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Original Article

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 PHASEOLOIDES*

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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 pentazocine, 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 analgesic 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

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INTRODUCTION

Entada phaseoloides , commonly known as box bean or St. Thomas' bean, 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, loins, 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 itches, vomiting, etc. [5]. The plant also reported having anthelmintic, 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, folkloric 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* Merrill [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. (Sikkim, India) and Cadila Pharmaceuticals

Ltd. (Gujarat, India) respectively. All other chemicals used were of analytical grade. All the primary antibodies (NFκB and IL-10) used for western blotting assay were procured from Santa Cruz Technology (Danvers, MA), while the primers (COX-1, COX-2, TNF-α, COMT and IL-6) used for Reverse transcriptase PCR were obtained from Imperial Life Science Pvt. Ltd. (Haryana, India).

Animals

Thirty numbers of healthy Swiss albino female mice (25-30 gm) and thirty numbers of wistar rats (150-220 gm) of either sex were obtained from the animal house of the Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science, Khanapara. 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/CPCSEA/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 polypropylene 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 were divided into 5 groups (n=6). 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 55±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]:

$$\text{Percentage Protection \%} = \frac{\text{Test Mean} - \text{Control Mean}}{\text{Control Mean}} \times 100$$

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 pentazocine (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, nuclease-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 $MgCl_2$. 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 annealing 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, India).

Western blotting assay

Immunoblotting assay of NFκB 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 Laemili 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 (Whatman PROTRAN). This step was followed by blocking of the membranes using 3% BSA (Hi media) 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 and washed with 1X TBST 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 Graph Pad 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. Repeated purification of fraction F3 on silica gel (60-120 mesh) using CHCl3/MeOH (12:1) yielded compound 4.

Oleic acid (1): Light yellow oil, ^{1}H-NMR (500 MHz, CDCl3) d: 5.35 (2H, m), 2.33(4H, m), 2.01 (4H, m), 1.64 (4H, m), 1.37e1.22 (H, m), 0.88 (3H, t, J ¼ 7.1 and6.2 Hz).13C NMR (CDCl3, 75 MHz) d: 180.55, 129.93, 129.63, 34.11, 31.91, 29.76, 29.66, 29.60, 29.53,29.37, 29.32, 29.14, 29.05, 27.20, 27.13, 24.64, 22.67. HR-ESI-MS m/z: 305.2456 (Calcd for C18H24O2Na: 305.2451).

Entadamide A (2): White amorphous powder, ^{1}H-NMR (500 MHz, CDCl3) d: 7.62 (1H,d,J¼ 14.5 Hz), 6.44(1H, br s), 5.69(1H, d, J ¼ 14.6 Hz), 3.71(2H,m), 3.46 (2H, m), 2.32 (3H,s). ^{13}C NMR (CDCl3, 75 MHz) d: 165.80, 143.67, 115.31, 62.46, 42.49, 14.60. HR-ESI-MS m/z: 162.0589 (Calcd for C6H12NO2S: 162.0583).

Entadamide A-beta-D-glucopyranoside (3): White amorphous powder, 1H-NMR (500 MHz, CD3OD) d: 2.33 (3H, s), 3.27 (1H, m), 3.29 (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, J ¼ 7.9 Hz), 5.85 (1H, d, J ¼ 14.58 Hz), 7.59 (1H, d, J ¼ 14.58 Hz). 13C NMR (75 MHz, CD3OD): d 165.58, 142.31, 114.83, 102.39, 75.8, 75.67, 72.97, 69.36, 68.23, 60.74, 38.92, 13.4. HR-ESI-MS m/z: 346.0946 (Calcd for C12H21NO7S: 346.0931).

Phaeoloidin (4): White amorphous powder, 1H-NMR (DMSO-d6, 500 MHz) δ: 9.00(1H, s, 4-OH), 6.94 (1H, d, J = 8.6 Hz, H-3), 6.58 (2H, m, H-5, H-6),4.99 (2H, brs, H-7), 4.53 (1H, d, J=7.4 Hz), 3.68 (dd, J = 11.8, 1.9 Hz, 1H), 3.61 (1H,d, J =15.7)&3.51(1H,d,J=15.7),3.46 (dd, J = 11.8, 5.6 Hz, 1H),3.20 (td, J = 5.7, 3.2 Hz, 4H), 3.14 (1H,dd, J= 7.4&8.5). 13C-NMR(DMSO-d6, 300 MHz)δ: 172.92 (C-8), 152.02 (C-4), 148.51(C-1), 125.88 (C-2), 117.45 (C-6), 117.03 (C-3), 113.97(C-5), 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-7). HRMS (ESI+) m/z: 329.0875 ([M-H]-;C14H18O9 calcd. 329.0873).

RESULTS

Acute toxicity study

No mortality or significant changes in behavior and body weight was observed following oral administration of MEEP at 2000 mg/kg. Accordingly 100, 200, and 400 mg/kg, p. o. were used in the study.

Pure compound isolated from *Entada phaseoloides*

Subjecting of MEEP to fractionation and purification led to the isolation of 4 compounds. The structures of isolated compounds were known by using IR, MS, 1D, and 2D NMR spectroscopic techniques. A comparison of the spectral data with those reported in the literature [30] led to identification and confirmation of the compounds as Oleic acid (1), Entadamide A (2), Entadamide A-b-D-glucopyranoside (3) and Phaseoloidin.

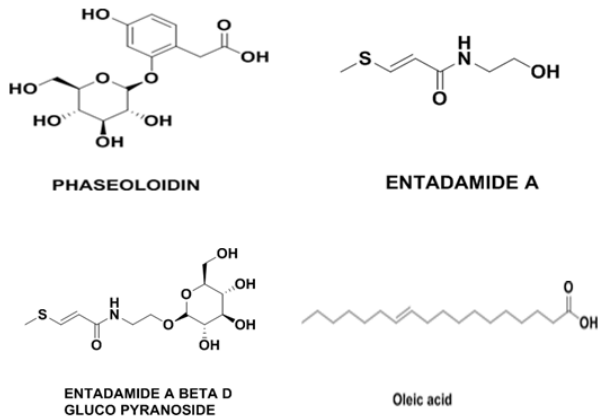

Fig. 1: Structures of pure compounds isolated from *Entada phaseoloides*

Peripheral analgesic activity study

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 analgesic activity of MEEP using the hot plate method 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: **Analgesic activity of MEEP on Eddy's hot plate in mice**

Groups	Treatment	Response mean±SEM (s) (Percent inhibition)			
		15 min	30 min	60 min	120 min
1.	Control	2.9±0.17	3.7±0.16	3.98±0.20	3.95±0.23
2.	Tramadol (22.8 mg/kg, i. p.)	4.1±0.18* (41.37 %)	5.08±0.17* (37.2 %)	6.05±0.20* (52.01 %)	7.75±0.27* (96.20 %)
3.	MEEP (100 mg/kg, p. o.)	4.2±0.23 (44.82 %)	4.7±0.22 (27.02 %)	4.4±0.10 (41.37 %)	5.1±0.23 (10.5 %)
4.	MEEP (200 mg/kg, p. o.)	3.9±0.32 (34.4 %)	4.8±0.24 (29.72 %)	4.5±0.21 (13.06 %)	5.7±0.36 (44.30 %)
5.	MEEP (400 mg/kg, p. o.)	4.7±0.26 (62.06 %)	4.6±0.17 (24.32 %)	6.15±0.61 (54.52 %)	5.9±0.31 (49.3 %)

*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 dosed dependent.

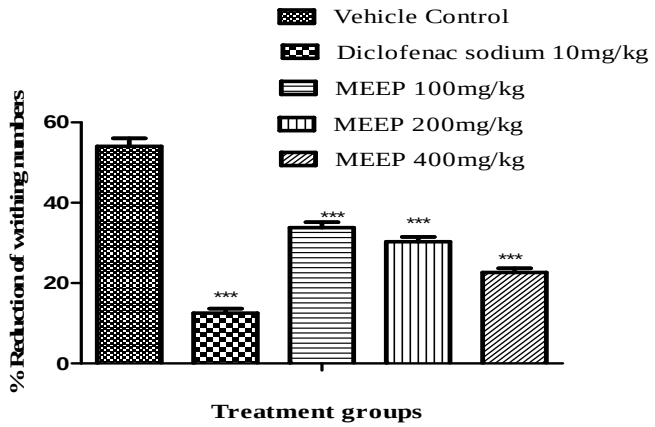

Fig. 2: Analgesic effects of the MEEP and diclofenac sodium on the acetic-acid-induced writhing response in mice. Each value represents as mean ± SEM. ***p < 0 .001 as compared with the control group (one-way ANOVA followed by "Dunnett's Multiple Comparison Test"

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.)	2.36±0.22	4.1±0.40* (70.8 %)	3.87±0.34* (48.84 %)	5.2±0.43* (92.5 %)	7.2±0.11* (148.27 %)
3.	MEEP (100 mg/kg, p. o.)	2.8±0.26	4.2±0.23* (75 %)	4.8±0.22* (84.61 %)	4.6±0.10* (70.37 %)	5.3±0.23* (82.75%)
4.	MEEP (200 mg/kg, p. o.)	2.8±0.28	4.6±0.32* (91.66 %)	3.6±0.24* (41.53 %)	5.02±0.21* (85.92 %)	5.7±0.35* (97.2 %)
5.	MEEP (400 mg/kg, p. o.)	2.74±0.32	4.04±0.34* (68.33 %)	4.38±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, n=6) (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 down-regulated in the MEEP treated rats as compared to the vehicle control group; the down-regulation of mRNA expression levels of TNF-α at 60 and 120 ($p<0.001$) min could also be seen in the MEEP treated rats compared to the vehicle control group. The mRNA expression levels of IL-6 at 60 and 120 ($p<0.001$) min and TNF-α at 60 and 120 ($p<0.001$) min were almost similar in the tramadol 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).

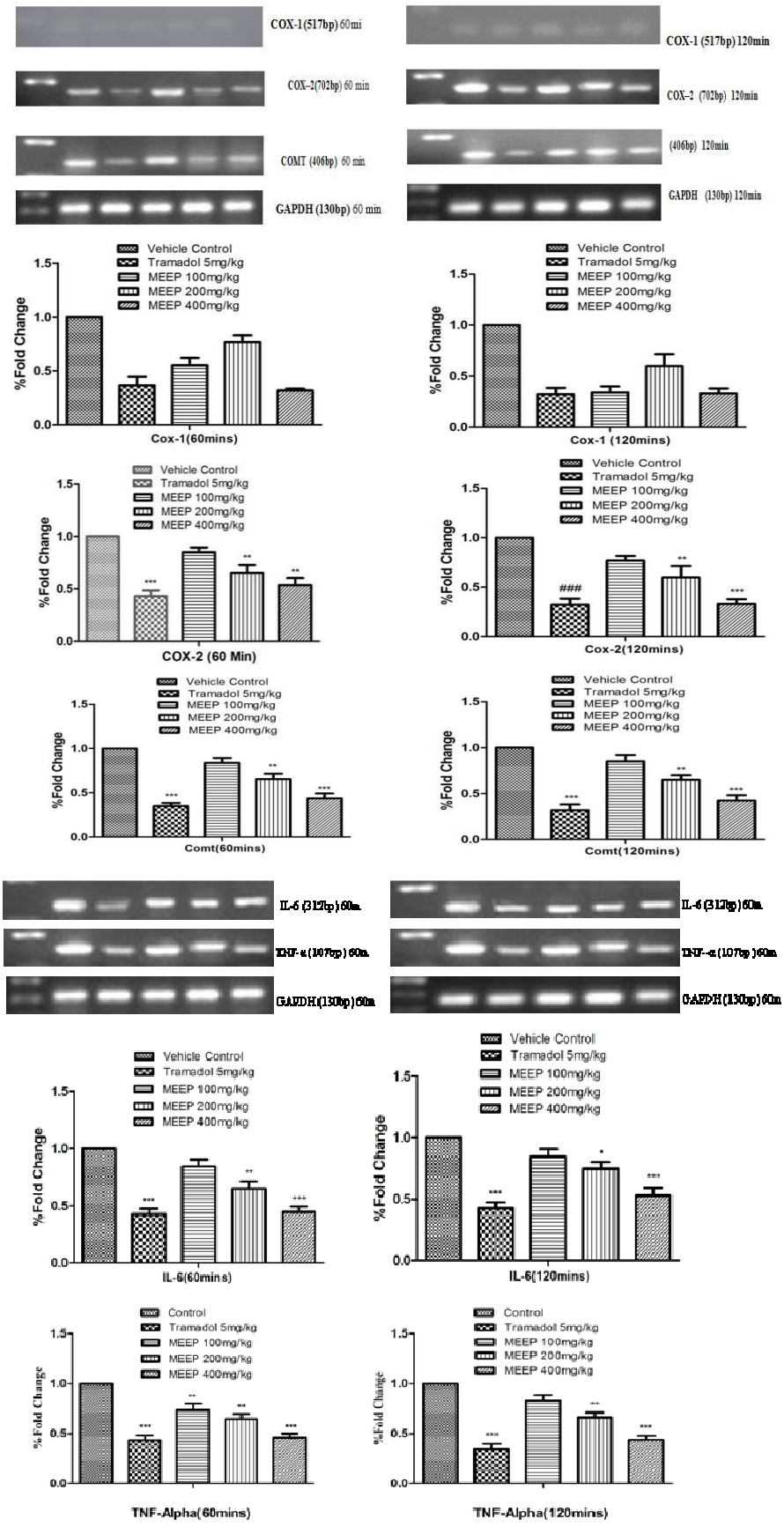

Fig. 3: Quantitative data expression of mRNA of COMT (60 min, 120 min), COX-1 (60 and 120 min), COX-2 (60 and 120 min), IL-6 (60 and 120 min), TNF-α (60 and 120 min) as fold change as compared with vehicle control group. Values are expressed as mean±SEM (*p<0.05, **p<0.01, ***p<0.001) compared with vehicle control group. Lane1 = Control, Lane2 = Tramadol, Lane3 = MEEP 100 mg/kg, Lane4 = MEEP 200 mg/kg, Lane5 = MEEP 400 mg/kg

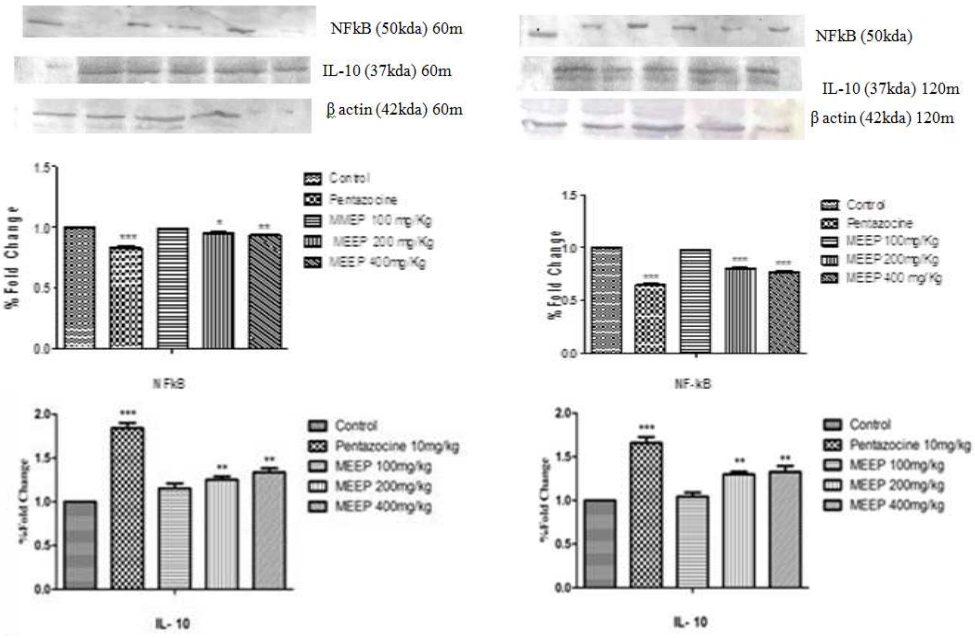

Fig. 4: Quantitative data expression of proteins levels (A) NFκB at 60 min, (B) NFκB at 120 min (C) IL-10 at 60 min and (D) IL-10,120 min. Values are expressed as percent fold change compared with control. Values represented as mean±SEM (n=6). *p<0.05, **p<0.01, ***p<0.001 compared with control group. Lane1= Protein Ladder, Lane2 = Control, Lane3 = Pentazocine 100 mg, Lane4 = MEEP 100 mg/kg, Lane5= MEEP 200 mg/kg, Lane6= 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 has made it obvious that MEEP exhibited anti-nociceptive activity by increasing the latency time of responses in mice in all tested doses following 60 and 120 min of administration. The percentage protection offered by MEEP at 400 mg/kg body weight is at par with that of tramadol following 1h of drug administration. In comparison to control, tramadol produced the highest analgesic activity among all tested samples whose peak activity was reached after 2 h.

The hot water immersion assay revealed that MEEP produced significant anti-nociceptive activity in a dose-dependent manner by increasing the latency time of responses in rats [39]. Among tested doses, 400 mg/kg exhibited more anti-nociceptive activity, and the potency was maximal at 2 h. In comparison to control, pentazocine produced the highest analgesic activity among all tested samples. 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 prostanoids 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 bonded 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 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 NFκB, 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, NFκB, IL-6, and TNF-α. The study corroborated the analgesic effects of this species, justified and supported its ethnopharmacological 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.

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