PHARMACOLOGICAL INVESTIGATIONS OF *NARAVELIA ZEYLANICA* IN VARIOUS *IN VITRO* AND *IN VIVO* MODELS OF INFLAMMATION

Ashoka Shenoy M [a *], Sridevi [b], Shastry C.S [a], Gopkumar P [c]

[a] Department of Pharmacology, Srinivas College of Pharmacy, Mangalore, India-574146
[b] Department of Pharmaceutics, Srinivas College of Pharmacy, Mangalore, India-574146
[c] Department of Pharmachemistry, Srinivas College of Pharmacy, Mangalore, India-574146

***Corresponding author**
Ashoka Shenoy M, Assistant Professor, Department of Pharmacology, Srinivas College of Pharmacy, Valachil, Parangipete Post, Mangalore Taluk-574 146.
Tel: +0824 2274722; fax: 0824 2274725; email ID:shenoyam@rediffmail.com

Summary

The objective of present study is to investigate the effect of lyophized aqueous extract of Naravelia *zeylanica* leaves in various *in vitro* and *in vivo* inflammatory models. N. *zeylanica* was studied for its *in vitro* inhibitory activity against 5-lipoxygenase (5-LO), cyclo-oxygenase (COX), leukotriene B4 (LTB4) and nitric oxide synthase (NOS). At doses 100 and 200 mg/kg, p.o. N. *zeylanica* was evaluated in acute pedal inflammation induced by carrageenan, histamine, serotonin and zymosan in rats and mice. Further, the effect of topical application of the extract (5 mg and 10 mg) on ear inflammation induced by various inflammatory agents like -*O*-tetradecanoyl-phorbol 13-acetate (TPA) or capsaicin or arachidonic acid or oxazolone or dinitrofluorobenzene (DNFB) was also investigated. *In vitro* evaluation of the extract revealed its inhibitory activity against the major inflammatory mediators 5-LO, COX, LTB4 and NOS. The extract significantly inhibited the pedal inflammation produced by carrageenan, histamine, serotonin and zymosan. Further, topical application of N. *zeylanica* significantly inhibited the ear inflammation induced by acute and multiple applications of TPA and acute application of capsaicin or arachidonic acid. However, the extract failed to inhibit ear inflammation induced by oxazolone or DNFB. To conclude N. *zeylanica* has anti-inflammatory activity possibly mediated through 5-LO and COX pathways.

Introduction

Life, disease and decay are inseparable. From his first awakening, man has sought to fight and control diseases. He turned to nature for inspiration and guidance (1). Herbs have been used as a source of drugs to combat diseases since time immemorial. The effectiveness, easy availability, low cost and non-toxic nature popularized herbal remedies. The rational design of novel drugs from traditional medicine offers new prospects in modern healthcare (2).

Naravelia zeylanica (Ranunculaceae) is a climbing shrub with tuberous roots. The plant is available rich all around south India. In ayurveda the plant has been extensively used by native peoples as an astringent, bitter, antipruritic and anti-inflammatory (6). It is also useful in pitta, helminthiasis, dermatopathy, leprosy, rheumatalgia, odontalgia, cephalalgia, colic inflammation, wound healing and ulcer protection (7). The root and stem have a strong penetrating smell and is used to relieve malarial fever and headache. Root and stem paste is applied externally for psoriasis, itches and skin allergies (8). The traditional medicine practioners using the leaf and stem juices for treating intestinal worms, psoriasis & dermatitis (9).

However, no phytochemical and pharmacological investigations of the leaves have been conducted so far to substantiate this practice. The current study aimed at exploring the anti-inflammatory potential as well as its mechanism of action of the leaves of N. *zeylanica*.

Materials and Methods

Plant material and extraction procedure

The fresh leaves of N.*zeylanica* were collected from Udupi, Karnataka, during month of September-October. It was authenticated by Dr. Gopalakrishna Bhat, Department of Botany, Poorna Prajna College, Udupi, and Karnataka, India. A voucher specimen (H.S.198) was deposited in the herbarium of our institute.

The fresh leaves were shade dried and powdered mechanically and stored in airtight containers.The aqueous extract of dried leaves was prepared as follows. One hundred grams of the leaves powder was soaked in 400 ml of distilled water for 16 h. The percolate was then decanted, centrifuged and filtered through Whatman (No.1) filter paper to obtain clear extract (300 ml). This process of extraction was repeated again with the same volume of distilled water. The percolates were pooled and lyophilized which yielded a brown colored powder (68% yield).

Drugs and chemicals

Arachidonic acid (AA), carrageenan, serotonin, histamine, 12-O-tetradecanoylphorbol 13-acetate (TPA), 2,4-dinitrofluorobenzene (DNFB), 4-ethoxymethylene-2-phenyl-2-oxazoline-5-one (oxazolone), Indomethacin, zymosan A (from Saccharomyces cervisiae) and dexamethasone were obtained from Sigma Chemical Co (ST. Louis, USA). 1-phenyl-3 pyrazolidinone (phenidone) was from Aldrich, Germany. 5-lipoxygenase (5-LO, human recombinant, specific activity ~ 100 units/mg) was purchased from Caymen Chemicals, Ann Arbor, USA. BWB70C and capsaicin were procured from Tocris, Avonmouth, UK. All other chemicals and reagents were of pure analytical grade and obtained from local suppliers.

Animals

Adult male Swiss albino mice (18-22 g) and Wistar rats (180-22*0* g) were procured from Indian Institute of Sciences. They are maintained under standard conditions (temperature 22 ± 2^{o}C, relative humidity 60±5% and 12 h light/dark cycle).The animals were housed in sanitized polypropylene cages containing sterile paddy husk as bedding. They had free

access to standard pellet diet and water *ad libitum*. The Institutional Animal Ethics Committee approved the experimental protocol. All the animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the "National Academy of Sciences" and published by the "National Institute of Health".

In vitro assays

5-lipoxygenase assays

For the evaluation of 5-LO inhibitory activity, the enzymatic activity of 5-LO was measured spectrophotometrically using human recombinant 5-LO and an incubation mixture containing 50 mM sodium phosphate, pH 7.4, 12 µg/ml phosphatidyl choline, 0.2 mM ATP, 0.2 mM CaCl2 and 20 µM AA. Different concentrations of N.*zeylanica* (dissolved in Milli Q water) or 1 µM of BWB70C, a specific 5-LO inhibitor [dissolved in 10% Dimethyl sulfoxide (DMSO), 0.1% final concentration] were added to the reaction mixture. The reaction was initiated by the addition of an aliquot enzyme (25 units), and the rate of conjugated diene formation was followed at room temperature for 2 min. Enzymatic activity was calculated from the highest linear rate of the diene formation at absorbance 234 nm and percentage inhibition was calculated relative to a control reaction containing Milli Q water or DMSO vehicle (10), (11).

Other in vitro assays

The enzyme inhibition assays for cyclo-oxygenases (COX -1 and COX -2), leukotriene B4 (LTB4) and nitric oxide synthase (NOS) were carried out by NovaScreen Biosciences Corporation, USA. The studies were done as per the standard protocols (12). The details of the receptor source, ligands and reference compounds used are listed in (table 1).

In vivo assays

Carrageenan, histamine or serotonin-induced rat pedal inflammation

Pedal inflammation in rat was produced according to the method of Winter *et al*, (13) Olajide *et al* (14) and Kaur *et al* (15). Rats were treated intraperitoneally with N.*zeylanica* (100 and 200 mg/kg) or Indomethacin (20mg/kg) or saline (10 ml/kg). Fifteen minutes after the treatment, rats were injected with 0.1 ml of carrageenan or histamine or serotonin (all 1% solution in saline) into the right hind paw under the sub plantar tissue. Paw volume measurements were carried out immediately before and various time points after phlogistic agent injection using a plethysmometer (Ugo Basile 7140).

Zymosan A-induced mouse paw inflammation

The method of Rouleau *et al* (16) was followed with minor modifications. Inflammation was induced in mice by sub plantar injection of 25 µl of 2% zymosan. A suspension in saline into the left hind paw. The animals were killed 4 h later and the hind paws were cut off at the ankle and weighed in an analytical balance. N.*zeylanica* (100 or 200 mg/kg,

p.o.) or Indomethacin (2*0* mg/kg, p.o.) or saline was injected 15 min prior to zymosan injection.

Acute single application of tetradecanoylphorbol acetate (TPA) induced mouse ear inflammation

The method of Recio *et al* (17) was followed. Inflammation was induced on the right ear by topical application of 2*0* µl of TPA in acetone (2.5 µg/ear) with a micropipette. Ear thickness was measured before and 4 h after induction of inflammation using a micrometer (Mitutoyo, Japan). N.*zeylanica* was dissolved in 80% acetone and applied topically (5 and 10 mg/ear in 20µl) simultaneously with TPA. The standard drug Indomethacin was administered at a dose of 0.10 mg/ear. The effect of the extract on inflammation was expressed as percent inhibition from the mice treated with 80% acetone.

Mouse ear inflammation-induced by multiple topical applications of TPA

The method of Stanley *et al* (18) was followed. Chronic inflammation was induced by topical application of 20 µl of TPA (2 µg/ear x 5 times) to both the inner and outer surface of both the ears of each mouse with a micropipette on alternate days. N.*zeylanica* (5 and 10 mg/ear) or dexamethasone (0.05 mg/ear) was applied topically twice daily for four days in the morning immediately after TPA application and 6 h later. The ear thickness was measured 4 h after the last TPA application.

Capsaicin-induced mouse ear inflammation

The method of Martione and Rodriguez (19) was followed with minor modifications. Inflammation was induced on the right ear by topical application of 2*0* µl of capsaicin in acetone (100 µg/ear) with a micropipette. Ear thickness was measured before and 1 h after induction of inflammation using a micrometer (Mitutoyo, Japan). N.*zeylanica* was dissolved in 80% acetone and applied topically (5 and 10 mg/ear in 2*0* µl) 15 min prior to capsaicin application. The standard drug Indomethacin was administered at a dose of 0.5 mg/ear. The effect of the extract on inflammation was expressed as per cent inhibition from the mice treated with 80% acetone.

Arachidonic acid (AA)-induced mouse ear inflammation

The method of Giner *et al* (20) was followed. N.*zeylanica* (5 and 10 mg/ear in 2*0* µl) dissolved in 80% acetone was applied 30 min before the application of AA (2 mg in 2*0* µl) in the right ear. Thickness of the ears was measured before and 1 h after the induction of inflammation using the micrometer. A reference group was treated with phenidone (1 mg/ear).

Oxazolone or dinitroflurobenzene (DNFB)-induced mouse ear inflammation

Mice were sensitized by topical applications on the shaven ventral abdomen of 50 µl of 2% solution of oxazolone in acetone or 0.2% (v/v) solution of DNFB in olive oil and

acetone mixture (1:4) on two consecutive days. Challenge was performed on Day 6 by application of either 30 µl of 2% oxazolone or 0.2% DNFB. N.*zeylanica* (5 and 10 mg/ear in 2*0* µl) or dexamethasone (0.5 mg/ear) was applied 30 min before the challenge. Ear thickness was measured 24 h after the challenge (21), (22).

Statistical analysis

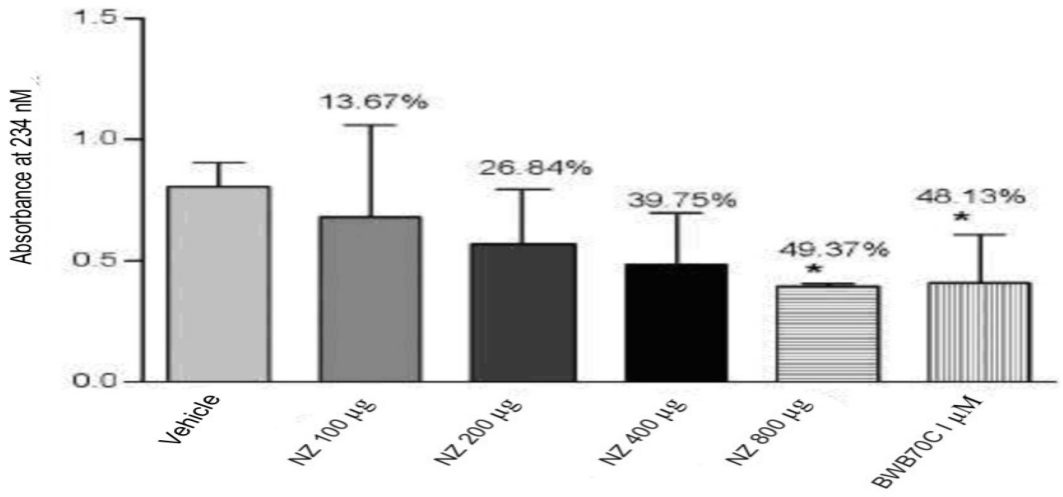
Inflammations are expressed as mean ± SEM. The inhibition percentages are calculated from the differences between the treated and control groups. Statistical significance of differences between the mean values was analyzed by ANOVA and Dunnett's test.

Results

5-lipoxygenase assays

N.*zeylanica* (100-800 µg/ml) caused a concentration -dependent inhibition of the human recombinant 5-LO. At the concentration of 800 µg/ml the inhibitory effect (49.37% inhibition) was found to be statistically significant (P< 0.05). The specific 5-LO inhibitor BWB70C exhibited 48.13% inhibition at 1µM (figure 1).

Figure 1: Effect of Naravelia zeylanica on human recombinant 5-lipoxygenase.



Each bar represents mean±SEM from 5 individual experiments. *P<0.05 and **P<0.001 compared with vehicle control (ANOVA followed by Dunnett's test). Values above the bars show percent inhibition compared to vehicle control.

Other in vitro assays

The percent inhibition exhibited by N.*zeylanica* (at 500µg/ml) on COX -1, COX - 2, NOS (constitutive-neuronal) and LTD4 are listed in (table1).

Table 1.Selected enzyme inhibition assays of N.*zeylanica

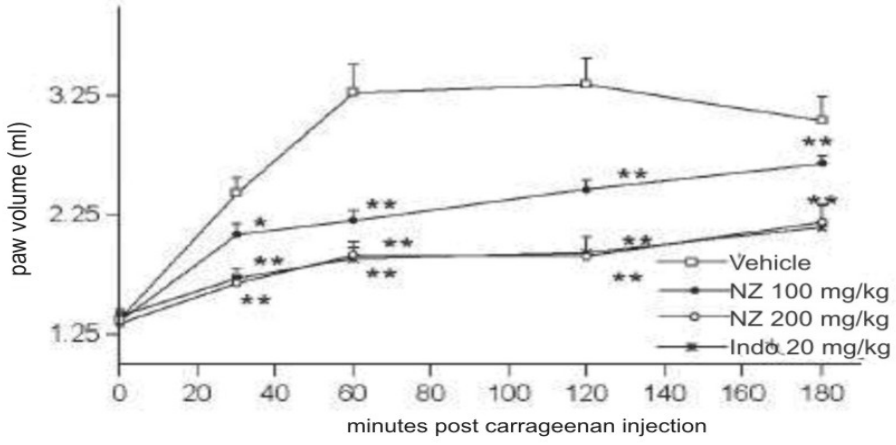
Receptor/enzyme	Source	Ligand	Reference compound	% inhibition at 500 µg/ml
NOS(constitutive neuronal)	Rat cerebellum	[^{3}H]Arginine	L-Arginine	84.11
COX-1	Bovine seminal vesicle	Arachidonic acid	SC560	29.02
COX-2	Bovine seminal vesicle	Arachidonic acid	DuP697	34.01
LTB_4	Guinea pig spleen	[^{3}H] LTB_4	LTB_4	79.02
*The radio-ligand binding studies were carried out by Nova Screen Biosciences Corporation USA				

Carrageenan, histamine, serotonin or zymosan A-induced paw inflammation

Intra plantar injection in the hind paw with carrageenan induced a progressive inflammation reaching a maximum at 3 h. Animals treated with aqueous extract of N.*zeylanica* at doses 100 and 200 mg showed a significant inhibition of inflammation in all phases of the experiment. The anti-inflammatory effect at the 200 mg/kg dose was comparable to that of Indomethacin (20 mg/kg, p.o) (figure 2).

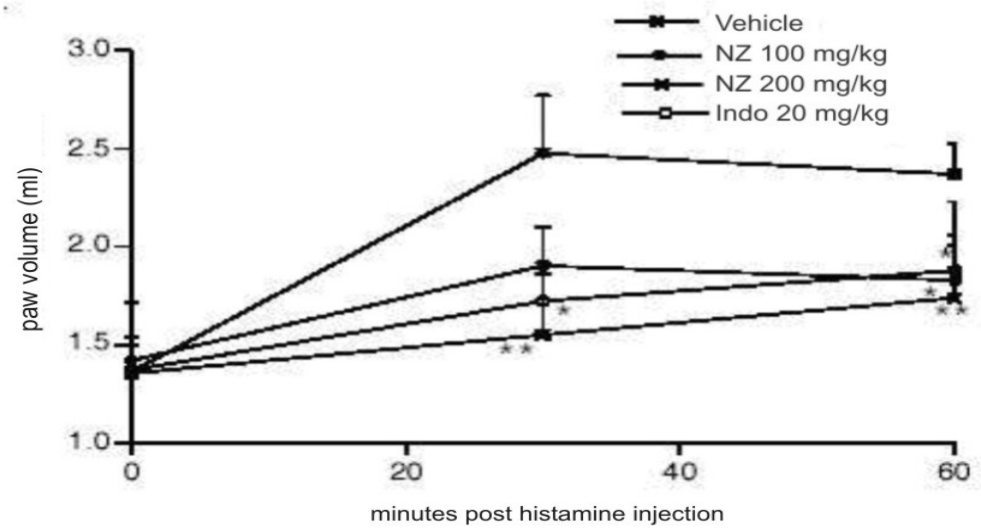
At the lower dose (100 mg/kg, p.o.), N.*zeylanica* extract did not exhibit significant inhibition of paw inflammation induced by intra plantar injection of histamine or serotonin or zymosan A. However, at 200 mg/kg dose N.*zeylanica* significantly inhibited the pedal inflammation caused by histamine or serotonin or zymosan A, which was similar to that of Indomethacin (2*0* mg/kg, p.o.) (figure 3), (figure 4), (figure 5).

Figure 2: Effect of Naravelia zeylanica on carrageenan induced pedal edema.



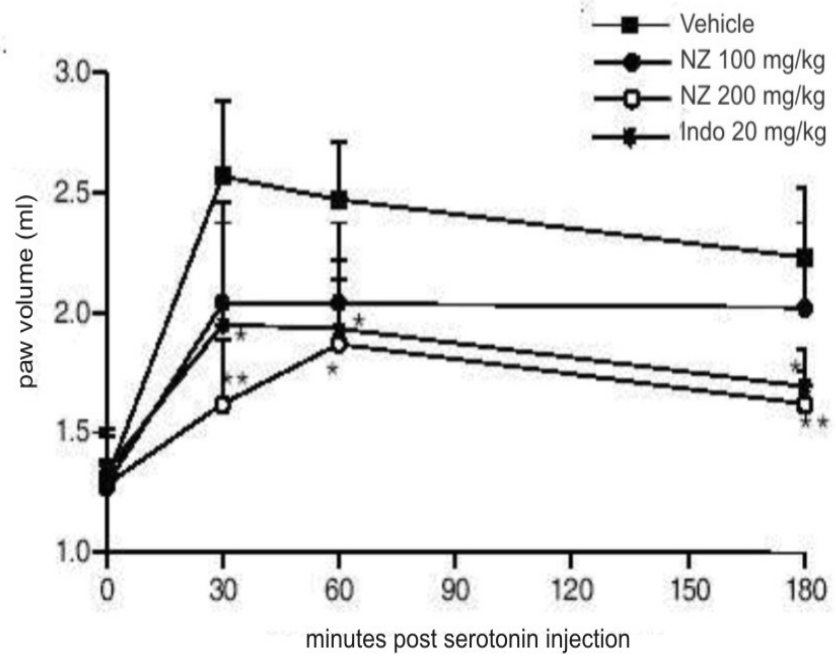
Each point represents mean±SEM. *P<0.05 and **P<0.001 compared with saline control (ANOVA followed by Dunnett's test).

Figure 3: Effect of Naravelia zeylanica on histamine induced pedal edema.



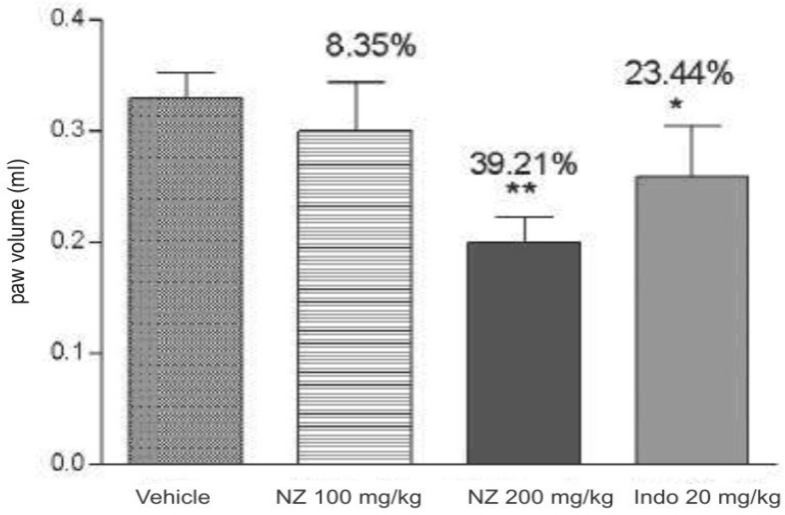
Each point represents mean±SEM. *P<0.05 and **P<0.001 compared with saline control (ANOVA followed by Dunnett's test).

Figure 4: Effect of Naravelia zeylanica on serotonin induced pedal edema.



Each point represents mean±SEM. *P<0.05 and **P<0.001 compared with saline control (ANOVA followed by Dunnett's test).

Figure 5: Effect of Naravelia zeylanica on zymosan A induced pedal edema.



Each point represents mean±SEM. *P<0.05 and **P<0.001 compared with saline control (ANOVA followed by Dunnett's test).

TPA-induced acute mouse ear inflammation

Topical application of aqueous extract of N.*zeylanica* inhibited the TPA-induced acute mouse ear inflammation by 37.31% and 52.08 % at doses 5 and 10 mg/ear respectively. The reference drug Indomethacin (0.5 *mg*/ear) exhibited 59.90% inhibition in this inflammatory model (table 2).

Capsaicin-induced acute mouse ear inflammation

Topical application of aqueous extract of N.*zeylanica* significantly (P< 0.001) inhibited the capsaicin-induced acute mouse ear inflammation by 26.13% and 41.15 % at doses 5 and 10 mg/ear respectively. The antiinflammatogenic effect exhibited by N.*zeylanica* was comparable to that of reference drug Indomethacin (0.5 mg/ear, 42.39% inhibition) in capsaicin-induced mouse ear inflammation (table 2).

Arachidonic acid (AA)-induced mouse ear inflammation

Administration of AA to mouse produced a short inflammatory response. Topical application of N.*zeylanica* extract showed a significant (P< 0.001) inhibition of AA-induced ear inflammation at doses 5 mg/ear (25.44% inhibition) and 10 mg/ear (40.40% inhibition) (table 2).

Mouse ear inflammation induced by multiple topical application of TPA

N.*zeylanica* at 5 mg/ear did not exhibit a significant inflammation reduction in prolonged inflammation induced by multiple applications of TPA to mouse ear. However, at the 10

mg/ear dose the extract showed mild antiinflammatogenic effect on TPA-induced chronic mouse ear inflammation. The glucocorticoid dexamethasone (0.5mg/ear) exhibited a statistically significant (P< 0.05) reduction of inflammation (table 2).

Table 2: Effect of aqueous extract of Naravelia *zeylanica* on ear inflammation by various agents

Treatment (mg/kg)	TPA	Capsaicin	Arachidonic acid	Multiple TPA
Acetone control	0.35±0.02	0.32±0.02	0.27±0.01	0.18±0.01
N.*zeylanica* (5)	0.22±0.01** (37.31)	0.24±0.02** (26.13)	0.20±0.01** (25.44)	0.17±0.00 (3.41)
N.*zeylanica* (10)	0.17±0.00** (52.08)	0.19±0.01** (41.15)	0.16±0.01** (40.40)	0.16±0.00* (10.98)
Indomethacin (0.5)	0.14±0.00** (58.90)	0.19±0.01** (42.39)	-	-
Phenidone (1)	-	-	0.14±0.00** (48.88)	-
Dexamethasone (0.05)	-	-	-	0.14±0.00* (19.32)
One-way F	49.14	15.07	32.92	2.91
ANOVA df	3.16	3.16	3.16	3.16
P	<0.001	<0.001	<0.001	<0.05
Ear thickness (in mm) is expressed as mean±SEM and percentage inhibition in parentheses. n=5 in each group. *P<0.05, **P<0.001 compared to acetone control. TPA=12-O-tetradecanoylphorbol 13-acetate.				

Oxazolone and DNFB-induced contact-delayed type hypersensitivity mouse ear inflammation

N.*zeylanica* failed to show any significant effect on oxazolone and DNFB-induced mouse ear swelling at 5 mg/ear dose. At 10 mg/ear dose a mild antiinflammatogenic effect was observed (8.59% inhibition in oxazolone and 10.23% inhibition in DNFB) (table 3).

Table 3. Effect of aqueous extract of Naravelia *zeylanica* on ear inflammation by various agents

Treatment (mg/kg)	Oxazolone	DNFB
Acetone control	0.26±0.01	0.34±0.03
N.*zeylanica* (5)	0.28±0.01 (0.00)	0.32±0.02 (6.04)
N.*zeylanica* (10)	0.24±0.01 (8.59)	0.31±0.02 (10.23)
Dexamethasone (0.5)	0.20±0.01 (22.35)	0.25±0.02* (26.51)
One-way F	0.69	2.87
ANOVA df	3.16	3.16
P	<0.05	<0.05
Ear thickness (in mm) is expressed as mean±SEM and percentage inhibition in parentheses. n=5 in each group. *P<0.05, **P<0.001 compared to acetone control. DNFB=2, 4-dinitrofluorobenzene		

Discussion

The present study established the anti-inflammatory activity of the aqueous extract of N.*zeylanica*. The extract produced marked inhibition of carrageenan-induced rat paw inflammation, a test which has a significant predictive value for anti-inflammatory agents acting by inhibiting the mediators of acute inflammation (14). Also, carrageenan-induced paw inflammation is a test largely used to study both steroidal and non-steroidal anti-inflammatory drugs. Carrageenan induces an inflammatory reaction in two different phases. The initial phase, which occurs between 0 and 2 h after injection of carrageenan, has been attributed to the release of histamine, serotonin and bradykinin on vascular permeability (*23*). The inflammation volume reaches its maximum approximately 3 h post-treatment after which it begins to decline (24). The late phase, which is also a complement-dependent reaction, has been shown to be due to overproduction of prostaglandin in tissues (25). N.*zeylanica* extract inhibited the inflammation from the first hour, acting on both the early as well the late phases. N.*zeylanica* extract also effectively inhibited the inflammation produced by histamine and serotonin, which suggests that the anti-inflammatory activity of N.*zeylanica* is possibly mediated by inhibiting the action of these mediators. In addition to these mediators, NO also plays an important role in carrageenan-induced paw inflammation (26). Inducible nitric oxide synthase (iNOS) expression and subsequent production of NO maintains the inflammation. In radio-ligand binding studies, at a concentration of 500 µg/ml N.*zeylanica* has an 84.10% inhibition of NOS (constitutive neuronal) (table 1), which may support to the present *in vivo* findings.

Zymosan, an insoluble fraction of yeast cell wall produces an inflammatory response through multiple factors which include generation of anaphylotoxins that induce histamine release from mast cells, biosynthesis of eicosanoids by neutrophil macrophages and generation and release of platelet-activating factors, oxygen free radicals and lysosomal enzymes (27),(28). N.*zeylanica* at 200 mg/kg dose significantly inhibited the zymosan-induced paw inflammation in mice, suggesting that N.*zeylanica* could involve inhibition of one or more of the above mentioned inflammatory mediators.

Topical application of TPA offers a model of skin inflammation appropriate for evaluating anti-inflammatory agents. TPA produces inflammation by activating phospholipase A2 (PLA2) which subsequently activates the release and metabolism of AA. The COX and 5-LO inhibitors are very effective in suppressing TPA-induced ear inflammation indicating the role of prostaglandins and leukotrienes respectively (17). Topical application of N.*zeylanica* extract significantly inhibited TPA-induced acute ear inflammation. *In vitro* inhibition of COX (table 1) and 5-LO (figure 1) by N.*zeylanica* could be corroborated with the results of the *in vivo* acute inflammatory model.

It is reported that, capsaicin-like molecules affect C-fiber thin primary afferent neurons that are connected to distinct sensory receptors (29). Stimulation of sensory nerves in the skin causes an inflammatory reaction comprising arteriolar dilatation, increase in vascular permeability and recruitment of leukocytes (30). This neurogenic inflammation arises from the release of vasoactive peptide transmitters such as substance P and calcitonin-gene related peptide (CGRP) from the peripheral endings of the afferent nerve fibers (31).

It is possible that the anti-inflammatory effect of N.*zeylanica* observed in this model could be related to inhibition of the above peptide mediators.

According to Young et al, (32) AA provokes a rapid intense inflammatory response in the mouse ear inflammation that is affected by lipoxygenase inhibitors, and the COX inhibitors are generally not active in this model. Effect of N.*zeylanica* on AA-induced ear inflammation could be correlated with its 5-LO inhibitory activity observed *in vitro* (figure 1).

Repeated application of TPA causes a measurable increase in the ear skin mastocyte population (20). Although glucocorticoids are the most active drugs against the chronic inflammation caused by repeated application of TPA, other pharmacological agents have also been found to be effective, e.g. 5-LO inhibitors (18). We thus propose that the mild inhibition by N.*zeylanica* could be due to its combined inhibition of 5-LO, LTB4 and COX.

Topical oxazolone or DNFB-induced delayed type hypersensitivity (mouse ear inflammation test) is applied as a tool to discover new drugs that could inhibit the inflammation and tissue destruction. It has been reported that non-steroidal anti-inflammatory drugs are fairly inactive in this model (21). No potential inhibitory effect of N.*zeylanica* was observed in the above models of chronic inflammation.

In conclusion, N.*zeylanica* a phytomedicine, exhibited anti-inflammatory activity in various *in vitro* and *in vivo* models of inflammation. These results suggest that cyclo-oxygenase and lipoxygenase pathways could be involved in the anti-inflammatory activity of N.*zeylanica.*

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