INVESTIGATING THE ROLE OF NF-κB, COX-1, COX-2, COMT, IL-10, IL-6 AND TNF-α IN MODULATING ANTI-NOCICEPTIVE ACTIVITY OF METHANOLIC EXTRACT OF ENTADA PHASEOLEOIDES

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ABSTRACT

Objective: To investigate the analgesic activity of methanolic extract of Entada phaseoloides (MEEP) along with its molecular mechanistic pathway.

Methods: Swiss albino female mice and Wistar rats of either sex were administered orally with MEEP extracts (100, 200 and 400 mg/kg) and pентazoncine, tramadol and diclofenac sodium, as standard drugs. Following administration, anti-nociceptive activity was evaluated using an acetic acid-induced writhing test, Eddy’s hot plate, and hot water immersion test. Serum was collected for molecular expression of various proteins and genes using Reverse Transcriptase PCR and Western Blotting.

Results: Acetic acid writhing test, a frequently used method to assess peripheral analgesic activity, revealed that MEEP reduced peripherally induced pain in a dose-dependent manner. Likewise, Eddy’s hot plate and hot water immersion methods, often implicated for testing central analgesic activities, showed that MEEP is bestowed with the capability to counteract analgesia in a dose and time-dependent manner. Pro-inflammatory cytokines and factors like COX-2, IL-6, TNF-α, and NF-κB that cause inflammatory responses and pain were significantly reduced, suggesting its anti-inflammatory and anti-inflammatory potential. This analgesic and the anti-inflammatory role played by MEEP is also supported by the up-regulation of anti-inflammatory cytokine IL-10 and COMT and COX-1 enzyme demonstrated no significant difference between the groups.

Conclusion: The study revealed the weak peripheral and potent central analgesic property MEEP by modulating pro-inflammatory and anti-inflammatory pathways.

Keywords: Analgesic, Cytokines, Diclofenac sodium, Entada phaseoloides, Pentazocine, Tramadol

INTRODUCTION

Entada phaseoloides, commonly known as box bean or St. Thomas’, has got multifarious healing properties against various ailments. Locally known as Gila (Sanskrit), Gila bean (English) or Hathibij (Hindi), etc. [1]. It is taxonomically placed in Leguminosae family of genus Entada. This plant had been mentioned in Chinese historical materials viz., “Nanfang Caomu Zhuang” and “Bencao Gangmu” [2]. Especially, the stems and seeds are important in conventional Chinese remedy for their effectiveness against various diseases. Seeds (Ketengzi) are used to treat haemorrhoids, constipation, and stomach-ache, while the stems (Guoganglong) are well known for treating rheumatoid arthritis, tetraplegia, and traumatic injury [3, 4]. This plant is popular and renowned amid many communities for its significant folkloric uses. The decoctions of dried vine materials are effective in treating rheumatic lumbar, leg pains, sprain, and contusions; powdered seeds were taken with water for malnutrition-related jaundice and edema, for relief from abdominal pain, a poultice of pound kernel seed is topically applied onto the affected area for colic and seed paste were used as counter-irritant to cure glandular swellings in the axilla, knees, and joints. South African community uses the seed to bite on during the ‘teething period’ in infants. Customarily, this plant has been used to cure cerebral hemorrhage, skin itchings, vomitings, etc. [5]. The plant also reported having antihelmintic, aphrodisiac [6], anti-inflammatory [7], anti-diabetic [8], and anti-tumor activity [9]. In the North-Eastern part of India, the paste of the bark is used as an ulcer protective agent [10]. Our previous investigations elucidated the anti-depressant, memory-enhancing [11] and stress relieving [12] activities of methanolic extract of Entada phaseoloides (MEEP). Different studies have unveiled the presence of various phytoconstituents, such as phenylacetic acid derivatives [13], sulfur-containing amides [14, 15], oleanane-type triterpene saponins [16, 17] in this plant.

Pain is a remarkable indication of inflammation, which is unduly stressful. Pain is generally treated with NSAIDs, opioids, and non-opioids analgesics, which have many undesired side effects. For the management of acute pain in clinical use, opioid analgesics are generally used as they have maximal analgesic potency [18-20]. Hence, as an alternative remedy, Ekdotic and traditional medicinal plants are tried; however, scientific validation of many such plants are still incomplete. The efficacy of the seeds of Entada Phaseoloides as a topical analgesic [21] and anti-inflammatory [22] agent has already been reported by others. The anti-nociceptive effect of Entada phaseoloides seeds formulation after topical application in arthritic wistar rats [22] and analgesic activity of seed kernel extracts and methanolic extract of Entada phaseoloides Merril [23] are reported.

However, the underlying molecular mechanism of its analgesic property is not yet reported. The present study is undertaken to evaluate the analgesic activity of MEEP and an attempt to investigate the role of various pro and anti-inflammatory factors involved in its anti-nociceptive activity.

MATERIALS AND METHODS

Pentazocine (an opioid narcotic analgesic), tramadol (a narcotic-like analgesic), and diclofenac sodium (Non-steroidal anti-inflammatory drug) were procured from Themis Medicare Ltd. (Uttarkhand India), Alkem Laboratories Ltd. (Silkim, India) and Cadila Pharmaceuticals
Acetic acid-induced writhing

Acute toxicity studies

Animals

Two universal models, viz. Eddy's hot plate and hot water immersion assay were performed in rodents.

Acute toxicity studies were conducted in Swiss albino mice (20-30g) following the Organization of Economic Corporation Development (OECD) Guidelines No. 423. The experimental protocol had been approved by the Institutional Animal Ethics Committee (IAEC) of the College of Veterinary Sciences, Assam Agricultural University, Khanapara [Ref no. 770/ac/CP/CSEA/FVSc, AAU/IAEC/15-16/367]. The experimental animals were maintained under standard laboratory conditions (12:12 h light/dark cycle) at ambient temperature (22-25°C) and kept in polycarbonate cages with free access to standard balanced ration, clean drinking water ad libitum.

Plant collection and identification

The dried seeds of Entada phaseoloides purchased from the local market of Sonapur during the month of April-May, 2016, were identified by taxonomist Dr. Iswar Chandra Barua, Principal Scientist, Department of Agronomy, Assam Agricultural University, Jorhat, Assam; a voucher specimen (AAU-NW-EVM-3) was deposited and kept at the herbarium of the Department of Agronomy, Assam Agricultural University, Jorhat-785013, Assam.

Preparation of methanolic extract

After collection, the kernel of the seeds was properly removed and dried under shade. The seeds were then finely ground to powder, weighed, and stored in an airtight container away from sunlight. Then, 250 gm of powdered seed was soaked in 1000 ml of methanol for 72 h was stirred after every 18 h with the help of a sterile glass rod. The mixture was then subjected to filtration (with the help of Whatman filter paper no. 1) and the solvent was removed using a rotary evaporator (BUCHI, R-210, Labortechnik AG, Meierseggstrasse Switzerland) under reduced pressure, leaving behind a dark brown residue (MEEP). The Extract so obtained is stored in an airtight container at 4°C. The percentage yield of the methanolic extract was 10.11% (w/w).

Acute toxicity studies

Acute toxicity studies were conducted in Swiss albino mice (20-30g) following the Organization of Economic Corporation Development (OECD) Guidelines No. 423. The extracts were administered orally to a group of mice (n=3) at the dose rate of 2000 mg/kg and the percentage mortality, if any, was recorded. For the next 14 d, animals were kept under observation to check if any abnormality or mortality occurred. Based on the results of the acute toxicity study, doses of 100, 200, and 400 mg/kg were chosen for carrying out the study.

Peripheral analgesic activity study

Acetic acid-induced writhing

The peripheral antinociceptive activity of MEEP was determined in female mice by the acetic acid-induced writhing test. Five groups (n=6) were taken for the study. Group 1 received distilled water orally and served as control. Group 2 received diclofenac sodium (10 mg/kg body weight i. p.), served as a standard group [24]. Group 3, 4, and 5 served as test groups and received MEEP (100, 200, and 400 mg/kg body weight, p. o., respectively). The writhes were induced by the intraperitoneal injection of 0.6% acetic acid (10 ml/kg). The numbers of writhes [muscular contractions] were counted 20 min post-injection of acetic acid i. p. The number of writhes in each group was compared with the control group and the percent reduction of writhes count was calculated as follows: (%Ncontrol –Ntest)/Ncontrol ×100, where N is the mean number of writhes for each group [25, 26].

Anti-nociceptive study

Two universal models, viz. Eddy’s hot plate and hot water immersion assay were performed in rodents.

Eddy's Hot plate test

Pre-screened (showing reaction time of 3-5 sec) Swiss albino female mice (n=5, divided into 5 groups). Group 1 received distilled water orally and served as control. Group 2 received tramadol (22.8 mg/kg body weight i. p.) and served as a standard group [12]. Group 3, 4, and 5 served as test groups and received MEEP (p. o.) at the doses 100, 200, and 400 mg/kg body weight, respectively. Eddy’s hot plate was maintained at 35±0.5 °C and reaction times (latency time for paw licking and jumping responses) were noted at 15, 30, 60, and 120 min following administration of the drug/extract. Cut off time for the experiment was 15 sec [27]. Percentage protection against thermal stimulus was calculated by the following formula [28]:

\[
\text{Percentage Protection} = \frac{\text{Control Mean} - \text{Drug Mean}}{\text{Control Mean}} \times 100
\]

Hot water immersion assay

Wistar rats were divided into 5 groups (n=6). Group 1 received distilled water orally and served as control. Group 2 received pentoxyfylline (10 mg/kg body weight i. p.) and served as a standard group [29]. Group 3, 4, and 5 served as test groups and received MEEP at the doses 100, 200, and 400 mg/kg body weight, p. o. respectively. Each rat was properly restrained in a rat holder leaving the whole tail extending out. The reaction time was determined by noting the tail withdrawal response (tail flick latency) when one-third of the tail was immersed in a beaker containing water maintained at 51±0.5 °C. The cut off time for immersion was 10 sec. The reaction time was evaluated at 60 min before and 15, 30, 60, and 120 min after the administration of the drug/extract. The idea of taking two standard drugs for the models is to compare the efficacy of our compound with both of them since both are opioid analgesics.

Molecular study

Reverse transcriptase-polymerase chain reaction (RT-PCR)

The mRNA expression levels of the pro-inflammatory genes (COX-1, COX-2, TNF-α, COMT and IL-6) were studied in whole blood using a thermocycler (Veriti, Applied Biosystems). Foster City, California, United States. The total RNA was isolated from the buffy coat by using TRizol (Ambion) and 1 µg of isolated RNA was reverse transcribed to cDNA by using the revert aid first-strand cDNA synthesis kit (Thermo Scientific). Waltham, Massachusetts, United States. The amplification reaction was carried out in a total volume of 10 µl using cDNA gene-specific primer, nuclearase-free water, and DreamTaq Green PCR Master Mix (2X). DreamTaq polymerase is supplied in 2X DreamTaq Green buffer, along with 0.4 mmol each of dATP, dCTP, dGTP and dTTP and 4 mmol MgCl2. The tubes were spun for 30 sec before loading into the thermocycler. The cyclic conditions include initial denaturation at 95 °C for 3 min, followed by 40 cycles each of which 30-sec denaturation at 95 °C, 30-sec annealing at 59 °C and 30-sec extension at 72 °C followed by final extension of 10 min at 72 °C. The amplified PCR products were run in agarose gel electrophoresis using 2% Agarose containing ethidium bromide in 1X TBE buffer to confirm the amplicons. The primers used for amplification were adopted from the National Centre for Biotechnology Information (NCBI) by Primer-BLAST (ILS primers, ILS).

Western blotting assay

Immunoblotting assay of NFkB and IL-10, the serum samples were considered as the source of protein. The protein content was determined using a Bradford assay. Samples for SDS-PAGE were prepared by mixing equal volume of serum and 2X Laemmi buffer (supplemented by 2-mercaptoethanol (Sigma, MO, USA) in the ratio 1:100, which was then heated at 90 °C for 5 min. Proteins electrophoretically separated on 12% SDS PAGE gels were transferred onto a nitrocellulose membrane (PROTRAN). This step was followed by blocking of the membranes using 3% BSA (HyClone) in 1X TBST. The membranes were then treated with primary antibodies (diluted in 1% BSA in the ratio 1:1000) at 4 °C overnight followed by 5 times wash [with 1X TBST] for 5 min each. After this, the membranes were probed with secondary antibodies at room temperature for 1 h.
temperature and washed with 1X TBSST for 5 times. Then TMB (Sigma, MO, USA) was applied and the membranes were incubated in dark for 5 min. The bands were analyzed using Image analyzer (Image J) software.

### Statistical analysis

Results obtained were expressed as mean±SEM. Statistical analysis was conducted by using a one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test, using GraphPad Prism software version 5.0 (San Diego, CA, USA). Results were considered to be statistically significant when p<0.05.

### Isolation of compounds from Entada phaseoloides

The methanolic extract (10 gm) of Entada phaseoloides was subjected to column chromatography (silica gel. 100-200 mesh, eluting with hexane/EtOAc mixture of increasing polarity) for obtaining 40 column fractions. Then the column fractions were analyzed with TLC (silica gel 60 F254, hexane: EtOAc, 60:40), and fractions with similar TLC patterns were amalgamated to give five major fractions (F1, F2, F3, F4, F5). Fractions F4 was subjected to repeated column chromatography eluting with EtOAc: hexane (19:81) to yield compound 1. Fraction F3 was subjected to Column chromatography (CC) on silica gel (100-200 mesh) using a hexane-EtOAc (10:0-6:4) to yield subfractions B1 and Compound 2. Subfraction B1 was then purified using preparative TLC with CHCl3: MeOH (90:10) to obtain compound 3. Repeat purification of fraction F3 on silica gel (60-120 mesh) using CHCl3/MeOH (12:1) yielded compound 4.

### Oleic acid [1]: Light yellow oil, 1H-NMR (500 MHz, CDCl3) δ: 5.35 (2H, m), 2.33 (4H, m), 0.14 (3H, 9H, s), 3.27 (1H, m), 2.39 (1H, m), 3.35 (1H, m), 3.37 (1H, m), 3.46 (1H, m), 3.72 (2H, m), 3.90 (1H, m), 3.96 (1H, m), 3.99 (1H, m), 4.29 (1H, d, 7.4 Hz), 5.85 (1H, d, 14.58 Hz), 7.59 (1H, d, 14.58 Hz). 13C NMR (CDCl3, 75 MHz) δ: 126.48, 124.97, 117.03, 115.49, 142.13, 114.83, 102.39, 75.67, 72.97, 69.36, 68.23, 60.74, 38.92, 13.4. HR-ESI-MS m/z: 305.2456 (Calcd for C18H24O2Na: 305.2451).

### Entadamide A (2): White amorphous powder, 1H-NMR (500 MHz, CDCl3) δ: 7.62 (1H,d, J = 14.5 Hz), 6.44 (1H, br s), 5.69 (1H, d, J = 14.6 Hz), 76.90 (C-5'), 76.49 (C-2'), 73.38 (C-3'), 69.74 (C-4'), 60.80 (C-6'), 34.93 (C-1'), 125.88 (C-2), 117.45 (C-6), 113.97 (C-3), 102.91 (C-1), 76.90 (C-5'), 76.49 (C-2'), 73.38 (C-3'), 69.74 (C-4'), 60.80 (C-6'), 34.93 (C-1'). 1H NMR (500 MHz, CD3OD) δ: 2.33 (3H, s), 3.27 (1H, m), 3.29 (1H, m), 3.34 (1H, m), 3.54 (1H, m), 3.59 (1H, m), 3.75 (2H, m), 3.90 (1H, m), 3.96 (1H, m), 4.08 (1H, m), 4.12 (1H, m), 4.18 (1H, m), 4.24 (1H, m), 4.80 (2H, brs, H-7), 4.53 (1H, d, J=7.4 Hz), 2.33 (4H, m), 2.01 (4H, m), 1.64 (4H, m), 1.37e1.22 (H, m), 1.17e1.22 (H, m), 1.09e1.12 (H, m). 13C NMR (CDCl3, 125 MHz) δ: 180.55, 165.80, 143.67, 115.31, 62.46, 42.49, 14.60. HR-ESI-MS m/z: 162.0589 (Calcd for C6H12NO2S: 162.0583).

### Acetic acid-induced writhing in mice

Results of the acetic acid-induced writhing test revealed that MEEP significantly reduces the writhes count as compared to the control group (Fig. 2). Diclofenac sodium, the standard drug, showed the highest protection against acetic acid-induced writhing (76.8%). The analgesic activity shown by MEEP against peripherally induced analgesia was dose-dependent, producing the highest analgesic activity at 400 mg/kg with percent protection of 58.1% against the peripherally induced sensation of pain. Hence, its effect on peripheral analgesia as compared to diclofenac sodium is not profound.

### Effect of MEEP on Eddy’s hot plate test in mice

The results of the acute toxicity study of MEEP using the hot plate test are presented in table 1. The latency times were recorded at 15, 30, 60, and 120 min post administration of the drug-extract. The percentage protection offered by the standard drug (tramadol) was the highest (96.20) at 2 h following drug administration, whereas, the percent protection offered by MEEP at the dose rate of 400 mg/kg body weight was 54.52, following 1 h of drug administration. However, at 1 h, the percent protection provided by 400 mg/kg body weight of MEEP (54.52) was higher than that of tramadol (52.01). The effect of tramadol lasted until 2 h.

### Table 1: Analytical activity of MEEP on Eddy’s hot plate in mice

| Groups | Treatment | 15 min | 30 min | 60 min | 120 min |
|--------|-----------|--------|--------|--------|---------|
| 1      | Control   | 2.90±0.17 | 3.70±0.16 | 3.98±0.20 | 3.95±0.23 |
| 2      | Tramadol  | 4.10±0.18 | 4.17±0.17 | 5.08±0.17 | 5.22±0.20 | 6.05±0.20 | 6.22±0.20 | 7.52±0.27 | 96.20% |
| 3      | MEEP (100 mg/kg, p.o.) | 4.20±0.23 | 4.47±0.22 | 5.08±0.22 | 5.22±0.22 | 6.05±0.22 | 6.22±0.22 | 7.52±0.27 | 96.20% |
| 4      | MEEP (200 mg/kg, p.o.) | 3.90±0.32 | 4.47±0.22 | 5.08±0.22 | 5.22±0.22 | 6.05±0.22 | 6.22±0.22 | 7.52±0.27 | 96.20% |
| 5      | MEEP (400 mg/kg, p.o.) | 4.70±0.26 | 4.67±0.17 | 5.46±0.24 | 5.67±0.24 | 6.46±0.24 | 6.67±0.24 | 7.87±0.27 | 96.20% |

*Values represent mean±SEM of n=6, *p<0.05,**p<0.01, ***p<0.001, compared to control (one-way ANOVA followed by "Dunnett's Multiple Comparison Test")
Hot water immersion induced analgesia in rats

The results of the analgesic activity of MEEP as assayed with hot water immersion are presented in Table 2. There was a significant difference between the vehicle (negative control) and test groups on the thermal stimulus in rats throughout the whole duration of the experiment. Significant (p<0.05) analgesic activity of the standard drug, pentazocine, at 15, 30, 60, and 120 min, as compared to the control animals was observed. Also, with respect to the control group, it was found significant in groups 3, 4, and 5.

Pentazocine significantly increased the response time of the animal to 7.2 sec (after 120 min), and showed a significant increase in the latency time of rats when compared with the control group. As can be seen in Table 2, there was a significant increase in latency time of MEEP in 100, 200, and 400 mg/kg, p.o. at 15, 30, 60, and 120 min; however, the maximum activity was recorded at 120 min. The percent protection offered by MEEP at 400 mg/kg body weight is at par with that of pentazocine at the same dose and interval of time (2 h). Its activity was also dose dependent.

Fig. 2: Analgesic effects of the MEEP and diclofenac sodium on the acetic-acid-induced writhing response in mice. Each value represents as mean±SEM. ***p<0.001 as compared with the control group (one-way ANOVA followed by “Dunnett’s Multiple Comparison Test”)

Table 2: Analgesic activity of MEEP and pentazocine in rats assayed using hot water immersion method

| Groups | Treatment (mg/kg) | Response mean±SEM (s) (percent protection) |
|--------|-------------------|------------------------------------------|
|        | Before            | After                                    |
|        | 60 min            | 15 min                                   | 30 min        | 60 min        | 120 min       |
| 1.     | Control           | 2.8±0.35                                 | 2.4±0.22      | 2.6±0.13      | 2.7±0.097     | 2.9±0.26      |
| 2.     | Pentazocine (10 mg/kg i.p.) | 2.3±0.22 (70.8%) | 4.1±0.40%* (48.84 %) | 3.87±0.34% (92.5 %) | 5.2±0.43% (92.5 %) | 7.2±0.11% (148.27 %) |
| 3.     | MEEP (100 mg/kg, p.o.) | 2.8±0.26 (75%) | 4.2±0.32% (91.66 %) | 4.8±0.22% (84.53 %) | 4.6±0.10% (70.37 %) | 5.3±0.23% (82.75 %) |
| 4.     | MEEP (200 mg/kg, p.o.) | 2.8±0.28 (75%) | 4.6±0.32% (91.66 %) | 3.6±0.24% (41.53 %) | 5.0±0.21% (85.92 %) | 5.7±0.35% (97.2 %) |
| 5.     | MEEP (400 mg/kg, p.o.) | 2.7±0.32 (68.33 %) | 4.0±0.34% (68.46 %) | 4.3±0.31% (68.46 %) | 5.07±0.09% (87.7 %) | 6.58±0.11% (126.89 %) |

*Numbers in parenthesis indicate percentage increase in reaction time when compared with control. *p<0.05 when compared with control (one-way ANOVA followed by "Dunnett’s Multiple Comparison Test")

Effects of MEEP on gene expressions of COX-1, COX-2, COMT, IL-6 and TNF-α at 60 and 120 min time intervals respectively by Reverse transcriptase PCR (RT-PCR)

The Reverse Transcriptase-PCR analysis of gene expression on COMT, COX-1, COX-2, IL-6, and TNF-α at 60 and 120 min time intervals is presented in Fig. 3. The mRNA expression levels of COMT at 60 and 120 (p<0.001) min were significantly up-regulated in the MEEP treated rats as compared to the vehicle control group. COX-1 enzyme demonstrated no significant difference between the groups. However, mRNA expression levels of COX-2 at 60 and 120 (p<0.001) min were significantly down-regulated in the MEEP treated rats as compared to the vehicle control group. The mRNA expression levels of IL-6 at 60 and 120 (p<0.001) min were also similar in the tamadol treated rats compared to the vehicle control group.

Immunoblotting assay of NFκB and IL-10 at 60 and 120 min in rats treated with MEEP

Protein expressions in the serum of control as well as test groups were assayed by western blotting. The results of the test groups as well as a standard drug (pentazocine) were compared with the control group. The results elucidated that there was down-regulation of NFκB and up-regulation of protein levels of IL-10 in the MEEP treated groups when compared with the control group. Dose and time-dependent significant down-regulation were observed in the protein levels of NFκB at 60 and 120 min (p<0.001) following extract administration. At 120 min time interval, there was significant down-regulation of the targeted NFκB gene, while protein expressions of IL-10 were significantly up-regulated in 400 mg/kg body weight treated rats when compared to the control (Fig 4).
Fig. 3: Quantitative data expression of mRNA of COMT (60 min, 120 min), COX-1 (60 and 120 min), COX-2 (60 and 120 min), IL-6 (60 and 120 min), TNF-α (60 and 120 min) as fold change as compared with vehicle control group. Values are expressed as mean±SEM (*p<0.05, **p<0.01, ***p<0.001) compared with vehicle control group. Lane1 = Control, Lane2 = Tramadol, Lane3 = MEEP 100 mg/kg, Lane4 = MEEP 200 mg/kg, Lane5 = MEEP 400 mg/kg.
DISCUSSION

Pain, a component firmly bonded with a multitude of clinical pathologies requires skillful and adequate management. There is a need to develop newer powerful analgesic agents with minimum side effects from natural sources [31]. Current therapies are vested with many side effects and thus have made researchers take an interest in hunting new products, and more particularly, herbal-based products. *Entada phaseoloides*, a plant that plentifully covers the cultivation of Himalayan belt and Arunachal Pradesh in particular, is gaining importance owing to its folklore and traditional medicinal properties. The current study was undertaken to explore the underlying mechanism of the analgesic activity of this plant, which is used traditionally to cure pain and inflammation for ages.

From the acute toxicity study, it is apparent that MEEP did not produce any toxicity at 2000 mg/kg body weight and thus considered safe. The antinociceptive activity of new drugs can be verified by assessing their effects centrally or peripherally, where the hot plate test used to evaluate the centrally acting analgesic effect and the acetic acid-induced writhing test used to evaluate the peripheral acting analgesic effect [32]. MEEP was tested for its ability to minimize or eliminate peripherally induced pain. A writhing test is a reliable method to assess the peripheral analgesic activity of any compound. It is a chemical method very often used for inducing pain of peripheral origin by using irritant principles like phenylquinone or acetic acid in female mice [33,34]. Plants that are effective in the writhing test have peripheral anti-nociceptive activity [35]. Our study noticeably showed the analgesic activity of MEEP against peripherally induced pain was dose-dependent and maximum analgesic activity was seen at 400 mg/kg with percent protection of 58.1%. Eddy’s hot plate and hot water immersion test are two frequently used standard pharmacological models for investigating central analgesic activity [36-38]. The results of Eddy’s hot plate test emphasized that MEEP showed a dose and time-dependent analgesic activity as compared to the control group. The data from Eddy’s hot plate test as well as the hot water immersion assay emphasized that MEEP showed a dose and time-dependent analgesic activity as compared to the control group. There might be some components in the MEEP that act centrally via the activation of opioid receptors [40]. The analgesic activity of MEEP could be due to its constituents like oleic acid [41] or entadamide [42] or both. We have taken two standard drugs tramadol and pentazocine in the eddy’s hot plate and hot water immersion model, respectively, to compare the efficacy with MEEP.

To further assess the antinociceptive and anti-inflammatory mechanisms of *Entada phaseoloides*, we conducted the molecular analysis. Under oxidative stress, ROS may initiate and exaggerate the inflammatory responses due to their capability to stimulate and regulate the inflammatory signaling cascades genes like NFκB and pro-inflammatory cytokines [43, 44]. The transcription factor NFκB plays a crucial role in regulating the expression of two significant anti-inflammatory factors, COX-2, and dynorphin that play dynamic role in augmenting the responses caused by nociceptive stimuli. COX-2, a major contributor to the synthesis of prostaglandin E2 brings about all the changes that happen to occur during inflammation, and thus induces the troubling sensation called ‘pain’ [45]. IL-10 is a potent anti-inflammatory cytokine housed in monocytes, macrophages, Th2, and B cells [46]. It inhibits NFκB activity [47] and promotes the degradation of mRNAs of pro-inflammatory cytokines [48]. In our study, as observed from the immunoblotting analysis, MEEP treated rats showed significant down-regulation of NFκB, whereas expression of the anti-inflammatory cytokine, IL-10 was significantly upregulated.

COMT is the key enzyme involved in the metabolism of the catecholamines: noradrenaline, adrenaline, and dopamine. COMT, an enzyme responsible for the regulation of pain perception, cognitive function, and mood, is an important regulator of catecholamine concentrations in the perception of pain [49, 50]. Lower COMT is associated with hyperalgesia brought about by stimulation of β2-
adrenergic receptors by catecholamines [51, 52]. Our study elucidated that there was significant up-regulation of the COMT gene at mRNA levels suggesting its crucial role being played in managing pain.

Cyclooxygenase enzymes play a pivotal role in prostaglandin synthesis that brings about the cascades of changes that take place during inflammation. It has two isotypes viz. COX-1 and COX-2. COX-1 is present throughout the body and plays a crucial role in regulating normal physiological functions of the body [53]. Blocking of this isotype can cause gastrointestinal side-effects. On the contrary, the expression of COX-2 gears up in the brain [54] and kidney [55] during basal conditions. Inflammation induces COX-2 and causes increased release of prostanooids culminating in peripheral nociception [56, 57]. Peripheral inflammation also induces pain in the neighboring uninjured tissue due to increased neuronal excitability [58]. Thus increased COX-2 activity is tightly bound with sensation of pain. COX-1 is constitutively active throughout the body and its activity predominates during normal physiological conditions [53]. In contrast, COX-2 expresses under basal conditions in the brain and kidney [53, 55]; however, it is markedly upregulated by a variety of inflammatory mediators. Because there is an induction of COX-2 at sites of inflammation, it’s believed that the therapeutic properties of NSAIDs account primarily for the inhibition of COX-2 [54, 59]. It was reported that there is no change in expression of COX-1 in normal rat paws compared to inflamed ones [60] and our study also visibly illustrated that MEEP causes significant down-regulation of COX-2.

Cytokines play a vital role in coordinating the immune system and the inflammatory response. Cytokines may be broadly classified as pro-inflammatory, (TNFα, IL-6, IL-8) or anti-inflammatory (IL-10, IL-4, and TGFβ) [61]. In our study, mRNA levels of IL-6 and TNF-α were significantly down-regulated. Hence pain perception in treated groups including MEEP and the standard group was reduced. On the other hand, anti-inflammatory cytokine IL-10 and COMT were increased.

The present study throws light on the fact that the MEEP has got central and weak peripheral analgesic activity. It also exemplified that the anti-nociceptive or anti-inflammatory activity occur following administration of MEEP might be either due to the down-regulation of some pro-inflammatory genes like NFkB, IL-6, and TNF-α or up-regulation of anti-inflammatory factors like IL-10 and the enzyme COMT.

CONCLUSION

As expected, our study showed analgesic activity of MEEP in experimental models of nociception at 400 mg/kg. MEEP possesses weak peripheral and potent central analgesic properties. It may be due to constituents like entadamide or oleic acid or both might contribute its analgesic property. The mechanism by which it brings about analgesia could be due to the up-regulation of anti-inflammatory factors like IL-10, COMT, or down-regulation of pro-inflammatory factors like COX-2, NFkB, IL-6, and TNF-α. The study corroborated the analgesic effects of this species, justified and supported its ethnomedicinal use scientifically as an analgesic and anti-inflammatory agent to treat pain and inflammation.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICTS OF INTERESTS

The authors declare that there are no conflicts of interest.

REFERENCES

1. Kirtikar KR, Basu BD, Blatter E. 4+ Vol. 2nd edition. Indian Medicinal Plants. Lalit Mohan Basu Publishers, Allahabad; 1935.
2. Mo S, Hui X, Chu G, Yang X, Wang L, Zheng C, et al. Phaseoloideside E, a novel natural triterpenoid saponin identified from Entada phaseoloides, induces apoptosis in EC-109 esophageal cancer cells through reactive oxygen species generation. J Pharmocol Sci 2013;122:163-75.
3. Fu XM, Zhang MQ, Shao CL, Li GQ, Bai H, Dai GL, et al. Chinese marine matura medica resources: status and potential. Mar Drugs 2016;14:46.
4. Nanjing University of Traditional Chinese Medicine. Dictionary of Chinese Materia Medica. Shanghai Shanghai Science and Technology Publishing House; 2006. p. 3563.
5. Entada phaseoloides, Australian Tropical Rainforest Plant; 2020.
6. Siddharaju P, Becker K, Malik HP. Chemical composition, protein fractionation, essential amino acid potential and antimetabolic constituents of an unconventional legume, Gila bean (Entada phaseoloides Merrill) seed kernel. J Sci Food Agric 2001;82:191-202.
7. Dawane JS, Pandit VA, Rajapadyhe BD. Experimental evaluation of the anti-inflammatory effect of topical application of Entada phaseoloides merill seeds as paste and ointment. N Am J Med Sci 2011;3:513-7.
8. Zheng T, Shu GW, Yang ZZ, Mo SS, Zhao Y, Mei Z, et al. Antidiabetic effect of total saponins from Entada phaseoloides (L.) Merr. In type 2 diabetic rats. J Ethnopharmacol 2012;139:814-21.
9. Liu WC. Anti-tumorous saponin extract from Entada phaseoloides. Chem Abst 1972;76:131-47.
10. Shankar R, Tripathi AK, Anuk G, Nezzay S, Rawat MS. Indigenous medicinal plants of northeast India in human health: literature note. J Drug Res Ayur Sci 2017;2:104-17.
11. Barua GC, Buragohain L, Purkayastha A, Saikia B, Babu KS, Kumari GS, et al. Entada phaseoloides attenuates scopolamine induced memory impairment, neuroinflammation and neuro-degeneration via BDNF/TRKB/NFkB p65 pathway in radial arm maze. Int J Pharm Pharm Sci 2018;10:29-38.
12. Barua GC, Buragohain L, Purkayastha A, Rizavi H, Gogoi SB, Rahman F, et al. Effect of seeds of Entada phaseoloides on chronic restrain stress in mice. J Ayur Integrat Med 2018;1:7-10. Doi:10.1016/j.jaim.2018.02.140.
13. Singh O, Ali M, Akhtar NJ. Phenolic acid glucosides from the seeds of Entada phaseoloides Merrill. Asian Nat Prod Res 2011;13:682-7.
14. Ikekami F, Sekine T, Duangteraprecha S, Matsushita N, Matsuda N, Ruanrungsni N, et al. Entamide C-a sulphur-containing amide from Entada phaseoloides. Phytochemistry 1999;28:881-2.
15. Ikekami F, Ohmiya S, Ruanrungsni N, Sakai S, Murakoshi I. Entamide D, a second new sulfur-containing amide from Entada phaseoloides. Phytochemistry 1997;26:1525-6.
16. Ionkova L, Momokov G, Proksch P. Effects of cyclotane saponins from hairy roots of Astragalus membranaceus Bge., on human tumor cell targets. Fitoterapia 2010;81:447-51.
17. Okada Y, Shibata S, Ikekawa T, Javellana AMJ, Kamo O. Entada saponins-III, a saponin isolated from the bark of Entada phaseoloides. Phytochemistry 1987;26:2789-96.
18. Ahmad F, Khan RA, Rasheed S. Preliminary screening of Entada phaseoloides Merrill. Australian Tropical Rainforest Plant; 2020.
19. Barua GC, Kirtikar KR, Basu BD, Blatter E. 4+ Vol. 2nd edition. Indian Medicinal Plants. Lalit Mohan Basu Publishers, Allahabad; 1935.
20. Marmitt DJ, Blatter E. A resource book for the Treatment of Cancer grant BT/PR16561/NER/95/232/2015 dated 09.01.2017.

AUTHORS CONTRIBUTIONS

All the authors have contributed equally.
21. Dawane JS, Pandit VA, Rajapadhye BD. Anti-nociceptive effect of *Entada phaseoloides* seeds formulation after topical application in arthritic wistar rats. J Clin Diagn Res 2013;7:2744-6.

22. Dawane JS, Pandit VA, Rajapadhye BD. Experimental evaluation of anti-inflammatory effect of topical application of *Entada phaseoloides* seeds as paste and ointment. N Am J Med Sci 2011;3:513-7.

23. Gupta R, Rath R, Thakurdesai P, Bodankar S. Anti-inflammatory and analgesic effects of methanolic extract of *Entada phaseoloides* seeds. Cell Tissue Res 2006;6:609-13.

24. Lahoti A, Kalra BS, Tekur U. Evaluation of the analgesic and anti-inflammatory activity of fixed-dose combination: non-steroidal anti-inflammatory drugs in experimental animals. Indian J Dent Res 2014;25:551-4.

25. Turner RA. Screening methods in pharmacology: analgesic agent. Vol. 13. Academic Press: London, UK; 1965.

26. Koster R, Anderson M, De J. Acetic acid for analgesic screening. Fed Proc 1959;18:412-7.

27. Eddy NB, Leimbach DJ. Synthetic analgesic dithienyl butanyl and dithinyl butylamines. J Pharmacol Exp Ther 1953;107:385-93.

28. Turner R, Hebborn P. Screening methods in pharmacology: analgesic agent. 2nd ed. London: Academic Press; 1965.

29. Verma A, Jana GK, Chakraborty R, Sen S, Sachan S, Mishra A. Analgesic activity of various leaf extracts of *Saraca indica* linn. Pharm Lett 2010;2:352-7.

30. Dong Y, Shi H, Yang H, Peng Y, Wang M, Li X. Antioxidant and anti-inflammatory activity of α-lipoic acid in gouty arthritis model. Neuroscience 1999;8:2497-507.

31. Lotta T, Vidgren J, Tilgmann C, Ulmanen I, Melin K, Jalkunen L, et al. Kinetics of human soluble and membrane bound catechol-O-methyltransferase: a revised mechanism and description of the thermolabile variant of the enzyme. Biochem 1995;34:4202-10.

32. Badhe SV, Phadke SV. Screening of cyclooxygenase inhibitors. Indian J Exp Biol 2003;41:1037-43.

33. Naima J, Islam MR, Pruma NM, Afin SR, Hosain MR, Hosain MK. Phytochemical screening and anti-inflammatory activity of *Mimoso diplostichis* leaves. Int J Pharm Sci Res 2019;10:3691-6.

34. Hameed JB, Ramadhan UH. Xanthine oxidase inhibitory, anti-inflammatory, antinociceptive activity of *Mimoso diplostichis* leaves. Cell Tissue Res 2006;6:609-13.

35. Kumar MA, Jitendra J. Anti-inflammatory and analgesic activity of bark extract of *Pterospermum acerifolium* linn. Int J Pharm Res 2009;1:32-7.

36. Sunday DO, Om U. Peripheral and central antinociceptive activities of the crude methanolic extract and fractions of *Monodora balsamina* linn. Asian J Pharm Clin Res 2010;3:15-9.

37. Carlsson KH, Jurna I. Depression by flupirtine a novel analgesic agent of motor and sensory responses of the nociceptive system in the rat spinal cord. Eur J Pharmacol 1987;143:89-97.

38. Chen VF, Li N, Jiao YL. Antinociceptive activity of petroleum ether fraction from the Methanol extracts of *Paederia scandens* in mice. Phytotherapy 2008;15:427-36.

39. Calixto JB, Beirith A, Ferraia J, Santos ARS. Naturally occurring anti-nociceptive substances from plants. Phytother Res 2000;14:401-18.

40. Berkowitz BA. The relationship of pharmacokinetics to pharmacological activity: morphine, methadone and naloxone. Clin Pharmacokinet 1976;1:219-30.

41. Ainsah O, Nabishah, Osman CB, Khalid BA*#*. Effects of naloxone, glycerylhydrazide, dexamethasone and deoxycorticosterone in repetitive stress. Clin Exp Pharmacol Physiol 1999;26:453-7.

42. Kamo KK, Mahberg PG. Morphin an alkaloids: biosynthesis in plant (Papaver spp.) tissue cultures in medicinal and aromatic plants I of biotechnology in agriculture and forestry. Springer 1988;4:251-63.

43. Komhoff M, Grone HJ, Klein T. Localization of cyclo-oxygenase-1 and -2 in adult and fetal human kidney: implication for renal function. Am J Physiol 1997;272:460-8.

44. Moncada S. Drugs that inhibit cyclo-oxygenase and its product, 6-oxo-PGF1α in vivo and in vitro. Prostaglandins 1978;15:955-67.

45. Barua et al. Int J Pharm Pharm Sci, Vol 12, Issue 8, 172-179