

ANALGESIC AND ANTI-INFLAMMATORY ACTIVITY OF SEED KERNEL EXTRACTS OF *ENTADA PHASEOLOIDES* MERRILL.

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ABSTRACT

Objective: To investigate analgesic and anti-inflammatory activities of seed kernel extracts of *Entada phaseoloides* Merrill in mice and rats.

Methods: Three extracts of different polarity of *Entada phaseoloides* Merrill seed kernels were prepared and tested for their analgesic and anti-inflammatory activities. Analgesic activity was evaluated using the hot plate and acetic acid-induced writhing in mice. Anti-inflammatory activity was evaluated using carrageenan induced paw edema and cotton pellet granuloma in rats.

Results: Pretreatment with ethanolic extract of *Entada phaseoloides* Merrill at dose 400 mg/kg p.o. showed significant ($P < 0.01$) analgesic effect in acetic acid induced pain model and significant ($P < 0.01$ and $P < 0.001$) anti-inflammatory effect in carrageenan induced rat paw edema and cotton pellet granuloma model respectively, while petroleum ether extract and aqueous extract of *Entada phaseoloides* Merrill showed weak analgesic and anti-inflammatory effects.

Conclusion: It is concluded that the ethanolic seed kernel extract of *Entada phaseoloides* Merrill possesses analgesic and anti-inflammatory activities.

Keywords: Analgesic, Anti-inflammatory, Carrageenan, Cotton pellet, *Entada phaseoloides* Merrill.

INTRODUCTION

Entada phaseoloides Merrill (Leguminosae) commonly called as Gila (Sanskrit), Gila bean (English), or Hathibij (Hindi) and traditionally used worldwide including India for nutritional and medicinal purpose [1]. The seeds are flat, orbicular with shining dark brown testa which is tough and torny [2]. The attractive seeds of *Entada phaseoloides* Merrill have also been used in for snuffboxes, matchboxes, beautiful lockets and in children games.

The soaked seed kernels are roasted/boiled and eaten as such or mixed with salt by Northeast Indian tribals. Occasionally, village people use the stem bark and seeds as natural shampoo to wash their hair and as a fish poison [3]. The seed kernel of *Entada phaseoloides* Merrill has been commonly used as herbal medicine for the treatment of hemostasis, detoxification [4] and stomach ulcer [5]. Some investigations suggested that it had anthelmintic, aphrodisiac [3], antidiabetic [6], anti-inflammatory [7] and molluscicidal activities [8]. The main active constituents of the plants are triterpene acid [9], phenylacetic acid esters [10, 11], triterpene saponins [12], phenolic acids [13, 14], chalcone glycosides [15] and sulfur-containing amides [16].

As a result of adverse side effects, like gastric lesions, caused by NSAIDs and tolerance and dependence induced by opiates, the use of these drugs as anti-inflammatory and analgesic agents have not been successful in all the cases. Therefore, new anti-inflammatory and analgesic drugs lacking those effects are being searched all over the world as alternatives to NSAIDs and opiates. During this process, the investigation of the efficacy of plant-based drugs used in the traditional medicine have been paid great attention because they are cheap, have little side effects and according to WHO still about 80% of the world population rely mainly on plant-based drugs [17].

The objective of the study was to evaluate the analgesic and anti-inflammatory activities of extracts of the seed kernels of *Entada phaseoloides* Merrill in rodents.

MATERIALS AND METHODS

Procurement of Plant material

Seed Kernels of *Entada phaseoloides* Merrill were obtained from a commercial supplier of Pune and then identified and authenticated

by Department of Botany, Agharkar Research Institute; Pune, India and voucher specimen (S - 130) is deposited at that Institute.

Drugs and Chemicals

Acetylsalicylic acid and diclofenac were provided as a gift sample from Emcure Pharmaceutical Ltd., Pune. Carrageenan (Sigma-Aldrich, St. Louis, MO, USA), Pentazocin (Ranbaxy, India), Acetic acid (Pure Chem. Ltd., India), petroleum ether (Merck, India), ethanol (Qualigens, India), formaldehyde (British Drug House, India) and Tween 80 (Research Lab, India) were purchased.

Extraction procedure

The seed kernels of the plants were powdered and subjected to successive extraction in soxhlet extractor as per standard procedure using petroleum ether (40-60°C) and ethanol at their boiling point for 48 h. The marc obtained from the ethanol extraction was further utilized for aqueous extraction by maceration for 48 h. The percentage yields of petroleum ether extract of *Entada phaseoloides* Merrill (PEEEP) was 4.5 g, while that of ethanol extract of *Entada phaseoloides* Merrill (EEEP) was 3.5 g and aqueous extract of *Entada phaseoloides* Merrill (AEEP) was 10 g. All the extracts were subjected to phytochemical and pharmacological screening.

Phytochemical analysis

Preliminary phytochemical studies of all the extracts were performed for carbohydrates, proteins, amino acids, fats and oil, steroids, volatile oil, glycosides, alkaloids, flavonoids, triterpenoids, tannins and phenolic compounds using standard procedures [18].

Experimental animals

Female Wistar rats weighing 200-250 g and female Swiss albino mice weighing 20-25 g were used for the study. The animals were procured from National Toxicology Centre, Pune. The animals were housed at $25 \pm 2^\circ\text{C}$, humidity ($60 \pm 10\%$) with 12 hour day and night cycle, with food and water *ad libitum*. The study was carried out as per CPCSEA norms after obtaining approval (CPCSEA/01/2011) from the Institutional Animal Ethical Committee of college.

Acute oral toxicity study

Healthy female Swiss albino mice (20-25 g) were used in acute toxicity study as per OECD guidelines-425. The animals were

fasted overnight and divided into groups with 5 animals in each group. Extracts (EEEEP, PEEEEP and AEEP) were administered orally at one dose level of 2000 mg/kg body weight. The mice

were observed continuously for behavioral and autonomic profiles for 2 hrs and for any sign of toxicity or mortality up to 48 hrs [19].

Treatment groups

Table 1: Treatment groups for analgesic and anti-inflammatory activity

Group	Treatment
1.	Vehicle control (2% Tween 80)
2.	Standard
3.	EEEEP 100 mg/kg
4.	EEEEP 200 mg/kg
5.	EEEEP 400 mg/kg
6.	PEEEP 100 mg/kg
7.	PEEEP 200 mg/kg
8.	PEEEP 400 mg/kg
9.	AEEP 100 mg/kg
10.	AEEP 200 mg/kg
11.	AEEP 400 mg/kg

Analgesic activity

Evaluation of analgesic activity by hot plate method in mice

The mice were divided into eleven groups of 6 mice each (Table 1). The animals were individually placed on the hot plate (Model- DS37, UGO Basile, Italy) maintained at $55 \pm 1^\circ\text{C}$, one hour after their respective treatments. The response time was noted as the time at which animals reacted to the pain stimulus either by paw licking or jump response, whichever appeared first. The cut off time for the reaction was 15 seconds [20, 21].

Evaluation of analgesic activity by acetic acid induced writhing method in mice

Acetic acid solution at a dose of 10 ml/kg (0.6%) was injected i.p. and the number of writhes during the following 15 min period was observed. Significant reductions in number of writhes by drug treatment as compared to vehicle treatment (vehicle control) animals were considered as a positive analgesic response. The mice were divided into eleven groups of 6 mice each (Table 1). The percent inhibition of writhing was calculated as follows:

$$\% \text{ Inhibition} = (\text{VC} - \text{VT} / \text{VC}) \times 100$$

VT, number of writhes in drug treated mice. VC, number of writhes in control group of mice [22, 23].

Anti-inflammatory activity

Evaluation of anti-inflammatory activity by carrageenan induced paw edema in rats

Female wistar rats (200-250 g) were divided into eleven groups of 6 rats each (Table 1). The test samples or vehicle were administered orally to experimental animals once a day for a period of 7 days. One hour after the last administration, acute paw edema was induced by subplanter injection of 0.1 ml of 1% freshly prepared carrageenan suspension in normal saline into the right hind paw of each rat. The paw volume was measured before (0 h) and at intervals of 1, 2, 3, 4, 5, 6 and 24 h after carrageenan injection using plethysmometer (Model- 7140, UGO Basile, Italy). The percentage of inhibition of edema was calculated for each group with respect to the control group as follows,

$$\% \text{ Inhibition of paw edema} = (\text{VC} - \text{VT} / \text{VC}) \times 100$$

VC and VT represent average paw volume of control and drug treated animals respectively [24-26].

Evaluation of anti-inflammatory activity by cotton pellet granuloma in rats

Eleven groups of animals were used in this model (Table 1). Subacute inflammation was produced using cotton pellets. Under

aseptic precautions, an incision was made on the back of each rat in each group and sterile cotton pellets (50 ± 1 mg) were implanted subcutaneously in the axilla under ether anesthesia. On the seventh day, animals were sacrificed; stomach and pellets were removed along with the granulation tissue and pellets were dried at 60°C for 24 hrs. The net dry weight of the granuloma was determined. Stomach was cut open in greater curvature and ulcer scoring was carried out. Histopathological studies were performed on stomach to confirm ulcer score [27, 28]. Stomach was stored in 10% formalin, embedded in paraffin section were cut by microtome and stained with hematoxyline and eosin stain (H & E) for histological examination using light microscopy for assessment of mucosal inflammation and congestion of blood vessels [29].

Statistical analysis

The data were analyzed by one way ANOVA followed by Dunnett's test, two way ANOVA followed by Bonferroni's post hoc test. All statistical analyses were performed using Graph Pad Prism software (San Diego, CA). Data was considered statistical significant at $P < 0.05$.

RESULT

Phytochemical screening

The PEEEEP showed presence of fats and oils, volatile oil, flavonoids, alkaloids, tannins and phenolic compounds. The AEEP showed presence of carbohydrates, proteins, amino acids, glycosides, alkaloids, tannins and phenolic compounds. The EEEEEP showed presence of fats and oils, steroids, volatile oil, saponins, glycosides, flavonoids, alkaloids, tannins and phenolic compounds.

Acute oral toxicity

All the three extracts of *Entada phaseoloides* Merrill (2000 mg/kg p.o.) did not produce any behavioral abnormalities and mortality. So the dose selected for further study was 100, 200 and 400 mg/kg for each extract.

Analgesic activity

Hot plate method: All the three extracts did not exhibit analgesic activity. In contrast, pentazocin at the dose of 30 mg/kg, p.o. significantly ($P < 0.001$) increased pain threshold of mice compared to the vehicle control group at 60 and 90 min.

Acetic acid writhing method: Intraperitoneal injection of acetic acid elicited the writhing response in vehicle control mice. The number of writhes counted in 15 min. was 64 ± 1.50 . EEEEEP 400 mg/kg produced a significant ($P < 0.01$) reduction in the number of writhes with peak effect 12.50 %, where as 100 mg/kg of acetylsalicylic acid showed 35.94 % of inhibition.

However there was no significant inhibition in the acetic acid induced writhing on treatment with PEEEEP (100, 200 and 400 mg/kg) and AEEP (100, 200 and 400 mg/kg). (Table 2)

Table 2: Effect of oral administration of *Entada phaseoloides* Merrill on acetic acid induced writhing in mice

Group	Treatment	No. of writhes (Mean ± SEM)	% Inhibition
1.	Vehicle control	64 ± 1.50	
2.	Acetylsalicylic acid 100 mg/kg	41 ± 2.00***	35.94
3.	EEEEP 100 mg/kg	61 ± 1.40	4.69
4.	EEEEP 200 mg/kg	59 ± 1.60	7.81
5.	EEEEP 400 mg/kg	56 ± 1.50**	12.50
6.	PEEEP 100 mg/kg	62 ± 1.70	3.13
7.	PEEEP 200 mg/kg	63 ± 2.10	1.56
8.	PEEEP 400 mg/kg	59 ± 1.30	7.81
9.	AEEP 100 mg/kg	62 ± 1.80	3.13
10.	AEEP 200 mg/kg	60 ± 1.90	6.25
11.	AEEP 400 mg/kg	58 ± 1.50	9.38

Data are expressed as mean ± S.E.M.; n=6 mice per group. One way ANOVA followed by Dunnett's test when compared with normal control *P<0.05, **P<0.01, ***P<0.001.

Anti-inflammatory activity

Carrageenan induced rat paw edema

The rats were pretreated with EEEP, PEEEP and AEEP for 7 days before the injection of carrageenan caused dose dependent inhibition of increase in paw edema from 1 h to 5 h. The inhibitory effect of the EEEP was recorded with a dose of 200 and 400 mg/kg at 4th h (11.39 %), (13.52 %) and at 5th h (20.28 %), (23.11 %)

respectively. Diclofenac (10 mg/kg) were administered 1 h before the injection of carrageenan caused significant (P<0.001) inhibition of increase in paw edema at 5th h. The inhibitory effect of the diclofenac at 10 mg/kg was recorded (16.55 %) at 4th h and (32.08 %) at 5th h. The inhibition elicited by the EEEP was comparable to that of diclofenac. However there was no significant inhibition in increase in paw edema on treatment with PEEEP (100, 200 and 400 mg/kg) and AEEP (100, 200 and 400 mg/kg). (Table 3)

Table 3: Effect of *Entada phaseoloides* Merrill on inhibition of right hind paws edema on carrageenan induced inflammation in rats

Treatment Group	Change in paw edema volume			% Inhibition at		
	1 h	4 h	5 h	1 h	4 h	5 h
Vehicle control	0.33 ± 0.024	0.94 ± 0.050	1.06 ± 0.068			
Diclofenac 10 mg/kg	0.31 ± 0.049	0.78 ± 0.054 ^{ns}	0.72 ± 0.051***	8.00	16.55	32.08
EEEEP 100 mg/kg	0.33 ± 0.022	0.92 ± 0.038	1.05 ± 0.037	2.50	1.60	1.10
EEEEP 200 mg/kg	0.32 ± 0.020	0.83 ± 0.054 ^{ns}	0.85 ± 0.051*	4.00	11.39	20.28
EEEEP 400 mg/kg	0.32 ± 0.028	0.81 ± 0.056 ^{ns}	0.82 ± 0.036**	5.00	13.52	23.11
PEEEP 100 mg/kg	0.33 ± 0.025	0.93 ± 0.058	1.05 ± 0.059	0.50	0.71	1.26
PEEEP 200 mg/kg	0.32 ± 0.038	0.92 ± 0.032	1.04 ± 0.029	3.00	2.14	2.04
PEEEP 400 mg/kg	0.32 ± 0.040	0.92 ± 0.062	1.04 ± 0.079	4.00	1.78	1.89
AEEP 100 mg/kg	0.34 ± 0.042	0.92 ± 0.077	1.05 ± 0.079	-2.50	1.42	1.42
AEEP 200 mg/kg	0.34 ± 0.039	0.92 ± 0.032	1.04 ± 0.035	-1.50	1.60	1.73
AEEP 400 mg/kg	0.33 ± 0.027	0.90 ± 0.039	1.02 ± 0.043	2.50	4.09	3.93

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test when compared with vehicle control *P<0.05, **P<0.01, ***P<0.001 and ^{ns} non significant.

Table 4: Effect of oral administration of *Entada phaseoloides* Merrill on cotton pellet granuloma in rats

Group	Treatment	Dry granuloma weight (mg) (Mean ± SEM)	% Inhibition
1.	Vehicle control	95 ± 3.40	
2.	Diclofenac 10 mg/kg	35 ± 3.30***	63.16
3.	EEEEP 100 mg/kg	96 ± 3.30	-1.05
4.	EEEEP 200 mg/kg	79 ± 2.50*	16.84
5.	EEEEP 400 mg/kg	66 ± 4.00***	30.53
6.	PEEEP 100 mg/kg	93 ± 3.50	2.11
7.	PEEEP 200 mg/kg	91 ± 3.70	4.21
8.	PEEEP 400 mg/kg	93 ± 2.70	2.11
9.	AEEP 100 mg/kg	100 ± 1.90	-5.26
10.	AEEP 200 mg/kg	95 ± 2.90	0
11.	AEEP 400 mg/kg	95 ± 3.50	0

Data are expressed as mean ± S.E.M.; n=6 rats per group. One way ANOVA followed by Dunnett's test when compared with normal control *P<0.05, **P<0.01, ***P<0.001.

Cotton pellet granuloma

EEEEP (400 mg/kg) and diclofenac (10 mg/kg) significantly (P<0.001) inhibited the granuloma formation, while EEEP (200 mg/kg) significantly (P<0.05) inhibited the granuloma formation compared with vehicle control group.

However there was no significant inhibition in granuloma formation on treatment with EEEP (100 mg/kg), PEEEP (100, 200 and 400 mg/kg) and AEEP (100, 200 and 400 mg/kg). (Table 4)

All the extracts at doses 400 mg/kg showed less ulcer score compared to the standard group (diclofenac 10 mg/kg).

Histopathology of stomach of normal rats showed intact gastric mucosa, no ulceration and congestion was observed. Diclofenac treated rats showed ulceration and congestion. EEEP, PEEEP and AEEP at 400 mg/kg treated rats showed lesser ulceration and no congestion. (Figure 1)

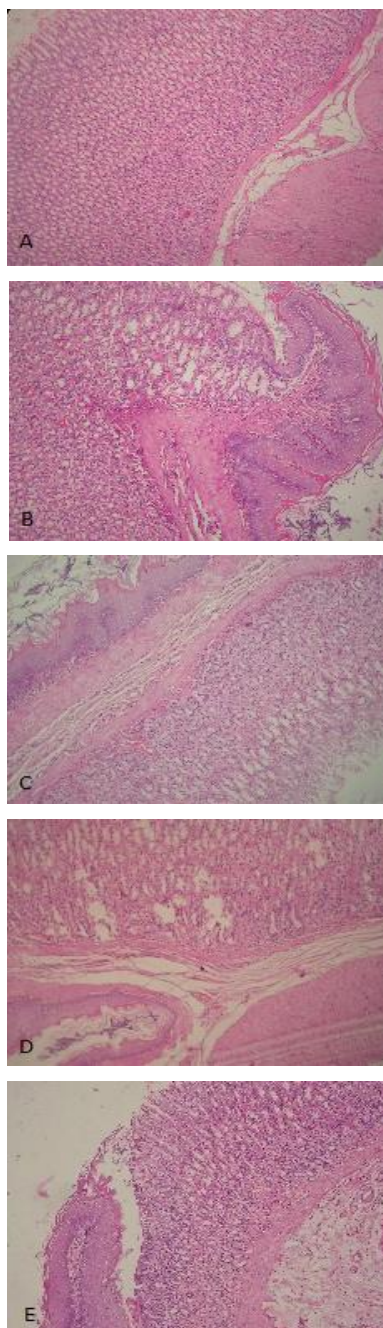


Fig. 1: Histopathology of stomach (A) Normal control (B) Diclofenac 10 mg/kg treated (C) EEEP 400 mg/kg treated. (D) PEEEP 400 mg/kg treated, (E) AEEP 400 mg/kg treated

DISCUSSION

Analgesic activity

The hot plate test was undertaken to verify if *Entada phaseoloides* Merrill would have any central analgesic effect. The results for the group-treated with *Entada phaseoloides* Merrill did not differ significantly from those obtained for the control group. On the other hand, the group-treated with pentazocin (30 mg/kg) showed a significant result. Hence, it is apparent that *Entada phaseoloides* Merrill has no analgesic effect on the central nervous system that would contribute to its peripheral analgesic effect.

The intraperitoneal injection of acetic acid elicited writhings (a syndrome characterized by a wave of abdominal musculature contraction followed by extension of the hind limbs). The writhing test is simple, reliable, and affords rapid evaluation of analgesic activity [30]. The induction of writhings by chemical substances injected i.p. results from the sensitization of nociceptors by prostaglandins and the test is useful for evaluation of mild analgesic non-steroidal anti-inflammatory drugs [31]. The inhibition of writhing induced by acetic acid in this study by *Entada phaseoloides* Merrill suggest a peripherally mediated analgesic activity based on the association of the model with stimulation of peripheral receptors especially the local peritoneal receptors at the surface of cells lining the peritoneal cavity [32]. The fact that the greatest number of writhes was produced at the 10–15 min time interval provides a basis for the modification of the acetic acid-induced writhing test such that the number of writhes is counted only for 5 min within the mentioned interval. This modification will enhance the rapidity associated with the model.

Anti-inflammatory activity

Carrageenan induced inflammation of rat paw is a significant predictive test for anti-inflammatory agents acting by mediators of acute inflammation [33]. Carrageenan is a family of linear sulphated polysaccharides extracted from the red seaweed marine alga *Chondrus crispus*. Lambda carrageenan is used in animal models of inflammation to test analgesics, because dilute carrageenan solution (1 %) injection causes swelling and pain [34]. According to Guang et al, development of edema induced by carrageenan is commonly correlated with early exudative stage of inflammation, involving release of histamine and serotonin from mast and basophil cells, and is characterized by increase in vascular permeability. Later there is release of bradykinin (an important mediator of pain and inflammation), prostaglandin and other cyclooxygenases products, and marked increase in cellular infiltration and subsequent release of acute inflammatory mediators such as myeloperoxidase and cytokines (IL-1 β , IL-6 and TNF- α) at the inflammatory site. Further neutrophils, macrophages, endothelial and other cells at the site of inflammation may produce reactive oxygen species (ROS) and reactive nitrogen species, which play a modulating role in the inflammatory response [35]. EEEP (400 mg/kg) inhibited the edema induced by carrageenan injection from 4th hour onwards, showing good inhibitory effect at 5th hour (23.11 %) indicating that the anti-inflammatory property of EEEP could be due to their effect on late phase of inflammation.

Cotton pellet induced granuloma formation is considered to be reliable experimental model for evaluation of effects of macrophage dysfunction and granuloma formation, central players in the formation, maintenance and progression of granulomas in various disease states [36, 37]. Efficacy of EEEP in this model is therefore depictive of inhibitory activity against macrophage activation, infiltration and aggression. EEEP was effective in both carrageenan-induced paw edema as well as cotton pellet granuloma and it can be assumed that it is effective in all the phases of inflammation i.e., acute, subacute and proliferative phases.

Evaluation of the ulcerogenic effect of the extracts on the rat stomach revealed a lesser ulceration of the gastric mucosa. Ulceration of the gastric mucosa by anti-inflammatory drugs usually indicates that prostaglandin synthesis inhibition may be involved in their mechanisms of action.

Inhibition of the synthesis of prostaglandin, a group of prostanoid mediators of inflammation and intact gastric mucosa is largely responsible for the anti-inflammatory and gastric ulceration effects of NSAIDs. Consequently, the irritant effect of the extract on the rat gastric mucosa suggests that the extract, like diclofenac, may inhibit prostaglandin synthesis [38].

Phytochemical analysis of the *Entada phaseoloides* Merrill seeds demonstrated the presence of saponins, flavonoids, terpenoids and steroids. Steroids are known to decrease inflammation and reduce the activity of the immune system, exerts anti-inflammatory effects [39]. Flavonoids are often used for their antioxidant effect against free radicals. There are also strong indications that they have antiviral, anti-inflammatory and anti-hypertensive properties [40]. We propose that the analgesic and anti-inflammatory activity of the EEEP seeds could be

due to combined effect of flavonoids, saponins, and steroids which are the major components of the ethanol extract of the plant seed kernels.

CONCLUSION

It is concluded that analgesic and anti-inflammatory properties of *Entada phaseoloides* Merrill can be considered an effective agent to treat inflammatory diseases. This plant, mainly its seed kernels, demonstrated a high activity for ethanol extract at dose 400 mg/kg. The study corroborated the analgesic effects of this species, justified and supported scientifically its ethnopharmacological use as an anti-inflammatory agent to treat pain and inflammation.

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Conflict of interest statement

We declare that we have no conflict of interest.

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