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ANTI-INFLAMMATORY AND ANTI-PYRETIC ACTIVITY OF ETHANOLIC EXTRACT OF *INDIGOFERA ASPALATHOIDES* (VAHL EX DC) LEAVES.

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Abstract: *Indigofera aspalathoides* has been used traditionally in the siddha medicine to treat elephantiasis, skin disorder, leprosy and cancer. The *in vitro* anti-inflammatory effect of the ethanolic extract of *Indigofera aspalathoides* leaves (EEIa) was subjected to HRBC membrane stabilization. *In vivo* anti-inflammatory activity was assessed by Carrageenan induced paw edema and *in vivo* anti pyretic activity was investigated by yeast induced pyrexia using Indomethacin and paracetamol as standards respectively. The effect of ethanolic leaf extract on HRBC membrane stabilization showed positive correlation exists between the concentration and the activity i.e. activity is directly proportional to concentration of the sample. EEIa at test doses of 100 and 150 mg/kg clearly demonstrated anti-inflammatory effects by reducing paw edema induced by carrageenan in a dose dependent manner in all the treated groups. Pyrexia was also found to be decreased in treated groups on dose dependent manner. The present study contributes towards validation of the traditional use of ethanolic extract of *Indigofera aspalathoides* leaves for the treatment of inflammatory and pyrexia related disorders.

Keywords: Inflammation, Pyrexia, Carrageenan, Neoplasm



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INTRODUCTION

The natural products are valuable sources of bioactive compounds^[1] and have been considered the single most successful discovery of modern medicines^[2]. A growing body of evidence suggests that many neoplasms are initiated by infections^[3]. Some recent reviews have discussed intimate connections between inflammation and cancer^{[4][5]}. Inflammation is known to contribute to physiological and pathological processes such as wound healing and infection by the activation and directed migration of leucocytes from the venous system to sites of damage^[3]. Inflammation functions at 3 stages of tumor development: Initiation, progression and metastasis. Tumor cells produce various cytokines and chemokines that attract leukocytes. The inflammatory component of a developing neoplasm may be included a diverse leukocyte population which contains neutrophils, dendritic cells, macrophages eosinophils and mast cells^[6]. Pyrexia or fever is caused as a secondary impact of infection malignancy or other diseased states^[7]. It is the body's natural function to create an environment where infectious agent or damaged tissues cannot survive^[7]. Normally the infected or damaged tissue initiates the enhanced formation of pro-inflammatory mediators (cytokines such as interleukin 1 β , α , β and TNF- α) which increase the synthesis of prostaglandin ϵ 2 (PG ϵ 2) near hypothalamic area and there by trigger the hypothalamus to elevate the body temperature^[8]. When body

temperature becomes high, the temperature regulatory system which is governed by a nervous feedback mechanism dilates the blood vessel and increases sweating to reduce the temperature when the body temperature becomes low hypothalamus protects the internal temperature by vaso - constriction. High fever often increases faster disease progression by increasing tissue catabolism, dehydration and existing complaint as found in HIV^[9]. Since medicinal plants are also use to treat burns, swelling and malaria, one can expect that they might possess analgesic, anti-inflammatory and antipyretic activities as well. One such plant from early history was *Indigofera aspalathoides* which belongs to Pappiloncea family, a low under shrub widely distributed in South India and Sri Lanka. In the traditional medicinal system, the leaves, flowers and tender shoot are said to be cooling and demulcent; they are used in the form of decoction for leprosy and cancerous affections^[10]. The leaves are also applied to abscesses. The whole plant is used in oedematous tumors and the ashes are used in preparations for dandruff's^[11]. Siddha physician traditionally uses leaves and flowers of this plant to treat elephantiasis, skin disorder, leprosy and cancer^[12]. To confirm its ethno botanical uses in the treatment of these ailments, we investigated the anti-inflammatory and antipyretic properties of ethanolic leaf extract of *Indigofera aspalathoides* in

experimentally-induced inflammation and fever in rodents.

MATERIALS AND METHODS:

Collection and Authentication of Plant material:

Indigofera aspalathoides was collected from the foot hills of Nagamalai, Madurai District, Tamil Nadu, in the month of August and September. The plant was identified taxonomically by Prof. Dr. G. V. S. Murthy, Scientist 'F' & Head of Office, Botanical Survey of India, Southern Regional Centre, T.N.A.U. Campus, Coimbatore, Tamil Nadu, India with a voucher specimen No.334 and herbarium was deposited for future reference.

Preparation of Plant Extract:

The collected leaves of *Indigofera aspalathoides* were shade dried and powdered. Removal of chlorophyll and de-waxing from powdered materials was done by treating with Petroleum ether (40- 60°C) by hot continuous percolation method in Soxhlet apparatus^[24] for 24 hrs. Then the marc was successively subjected to ethanol extraction for 24 hrs to obtain the corresponding fraction. The extract was concentrated and dried in desiccators.

In-vitro anti inflammatory activity by HRBC membrane stabilization method:

The HRBC membrane stabilization has been used as a method to study the anti-inflammatory

Activity^[13]. Fresh human blood was collected and mixed with equal volume of sterilized Alsever solution (2% dextrose, 0.8% sodium citrate, 0.5% citric acid and 0.42% sodium chloride in distilled water). The blood was centrifuged at 3000 rpm for 15 minutes, this packed cells were washed with iso saline (0.85% Ph 7.4) and a 10% v/v suspension was made with iso-saline. The assay mixture contains the drugs (at various concentrations as mentioned) 1 ml phosphate buffer (0.15M, ph-7.4) 2 ml of hypo saline (0.36%) and 0.5 ml HRBC suspension. Hydrocortisone sodium was used as the reference drug. Instead of hypo saline 2 ml of distilled water was used in the control. All the assay mixtures were incubated at 37°C for 30 minutes and centrifuged. The hemoglobin content in the supernatant solution was estimated using colorimeter at 540 nm or spectrophotometer at 560 nm. The percentage of hemo-lysis was calculated by assuming the hemolysis produced in the presence of HRBC membrane stabilization or protection which was calculated using the formula,

OD of drug treated sample

$$\% \text{ Protection} = 100 - \frac{\text{OD of drug treated sample}}{\text{OD of Control}} \times 100$$

OD of Control

Pharmacological Procedures:

Animals:

Wistar albino female rats (150-200g) were used in the study. All animals obtained were from agricultural university, Mannuthy, Trissur, Kerala and housed for at least one week in the laboratory animal room before testing. The experiment were conducted in accordance with the internationally accepted principles for laboratory animal use and the experimental protocols duly approved by the institutional animal ethical committee (IAEC Approval No: KMCRET/Ph.d/ 15/ 2012-13) of KMCH college of pharmacy, Coimbatore Tamil Nadu, India.

Groups:

Group: 1: Vehicle treatment water/propylene glycol.

Group: 2: Drug treatment (Indomethacin for anti-inflammation/paracetamol for anti pyretic activity)

Group: 3: Extract treatment which received EEIa 100mg/kg.

Group: 4: Extract treatment which received EEIa 150mg/kg.

Evaluation of *In vivo* Anti-Inflammatory Activity:

The animals were starved overnight. To insure uniform hydration the rats received 5 ml of water by stomach tube (controls) or the test drugs dissolved or suspended in the same volume.

Thirty minutes later the rats were challenged by a subcutaneous injection of 0.05 ml of 1% solution of carrageenan in to the plantar side of the left hind paw. The paw was marked with ink at the level of the lateral malleolus and immersed in mercury up to this mark. The paw volume was measured plethysmographically immediately after injection again 3 and 6 h and eventually 24 h after challenge. The increase of paw volume after 3 or 6 h is calculated as percentage compared with the volume measured immediately after injection of the irritant for each animal. The difference of average values between treated animals and control groups was calculated for each time intervals and statistically evaluated. Depending on the irritant steroidal and non-steroidal anti-inflammatory drugs have a pronounced effect in the paw edema test. With carrageenan as irritant doses of 50-100mg/kg Indomethacin have been found to be effective^[14].

Evaluation of *In vivo* Anti-Pyretic Activity:

Animals were selected for the experiment after confirmation of approximate constant rectal temperature for 7 days. The anti pyretic activity of the ethanolic extract of *Indigofera aspalathoides* was evaluated based on Brewer's yeast induced pyrexia in rats. Pyrexia was induced by subcutaneous injection of 10 ml /kg of 15 % w/v Brewer's yeast suspension in 0.09% of saline below the nape of the neck. The rectal temperature of each rat was measured using a digital thermometer before injection of yeast. At 18 h following yeast injection induction of fever was confirmed by measuring rectal temperatures of the rats. Different groups were treated with the vehicle, EEIa (100mg and 150mg) and paracetamol (150mg/kg). After dosing the respective group, the rectal temperature was recorded over a period of 1 -6 h^[15].

Reagents:

Carrageenan (Cg) and Hydrocortisone were purchased from Sigma Chemical Co. Other drugs and reagents used were of analytical grade.

Statistical Analysis:

Values were expressed as mean \pm SEM. Statistical significance of weight or volume change was determined by ANOVA, followed by Dunnet's *t*-test; values with $P < 0.05$ and $p < 0.01$ were considered as statistically significant. Graph Pad Prism

version 6.0, Graph Pad Software Inc., was used for statistical analysis.

Results and Discussion:**Effect of EEIa on HRBC membrane stabilization and carrageenan-induced paw edema in Rats:**

The inhibition of hypotonicity induced HRBC membrane lysis i.e., stabilization of HRBC membrane was taken as a measure of the anti inflammatory activity. The percentage of membrane stabilization for ethanolic extracts and Hydrocortisone were done from 10mg -100 mg /ml. Ethanolic extracts of *Indigofera aspalathoides* were effective in inhibiting the salinity induced hemolysis of HRBC at different concentrations (10-100mg/ml). With the increasing concentration the membrane hemolysis is decreased and membrane stabilization / protection increased as shown in Fig.1, thus anti inflammatory activity of the extracts was concentration dependent. As shown in Fig. 2, sub plantar injection of carrageenan noticeably induced paw edema in rats, which persisted for over 6 h. However, EEIa was found to be effective as an anti-inflammatory agent at a higher dose (150mg/kg). The percentage of paw edema in rats was significantly reduced by administration of 150 mg/kg EEIa, which lasted for 6 h, when compared with the control group of rats given distilled water.

Indigofera aspalathoides leaves extracts exhibited membrane stabilization effect by

inhibiting hypotonicity induced lysis of erythrocyte membrane. The erythrocyte membrane is analogous to the lysosomal membrane^[16] and its stabilization implies that the extract may as well stabilize lysosomal membranes. Stabilization of lysosomal membrane is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil such as bactericidal enzymes and proteases, which cause further tissue inflammation and damage upon extra cellular release. Some of the NSAIDs are known to possess membrane stabilization properties which may contribute to the potency of their anti-inflammatory effect.

The present study established the anti-inflammatory activity of EEIa in a carrageenan induced model of acute inflammation. The anti-inflammatory activity gradually increased with time and maximal action was evident at the 5th hour possibly due to delayed oral absorption. EEIA showed maximum activity and retained its efficacy up to 24 h. The carrageenan rat paw edema test is suitable for evaluation of anti-inflammatory drugs and is used frequently to assess the antiedematous effect of natural products^[17]. As we know, prostaglandins play an important role in pain progress in chemical nociception models^{[18][19]} and are the target of action of commonly used anti-inflammatory drugs. Several inflammatory mediators, such as sympathomimetic amines, tumor necrosis factor-1 (TNF- α),

interleukin-1 (IL-1) and interleukin-8 (IL-8), are also involved in the nociceptive response to chemical stimulus in rats^[20]. There are two phases of carrageenan-induced inflammatory reaction: early phase (0–2.5 h after injection of carrageenan) results from serotonin, brady-kinin and histamine liberation, while late phase is associated with the release of prostaglandins^[21]. The carrageenan-induced hind paw edema in rat is known to be sensitive to COX inhibitors, but not to 5-Lipo oxygenase inhibitors, and has been used to evaluate the effect of non-steroidal anti-inflammatory agents which primarily inhibit the COX involved in prostaglandins synthesis. It has been demonstrated that the suppression of carrageenan-induced hind paw edema after the third hour correlates reasonably with therapeutic doses of most clinically effective anti-inflammatory agents^[22]. Thus the ethanolic extract of the leaves of *Indigofera aspalathoides* inhibited all the three phases of carrageenan induced inflammation. This may indicate that the active constituent(s) act by affecting the synthesis, release and action of inflammatory mediators involved in carrageenan induced edema.

Effect of EEIa on Yeast-induced pyrexia:

Fig.3 shows the effect of the extract against yeast-induced pyrexia. There was a dose dependent reduction in the temperature of rats treated with the leaf extract. The reductions caused by the extract was significant ($P < 0.005 - 0.001$) when

compared to control and comparable to that of the standard drug, paracetamol (150mg/kg). Pyrexia or fever is caused as a secondary impact of infection, tissue damage, inflammation, graft rejection, malignancy or other diseased state. It is the body's natural defence to create an environment where infectious agent or damaged tissue cannot survive. Normally the infected or damaged tissue initiates the enhanced formation of pro inflammatory mediators (cytokines like interleukin 1β , α , β and TNF- α), which increase the synthesis of prostaglandin E2 (PGE2) near pre optic hypothalamus area and thereby triggering the hypothalamus to elevate the body temperature^[15]. As the temperature regulatory system is governed by a nervous feedback mechanism, when body temperature becomes very high it will dilate the blood vessels and increase sweating to reduce the temperature; but when the body temperature becomes very low, hypothalamus will protect the internal temperature by vasoconstriction. High fever often increases faster disease progression by increasing tissue catabolism, dehydration, and existing complaints, as found in HIV, when fever during sero-conversion, results in disease progression^[23]. Most of the antipyretic drugs inhibit COX-2 expression to reduce the elevated body temperature by inhibiting PGE2 biosynthesis^[13]. Moreover, these synthetic agents irreversibly inhibit

COX-2 with high selectivity but are toxic to the hepatic cells, glomeruli, cortex of brain and heart muscles, whereas natural COX-2 inhibitors have lower selectivity with fewer side effects^[23]. Search for herbal remedies with potent antipyretic activity received momentum recently as the available antipyretics, such as paracetamol, Nimesulide etc, have toxic effect on the various organs of the body^[23]. The results showed that ethanolic extract of *Indigofera aspalathoides* possesses a significant antipyretic effect in maintaining reducing yeast-induced elevated body temperature in rats and their effects were comparable to that of the standard antipyretic drug paracetamol.

Conclusion:

In conclusion, our findings demonstrate that the ethanolic extract of *Indigofera aspalathoides* has the favorable anti-inflammatory and antipyretic activities, which are involved in possible inhibition of the central synthesis of prostaglandins, and affirm the claim by traditional medicine practitioners that *Indigofera aspalathoides* can be used to treat Inflammations related disorders. However, further studies are necessary to fully elucidate the mechanism of action of the plant.

Table: 1: *In vitro* Anti- Inflammatory activity of *Indigofera aspalathoides* leaves

S. No	Medium	Concentration of the leaf (mg/ml)	Standard (Hydrocortisone)	Treated (EEIa)
		10	90.20±0.08	36.72±0.10**
		20	91.57±0.11	38.62±0.23**
		30	92.23±0.12	40.04±0.11**
		40	92.77±0.06	42.57±0.20**
1.	Hypo Saline	50	93.39±0.15	43.47±0.16**
		60	94.48±0.14	44.49±0.16**
		70	94.64±0.1	46.42±0.20**
		80	95.30±0.09	48.40±0.10**
		90	96.73±0.08	51.56±0.14**
		100	97.44±0.08	52.75±0.14**
		10	86.36±0.11	34.53±0.12**
		20	85.55±0.19	36.68±0.11**
		30	92.22±0.12	39.39±0.12**
		40	89.50±0.15	40.64±0.03**
2.	Distilled Water	50	90.45±0.21	40.64±0.03**
		60	91.47±0.12	42.58±0.11**
		70	92.58±0.20	44.74±0.09**
		80	93.67±0.09	46.58±0.07**
		90	94.59±0.21	47.66±0.10**
		100	95.53±0.19	49.30±0.13**

Data expressed as mean ± SEM of three determinations. **P < 0.01 relative to control.

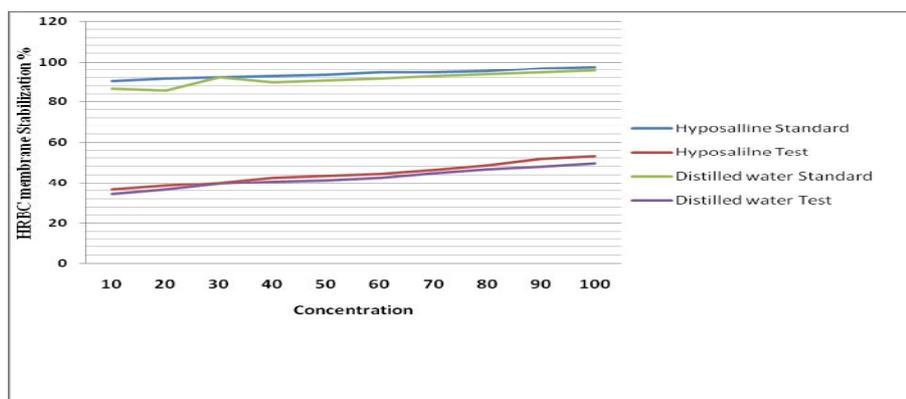


Fig: 1: Impact of ethanolic extract of *Indigofera aspalathoides* leaves on HRBC membrane stabilization.

Table: 2: *In vivo* Anti- Inflammatory activity of Etahnolic Extract *Indigofera aspalathoides* leaves

Group	Dose	Paw Volume (ml) after Induction of Carrageenan						
		0 hr	1 hr	2 hr	3 hr	4 hr	5 hr	6 hr
Control	Vehicle	1.053±0.05	1.545±0.05	1.737±0.02	1.757±0.02	1.822±0.03	1.822±0.02	1.805±0.02
	5ml/kg							
Standard	Indomethacin	0.99±0.27	1.573±0.013 ^{ns}	1.858±0.35*	1.848±0.03*	1.732±0.02*	1.533±0.01*	1.247±0.02 ^{ns}
	20mg/Kg							
EEIa	100 mg/kg	1.037±0.009	1.870±0.01 ^{ns}	1.870±0.02**	1.822±0.02**	1.752±0.02**	1.543±0.013**	1.447±0.01*
EEIa	150 mg/kg	1.042±0.01	1.443±0.02 ^{ns}	1.885±0.02**	1.850±0.03*	1.727±0.02*	1.460±0.01*	1.367±0.01*

Anti-inflammatory effects of EEIa on carrageenan induced inflammation are indicated in table.

P<0.001, P<0.01, P<0.05, Control compared with Standard, One way ANOVA followed

Dunnet: compare all column Vs control column was performed

All values are expressed as Mean ± SEM. (n=6 animal in each group).

1. ns -not significant. 2. * - significant.

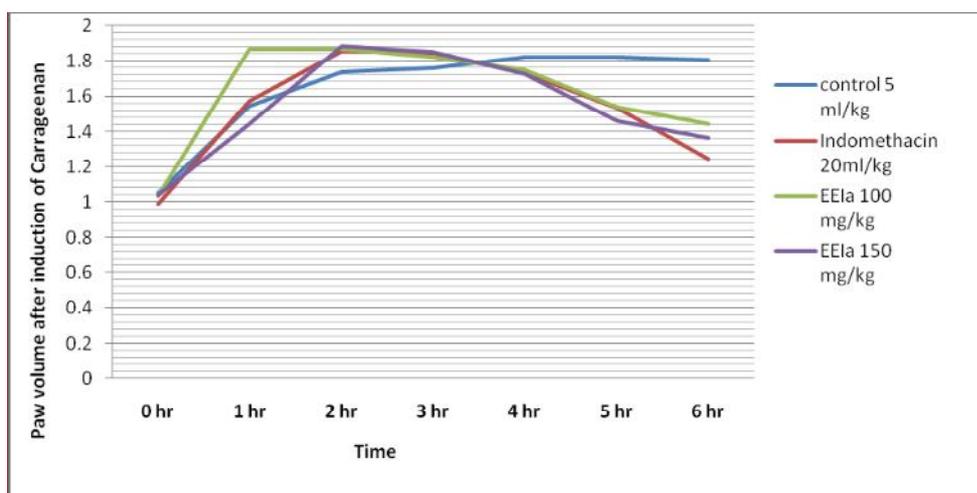


Fig: 2: Effects of EEIa on carrageenan-induced paw edema in rats. When 0.5% of 0.1 ml of freshly prepared carrageenan was injected sub plantarly into the left hind paws of female rats, acute inflammation was induced. Reduction of paw volume was measured every one hour interval after administration of plant extract

Table: 3: *In vivo* Anti- pyretic activity of *Indigofera aspalathoides* leaves

Anti-pyretic activity effects of the EEIa on yeast induced pyrexia are indicated in the table.

Group	Dose	Rectal temperature in °C at various times							
		18 h	18 h	19 h	20 h	21 h	22 h	23 h	24 h
Control	5 ml/kg	37.23±0.09	38.45 ± 0.07	38.60 ± 0.06	38.57 ± 0.03	38.63 ± 0.02	38.75±0.04	38.87 ± 0.03	39.02± 0.05
Paracetamol	150mg/kg	38.85±0.11	38.88±0.14 ^{ns}	36.74±0.19 ^{**}	36.52±0.22 ^{***}	36.21±0.1512 ^{***}	36.15±0.16 ^{**}	36.08±0.17 ^{***}	36.00±0.19 ^{***}
EEIA	100mg/kg	37.26±0.05	37.87±0.22 ^{ns}	37.76±0.25 ^{ns}	37.45±0.3 [*]	37.37±0.33 ^{**}	37.43±0.3 ^{***}	37.27±0.33 ^{***}	37.22±0.3 ^{***}
EEIA	150mg/kg	37.30±0.45	37.88±0.41 ^{ns}	37.52±0.48 [*]	37.29±0.314 ^{**}	37.15±0.2 ^{***}	37.20±0.308 [*]	37.09±0.30 ^{***}	37.03±0.3 ^{***}

$P < 0.001$, $P < 0.01$, $P < 0.05$, Control compared with Standard,

One way ANOVA followed Dunnett: compare all column Vs control column was

performed, All values are expressed as Mean \pm SEM. (n=6, animal in each group).

1. ns -not significant.

2. * - significant

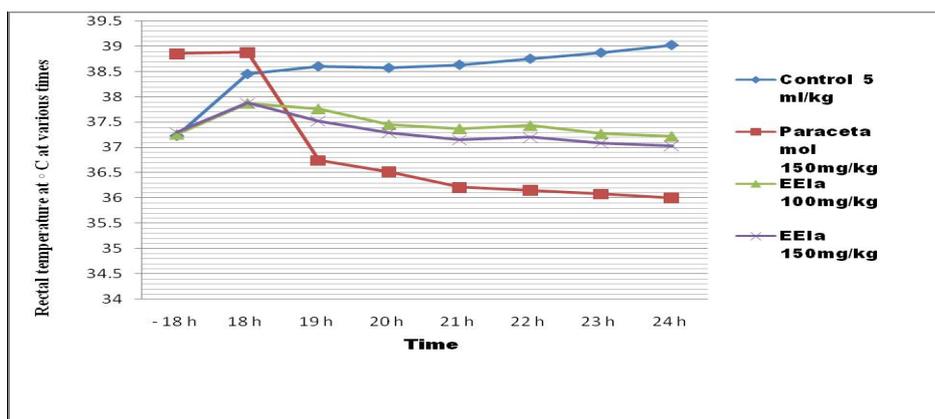


Fig. 3. Time course of the effects of EEIa on the change of the temperature of rats. The rats were injected with Brewer's yeast, after 18 h incubation, oral administration of EEIa or paracetamol was given. The temperature was measured every 1 h.

References:

1. Molinski TF: Development in marine natural products receptor specific bioactive compounds. J Nat Prod 1993; 56:1-8.

2. Grabley S and Thiericke R: Bioactive agents from natural resources-Trends in discovery and application. Adv Bio chem eng Bio technol 1999; 64:101-54.

3. Coussens LM and Werb Z: Inflammation and cancer. Nature 2002; 420: 860-7.

4. Balkwill F and Mantovania: Cancer and inflammations-Implication for pharmacology and therapeutics. Clin pharmacol ther 2010; 87:401-6.

5. Mantovani A, Garlanda C and Allavena P: Molecular pathways and targets in cancer related inflammation. Am Med 2010; 42:161-70.

6. Grivennikov SI and Karin M: Inflammation and oncogenesis; A Vicious Connection. Curr opin Genet Dev 2010; 20:65-71.

7. Chattopadhyay D, Arunachalam G and Ghosh *et al*: Antipyretic activity of *Alstonia macrophylla* wall DC; An ethno medicine of Andaman Islands. J. Pharm Pharmaceutical Sci 2005; 8:558-564.

8. Spacer CB and Breder CD: The Neurologic basis of fever. N Eng J Med 1994; 330: 1880-1886.

9. Veugelers PJ, Kaldor JM and Strathda SA *et al*: Incidence and Prognostic significance of symptomatic primary human immunodeficiency virus type 1 infection in men. *J. Infect. Dis* 1997; 176:112-117.
10. Kirtikar KR and Basu D: *Glossary of Indian Medicinal plants* 1975 Vol I, m/s Periodical Experts, New Delhi: 338.
11. *The Wealth of India: A Dictionary of Indian Raw Materials and industrial Products, Raw Materials. Vol V, H-K, Publication and Information Direction, Council of Scientific and Industrial Research New Delhi, India 1959:176.*
12. Nandakarni AK : *Indian Materia Medica*. Popular prakashan 1976:112.
13. Gandhidasan R, Thamarachelvam A and Baburaj S: Anti inflammatory action of *Lanuca coromandelica* HRBC membrane stabilization. *Fitotherpia* 1991; 62:82-83.
14. Winter C, Risley EA and Nuss GW: Carrageenan-induced edema in hind paws of the rat as an assay for anti-inflammatory drugs. *Proc Soc Exp Biol Med* 1962; 111:544–547.
15. Loux JJ De Palma PD and Yankel SL: 1972. Antipyretic testing of aspirin in rats. *Toxicol Appl Pharmacol* 1972; 22 (4): 672-675.
16. Chou CT: The anti-inflammatory effect of *Tripterygium wilfordii* Hook F on adjuvant-induced paw edema in rats and inflammatory mediators release. *Phytother Res* 1997; 11:152-154.
17. Basu A and Nag Chaudhuri AK: Preliminary studies on the anti-inflammatory and analgesic activities of *Calotropis procera* root extract. *Journal of Ethno pharmacology* 1991; 31: 319–324.
18. Santos AR, Vedana EM and De Freitas GA: Anti-nociceptive effect of meloxicam in neurogenic and inflammatory nociceptive models in rats. *Inflammation Research* 1998b; 47: 302–307.
19. Zheng CJ, Tang WZ, Huang BK, Han T, Zhang QY, Zhang and Qin LP: Bioactivity-guided fractionation for analgesic properties and constituents of *Vitex negundo* L. seeds. *Phytomedicine* 2009; 16: 560–567.
20. Ribeiro RA, Vale ML, Thomazzi SM, Paschoalato ABP, Poole S, Ferreira SH and Cunha FQ: Involvement of resident macrophages and mast cells in the writhing nociceptive response induced by zymosan and acetic acid in rats. *European Journal of Pharmacology* 2000; 387:111–118.
21. Antonisamy P and Ignacimuthu S: Immunomodulatory, analgesic and antipyretic effects of violacein isolated from *Chromo bacterium violaceum*. *Phytomedicine* 2010; 17: 300–304.
22. Panthong A, Supraditaporn W, Kanjanapothi D, Taesotikul T and Reutrakul V: Analgesic, anti-inflammatory and venotonic effects of *Cissus quadrangularis* Linn. *Journal of Ethno pharmacology* 2007; 110: 264–270.

23. Guyton AC, Hall JE: Text book of medical physiology. 9th ed. Philadelphia: W.B. Saunders Company 1998: 920-922.

24. Harborne JB: Phytochemical methods 11th Edn in Chapman &Hall New York, 1984.