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Anti-Inflammatory Activity of *Vitex leucoxylon* L. Bark Extracts Against Freund's Complete Adjuvant Induced Arthritis in Sprague Dawley Rat

Alluri V. Krishnaraju, Chundi B.M. Rao, Dodda Sundararaju,
Krishanu Sengupta and Golakoti Trimurtulu
Laila Impex R and D Centre, Unit-I, Phase-III, Jawahar Autonagar,
Vijayawada-520007 Andhra Pradesh, India

Abstract: Problem statement: Anti-inflammatory agents, particularly steroids and cyclooxygenase inhibitors are often associated with adverse side effects including, GI irritation, ulcers, hypertension and cardiac abnormalities. A safe and effective intervention is essential for the treatment of inflammatory disorders. **Approach:** Abstract *Vitex leucoxylon* L., a medicinal plant of the verbenaceae family, used in traditional medicine for relieving headache and catarrh. VL-89/185A, was obtained by bio activity-guided fractionation using 5-Lipoxygenase inhibitory activity. VL-89/185A was further tested against Freund's Complete Adjuvant (FCA) induced arthritis in Sprague Dawley (SD) rat. **Results:** Oral supplementation of VL-89/185A resulted in significant anti-inflammatory effects as indicated by reduction in paw edema both at 100 and 250 mg kg^{-1} doses when compared to untreated control rats. Furthermore, treatment with VL-89/185A significantly reduced circulating proinflammatory cytokines TNF-α and IL-1β. The safety of VL-89/185A was established (LD_{50} >5000 mg kg^{-1} body) by acute oral toxicity limit test according to OECD guidelines 425. **Conclusion:** The safety and efficacy profiles indicated that VL-89/185A is a safe intervention for inflammatory disorders.

Key words: Arthritis, IL-1β, 5-lipoxygenase, TNF-α, *Vitex leucoxylon*

INTRODUCTION

Vitex leucoxylon L., commonly known as lokki, is a medicinal plant of the Verbenaceae family. It is widely distributed in Eastern Ghats and Deccan plateau in India. The leaves of *V. leucoxylon* are used in traditional medicine for relieving headache, fever and catarrh[1]. General pharmacological studies revealed anti-psychotic, anti-depressant, analgesic, anti-inflammatory, anti-parkinsonian and anti-microbial activities of aqueous and ethanolic extracts of leaves of *V. leucoxylon*[2]. Sarma *et al*.[3] have studied the anti-inflammatory and wound healing properties of the crude alcoholic extract of the leaves in acute inflammation model[3]. The roots and bark are astringent and the roots are reported to be used as a febrifuge. β-Sitosterol, dimethyl terphthalate, vitexin, isovitexin, agnuside and aucubin were isolated from the leaves or barks of *V. leucoxylon*[4].

Inflammation is typically a protective mechanism that is triggered in response to noxious stimuli, trauma or infection to guard the body and to hasten-up the recovery process. However, inflammation that is unchecked leads to chronic inflammatory disorders. Arachidonic Acid (AA) metabolism plays a crucial role in inflammatory process and associated diseases. Some of the anti-inflammatory drugs inhibit the lipoxygenase pathway and some inhibit cyclooxygenase pathway and these two pathways can be used for potential interventions against inflammation. Unfortunately most of the anti-inflammatory drugs, particularly steroids and cyclooxygenase inhibitors are often associated with adverse side effects including, GI irritation, ulcers, hypertension and cardiac abnormalities[5-6].

The use of herbal remedies for arthritis treatment has been gaining momentum in recent years[7]. There has been some concern over the use of COX-2 inhibitors for therapeutic intervention, especially since some of the products based on COX-2 were either withdrawn or made to carry warning by the US FDA[8,9]. 5-Lipoxygenase (5-LOX) inhibitors of herbal origin on the other hand are reported to offer significant relief and devoid of adverse effects 5-LOX inhibitors are thus becoming first choice of treatment for chronic inflammatory disease such as arthritis[10,11]. Tumor necrosis factor α (TNF-α) is a pleotropic inflammatory

Corresponding Author: Alluri V. Krishnaraju, Laila Impex R and D Centre, Unit-I, Phase-III, Jawahar Autonagar, Vijayawada-520007 Andhra Pradesh, India Tel: +91 866 2545244 Fax: +91 866 2546216

cytokine produced by the immune system that suppresses tumor cell proliferation. Subsequent studies established that TNF-α is a key mediator of inflammation[12-13]. Interleukin-1, another important cytokine produced mainly by blood monocytes, mediates the panoply of host reactions collectively known as acute phase response. It is also known as Endogenous Pyrogen (EP), mononuclear cell factor and lymphocyte activating factor. Both IL-1α and IL-1β can trigger fever by enhancing prostaglandin E_2 (PGE_2) synthesis by the vascular endothelium of the hypothalamus and can stimulate T cell proliferation. The cytokine interleukin 1 beta (IL-1β) is a potent mediator in response to infection and injury[14].

V. leucoxylon leaf extracts were previously tested for anti-inflammatory activity using carrageenan induced paw edema. However the bark extracts *V. leucoxylon* were not tested using arthritis or chronic inflammatory models to prove the efficacy of *V. leucoxylon.* Hence, we sought to study the 5-lipoxygenase inhibitory activity of *V. leucoxylon* bark extracts and its anti-arthritis potential against FCA induced arthritis.

MATERIALS AND METHODS

Plant material and chemicals: The plant material (bark of *V. leucoxylon* L) was collected from Raapur reserve forest Veligonda range, Nellore District, (South-Eastern Ghats) of Andhra Pradesh, in September 2005 and identified by Dr. K. Narasimha Reddy. A voucher specimen (No. LIH 6577) was deposited in the raw drug specimen depository of the Taxonomy Division at Laila Impex R and D Centre, Vijayawada, India. Powdered material (750 g) of *V. leucoxylon* bark, was extracted with hexane (2 L), ethyl acetate (1.75 L) and methanol (1.75 L) using a Soxhlet apparatus and the spent material was then successively extracted with aqueous methanol (80%, 2 L) and water (2 L). The extracts were filtered, concentrated, independently and dried under reduced pressure to obtain, hexane (8.2 g), ethyl acetate (4.5 g), methanol (40 g), aqueous methanol (42 g) and water (38 g) extracts and then used as test substances. A Potent 5-lipoxygenase (5-LOX) inhibitory fraction obtained through bioactivity guided fractionation was used for *in vivo* efficacy and toxicity studies. Aqueous methanol extract of *V. leucoxylon* (VL-89/185A) bark was found to be the most potent 5-LOX inhibitor. It was used for *in vivo* study. NDGA, ethanol, linoleic acid, potassiumdihydrogenphosphate, dipotassiumhydrogenphosphate and all other reagents unless otherwise mentioned were obtained from Sigma Chemical Company (St. Lousis, MO).

***In vitro* 5-Lipoxygenase inhibition:** 5-LOX enzyme inhibitory activity of *V. leucoxylon* extracts was measured using the method of Reddanna *et al.*[15] modified by Ulusu *et al.*[16] The assay mixture contained 80 μM linoleic acid and 10 μl potato 5-LOX in 50 mM phosphate buffer (pH 6.3). The reaction was initiated by the addition of enzyme buffer mix to linoleic acid and the enzyme activity was monitored as the increase in absorbance at 234 nm. The reaction was monitored for 120 sec and the inhibitory potential of the test substances was measured by incubating various concentrations of test substances for two minutes before addition of linoleic acid. All assays were performed in triplicate. Percentage inhibition was calculated by comparing slope of test substances with that of enzyme activity. The data was shown in Fig. 1.

FCA induced arthritis in sprague dawley rats: Male and female SD rats obtained from National Institute of Nutrition (NIN), Hyderabad, were housed under standard laboratory conditions with free access to food and water. The temperature (20-24°C), relative humidity (45-70%) and a 12-h light/dark cycle were maintained. The IAEC of Laila Impex R and D Centre has approved this protocol (LI 061006B). All animal procedures were performed in strict compliance with the guidelines issued by the CPCSEA. Adjuvant induced arthritis experiment was done according to the method of Chang *et al.*[17], which was modified by Roy *et al.*[18] Briefly, the healthy SD rats were selected, acclimatized and then orally supplemented with VL-89/185A at doses 100 or 250 mg kg^{-1} body weight or prednisolone at 10 mg kg^{-1} in 10 mL kg^{-1} of 0.5% CMC for four weeks. The control group received the same volume of vehicle (0.5% CMC) orally. On 14th day of treatment, 50 μL Freund's Complete Adjuvant (FCA) was injected subcutaneously in to the sub-plantar region of left hind paw. Blood samples were drawn on 3rd, 7th and 14th day after induction, centrifuged in refrigerated centrifuge and the serum aliquots were frozen at -80°C for analysis of pro-inflammatory cytokines. The paw volume was measured by independent observers with no knowledge of treatment allocation before and after the injection of Freund's adjuvant and continued daily there after for 14 days using water displacement plethysmometer (Ugo basile, Italy). The difference in volume (V_{13}) between paw volume on day 13 after injection and that on the day (V_0) of injection is calculated to estimate inflammatory response. Percent inhibition = [(Mean Edema of control gp-Mean Edema of treated gp)/Mean Edema of control gp]×100. The data was shown in Fig. 2.

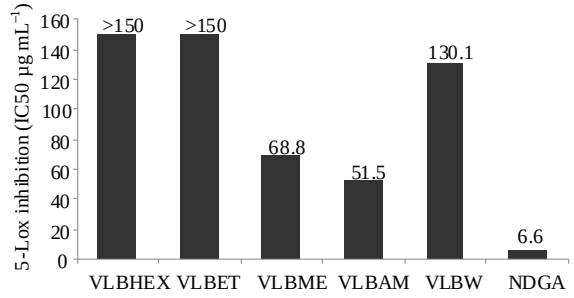

Fig. 1: Bar diagrammatic representations of *in vitro* 5-lipoxygenase inhibitory activity. The bars represent, *V. leucoxylon*bark hexane, ethyl acetate, methanol, aqueous methanol and water extracts; and a positive control nordihydroguiaritic acid. Each bar represents 50% enzyme inhibitory concentration (IC_{50} in µg mL^{-1})

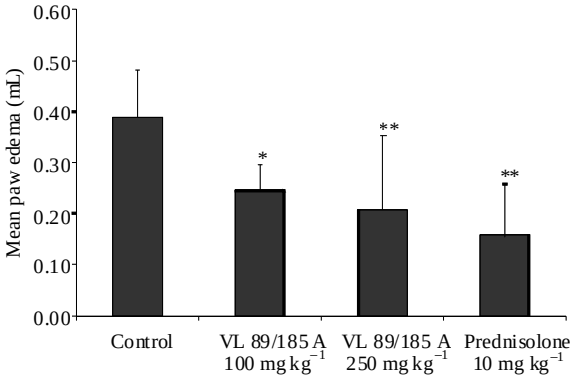

Fig. 2: Bar diagrammatic representations of paw edema in different group of animals. After 13 days of FCA challenge, paw volumes were measured using water displacement plethysmometer and edema was calculated by subtracting initial paw volume V_0 from final paw volume V_{13}. The bars represent, control, VL 89/185A 100, 250 and prednisolone 10 mg kg^{-1}. Each bar represents mean±SEM. N = 6, *: p<0.05 and **: p<0.005 (Vs control)

Measurement of serum cytokineș: The serum TNF-α or IL-1β concentrations were measured as per the instructions provided with the rat cytokine ELISA development kits (R and D Systems, USA). Briefly, 0.1 µg of either TNF-α or IL-1β capture antibody was coated onto each well of 96-well ELISA plates (Maxisorp, NUNC, Denmark). The non-specific sites in the reactive wells were blocked with 1% BSA and thereafter 50 µL of rat serum sample from each animal

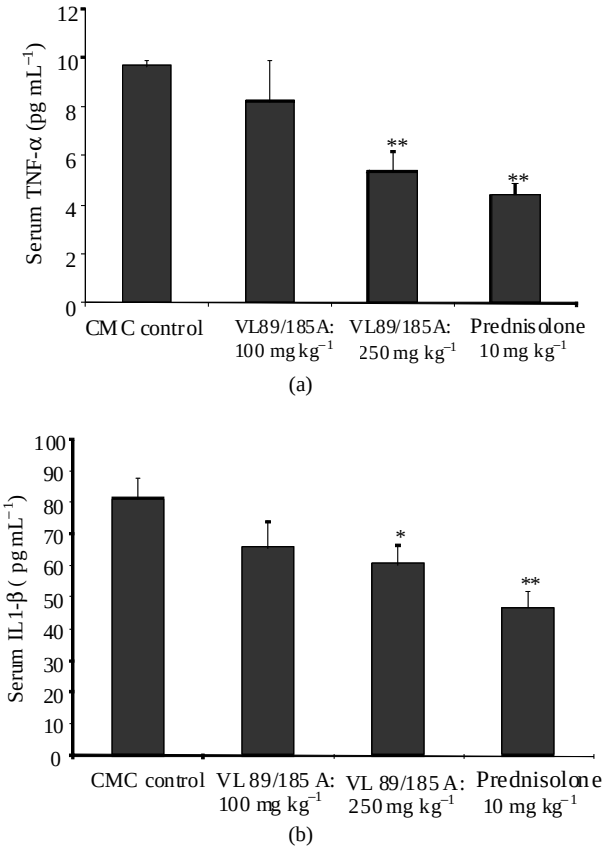

Fig. 3: Bar diagrammatic representations of serum TNF-α (A and IL-1βB concentrations in different group of animals. After 14 days of FCA challenge, serum TNF-α and IL-1β were quantitatively measured by enzyme-immuno assay kit (R and D Systems, USA). The bars represent, control, VL 89/185A 100, 250 and prednisolone 10 mg kg^{-1}. Each bar represents mean±SEM. N = 4,*: p<0.05 and **: p<0.005 (Vs control)

was added separately to each well and incubated for 2 h at room temperature. The specifically bound TNF-α or IL-1β was probed with respective detection antibodies and the antigen-antibody reactions were detected with avidin-peroxidase conjugate. Finally, the specific color reactions were developed by adding TMB substrate and were read at 405 nm in a micro plate ELISA reader (BioRad, USA). The mean of each group was estimated and the data is shown in Fig. 3.

Acute oral toxicity: Acute oral toxicity of VL-89/185A was studied according to OECD guidelines, #425; "acute oral toxicity up-and-down procedure"[19]. The

study protocol (LI 050403) was approved by Institutional Animal Ethics Committee (IAEC) and the study was conducted at the animal facility of Laila Impex R and D Centre, which was registered by CPCSEA (Reg No 204/CPCSEA). Three Female Sprague-Dawley rats (age: 9-10 weeks), obtained from National Institute of Nutrition (Hyderabad, India), were allowed free access to standard rodent diet supplied by Tetragon Chemie (Bangalore, India) and provided filtered U.V. exposed water *ad libitum*. The animals were acclimated to laboratory conditions for 7 days prior to initiation of dosing. The animal room was kept at a controlled temperature (20-24°C), humidity (45-70%) and light (12 h light 12 h^{-1} dark). A limit test was performed on three female Sprague-Dawley (SD) rats. Dose formulation of VL-89/185A was prepared prior to administration, by suspending VL-89/185A in 0.5% CMC in water to obtain a final concentration of 500 mg mL^{-1} to allow a constant dosage volume of 10 mL kg^{-1} $body^{-1}$ weight. Female rats were administered VL-89/185A by oral gavage. The rats were fasted overnight prior to dosing and returned to feeding 3 h after dosing. On the day of dosing, all the animals were observed for mortality and signs of intoxication at 1, 3, 4 and 5 h following dosing and thereafter they were observed twice a day for 14 days. Body weights of rats were individually recorded before dosing (day 0) and thereafter continued at weekly intervals. All animals were sacrificed at the end of the observation period and subjected to a complete gross necropsy.

Statistical analysis: The *in vivo* data were analyzed using ANOVA, all values reported as mean±SEM from 5-6 samples. Statistical significance was set at $p<0.05$.

RESULTS

5-Lipoxygenase enzyme inhibitory activity: According to the results shown in Fig. 1, it is quite evident that methanol and aqueous methanol and water extract of *V. leucoxylon* bark exhibited dose dependent 5-lipoxygenase inhibitory activity with IC_{50} values 68.8, 51.5 and 130.1 μg mL^{-1} respectively. The methanol and aqueous methanol extracts exhibited moderate 5-Lipoxygenase inhibitory activity, when compared with known standard NDGA.

FCA induced arthritis: Oral supplementation of *V. leucoxylon* active fraction VL-89/185A resulted in dose dependent and statistically significant efficacy against Freund's Complete Adjuvant induced arthritis in SD rats. Both 100 and 250 mg kg^{-1} doses of VL-89/185A showed significant inhibition of paw edema with percentage inhibitions 36.92 and 46.41% respectively. The positive control, prednisolone (10 mg kg^{-1}) exhibited better inhibition (59.74%) in paw edema. FCA injected, vehicle control rats showed significant elevation of proinflammatory cytokine TNF-α on 14th day after induction relative to healthy control animals. Oral supplementation of VL-89/185A resulted in statistically significant reduction of TNF-α in serum at a dose of 250 mg kg^{-1}. The positive control prednisolone exhibited highly significant reduction of TNF-α in serum at a dose of 10 mg kg^{-1} Fig. 3a. Circulating levels of IL-1β were higher in FCA induced animals. Oral treatment of FCA induced rats with VL-89/185A had dose dependent effect on circulating IL-1β at doses of 100 and 250 mg kg^{-1}. FCA induced rats treated with prednisolone (10 mg kg^{-1}) resulted in highly significant reduction of circulating IL-1β as shown in Fig. 3b.

Acute oral toxicity study: In the present study, single oral administration of VL-89/185A was made to female SD rats to assess its systemic toxicity, as a limit test. VL-89/185A, at the limit dose level of 5,000 mg kg^{-1} body weight did not caused mortality in any of the three treated test animals (three female SD rats) and did not induced any signs of evident toxicity following dosing and during the post treatment observation period of 14 days. The body weight gain found to be normal and no gross pathological alterations were encountered in any of the rats, as evident at terminal necropsy. Based on these results the median lethal dose (LD_{50}) of VL-89/185A after single oral administration in female SD rats was found to be greater than 5,000 mg kg^{-1} body weight under the conditions of this study.

DISCUSSION

Many Plant derived compounds have tested for their ability to block leukotriene synthesis in isolated cells from rat, mouse or human sources. The polyphenol, nordihydroguaretic acid (NDGA) from the Mexican desert plant *Larrea divaricata* was the first plant-derived 5-Lipoxygenase inhibitor[20]. Plant derived chemical constituents like flavanoids, coumarins, Quinones, pentacyclic triterpenes, sesquiterpenes, alkaloids and polyacetylates have been reported to be 5-Lipoxygenase inhibitors[21,22]. In the present study, VL-89/185A from barks of *V. leucoxylon* showed 5-lipoxygenase inhibitory activity. Bio-assay (5-Lipoxygenase inhibition) guided fractionation yielded VL-89/185A as the most active fraction. This active fraction was used for in vivo anti-inflammatory study.

Freund's Complete Adjuvant (FCA) induced arthritis and collagen induced arthritis are the two animal models which are extensively used to study anti-arthritis potential of therapeutic interventions. Paw swelling is one of the major factors in assessing the degree of inflammation and efficacy of test drugs[23,24]. The anti-inflammatory efficacy of some plants of vitex genus including *V. altissima* were evaluated in rat models of paw edema[21,25]. In the present investigation, various bark extractives and fractions were screened by *in vitro* 5-LOX enzyme inhibitory assay. The most potent 5-LOX inhibitory fraction (VL-89/185A) was then evaluated for *in vivo* efficacy against FCA induced arthritis in rats. VL-89/185A obtained from the bark showed dose dependent efficacy with 36.92 and 46.41% inhibition of paw edema at 100 and 250 mg kg^{-1} doses respectively. Where as standard prednisolone showed 59.74% inhibition at a dose of 10 mg kg^{-1} body weight. Elevated level of TNF-α has been reported in arthritis patients and in experimentally induced arthritis Philippe *et al*.[26] TNF-α and IL-1β are potent proinflammatory cytokines capable of inducing multiple signaling cascades that can serve in host defense and paradoxically contribute to inflammatory tissue injury[27]. Oral supplementation of VL-89/185 A obtained from the bark of *V. leucoxylon* resulted in dose dependent and statistically significant reduction of circulating TNF-α and IL-1β. As the active fraction has shown significant anti-inflammatory activity, an acute oral toxicity study was initiated. Acute oral toxicity limit test of VL-89/185A revealed that it is non-toxic up to 5000 mg kg^{-1} body weight in female SD rats

CONCLUSION

VL-89/185A exhibited significant inhibition of paw edema in FCA induced arthritis. The reduction in circulating cytokines TNF-α and IL-1β further confirmed its efficacy. Based on foregoing preclinical efficacy and safety data, the 5-LOX inhibitory fraction VL-89/185A obtained from *V. leucoxylon* bark extract can be considered as safe and effective intervention for inflammatory diseases including arthritis.

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