were treated with 1.25, 2.5 and 5 g/kg of MFL, 0.67 mg/kg of chlorpheniramine and 5 ml/kg of water, respectively. After 1 h, these rats were subcutaneously injected with 0.05 ml of 200 μg/ml histamine dihydrochloride into the fur removed area of the skin (Spector, 1956) under mild ether anaesthesia and the area of the wheal formed after 1.5 min was calculated.

2.6.5. Sedative activity

Rats of either sex ($n = 9$/group) were treated either with 5 g/kg of MFL or 5 ml/kg of water. After 1 h, all these rats were tested on rat hole-board apparatus to determine sedation as described by File and Wardill (1975). The rats were individually placed in the centre of rat hole-board apparatus and the number of rears, number of head dips, locomotory activity and the number of faecal boluses produced were recorded for 7.5 min. The time spent per head dip was then calculated.

2.6.6. Muscle strength and co-ordination

Rats of either sex ($n = 9$/group) were treated either with 5 g/kg of MFL or water. One hour later, these rats were subjected to bar holding test (to evaluate muscle strength) followed by the bridge test (to evaluate muscle co-ordination) and the latency to fall and slide off was determined, respectively (Plaznic et al., 1993).

2.7. Toxicity

Male rats ($n = 6$/group) were treated either with 5 g/kg/day of MFL or 5 ml/kg/day of water for 14 consecutive days. After the treatment, these rats were continuously observed for 1 h for overt clinical signs of acute toxicity or stress. They were daily observed for overt signs of toxicity or stress during the period of treatment. The rats were weighed using an animal balance (MP 6000, Chyo Balance Corporation, Tokyo, Japan), prior to the start of the experiment and on day 1 of the post-treatment. On day 1 of the post-treatment, blood was taken out from the tail under mild ether anaesthesia, serum separated out. The serum activities of aspartate and alanine transaminases, serum concentrations of glucose, urea and creatinine were determined using Randox assay kits. Then, the rats were killed, stomachs were taken out, opened and examined for macroscopic haemorrhagic gastric lesions.

2.8. Analysis of data

Data are given as means±S.E.M. Statistical analyses were done by using Student's *t*-test, linear regression analysis and Pearson's correlation analysis, one-way ANOVA followed by Tukey's Family Error Rate test. $P = 0.05$ was considered as significant.

3. Results

3.1. Anti-inflammatory activity

3.1.1. Carrageenan-induced paw oedema test

When compared with the control, treatment with MFL significantly ($P < 0.05$) and dose-dependently reduced the paw oedema only at the 2 h after carrageenan injection while there was no significant suppression at 4 h. There was no significant difference between the paw oedema of rats treated with MFL at preflowering and flowering stages of the tree (data not shown). However, 5 mg/kg indomethacin significantly suppressed paw oedema at both 2 and 4 h (Table 1). The suppression of paw oedema by MFL was inversely dose related ($r^2 = 1$, $P < 0.01$). The EC_{50} for the suppression of paw oedema by MFL was 2 g/kg.

3.1.2. Formaldehyde-induced paw oedema test

The treatment with 2.5 and 5 g/kg/day of MFL for 7 days significantly ($P < 0.05$) reduced the paw oedema from days 4 to 6 when compared with the control. By day 7 of the treatment, the paw oedema in control and treated groups had almost disappeared. However, the treatment with 1.25 g/kg/day did not significantly suppressed the

Table 1
Effect of oral treatment of *Vitex negundo* mature fresh leaves (MFL) on the carrageenan-induced paw oedema of rats

Dose	Paw oedema (ml)	
	2 h	4 h
5 ml/kg water	0.52 ± 0.05	0.49 ± 0.02
1.25 g/kg MFL	0.23 ± 0.03**	0.45 ± 0.04
2.5 g/kg MFL	0.28 ± 0.04**	0.49 ± 0.06
5 g/kg MFL	0.38 ± 0.03*	0.43 ± 0.04
5 mg/kg indomethacin	0.11 ± 0.02**	0.28 ± 0.03**

Values are means ± S.E.M. ($n = 9$).
* $P < 0.05$ as compared with the control (Student's *t*-test).
** $P < 0.01$ as compared with the control (Student's *t*-test).

paw oedema (Table 2). The paw oedema suppression showed a bell-shaped dose–response relationship.

3.2. Analgesic activity

3.2.1. Hot plate and tail flick tests

As compared with the controls, the treatment with MFL produced a significant and dose-dependent ($r = 0.92$, $P < 0.05$) increase in the reaction time of rats at 1 h after treatment in the hot plate test showing an analgesic effect. A significant difference could not be observed between the reaction times of rats treated with MFL at preflowering and flowering stages of the tree (data not shown). The increase of the reaction time was significant only with the 2 and 5 g/kg doses. The EC_{50} for the increase in reaction time was 4.1 g/kg. However, aspirin produced a significant increase in the reaction time at 1 and 3 h after treatment. There was no significant increase in the reaction time with 1.25 g/kg in the hot plate test and any of the doses in the tail flick test (Table 3). The treatment with 5 mg/kg of naloxone did not significantly reduce the reaction time of MFL-treated rats excluding an opioid receptor-mediated action (reaction time: MFL + naloxone versus MFL + saline: 12.8 ± 1.4 s versus 13.6 ± 0.9 s).

3.2.2. Formalin test

The treatment with MFL significantly ($P < 0.05$) reduced the licking time of the formalin-injected paw both in the early and late phases of the test as compared with the control. The frequency of licking was significantly ($P < 0.05$) reduced only at the late phase. However, aspirin significantly ($P < 0.05$) reduced the licking time and frequency at both the phases of the test. The licking time was significantly ($P < 0.05$) higher at the early phase than the late phase in both MFL- and aspirin-treated rats (Table 4).

3.3. Mechanisms of the anti-inflammatory and analgesic activities

3.3.1. PG synthesis inhibition activity

The treatment with MFL does-dependently reduced the amplitude and frequency of spontaneous contractions of isolated dioestrous rat uterus indicating a PG synthesis inhibition (Table 5). The percent inhibition was inversely proportional to the concentration (for amplitude, $r = -0.98$, $P < 0.05$; for frequency, $r^2 = 0.88$, $P < 0.05$). For aspirin, percent inhibition of amplitude ($r = 0.96$, $P < 0.05$) and frequency ($r = 0.98$, $P < 0.05$) was directly proportional to the concentration. The EC_{50} for the inhibition of amplitude: MFL versus aspirin: 228.1 μg/ml versus 3.0 μg/ml and the frequency: MFL versus aspirin: 257.7 μg/ml versus 3.6 μg/ml. The latent period for the initiation of the inhibition: MFL versus aspirin: 228.0 ± 30.6 s versus 202.8 ± 33.0 s.

3.3.2. Membrane stabilising activity

MFL dose-dependently inhibited the heat-induced haemolysis of rat erythrocytes in vitro indicating a membrane stabilising activity as seen with aspirin (Table 6). However, the activity was inversely related to the concentration ($r^2 = 0.9$, $P < 0.05$) with MFL while with aspirin it was directly related to the concentration. EC_{50} for the membrane stabilising activity: MFL versus aspirin: 2.6 mg/ml versus 44.4 μg/ml.

3.3.3. Antioxidant activity

MFL showed a dose-dependent ($r^2 = 0.68$) antioxidant activity as indicated by the antioxidant index. However, BHT showed a higher antioxidant index than MFL (Table 7). The dose–response relationship was curvilinear ($r^2 = 0.68$) and the EC_{50} for the antioxidant activity was 2.3 mg/ml MFL.

Table 2
Effect of oral treatment of mature fresh leaves (MFL) of *Vitex negundo* on the formaldehyde-induced paw oedema of rats

Dose	Paw oedema (ml)						
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
5 ml/kg/day water	0.26 ± 0.03	0.24 ± 0.05	0.22 ± 0.05	0.46 ± 0.05	0.39 ± 0.02	0.31 ± 0.05	0.16 ± 0.03
1.25 g/kg/day MFL	0.31 ± 0.03	0.35 ± 0.04	0.15 ± 0.05	0.47 ± 0.08	0.36 ± 0.03	0.29 ± 0.04	0.09 ± 0.04
2.5 g/kg/day MFL	0.41 ± 0.03	0.21 ± 0.04	0.25 ± 0.04	0.26 ± 0.03**	0.18 ± 0.02**	0.13 ± 0.02**	0.11 ± 0.03
5.0 g/kg/day MFL	0.46 ± 0.04	0.22 ± 0.04	0.32 ± 0.04	0.31 ± 0.05*	0.24 ± 0.05**	0.18 ± 0.04*	0.11 ± 0.03

Values are means ± S.E.M. ($n = 9$).
* $P < 0.05$ as compared with the control (Student's *t*-test).
** $P < 0.01$ as compared with the control (Student's *t*-test).

Table 3
Effect of oral treatment of mature fresh leaves (MFL) of *Vitex negundo* on the reaction time of rats

	Reaction time(s)					
	Hot plate			Tail flick		
	Pre-treatment	1 h	3 h	Pre-treatment	1 h	3 h
5 ml/kg water	11.8 ± 0.8	11.1 ± 0.7	12.3 ± 0.9	1.5 ± 0.2	1.8 ± 0.2	1.6 ± 0.1
1.25 g/kg MFL	12.2 ± 1.0	9.1 ± 0.8	11.1 ± 0.8	1.7 ± 0.2	1.4 ± 0.8	1.8 ± 0.2
2.5 g/k MFL	10.9 ± 0.5	15.2 ± 1.5*	11.3 ± 2.4	1.8 ± 0.1	1.9 ± 0.2	1.7 ± 0.1
5 g/kg MFL	12.2 ± 0.6	18.8 ± 1.6**	13.3 ± 1.6	1.5 ± 0.2	1.2 ± 0.1	1.3 ± 0.1
100 mg/kg aspirin	9.5 ± 1.2	15.3 ± 1.7*	16.4 ± 1.4*	1.5 ± 0.1	1.3 ± 0.1	1.1 ± 0.1

Values are means ± S.E.M. ($n = 6$).
* $P < 0.05$ as compared with the control (Student's *t*-test).
** $P < 0.01$ as compared with the control (Student's *t*-test).

Table 4
Effect of oral treatment of mature fresh leaves (MFL) of *Vitex negundo* on the licking time and frequency of rats in the formalin test

Dose	Early (first) phase (0–5 min)		Late (second) phase (20–25 min)	
	Licking time (s)	Licking frequency (min^{-1})	Licking time (s)	Licking frequency (min^{-1})
5 ml/kg water	59.3 ± 5.3	12.4 ± 1.4	48.8 ± 7.4	10.1 ± 1.2
1.25 g/kg MFL	37.9 ± 4.5*	8.4 ± 0.8	21.9 ± 5.1**	6.0 ± 1.9*
2.5 g/kg MFL	43.0 ± 6.1*	12.0 ± 1.4	14.8 ± 5.2**	9.0 ± 4.0
5 g/kg MFL	39.4 ± 6.0*	8.8 ± 1.0	14.2 ± 4.1**	4.1 ± 0.9**
100 mg/kg aspirin	20.8 ± 3.0**	7.0 ± 1.5**	8.3 ± 2.2**	4.9 ± 1.5**

Values are means ± S.E.M. ($n = 9$).
* $P < 0.05$ as compared with the control (Student's *t*-test).
** $P < 0.01$ as compared with the control (Student's *t*-test).

3.3.4. Antihistamine activity

As compared with the control treatment with MFL significantly ($P < 0.01$) and dose-dependently reduced the area of the wheal formed on the rat skin by the injection of histamine indicating an antihistamine activity. A 0.67 mg/kg of chlorpheniramine also significantly ($P < 0.01$) reduced the area of the wheal (Fig. 1). The antihistamine activity of MFL is inversely dose related ($r = -0.98$, $P < 0.05$). EC_{50} for the antihistamine activity was 0.99 g/kg of MFL.

3.3.5. Sedative activity

The treatment with MFL failed to significantly alter the parameters of rat hole-board test compared to control (data not shown), showing that there is no sedative action in MFL.

3.3.6. Muscle relaxation and co-ordination

When compared with the control, the treatment with MFL failed to significantly alter the latency to fall in the bar holding test (control versus treatment: 60.0 ± 0.1 versus

Table 5
Prostaglandin synthesis inhibition activity of different concentrations of mature fresh leaves (MFL) of *Vitex negundo* and aspirin as indicated by the reduction of spontaneous contractions of isolated rat uterus at dioestrus with respect to normal contraction

Concentration (μg/ml)	% reduction of amplitude	% reduction of frequency
100 MFL	71.3 ± 3.1	70.1 ± 9.0
200 MFL	57.5 ± 6.8	50.9 ± 6.3
300 MFL	51.1 ± 15.1	48.9 ± 7.6
400 MFL	37.2 ± 6.5	33.8 ± 10.0
2 aspirin	36.9 ± 3.5	19.4 ± 4.0
4 aspirin	65.4 ± 9.9	50.8 ± 8.0
6 aspirin	79.7 ± 5.7	70.8 ± 4.2

Values are means ± S.E.M. ($n = 3$/concentration).
There was a significant relationship ($P < 0.05$) between the concentration and percent reduction (linear regression, Pearson's correlation).

Table 6
Membrane stabilising effect of different concentrations of *Vitex negundo* mature fresh leaves (MFL) and aspirin as indicated by the inhibition of heat-induced haemolysis of rat erythrocytes in vitro

Concentration	% inhibition
2.5 mg/ml MFL	31.2 ± 0.9
5 mg/ml MFL	26.8 ± 2.8
7.5 mg/ml MFL	16.5 ± 2.5
10 mg/ml MFL	6.6 ± 2.6
5 μg/ml aspirin	17.6 ± 1.6
10 μg/ml aspirin	20.9 ± 1.8
15 μg/ml aspirin	25.9 ± 0.5
20 μg/ml aspirin	31.9 ± 0.6

Values are means ± S.E.M.
There was a significant ($P < 0.05$) relationship between the concentration and percent inhibition (linear regression analysis).

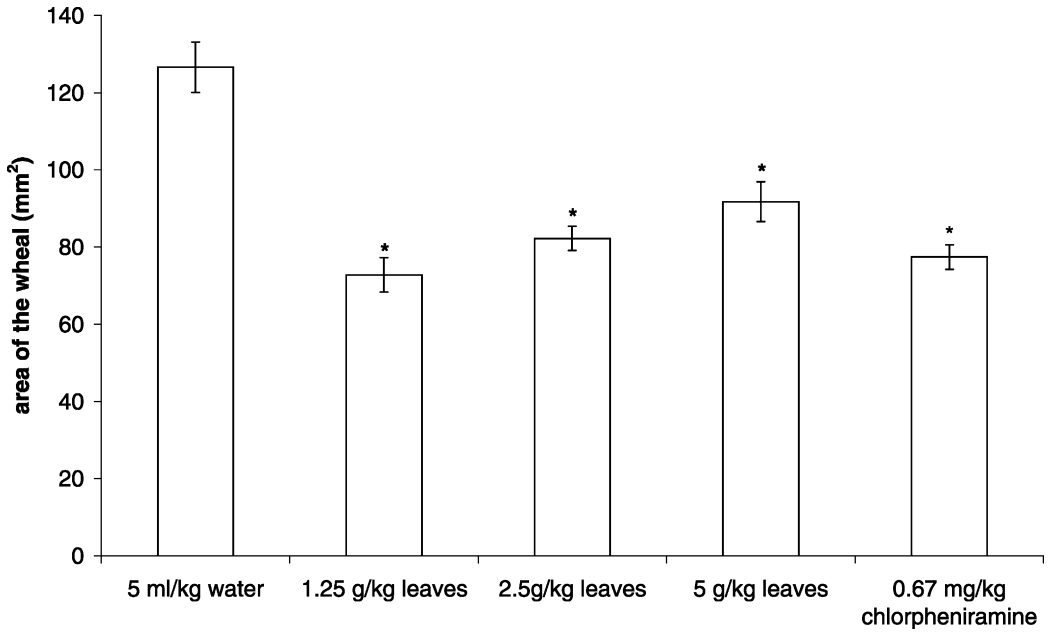

Fig. 1. Antihistamine activity of *Vitex negundo* mature fresh leaves as indicated by the area of the wheal. Data are given as means ± S.E.M. $^{*}P < 0.01$ as compared with the control (one-way ANOVA, Tukey's Family Error Rate test).

56.9 ± 2.1) or latency slide off in the bridge test (control versus treatment: 58.3 ± 1.7 versus 55.6 ± 2.2).

3.4. Toxicity

The treatment with 5 g/kg/day of MFL for 14 days failed to produce any overt clinical signs of toxicity or stress. The treatment also did not significantly alter the body weights (control versus treatment: 232.0 ± 8 g versus 251.8 ± 5.6 g), serum creatinine (control versus treatment: 1.7 ± 0.3 mg/dl versus 1.1 ± 0.2 mg/dl), urea (control versus treatment: 33.6 ± 3.0 mg/dl versus 38.6 ± 1.2 mg/dl), random glucose (control versus treatment: 136.2 ± 8.0 mg/dl versus 141.5 ± 6.6 mg/dl) and the activity of ALT (control versus treatment: 12.0±2.1 U/l versus 15.0±2.0 U/l). However, the treatment caused a significant ($P < 0.05$) increase in serum activity of AST (control versus treatment: 24.3 ± 5.1 U/l versus 74.7 ± 5.5 U/l). The treatment also did not cause haemorrhagic lesions in the gastric mucosa after 14 days of treatment.

Table 7
Antioxidant activity of different concentrations of mature fresh leaves (MFL) of *Vitex negundo*

Concentration (mg/ml)	Antioxidant index
1.25 MFL	40.2 ± 0.1
1.875 MFL	38.8 ± 1.2
2.5 MFL	33.7 ± 0.5
3.125 MFL	45.6 ± 0.1
0.1 BHT	57.4 ± 1.2

Values are means ± S.E.M.

4. Discussion

Experimental investigations revealed that the MFL of *Vitex negundo* have dose-dependent activity against inflammation as revealed in the carrageenan and formaldehyde models. Further, hot plate test and the formalin test revealed that MFL can also suppress acute pain. However, the anti-inflammatory activity is 1.7 times lower than indomethacin in the carrageenan model while the analgesic activity is 1.2 times lower than aspirin in the formalin test. MFL demonstrated a dose-dependent PG synthesis inhibition, membrane stabilising, antihistamine and antioxidant activities. The inverse dose–response relationship shown by acute anti-inflammatory, antihistamine, PG synthesis inhibition and membrane stabilising activities may be due to reduction of the effectiveness of the active principle at its high concentrations. However, further investigations are needed to confirm this suggestion. This type of relationship indicates that the concentrations used in these tests are within the therapeutic window of MFL in which certain drugs exert their maximum curative effect (Tripathi, 1994).

According to Vinegar et al. (1987) and Antonio and Brito (1998) in the carrageenan model, the early phase (1–2 h) is mainly mediated by histamine, serotonin and the increase of PG synthesis in the surroundings of the damaged tissues while the late phase is mainly mediated by bradykinin, leukotrienes, polymorphonuclear cells and PGs produced in tissue macrophages. In this experiment, the suppression of inflammation at the early phase of inflammation can be contributed by PG synthesis inhibition and antihistamine activities shown by MFL. The lack of anti-inflammatory activity at the second phase may indicate the short duration

of action of MFL and the increase of the leukotriene at the second phase caused by the inhibition of PG synthesis in the first phase because inhibition of PG synthesis diverts the reaction towards increase in leukotrienes synthesis (Mayes, 1996). Although opioid receptor-mediated activities can suppress the inflammation in the carrageenan-induced paw oedema (Planas et al., 2000), such activity can be excluded here because the naloxone test revealed that the leaves do not act via opioid receptors.

In the formaldehyde-induced paw oedema model, the anti-inflammatory activity was evident from days 4 to 6 of the treatment, indicating that MFL is effective against the establishment of chronic inflammation which happens at the later stage of acute inflammation (Hanna and Poste, 1991). This action can be contributed by the PG synthesis inhibition, membrane stabilising and antioxidant activities of MFL as reduced PG synthesis and oxidation and the membrane stabilisation play key roles in countering the establishment of chronic inflammation (Perez et al., 1995; Rang et al., 1995). As seen in this experiment, the ability of a drug to suppress inflammation when it is applied after the onset of inflammation is likely to be due to the genuine anti-inflammatory activity of the drug (Duwiejua et al., 1994). These observations provide the support for the use of MFL of *Vitex negundo* as anti-inflammatory agent in the Ayurveda medicine while the strong antihistamine activity confirms the use of MFL to counter skin itching.

The analgesic activity shown only in the hot plate test reveals that the activity is supraspinally mediated (Hough et al., 1999) and can be brought about by the PG synthesis inhibition activity of MFL.

In the formalin test, the pain in the early phase is caused due to the direct stimulation of the sensory nerve fibres by formalin while the pain in the late phase is due to inflammatory mediators, like histamine, PGs, serotonin and bradykinin (Murray et al., 1988; Tjolsen et al., 1992). The pain suppression at the early phase by MFL as well as aspirin may be due to a local anaesthetic activity caused by the membrane stabilising activity of them because membrane stabilising agents produce local anaesthesia reducing the sensation of acute pain (Rang et al., 1995). The considerably higher pain suppression at the late phase than the early phase could be due to the concerted action of PG synthesis inhibition, membrane stabilisation and antihistamine activities of MFL. Formalin develops a hyperalgesic state in the late phase of the test (Kaufmann et al., 1997). The pain suppression at the second phase therefore indicates the antihyperalgesic activity of the leaves which is useful in controlling acute pains. NSAIDs when acting supraspinally reduce the pain at both the phases of the formalin test (Martindale et al., 2001) which was also observed in this experiment with MFL as well as with aspirin.

Sedatives produce analgesia (Rang et al., 1995). However, MFL did not have sedative action in the rat hole-board test. Therefore, a contribution from sedation to the analgesic activity can be excluded. As naloxone failed to reverse analgesic activity in the hot plate test, analgesic action operating through opioid receptors can be ruled out. Muscle relaxants can produce false positive results in the hot plate test indicating analgesic activity (Gracioso et al., 1998). However, there was no muscle relaxant activity induced by MFL in the bar holding test. Stress can produce analgesia (Ganong, 1995). There was no sign of stress observed in the rats treated with the MFL.

The anti-inflammatory and analgesic activities of the leaves did not disappear after the flowering of the tree in contrast to *Anisomeles indica* which lost these activities after flowering of the plant (Dharmasiri et al., 2002, 2003).

The treatment of MFL for 14 days did not produce detectable toxic effect in terms of body weight, serum concentrations of urea, creatinine, glucose and serum activity of ALT. The treatment did not cause gastric lesions which is a beneficial effect compared to the modern NSAIDs. The reason for the increase of serum activity of AST has to be further investigated. Therefore, people should be cautious when the leaves are used in oral preparations.

In conclusion, these observations provide evidence for the anti-inflammatory and analgesic properties of mature fresh leaves of *Vitex negundo* claimed in Ayurveda medicine. Also, it uncovered some of the possible mechanisms of these actions. Further studies will be undertaken to correlate the pharmacological activities with the chemical constituents.

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