ANTI-INFLAMMATORY AND ANALGESIC ACTIVITIES OF ETHANOL EXTRACT OF *INDIGOFERA TRITA* LINN.

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Summary

The primary aim of the present study was to evaluate the anti-inflammatory and analgesic activities of the ethanol extract of *Indigofera trita* (EIT). Secondary aim was to expose total phenol and total flavonoid contents of the extract. The anti-inflammatory activity of the extract was evaluated by using carrageenan-induced rat paw edema and cotton pellet granuloma method. The analgesic activity of the extract was evaluated for its central and peripheral pharmacological actions by using Eddy's hot plate method and acetic acid-induced writhing respectively. The study was carried out using dose of 200 & 400 mg/kg orally. Total phenol content was determined by using Folin-Ciocalteu reagent method and total flavonoids content by using aluminium chloride method. The pharmacological screening of the extract showed significant ($P<0.001$-0.01) dose-dependent anti-inflammatory activity with good analgesic profile. The findings of anti-inflammatory and analgesic activity harmonize with total phenol and flavonoid contents.

Keywords: *Indigofera trita*, total phenol content, total flavonoids content, analgesic, anti-inflammatory.

Introduction

In Indian system of medicine, a large number of drugs of either herbal or mineral origin have been advocated for various types of diseases and other different unwanted conditions in humans [1]. Ayurvedic medicines are largely based upon herbal and herbomineral preparations and have specific diagnostic and therapeutic principles [2]. Inflammation is a disorder involving localized increases in the number of leukocytes and a variety of complex mediator molecules [3]. 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 diseases [4, 5].

Medicinal plants are believed to be an important source of new chemical substances with potential therapeutic effects [6, 7]. The research into plants with alleged folkloric use as pain relievers, 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 trita Linn. (Family: Fabaceae) is an under shrub with wide distribution, mostly found in India, Ceylon, South Africa and North Australia. The plant is known as Kattuavuri and Punal Murungai in Tamil. The entire plant is traditionally used for various ailments including liver disorders and tumors [8, 9]. It is found to be active against transplantable tumors [10]. The plant also possesses strong antioxidant and hepatoprotective activity [11]. Hence, the study was designed to investigate the anti-inflammatory and analgesic activities of ethanol extract of *Indigofera trita* (EIT) on various experimental models.

Materials and Methods

I. Collection and Extraction

Entire plant of *Indigofera trita* were collected in and around the foothill of Shevaroys in Salem district, Tamil Nadu, India, in the month of February 2008 and authenticated by botanist, Botanical Survey of India, Coimbatore, Tamilnadu, India. The entire plants were shade dried and pulverized. The powder was treated with petroleum ether for dewaxing and removal of chlorophyll. Later, it was packed (250 g) in a soxhlet apparatus and subjected to continuous hot percolation for 8 h using 450 ml of ethanol (95 %v/v) as solvent. The extract was concentrated under vacuum and dried in a dessicator (yield, 11.25 g, 4.5 %w/w).

II. Preliminary Phytochemical Screening

The extract was screened for the presence of various constituents employing standard screening test [12]. Conventional protocol for detecting the presence of steroids, alkaloids, tannins, flavonoids, glycosides, flavonoids etc., was used.

III. 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. They were housed in microlon boxes in a controlled environment (temperature 25 ± 2 ºC and 12 h dark/light cycle) with standard laboratory diet and water *ad libitum*. The experiments were performed in accordance with the guidelines established by the European community for the care and use of laboratory animals and were approved by Institutional Animal Ethical Committee (IAEC).

IV. Chemicals

Pentazocin (Ranbaxy, India), Aceclofenac and aspirin (Micro Labs, India), kappa carrageenan type III, quercetin (Sigma, St. Louis, USA), Folin-Ciocalteu reagent, sodium bicarbonate, gallic acid, aluminium chloride and acetic acid (Merck Co.) were used in the studies.

V. Methodology:

1. Anti-inflammatory activity

a. Carrageenan-induced rat paw edema

The rats were divided into four groups (n = 6). The different groups were treated orally with EIT (200 & 400 mg/kg), Aceclofenac (10 mg/kg), and vehicle control (5% gum acacia, 1 ml/100g of body weight). The administration of extract and drugs was 1 hr 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, 2 and 3 h after the carrageenan injection by using plethysmometer. The anti-inflammatory effect of EIT was calculated by the following equation: -

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

Where Vt represents the paw volume in drug treated animals and Vc represents the paw volume of control groups animals [13].

b. Cotton pellet-induced granuloma

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 (5% gum acacia, 1 ml/100 g of body weight). EIT at the concentration of 200 & 400 mg/kg was administered orally to groups II, III animals for seven consecutive days from the day of cotton pellet implantation. Group IV animals received aceclofenac at a dose of 10 mg/kg for the same period. On 8th day the animals were anaesthetized and the pellets together with the granuloma tissues were carefully removed and made free from extraneous tissues. The wet pellets were weighed and then dried in an oven at 60 °C for 24 h to constant weight, after that the dried pellets were weighed again. Increment in the dry weight of the pellets was taken as a measure of granuloma formation. The antiproliferative effect of EIT was compared with control [14].

2. Analgesic Activity

a. Acetic Acid Induced Writhing Test

The writhing test in mice was carried out using the method of Koster et al. [15]. The writhes were induced by intraperitoneal injection of 0.6 %v/v acetic acid (80 mg/kg). Two different doses of EIT (200 & 400 mg/kg) were administered orally to the group II and group III of six animals each. Group I served as control (5 % gum acacia, 1 ml/100 g of body weight) and group IV animals received aceclofenac at a dose of 10 mg/kg. The extract and standard drug was 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.

b. Hot Plate Method

The hot plate method in rats was performed by the method of Eddy and Leimbach [16] . 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 (5 % gum acacia, 1 ml/100 g of body weight), group II and group III received EIT at a dose of 200 & 400 mg/kg orally. Group IV received pentazocin at a dose of 5 mg/kg. All the treatments were given 30 min before the thermal stimulus and the response was determined at 60, 120 and 180 min.

3. Estimation of total phenol content

Total phenol content was determined by Folin-Coicalteu method [17]. A diluted extract (0.5 ml, 1:10 v/v) or phenolic standard (gallic acid) was mixed with Folin-Ciocalteu reagent (5 ml, 1:10 diluted with distilled water) and aqueous sodium carbonate (4 ml, 1M). Solutions were heated in a water bath at 45 ˚C for 15 minutes. The total phenols were determined colorimetrically at 765 nm. The standard curve was prepared by using 0, 50, 100, 150, 200 and 250 mg/L solutions of gallic acid in methanol: water (50:50, v/v). Total phenols were expressed as gallic acid equivalents (mg/g dry mass) which is a common reference compound.

4. Total flavonoid content

Total flavonoid content was estimated by aluminium chloride colorimetric method reported by Chia-Chi Chang et al. [18]. Ten milligram of quercetin was dissolved in 80 % ethanol and then diluted to 25, 50 and 100 µg/ml. the diluted standard solutions (0.5 ml) were separately mixed with 1.5 ml of 95 % ethanol, 0.1 ml of 10 % aluminium chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of diltilled water. After incubation at room temperature for 30 minutes the absorbance of the reaction mixture was measured at 415 nm with Perkin Elmer Lambda 25 UV-Visible spectrophotometer. The amount of 10 % aluminium chloride was substituted by the same amount of distilled water in blank. Similarly 0.5 ml of ethanol extract was reacted with aluminium chloride for determination of flavonoid content as described above. The results were expressed quercetin equivalents as mg/g of dry mass.

Statistical Analysis

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

Results

Preliminary Phytochemical Screening

Preliminary phytochemical screening of the ethanol extract of *Indigofera trita* revealed the presence of alkaloids, glycosides, triterpenes, flavonoids and phenolic compounds. Further separation of the specific phytochemical is in progress.

Carrageenan induced rat paw edema

The result of EIT against carrageenan-induced paw edema is shown in Table 1. EIT (200 & 400 mg/kg) gave significant ($P<0.001$) reduction of rat paw edema at all assessment times in dose dependent manner. The extract showed maximum inhibition of 52 % at the dose of 400 mg/kg after 3 h of drug treatment in carrageenan-induced paw edema whereas the standard drug showed 58 % of inhibition.

Effect of EIT on Cotton pellet granuloma

The effects of EIT and aceclofenac on the proliferative phase of inflammation are shown in Table 2. A significant ($P<0.01$) reduction in the weight of the cotton pellets was observed with EIT (200 & 400 mg/kg) compared with the vehicle treated rats. However the degree of reduction was less than the effect caused by aceclofenac.

Effect of EIT on Hot plate method

The animals pretreated with EIT (200 & 400 mg/kg) showed a dose dependent increase in latency of response in the hot plate method. The increase in the latency responses were significant (P<0.01). The results were showed in Table 3.

Effect of EIT on acetic acid induced writhings in mice

The extract (200 & 400 mg/kg) dose dependently reduced acetic acid induced writhings in mice. The reduction was statistically significant (P<0.01) when compared to control. The results were showed in Table 4.

Total phenol and flavonoid content

Total phenol and total flavonoid contents were determined by previously reported methods. Total phenol content of ethanol extract of *Indiogofera trita* was found that 325 ± 1.75 mg of gallic acid equivalent /g of dry mass. Total flavonoid content was found that 169 ± 1.02 mg of quercetin equivalent /g of dry mass. The results were shown in Table 5.

Discussion

The most widely used primary test for screening of anti-inflammatory agents is carrageenan induced edema in the rat paw hind paw [13]. 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-like substances [19]. Based on this, it could be argued 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 [20].

Ueno et al.,[21] found that the injection of carrageenan into the rat paw induces the liberation of bradykinin, which later induces the biosynthesis of prostaglandins and other autocoids, 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 [22]. There fore, it is suggested that the mechanism of action of EIT may be related to prostaglandin synthesis inhibition.

The cotton pellet granuloma method has been widely employed to assess the transductive, exudative and proliferative components of chronic inflammation and 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 [20, 23]. Administration of EIT at the doses of 200 & 400 mg/kg significantly reduced the granulomatous tissue formation when compared to control

It is known that non-steroidal anti-inflammatory drugs usually do not increase the pain threshold in normal tissues, whereas local anesthetics and narcotics do [24]. However, the hot

plate test was undertaken to verify if EIT would have any central analgesic effect. The results for the group treated with EIT showed significant activity when compared to control group and nearly equal to the group treated with pentazocin (5 mg/kg). Hence, it is assumed that EIT has significant analgesic effect on the central nervous system.

With respect to the writhing test, the research group of Deraedt et al [25] described the quantification of prostaglandins by radioimmunoassay in the peritoneal exudates of rats, obtained after intraperitoneal injection of acetic acid. They found high levels of prostaglandins $PGE_{2\alpha}$ and $PGF_{2\alpha}$ during the first 30 min after acetic acid injection. Nevertheless, it was found that the intraperitoneal administration of acetic acid induces the liberation not only of prostaglandins, but also of the sympathetic nervous system mediators [26, 27]. Thus the results obtained for the writhing test using acetic acid are similar to those obtained for the edematogenic test using carrageenan, since EIT was effectively inhibiting the writhings in mice in dose dependent manner. The results were comparable with the group treated with aspirin. Therefore, anti-inflammatory substances may also be involved in the peripheral analgesic activity.

Preliminary phytochemical screening indicated the presence of flavonoids in EIT. Selected phenolic compounds and flavonoids were shown to inhibit both the cyclooxygenase and 5-lipxygenase pathways [28-30]. This inhibition reduces the release of arachidonic acid. The exact mechanism by which flavonoids inhibit these enzymes is not clear. Quercetin, in particular, inhibits both cyclooxygenase and lipoxigenase activities, thus diminishing the formation of these anti-inflammatory metabolites [31, 32].

Another anti-inflammatory feature is the ability of flavonoids to inhibit eicosanoid biosynthesis [33, 34]. Eicosanoids, such as prostaglandins, are involved in various immunological responses and are the end products of the cyclooxygenase and lipoxigenase pathways. Flavonoids also inhibit both cytosolic and membranal tyrosine kinases which play key roles in the signal transduction pathway that regulates cell proliferation [35]. Another anti-inflammatory property of flavonoids is their suggested ability to inhibit neutrophils degranulation. This is the direct way to diminish the release of arachidonic acid by neutrophils and other immune cells [36, 37]. From the above discussion, the ethanol extract of roots of *Indigofera trita* exhibited significant anti-inflammatory and analgesic activity.

Conclusion

All these data obtained in this study point to possibly developing the ethanol extract of *Indigofera trita* as a novel and potential agent in the management of inflammation and pain which are probably mediated via inhibition of various autocoids formation and release. Further detailed investigation is underway to determine the exact phytoconstituents that are responsible for these activities.

Acknowledgements

The authors are thankful to Dr. M. Karunanidhi, Chairman, Angammal Educational Trust, Tiruchengodu, India and Principal, Swamy Vivekanandha College of Pharmacy, Tiruchengodu, India for providing infrastructural facilities to carry out this study.

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Table 1. Effect of Ethanol Extract of *Indigofera trita* on Carrageenan Induced Rat Paw Edema

Treatment	Dose/kg	Paw volume in ml				% inhibition after 3 h.
		0 h	1 h	2 h	3 h	
Control (Normal saline)	2 ml	0.17 ± 0.002	0.22 ± 0.007	0.29 ± 0.003	0.5 ± 0.006	-
Aceclofenac	10 mg	0.10 ± 0.006	0.12 ± 0.005	0.14 ± 0.008	0.21 ± 0.130***	58
EIT	200 mg	0.16 ± 0.001	0.22 ± 0.007	0.23 ± 0.010	0.26 ± 0.006***	48
EIT	400 mg	0.12 ± 0.006	0.14 ± 0.011	0.17 ± 0.008	0.24 ± 0.004***	52

N=6
***P< 0.001 Vs control
Data were analyzed by one way ANOVA followed by Dunnett test

Table 2. Effect of Ethanol Extract of *Indigofera trita* on Cotton Pellet Granuloma

Treatment	Dose/kg	Weight of cotton pellets (mg) (wet)	Percentage inhibition	Weight of cotton pellets (mg) (dry)	Percentage inhibition
Control (Normal saline)	2 ml	183.17 ± 14.3	-	48.62 ± 3.6	-
Aceclofenac	10 mg	78.25 ± 6.3**	57.25	23.54 ± 2.4**	51.57
EIT	200 mg	121.16 ± 12.1**	33.85	34.42 ± 2.4**	29.20
EIT	400 mg	87.487 ± 7.4**	52.25	26.71 ± 2.1**	45.06

N=6

**P< 0.01 Vs control

Data were analyzed by one way ANOVA followed by Dunnett test

Table 3. Effect of Ethanol Extract of *Indigofera trita* on Thermic Stimulus Induced (Hot Plate) Pain in Rats

Treatment	Dose/kg	Reaction times in seconds of time (hr)			
		0 hr	1 hr	2 hr	3 hr
Control (Normal saline)	2 ml	2.4 ± 0.15	2.32 ± 0.40	2.45 ± 0.16	2.36±0.14
Pentazocin	5 mg	2.3 ± 0.4	7.5 ± 0.22**	9.72 ± 1.10**	7.84 ± 0.14**
EIT	200 mg	2.5 ± 0.22	5.3 ± 0.23**	8.06 ± 0.75**	6.9 ± 1.10**
EIT	400 mg	2.6 ± 0.6	6.83 ± 0.30**	8.76 ± 0.36**	7.20 ± 0.36**

N=6

**P<0.01 Vs control

Data were analyzed by one way ANOVA followed by Dunnett test

Table 4. Effect of Ethanol Extract of *Indigofera trita* on Chemical Stimulus Induced (Writhing Test) Pain in Rats

Treatment	Dose/kg	No of writhing (20 min.)	Percentage Inhibition
Control	-	79.8 ± 2.45	-
Aspirin	300 mg	26.5 ± 1.72**	66.79
EIT	200 mg	62.7 ± 1.67**	21.42
EIT	400 mg	53.5 ± 1.58**	32.95

N=6

**P< 0.01 Vs control

Data were analyzed by one way ANOVA followed by Dunnett test

Table 5. Total Phenol and Total Flavonoid Contents of Ethanol Extract of *Indigofera trita*

Extract	Total Phenol Content (mg of Gallic acid equivalent/ mg)	Total Flavonoid Content (mg of Quercetin equivalent /mg)
Ethanol Extract of *Indigofera trita*	325 ± 1.75	169 ± 1.02