

Beneficial effects of methanolic extract of *Indigofera linnaei* Ali. on the inflammatory and nociceptive responses in rodent models

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Indigofera linnaei Ali. (Tamil Name: Cheppu Nerinjil) belongs to the family Fabaceae, used for the treatment of various ailments in the traditional system of medicine. In the present study, the beneficial effects of methanol extract of whole plant of *I. linnaei* (MEIL) were evaluated on inflammation and nociception responses in rodent models. *In vitro* nitric oxide (NO), lipoxygenase (LOX) and cyclooxygenase (COX) inhibitory activities were also performed to understand the mode of action. MEIL at the dose of 200 & 400 mg/kg, p.o. significantly inhibited carrageenan induced rat paw volume and reduced the weight of granuloma in cotton pellet granuloma model. The results obtained were comparable with the standard drug aceclofenac. The anti-nociceptive effect of MEIL in mice was evaluated in hot plate and acetic acid induced writhing model. The plant extract significantly reduced the number of writhes and the analgesic effect was higher than that of the standard drug aspirin. However, the extract fails to increase the latency period in hot plate method suggesting that the extract produce nociception by peripheral activity. The extract produced inhibitory effect on NO, LOX and COX in concentration dependent manner. The extract exhibited pronounced and selective COX-2 inhibition. Altogether, these results suggested that the methanol extract of *Indigofera linnaei* could be considered as a potential anti-inflammatory and analgesic agent.

Uniterms: *Indigofera linnaei*/pharmacognosy. *Indigofera linnaei*/anti-inflammatory activity. *Indigofera linnaei*/analgesic activity. *Indigofera linnaei*/anti-nociceptive effect. Nitric oxide/scavengers. Cyclooxygenase/inhibitors. Lipoxygenase/inhibitors. Medicinal plants.

Indigofera linnaei Ali pertence à família Leguminosae e é utilizada para o tratamento de várias doenças na medicina tradicional. No presente estudo, os efeitos benéficos do extrato metanólico da planta inteira de *I. linnaei* (MEIL) foram avaliados em respostas inflamatórias e nociceção em modelos de roedores. Testes *in vitro* de atividade inibitória do óxido nítrico (NO), lipoxigenase (LOX) e ciclooxigenase (COX) também foram realizados para compreender o modo de ação. MEIL nas doses de 200 e 400 mg/kg, p.o. inibiu significativamente o volume da pata de rato induzido por carragenana e reduziu o peso do granuloma no modelo de pélete de algodão. Os resultados obtidos foram comparáveis ao do fármaco padrão, aceclofenaco. O efeito anti-nociceptivo de MEIL foi avaliado em camundongos no modelo de placa quente e de contorção induzida por ácido acético. O extrato da planta reduziu significativamente o número de contorções e o efeito analgésico foi maior do que o do fármaco padrão, ácido acetilsalicílico. Porém, o extrato não conseguiu aumentar o período de latência no método da placa quente, sugerindo que este produz nociceção por atividade periférica. O extrato produziu efeito inibitório sobre o NO, LOX e COX dependente da concentração. O extrato exibiu inibição acentuada e seletiva da COX-2. No seu conjunto, estes resultados sugerem que o extrato metanólico de *Indigofera linnaei* poderia ser considerado como agente anti-inflamatório e analgésico potencial.

Unitermos: *Indigofera linnaei*/farmacognosia. *Indigofera linnaei*/atividade anti-inflamatória. *Indigofera linnaei*/atividade analgésica. *Indigofera linnaei*/efeito anti-nociceptivo. Óxido nítrico/sequestrantes. Lipoxigenase/inibidores. Ciclooxigenase/inibidores. Plantas medicinais.

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INTRODUCTION

Inflammation is a protective response of the body towards various injurious stimuli like infections and trauma (Vijayalakshmi *et al.*, 2011; Yonathan *et al.*, 2006). At the same time it is accompanied with pain, redness, swelling and malfunctioning of the affected part of the body (Amira *et al.*, 2012). Inflammatory process involves the release of various chemical mediators that are responsible for signs and symptoms associated with such conditions. Prostaglandins are ubiquitous substances that indicate and modulate cell and tissue responses involved in inflammation. Their biosynthesis has also been implicated in the pathophysiology of cardiovascular diseases, cancer, colonic adenomas and Alzheimer's disease. To alleviate the pain and other associated symptoms various anti-inflammatory agents are used, most of which are synthetic drugs, associated with various side effects such as peptic ulcer, bleeding etc. (Dharmasiri *et al.*, 2003; Bepary *et al.*, 2008). Based on ethnopharmacological uses, many medicinal plants have attracted considerable interest, particularly in the treatment of various disease conditions including chronic inflammatory diseases (Moro *et al.*, 2012). The research into medicinal plants with alleged folkloric use as analgesics, anti-inflammatory agents, should therefore be viewed as a fruitful and logical research strategy in the search for new analgesic and anti-inflammatory drugs.

Indigofera linnaei Ali (Syn: *Indigofera enneaphylla* L.) belongs to the family Fabaceae and is a reputed indigenous medicine. It is a small trailing, much branched annual or biennial herb, distributed throughout India. The juice of the plant is used as antiscorbutic and diuretic, for burns and epilepsy. It has long been used by the ethnic people and native medical practitioners to treat rheumatism, arthritis, inflammation, tumor and liver diseases (Wealth..., 1992). Literature review reveal that three nitropropanoyl esters of glucose namely 1,2,6-tri-*O*-(3-nitropropanoyl)- β -D-glucopyranose, 2,3,4,6-tetra-*O*-(3-nitropropanoyl)- α -D-glucopyranose and 3,4,6-tri-*O*-(3-nitropropanoyl)- α -D-glucopyranose were isolated from the aerial parts of *Indigofera linnaei* (Majak *et al.*, 1992). The plant exhibit wound healing activity in rats (Hemalatha *et al.*, 2001). A new isoflavone namely 7,8-methylenedioxy-4'-methoxyisoflavone has been isolated from the entire plant of *Indigofera linnaei* (Rajendraprasad, Chakradhar, 2004). In our earlier studies, the plant extract exhibited a potent free radical scavenging and antioxidant activity (Kumar *et al.*, 2011a) and anticancer activity (Kumar *et al.*, 2011b). In continuation of our previous works on this plant,

the present study was aimed to evaluate the beneficial effects of methanol extract of *Indigofera linnaei* (MEIL) on inflammatory and nociceptive responses in rodent models.

MATERIAL AND METHODS

Chemicals

Kappa carrageenan type III, acetylsalicylic acid, linoleic acid, lipoxygenase (lipoxidase from soyabean), Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, naphthylethylenediamine dihydrochloride, LPS were purchased from Sigma Chemicals Co., (St. Louis, USA). Pentazocin was obtained from Ranbaxy Laboratories Ltd. (New Delhi, India). Aceclofenac was obtained from Micro Labs (Hosur, India). All other chemical used were of analytical grade. Murine monocytic macrophage cell line RAW 264.7 was procured from National Centre of Cell Science (Pune, India).

Plant material and Extraction

Entire plants of *I. linnaei* were collected from the foothills of Yercaud in the month of November 2014. The plant was authenticated by Dr. G.V.S. Murthy, Joint Director, Botanical Survey of India, Coimbatore, Tamilnadu, India. A voucher specimen is preserved in our laboratory for future reference (Voucher No. P. Ch. IL 002). The plant material was shade dried, pulverized and extracted (500 g) by cold maceration process with 80% methanol at room temperature for 72 h. The extract was filtered and concentrated to dryness under reduced pressure and controlled temperature (40 °C to 50 °C) in a rotary evaporator. The extract was a dark yellowish brown solid weighing 50.2 g (yield, 10.4 %) and was preserved in a vacuum desiccator until further use.

Animals

Male Wistar albino rats (150-200 g) and Swiss albino mice (20-25 g) were procured from Venkatershwara Enterprises, Bangalore, Karnataka, India, and used throughout the study. The animals were housed in microcolon boxes in a controlled environment (temperature 25 ± 2 °C and 12 h dark/light cycle) with standard laboratory diet and water *ad libitum*. The study was conducted after obtaining Institutional Animal Ethical Committee (IAEC) clearance (Proposal No: SVCP/IAEC/Ph.D/01/2014-15 dt 24.1.2015).

***In vitro* anti-inflammatory activities of MEIL**

Cell culture and stimulation

The murine monocytic macrophage cells RAW 264.7 was grown in a plastic culture flask in Dulbecco's Modified Eagle's Medium (DMEM) with L-glutamine supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin solution under 5% CO₂ at 37 °C. After 4-5 days, cells were removed from the culture flask and centrifuged for 10 min at 1500 rpm. The medium was then removed and the cells re-suspended with fresh DMEM. The cell concentration was adjusted to 1.10⁶ cells/mL in the same medium. 100 µL of the above concentration were cultured in a 96-well plate for one day to become nearly confluent. Concentrations ranging from 10-100 µg/mL of the extract or standard (Aceclofenac) were prepared in DMEM to give a volume of 100 µL in each well of a microtitre plate. Then wells were cultured with the vehicle, test extract and standard in the presence of 1 µg/mL of LPS for 24 h.

Nitric oxide inhibitory assay

The nitrite concentration in the culture medium was measured as an indicator of NO production based on the Griess reaction (Hu *et al.*, 2008). 100 µL of each supernatant was mixed with the same volume of Griess reagent (1% sulphanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water) and the remaining mixture was then incubated at room temperature for 10 min. The absorbance at 540 nm was measured using a microtitre plate reader. Nitrite concentration in the supernatant was determined by comparison with a sodium nitrite standard curve.

5-Lipoxygenase Inhibitory assay

The 5-lipoxygenase inhibitory activity was used as an indicator of the anti-inflammatory activity. The assay was done using linoleic acid as substrate and 5-lipoxygenase as enzyme. A total volume of 200 µL assay mixture containing 160 µL sodium phosphate buffer (100 mM, pH 8.0), 10 µL test extract (10-100 µg in 100 mM Tris buffer, pH 7.4) and 20 µL of lipoxygenase enzyme was used. The contents were incubated at 25 °C for 10 min. The reaction was then initiated by the addition of 10 µL linoleic acid solution. The change in absorbance was observed after 6 min at 234 nm (Komatou *et al.*, 2010). All reactions were performed in triplicates in 96-well microplates. Aceclofenac was used as reference standard. The percentage inhibition was calculated by the following formula:

$$\text{Inhibition (\%)} = [A_{\text{Control}} - A_{\text{Test}} / A_{\text{Control}}] \times 100$$

Cyclooxygenase inhibitory assay

In vitro COX-2 inhibitory activities of MEIL have been evaluated using 'COX (Ovine) inhibitor screening kit' (Cayman Chemical Company, Ann Arbor, MI) with 96-well plates. All the reagents added were prepared just before use. This screening assay directly measures PGF₂α produced by SnCl₂ reduction of COX-derived PGH₂. COX-1 and COX-2 initial activity tubes were prepared taking 950 µL of reaction buffer, 10 µL of heme and 10 µL of COX-1 and COX-2 enzymes in respective tubes. Similarly, COX-1 and COX-2 inhibitor tubes were prepared by adding 20 µL of the extract at various concentrations (10-100 µg/mL) in each tube in addition to the above ingredients. The background tubes corresponding to inactivated COX-1 and COX-2 enzymes obtained after keeping the tubes containing enzymes in boiling water for 3 min along with vehicle control. Reactions were initiated by adding 10 µL of arachidonic acid in each tube and quenched with 50 µL of 1 M HCl. PGH₂ thus formed was reduced to PGF₂α by adding 100 µL of SnCl₂. The prostaglandin produced in each well was quantified using prostaglandin specific antiserum that binds with major prostaglandins and reading the plate at 405 nm.

***In vivo* anti-inflammatory activities of MEIL**

Carrageenan-induced rat paw edema

The rats were divided into four groups (n = 6) and were treated orally with MEIL (200 & 400 mg/kg), aceclofenac (10 mg/kg), and vehicle control (0.3% CMC, 1 mL/kg/p.o.). The administration of extract and standard drug was 1 h prior to injection of 0.1 ml of 1% freshly prepared suspension of carrageenan in normal saline in the right hind paw sub plantar of each rat. The paw volume was measured initially and then at 1, 3 and 5 h after the carrageenan injection by using plethysmometer (Sulaiman *et al.*, 2010). The anti-inflammatory effect of MEIL was calculated by the following formula:

$$\text{Anti-inflammatory activity (\%)} = (1 - V_t / V_c) \times 100$$

where V_t represents the paw volume in drug treated animals and V_c represents the paw volume of control groups animals.

Cotton pellet granuloma method

The animals were divided into four groups of six animals in each group. The rats were anaesthetized and sterile cotton pellets weighing 10 ± 1 mg were implanted subcutaneously into both sides of the groin region of

each rat. Group I served as control and received the vehicle (0.3% CMC, 1 mL/kg/p.o.). Group II animals received aceclofenac at a dose of 10 mg/kg for the same period. MEIL at the concentration of 200 & 400 mg/kg was administered orally to groups III and IV animals for seven consecutive days from the day of cotton pellet implantation. On 8th day the animals were killed by giving excess anesthesia and the pellets together with the granuloma tissues were carefully removed and made free from extraneous tissues. The wet pellets were weighed initially and then dried in an oven at 60 °C for 24 h to constant weight and were weighed again. Increment in the dry weight of the pellets was taken as a measure of granuloma formation. The antiproliferative effect of MEIL was compared with control (Jothimanivannan *et al.*, 2010).

***In vivo* antinociceptive activity of MEIL**

Acetic acid induced writhing test

The writhes were induced by intraperitoneal injection of 0.6 % (v/v) acetic acid (80 mg/kg). Group I served as control (0.3% CMC, 1 mL/kg/p.o.) and group II animals received aspirin at a dose of 300 mg/kg. Two different doses of MEIL (200 & 400 mg/kg) were administered orally to the group III and group IV animals. The extract and standard drug were administered 30 min before chemical stimulus. The number of muscular contractions was counted over a period of 20 min and is expressed as writhing numbers (Purnima *et al.*, 2009).

Hot plate method

the hot plate method in rats was performed by using Eddy's hot plate method. The evaluated parameters were the latency time for paw licking and jumping responses on exposure to the hot plate surface, kept at 55 ± 1 °C. The animal was kept in the hot plate until it lifted one of its hind paws. For this method, the animals were divided into four groups of six animals each. Group I served as control (0.3% CMC, 1 mL/kg/p.o.). Group II received pentazocin at a dose of 5 mg/kg. Group III and IV received MEIL at a dose of 200 & 400 mg/kg/p.o. respectively. All the treatments were given 30 min before the thermal stimulus and the response was determined at 60, 120 and 180 min (Nikajoo, 2009).

Statistical analysis

All values were expressed as mean ± SEM. Statistical analysis was performed with one way analysis of variance (ANOVA) followed by Tukey Kramer Multiple Comparison test. P values <0.05 were considered to be statistically significant when compared to control.

Determination of IC₅₀ values and statistical analysis were done by using GraphPad Prism v5.01 software.

RESULTS AND DISCUSSION

Inflammation is a reaction occurring in several types of tissue injuries, infections or immunological stimulation as a defense against foreign or altered endogenous substances. The process of inflammation comprises of a series of changes of the terminal tissues, which tend to eliminate the injurious agents and to repair the damaged tissue. Nitric oxide is a short-lived free radical produced from L-arginine by nitric oxide synthase (NOS) that mediates diverse functions by activating on various cells through interactions with different molecular targets. Although nitric oxide acts as an essential multifunctional mediator in various biological systems, excessive production of nitric oxide by inducible nitric oxide synthase (iNOS) is involved in various types of inflammation and multistage carcinogenesis at inflammatory sites (Lee *et al.*, 2009; Wang *et al.*, 2013). In the present study, a dose dependent and gradual increase in the percentage inhibition was observed with MEIL. Higher percentage inhibition (95.7) was observed at 100 µg/mL concentration of MEIL. The IC₅₀ value was found to be 60.67 µg/mL for MEIL. Aceclofenac at a concentration range of 10-100 µg/mL was employed and served as reference standard. The lowest dose (10 µg/mL) produced an inhibitory percentage of 20.1 and the highest concentration produced 97.03% inhibition. Like MEIL, a dose dependent increase in the percentage of inhibition was observed in all the concentrations tested. The IC₅₀ value of aceclofenac against nitric oxide inhibitory assay was found to be 79.43 µg/mL. The results reveal that the extract inhibits the production of nitric oxide as stimulated by LPS in mouse macrophage cell line RAW 264.7. The results obtained are presented in Table I.

Lipoxygenases (LOX) are members of a class of non-heme iron-containing dioxygenases that catalyze the addition of molecular oxygen to fatty acids containing a *cis, cis*-1,4-pentadiene system to give an unsaturated fatty acid hydroperoxides. It has been proved that these LOX products play a key role in variety of disorders such as bronchial asthma, inflammation and tumor angiogenesis. In human tissues, LOX is expressed in platelet, eosinophils, neutrophils, colonic tissues, lung tissues and bone marrow cells. Neutrophils contain 5-LOX which converts the arachidonic acid to 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HPETE). This 5-HPETE is converted into a series of leukotrienes and the nature of the final product varies according to the tissue. Leukotrienes are mediators of

allergic response and inflammation. Inhibitors of 5-LOX are used in the treatment of inflammation (Shah *et al.*, 2013). Various concentrations (10-100 $\mu\text{g/mL}$) of MEIL were analyzed for 5-lipoxygenase inhibitory activity. A proportionate increase in the percentage of lipoxygenase was observed for MEIL. The IC_{50} value of MEIL was found to be 59.99 $\mu\text{g/mL}$. Maximum inhibition (97.6%) by MEIL was observed at the concentration of 100 $\mu\text{g/mL}$. Aceclofenac at the concentration of 100 $\mu\text{g/mL}$ exhibits a percentage inhibition of 97.8 and the IC_{50} value was found to be 45.97 $\mu\text{g/mL}$. A gradual increase in percentage of inhibition was observed for increasing concentration of aceclofenac. However the concentration of MEIL required for inhibiting lipoxygenase enzyme was higher than that of the standard drug aceclofenac. The methanolic extract of *I. linnaei* inhibits 5-LOX in concentration dependent manner and this inhibitory activity might be attributed to the anti-inflammatory potential of the extract. The results are presented in Table I.

The enzyme cyclooxygenase (COX) has been played as a key role in inflammatory process. It is a membrane bound glycoprotein found in the endoplasmic reticulum of prostanoid forming cells. It occurs catalytically in two active forms i.e. COX-1 and COX-2. These two isoforms of COX are almost identical in structure but have important differences in substrate and inhibitor selectivity and in their intracellular locations (Gacche *et al.*, 2011). Among these two isoforms, COX-2 involved in mediating the inflammatory process produces PGE_2 from endogenous arachidonic acid. COX-2 is inducible and

dramatically up-regulated by a variety of stimuli such as cytokines, mitogens, oncogenes, growth factor and tumor promoters and is detectable in only certain type of tissues. Non-steroidal anti-inflammatory drugs (NSAIDs) are COX inhibitors and they also inhibit COX-1. This is problematic because COX-1 is essential for the repair and maintenance of stomach linings, which result in varying degrees of gastric ulcerations, perforation or obstructions (Gately, 2000). So there is a need for agents which inhibit COX-2 selectively without affecting COX-1. In the present study, the methanolic extract of *I. linnaei* was screened for its inhibitory efficacy against COX-1 and COX-2.

The *in vitro* cyclooxygenase inhibitory study demonstrated that the plant extract had good and selective inhibitory activity against COX-2 as compared to COX-1. The results obtained revealed that MEIL selectively inhibited COX-2 catalysed prostaglandin biosynthesis with an IC_{50} value of 15.82 $\mu\text{g/mL}$, compared with COX-1 derived prostaglandin synthesis which showed an IC_{50} value of 76.48 $\mu\text{g/mL}$. The resulting COX-2 selectivity was 4.83 (Table II). A comparison was also made with COX-1/COX-2 selectivity ratio of some traditional NSAIDs (Indomethacin and aceclofenac) and Celecoxib, a synthetic selective COX-2 inhibitor, obtained in the same assay system. Indomethacin and aceclofenac non-selectively inhibited COX-1/2 ratio. The percentage inhibition produced by MEIL against COX-1 ranged between 12.6 and 65.4, whereas it was 30.17 to 94.47 for COX-2. Maximum enzyme inhibitory effect was observed at 100 $\mu\text{g/mL}$ for both COX-1 and COX-2. The extract inhibits both isoforms

TABLE I - Nitric oxide and 5-lipoxygenase Inhibitory activity of MEIL

Sample	Concentration ($\mu\text{g/mL}$)	NO Inhibitory Activity		5-lipoxygenase Inhibitory Activity	
		Enzyme Inhibition (%)	IC_{50} ($\mu\text{g/mL}$)	Enzyme Inhibition (%)	IC_{50} ($\mu\text{g/mL}$)
MEIL	10	20.73 \pm 0.75		19.9 \pm 0.96	
	20	26.3 \pm 0.9		24.8 \pm 1.16	
	40	36.83 \pm 1.57	60.67	39.27 \pm 1.7	59.99
	60	54.87 \pm 3.48		61.87 \pm 1.36	
	80	77.7 \pm 2.14		91.87 \pm 2.62	
	100	95.7 \pm 1.55		97.6 \pm 1.76	
Aceclofenac	10	20.1 \pm 1.38		21 \pm 1.13	
	20	24.56 \pm 4.15		32.4 \pm 1.53	
	40	37.9 \pm 2.23	79.43	49.83 \pm 1.45	45.97
	60	60.93 \pm 2.04		73.4 \pm 1.69	
	80	84.15 \pm 1.49		92.36 \pm 2.75	
	100	97.03 \pm 1.43		97.8 \pm 1.6	

Data were expressed as Mean \pm SD (n = 3); Average of three independent determinations.

of cyclooxygenase in concentration dependent manner. At the same time, COX-2 selectivity ratio is more than the standard NSAIDs such as indomethacin and aceclofenac. The results obtained indicate that the extract is having selective COX-2 inhibition.

In the present study, the anti-inflammatory and anti-nociceptive activities of the methanolic extract of *Indigofera linnaei* (MEIL) has been established in both acute and chronic inflammation models as employed. Carrageenan induced rat paw edema is a suitable test for evaluating anti-inflammatory drugs which has been used to assess the anti-edematous effect of natural products (Panthong *et al.*, 2003). The development of edema in the paw of the rat after injection of carrageenan is believed to be biphasic event. The initial phase observed during the first hour is attributed to the release of histamine and serotonin; the second phase is due to the release of prostaglandin, protease and lysosome (Paviaya *et al.*, 2013). Based on this, it could be hypothesized that the suppression of the first phase may be due to inhibition of the release of early mediators, such as histamine and serotonin, and the action in the second phase may be explained by an inhibition of cyclooxygenase (Gobianand *et al.*, 2010). Previous studies suggest that the injection of carrageenan into the rat paw induces the liberation of bradykinin, which later induces the biosynthesis of prostaglandins and other autocooids, which are responsible

for the formation of the inflammatory exudates. Besides, in the carrageenan induced rat paw edema model, the production of prostanoids has been through the serum expression of COX-2 by a positive feedback mechanism (Ueno *et al.*, 2000).

Sub-plantar injection of carrageenan in rats showed to a time-dependent increase in paw volume (Table III), which was observed at 1 h and maximum swelling at 5 h after administration of carrageenan injection in the control group. However, carrageenan induced inflammation was significantly ($p < 0.001$) reduced in all phases of the experiment by the treatment with MEIL 200 and 400 mg/kg, as well as aceclofenac. No significant difference in reduction was observed by MEIL when compared to the standard drug aceclofenac, which indicates the equipotent nature of the extract. Maximum inhibition was observed at the dose of 400 mg/kg. The results reveal that the methanol extract of *I. linnaei* had a significant anti-inflammatory effect on the tested dose levels. The extract at a dose of 400 mg/kg, had higher anti-inflammatory potential than aceclofenac (NSAID), a cyclooxygenase inhibitor, while at the dose of 200 mg/kg the activity was similar to that of aceclofenac. Therefore, the results obtained suggest that the mechanism of action of MEIL may be related to the inhibition of prostaglandin biosynthesis.

The cotton pellet granuloma method has been widely employed to assess the transductive, exudative

TABLE II - Comparison of IC₅₀ values of various COX inhibitors with MEIL

Sample/Drugs	IC ₅₀ (µg/mL)		COX-2 Selectivity (COX-1/COX-2)*
	COX-1	COX-2	
MEIL	76.48	15.82	4.83
Celecoxib	24.16	0.48	50.33
Indomethacin	0.006	0.38	0.015
Aceclofenac	0.08	0.26	0.3

*Ratio of the IC₅₀ values for COX-1 and COX-2 can be used as an indication of the COX-2 selectivity of inhibitors. A COX-1/COX-2 ratio of more than 1 indicates preferential COX-2 selectivity.

TABLE III - Effect of MEIL on Carrageenan Induced Paw Edema in Rats

Design of Treatment	Dose (mg/kg)	Paw Volume (mL)		
		1 h	3 h	5 h
Control	-	0.37 ± 0.01	0.52 ± 0.03	0.95 ± 0.03
Aceclofenac	10	0.24 ± 0.01 (35.13)	0.30 ± 0.02 ^a (42.3)	0.28 ± 0.01 ^a (70.52)
MEIL	200	0.27 ± 0.01 (27.02)	0.35 ± 0.01 ^a (32.69)	0.31 ± 0.02 ^a (67.37)
MEIL	400	0.26 ± 0.012 (29.73)	0.31 ± 0.02 ^a (40.38)	0.25 ± 0.06 ^a (73.68)

n=6; Data were expressed as Mean ± SEM; * $p < 0.001$ vs Control; Data were analyzed by One way ANOVA followed by Tukey Kramer Multiple Comparison test. Percentage inhibition is given in parenthesis.

and proliferative components of chronic inflammation is a typical feature of established chronic inflammatory reaction. The fluid absorbed by the pellet greatly influences the wet weight of the granuloma and dry weight correlates well with the granuloma of the granulomatous tissue formed (Joseph *et al.*, 2010). In the present investigation, the methanol extract of *I. linnaei* registered profound anti-inflammatory activity against cotton pellet granuloma in rats (Table IV). The extract exhibited a significant ($p < 0.001$) anti-inflammatory activity and the results were comparable with that of standard drug aceclofenac. The extract at the dose of 400 mg/kg showed the maximum granuloma inhibition, which is equal to aceclofenac.

In the writhing test, high levels of prostaglandins PGE₂ and PGF₂ was observed during the first 30 min after acetic acid injection. Nevertheless, it was found that the intraperitoneal administration of acetic acid induces not only the liberation of prostaglandins, but also of the release of pro-inflammatory cytokines, such as tumor necrosis factor- α (TNF- α), interleukine-1b (IL-1b) and IL-8 from resident peritoneal macrophages and mast cells (Mothana, 2011). The oral administration of MEIL induced the analgesic activity significantly and the results are presented in Table V. The treatment of animals with MEIL at the dose of 200 and 400 mg/kg, produced a significant ($p < 0.001$) and dose dependent inhibition in abdominal writhes produced by acetic acid. The inhibition by MEIL at the dose of 400 mg/kg was nearly similar

to that produced by the standard drug aspirin. Thus, the results obtained for the writhing test using acetic acid are similar to those obtained for the edematogenic test using carrageenan, since MEIL effectively inhibited the writhings in mice in dose dependent manner. The results were comparable with the group treated with standard drug aspirin, which indicate that the antinociceptive effect of *I. linnaei* might be attributed to the inhibition of the synthesis of some pro-inflammatory mediators such as prostaglandins and cytokines.

The hot plate test is a classical approach widely applied in the analgesic investigations for several decades. The hot plate test when associated with the writhing test, can usually distinguish central from peripheral effects (Srinivasan *et al.*, 2003). Some analgesic drugs such as aspirin usually have few effects in the hot plate test indicating their peripheral analgesic activities. But some other analgesic drugs such as ibuprofen and morphine can decrease prostaglandin synthesis via central inhibition of cyclooxygenase (Zhu *et al.*, 2011) or bind to specific opioid receptors in the central nervous system, exhibiting both their peripheral and central analgesic activities. However, the hot plate test was undertaken to verify if MEIL would have any central analgesic effect and the results are presented in Table VI. In this analgesic testing model, pentazocine at a dose of 5 mg/kg, significantly prolonged the reaction time of animals with relatively extended duration of

TABLE IV - Effect of MEIL on Cotton Pellet Granuloma in Rats

Design of Treatment	Dose (mg/kg)	Wet weight (mg)	Inhibition (%)	Dry weight (mg)	Inhibition (%)
Control	-	203.8 \pm 6.24	-	56.17 \pm 2.17	-
Aceclofenac	10	63.17 \pm 2.19 ^a	69	25.5 \pm 2.08 ^a	54.6
MEIL	200	69.67 \pm 2.67 ^a	65.81	28.8 \pm 2.13 ^{a,b}	48.72
MEIL	400	58.47 \pm 1.88 ^a	71.31	24.3 \pm 1.75 ^a	56.73

n=6; Data were expressed as Mean \pm SEM; ^ap < 0.001 vs Control; ^bp < 0.01 vs Aceclofenac; Data were analyzed by One way ANOVA followed by Tukey Kramer Multiple Comparison test.

TABLE V - Effect of MEIL on Chemical Stimulus Induced Pain in Mice

Design of Treatment	Dose (mg/kg)	No. of writhings	Inhibition (%)
Control	-	105.66 \pm 3.20	-
Aspirin	300	49.67 \pm 2.99 ^a	53
MEIL	200	66.83 \pm 2.23 ^{a,b}	36.75
MEIL	400	50 \pm 2.15 ^a	52.68

n=6; Data were expressed as Mean \pm SEM; ^ap < 0.001 vs Control; ^bp < 0.001 vs Aspirin; Data were analyzed by One way ANOVA followed by Tukey Kramer Multiple Comparison test.

TABLE VI - Effect of methanolic extract of *Indigofera linnae* on Thermal Stimulus Induced Pain in Mice

Design of Treatment	Dose (mg/kg)	Reaction Time (Sec.)			
		0 h	1 h	2 h	3 h
Control	-	2.53 ± 0.14	2.45 ± 0.08	2.53 ± 0.1	2.58 ± 0.09
Pentazocine	5	2.4 ± 0.13	7.41 ± 0.35 ^a	9.95 ± 0.41 ^a	8.38 ± 0.25 ^a
MEIL	200	2.5 ± 0.13	3.21 ± 0.28 ^b	3.12 ± 0.15 ^b	2.71 ± 0.18 ^b
MEIL	400	2.5 ± 0.14	3.28 ± 0.18 ^b	3.02 ± 0.2 ^b	2.88 ± 0.31 ^b

n=6; Data were expressed as Mean ± SEM; ^ap < 0.001 vs Control; ^bp < 0.001 vs Pentazocine; Data were analyzed by One way ANOVA followed by Tukey Kramer Multiple Comparison test.

stimulation, confirming centrally mediated activity. The results obtained from the extract treated groups revealed no significant activity when compared to control group and a highly significant difference was observed with the group treated with pentazocin (5 mg/kg). Hence, it is assumed that MEIL has no significant analgesic effect on the central nervous system.

Recently our biological studies on methanol extract of *I. linnaei* reported potent antioxidant and free radical scavenging activities as well as a good anticancer activity (Kumar *et al.*, 2011a; Kumar *et al.*, 2011b). Many *Indigofera* species such as *I. colutea*, *I. macrocalyx*, *I. nigritiana*, *I. puchra*, *I. tinctoria*, *I. trita* and *I. cassioides* exhibited potent anti-inflammatory, analgesic and lipooxygenase inhibitory activities (Bakaso *et al.*, 2013; Kumar *et al.*, 2009; Kumar *et al.*, 2013). Reports of the previous studies and the traditional use of this medicinal plant encouraged us to extend our evaluation using several *in vitro* and *in vivo* models of inflammation and nociception in rodent models. It is important to point out that this work represents the first report on the anti-inflammatory and antinociceptive activities of *I. linnaei*.

Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) participate in normal cell function as well as in pathological disorders such as inflammation. Several studies have demonstrated the participation of ROS and RNS in inflammation models. Preliminary phytochemical and quantitative analysis of MEIL indicated the presence of rich amount of flavonoids and phenolic compounds in MEIL (Kumar *et al.*, 2011a). Anti-inflammatory and antinociceptive activities as well as antioxidant potential of many plants have been attributed to their flavonoids (Garcia-Mediavilla *et al.*, 2007; Gonzalez *et al.*, 2011; Sharififar *et al.*, 2009) or to their high triterpene (Ammon, 2006; Mothana *et al.*, 2011). Flavonoids are known as important secondary plant products in many medicinal plants. They have attracted much attention in relation to their wide range of activities in the prevention of cancer, inflammation

and coronary heart disorders (Garcia-Mediavilla *et al.*, 2007). Previous reports suggest that these compounds may inhibit several enzymes, which are activated in certain inflammatory conditions. Prostaglandins and nitric oxide (NO) biosynthesis is involved in inflammation. Moreover, inducible nitric oxide synthase (iNOS), cyclooxygenase (COX-2) and lipooxygenase are responsible for the production of large amounts of these pro-inflammatory mediators (Garcia-Mediavilla *et al.*, 2007). Different studies confirm that flavonoids like quercetin and kaempferol significantly decrease the levels of iNOS, COX-2, lipooxygenase and reactive C-protein (RCP) in a concentration dependent manner (Morales *et al.*, 2006; Tunon *et al.*, 2009). Furthermore, some investigated flavonoids such as kaempferol, quercetin and daidzein also inhibit the activation of the signal transducer and activator of transcription 1 (STAT-1), another important transcription factor for iNOS. Luteolin 7-glucoside, kaempferol and quercetin effectively inhibit lipopolysaccharide (LPS)-induced PGE₂ production. Four flavonoids significantly inhibit LPS-induced COX-2 expression, while mPGES-1 expression was downregulated by kaempferol and isorhamnetin (Hamalainen *et al.*, 2011). The observed beneficial effects of methanol extract of *I. linnaei* on inflammatory and nociceptive responses in rodents may be due to the presence of these phytochemicals.

CONCLUSION

All the data obtained in the present work demonstrate the possible development of methanol extract of *Indigofera linnaei* as a novel and potential drug in the management of inflammation and pain, which are probably mediated via inhibition of various autacoids formation and release. Further detailed investigation is underway to determine the exact phytoconstituents that are responsible for these activities.

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