EVALUATION OF ANALGESIC ACTIVITY OF HYDROETHANOL EXTRACT OF *PLANTAGO EROSA EX* ROXB

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Summary

Plantago erosa ex Roxb (Plantaginaceae) locally known as *"Singapat"* is a perennial herb traditionally used in folk remedy as astringent, antitoxic, antimicrobial, demulcent, expectorant and diuretic, applied externally for insect bites, poison-ivy rashes, minor sores, boils and is also known to be able to cure snakebite. The present study involves the evaluation of the anti-nociceptive and analgesic activity of *Plantago erosa* in various animal models of algesia and nociception. Acetic acid induced writhing and formalin-induced paw licking model were used for anti-nociceptive activity and hot plate and tail flick models were used for analgesic activity. These activities of hydroethanol extract of *Plantago erosa* (PEHE) were assessed at the doses 50,100 and 200 mg/kg p.o. In acetic acid induced writhing method, the number of writhing decreased significantly in the PEHE treated group dose dependently. In formalin-induced paw licking test, administration of PEHE completely abolished the early phase and dose dependently decreased the reaction time in the late phase. In hot plate and tail flick test, there was increase in the reaction time in PEHE treated group which was dose and time dependent with maximum effect at 200mg/kg p.o. at 60 minutes. Presence of phytoconstituents *viz.* tannins, diterpenes, triterpenes and steroids in PEHE might account for its analgesic activity. From the study, it can be concluded that PEHE acts through central and peripheral mechanism as well.

Key words: Acetic acid, analgesic, Eddy's hot plate, hydroethanol extract, *Plantago erosa*, tail flick.

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Introduction

Pain is defined as neuralgia, an unpleasant sensory experience associated with tissue damage, such as injury, inflammation or cancer, but severe pain can arise independently of any obvious predisposing cause, or persist long after the precipitating injury has healed [1]. There are innumerable drugs used for analgesic effect however majority of them are synthetic in nature such as, NSAIDs and opiates. Due to the adverse side effects, like gastric lesions, caused by NSAIDs and tolerance and dependence induced by opiates, the use of these drugs as analgesic agents have not been successful in all the cases. Prolonged use of these drugs causes many side and toxic effects. Investigation of the efficacy of plant-based drugs used in the traditional medicine have been paid great attention because they are cheap, having least side effects [2].

Medicinal herbs constitute the basis of traditional medicinal practice worldwide [3]. The World Health Organization (WHO) has shown that, over 80% of the population in traditional medicinal system depends on these medicinal plants. They have also been used in the development of new drugs and continue to play an invaluable role in the drug discovery process [4,5].

The rich biodiversity of North East India cannot be neglected as several native plants of this region have a long tradition of being used as therapeutic agents. Considering the abundance of invaluable medicinal plants available in this region and its diverse use in various disorders, it is expected that screening and scientific evaluation of plant extracts for their different activity may provide us with new drug molecule(s) which can not only combat various side effects of the commercially available synthetic drugs but also help in reducing the cost of medication.

Plantago erosa ex Roxb (Plantaginaceae) locally known as *"Singapat"* is a perennial herb which is traditionally used in folk remedy as astringent, antitoxic, antimicrobial, demulcent, expectorant and diuretic, applied externally for insect bites, poison-ivy rashes, minor sores, boils and is also known to be able to cure snakebite [6]. Literature survey showed that the aqueous extract of *P. erosa* possess anti inflammatory and analgesic activities [7], *Plantago erosa* and *P. asiatica* have been reported for its *in vitro* cytotoxic, antiviral and immunomodulatory effects [8]. A composition containing the herbs *Plantago major* and *Piper methysticum* has been patented for its use in producing a diminished desire for tobacco (i.e., nicotine) without the use of nicotine itself. It also advantageously provides anti-depressive and anti-anxiety effects without sedative or hypnotic effects (United States Patent 6303647).

In our previous study, the anti-inflammatory activity of methanol extract of *Plantago* erosa has been reported [9]. Anxiolytic activity of the hydroethanol extract of *Plantago erosa* has been also been reported [10] by us. In this study, analgesic activity and anti-nociceptive activity of hydroethanol of *Plantago erosa* was evaluated.

Materials and Methods

Plant Material

The leaves of *Plantago erosa* were collected during the month of July-Sept, 2010 from the medicinal garden of the Department of Pharmacology & Toxicology, C.V.Sc. Khanapara, Guwahati and were identified by Botanical Survey of India, Shillong, Meghalaya and a voucher specimen (No AAU/CVSC/PHT/ 07-08/ 04) has been deposited therein.

Preparation of plant extract

Fresh leaves of *Plantago erosa* were cleaned and dried under shade in clean dust free environment, grinded and stored in air-tight container. They were (250 g) soaked in 1000 ml of hydroethanol (50:50) for 72 h in separate beakers. The plant material was stirred every 18 h using a sterile glass rod. The solvent was filtered every 3rd day using muslin cloth and What man's filter paper No 1. The filtrate obtained was concentrated in Rotary Evaporator (Equitron, Roteva) at 50-60°C under reduced pressure leaving a dark brown residue. The *Plantago erosa* hydroethanol extract (PEHE), thus obtained was transferred to a Petri dish and kept over water bath (50°C) until the solvent gets completely evaporated. It was stored at 4°C for future use. Recovery was 28.62% (w/w).

Animals

Swiss Albino mice (20-30 g) and Wistar rats (150- 200 g) of either sex were used for the study. The animals had free access to food and water. They were fasted overnight before the experiment. The animals were housed in animal room, with alternating light-dark cycle of 12 hours each. The animals were acclimatized to the laboratory conditions for at least five days prior to the experiments. All the experiments were conducted between 0900 h – 1800 h. The study was conducted after obtaining the approval of the Institutional Animal Ethics Committee **(**No: 770/03/ac/CPCSEA/FVSc, AAU/IAEC/06/22).

Chemicals and drugs

Indomethacin was purchased from Jagsonpal Pvt Ltd, Amar Nagar, Faridabad, India. Morphine sulphate was procured from Drugs India Pvt. Ltd. Dispur, Guwahati-5. Ethanol, acetic acid and formaldehyde were procured from Merck India Ltd, Mumbai. All the chemicals and solvents were of analytical grade.

Phytochemical screening

The freshly prepared PEHE was subjected to phytochemical screening tests for presence of various constituents as per standard methods [11].

Acute toxicity studies

Acute toxicity study was carried out according to the Organization of Economic Corporation Development (OECD) guidelines No. 425. PEHE was administered orally in doses of 100, 200, 400, 800, 1000 and 2000 mg/kg p.o. in different groups of mice (n=3) and percentage mortality was recorded for a period of 24 hours [12]. Based on the above toxicity study, direct limit test was done. Initially, a particular dose on the basis of the above study was administered to single female rat and the rat was observed for 48 hours with close surveillance up to initial 4 hours (same as in case of first rat); after 48 hours (of the second administration), same dose was administered to 2 more female rats and observation was done similarly. The rats were observed for 14 days and the weight of the animals was recorded on 7^{th} and 14^{th} day.

Evaluation of anti-nociceptive activity:

Acetic acid induced writhing model

The animals were pretreated with control (distilled water 10ml/kg p.o.), PEHE (50, 100, 200 mg/kg p.o. body weight) and standard drug indomethacin (10mg/kg p.o.). Thirty minutes after administration, the animals were injected with 0.7% aqueous acetic acid (10mg/kg i.p.) and number of writhes per animal was counted for the 20 minutes. Percent reduction in writhing syndrome was calculated and compared with the standard drug. Percent reduction indicates the percentage protection against abdominal constriction which was taken as an index of analgesia [13].
It was calculated as:

$$\frac{(W_c - W_t)}{W_c} \times 100$$

where, W_c = number of writhing of the control group
W_t = number of writhing of the treated group

Formalin- induced paw licking model

The animals were pretreated with control (distilled water 10ml/kg p.o.), PEHE (50, 100, 200 mg/kg p.o.) and standard drug indomethacin (10mg/kg p.o.). Fifteen minutes after treatment, 20 µl of 1% formalin was injected subcutaneously under dorsal surface of the hind paw and the time spent for licking the paw injected with formalin was counted for 30 minutes after formalin injection and considered as indicative of the pain stimuli. The formalin test has two distinctive phases possibly reflecting different types of pain. The first phase peaked at 5 minutes and the second phase at 20-30 minutes after formalin injection. This represented neurogenic and inflammatory responses, respectively [14].

Evaluation of analgesic activity:

Eddy's hot plate model

The animals were pretreated with control (distilled water 10ml/kg), PEHE (50, 100, 200 mg/kg body weight) and standard drug morphine (1.5 mg/kg i.p.). Thirty minutes after administration, the animals were placed on the hot plate (55± 0.5^0 C). Reaction time was recorded at 0, 30, 60, 90 and 120 minutes when the animals licked their paws and tried to escape. A cut off time of 20 seconds was considered positive [15].

Tail flick model

The animals were pretreated with control (distilled water 10ml/kg p.o.), PEHE (50, 100, 200 mg/kg p.o.) and standard drug morphine (1.5 mg/kg i.p.). Thirty minutes after administration, the tail of the animals were placed on hot wire of the Nichrome analgesiometer. Reaction time was noted when the animal withdraws the tail from the heated wire. The reaction times at 0, 30, 60, 90 and 120 minutes were recorded, following the administration of drugs [15].

Results

Phytochemical screening

The phytochemical screening of PEHE showed the presence of tannins by ferric chloride and gelatin test; diterpenes and triterpenes by Salkowski's and Liberman Buchardt's test and steroids by Salkowski's and Liberman Buchardt's test

Acute toxicity studies

Oral administration of PEHE up to 2 g/kg did not produce any toxic effects in the normal behavior of the mice. No mortality was observed and the extract was found to be safe at the given dose.

Acetic acid induced writhing model

PEHE caused significant ($P<0.01$) and dose dependent reduction in the number of writhing in mice. The maximum effect was observed at 200 mg/kg oral dose, with percent reduction of 83.33%, which was comparable to the standard drug Indomethacin (89.18%). The result is shown in Table 1.

Table 1 — Analgesic activity of PEHE in acetic acid induced writhing syndrome model.

Treatment groups	Dose mg/kg (p.o.)	Number of writhings	Percent Reduction (%)
Control	-	77.00± 1.67	0.00
PEHE	50	15.33±0.61[a]	80.09
	100	13.83±0.54[b]	82.03
	200	12.83±0.60[c]	83.33
Indomethacin	10	8.33±0.66[d]	89.18

Values are given as mean ± SEM of 6 animals

Means bearing the different superscript differ significantly P<0.01 as compared to control.

Formalin- induced paw licking model

Administration of PEHE, at 50mg/kg p.o. showed paw-licking time of 16.52 seconds, standard drug Indomethacin (10 mg/kg p.o.) showed paw licking time of 20.4 seconds. It is interesting to note that higher doses of PEHE at 100 and 200 mg/kg p.o. completely abolished the early phase indicated by absence of licking of paw after the formalin injection. In the late phase, the administration of PEHE decreased the duration of paw licking dose dependently from 79.48 seconds p.o. to 59.99 seconds (50 mg/kg to 200 mg/kg p.o.). The effect of PEHE at 200 mg/kg p.o. was higher (59.99 seconds) than the standard drug Indomethacin (35.50 seconds). The result is shown in Table 2. In this model, PEHE showed significant and better anti-nociceptive activity than the standard drug.

Table 2 — Analgesic activity of PEHE in formalin induced paw licking.

Treatments	Dose mg/kg (p.o.)	Early phase (secs)	Late phase (secs)
Control	-	58.39±1.01	142.48±1.14
PEHE	50	$16.52\pm0.97_{A}^{a}$	$79.48\pm2.61_{B}^{a}$
	100	0.00	$73.19\pm0.63_{A}^{a}$
	200	0.00	$59.99\pm1.10_{A}^{b}$
Indomethacin	10	$20.40\pm1.01_{A}^{a}$	$35.50\pm2.69_{B}^{c}$

Values are given as mean ± SEM of 6 animals

Means bearing the different superscript differ significantly P<0.01 as compared to control.

Eddy's hot plate model

The reaction time of PEHE treated group increased significantly (P<0.01) and the maximum effect was observed at the dose 200 mg/kg p.o. at 60 min with a reaction time of 20.53 seconds, which is better than the standard drug morphine (1.5 mg/kg i.p.) at 60 min which showed a reaction time of 12.3 seconds (Table 3).

Table 3 — Analgesic activity of PEHE in hot plate test

Treatments groups	Dose mg/kg	Reaction time (secs)				
		0 min	30 min	60 min	90 min	120 min
Control (p.o.)	-	$4.99\pm0.19_A^a$	$5.03\pm0.019_A^a$	$5.70\pm0.31_A^a$	$5.31\pm0.19_A^a$	$4.69\pm0.21_A^a$
PEHE (p.o.)	50	$12.81\pm1.96_A^b$	$16.50\pm1.93_B^b$	$16.65\pm2.48_B^c$	$19.53\pm1.98_C^b$	$20.86\pm1.21_C^b$
	100	$15.3\pm1.60_A^b$	$17.28\pm1.59_B^b$	$19.68\pm0.88_C^b$	$20.26\pm1.97_C^b$	$17.31\pm1.26_B^b$
	200	$15.1\pm1.71_A^b$	$17.7\pm1.50_B^b$	$20.53\pm0.82_C^b$	$19.05\pm1.97_C^b$	$18.43\pm1.70_B^b$
Morphine (i.p.)	1.5	$8.55\pm0.25_A^c$	$10.13\pm0.09_B^c$	$12.3\pm0.15_B^c$	$13.8\pm0.35_C^c$	$14.95\pm0.20_C^c$

Values are given as mean ± SE of 6 animals

Means bearing the different superscript in rows and different subscript differ significantly P< 0.01.

Tail flick model

In the tail flick model, the maximum effect was observed at the dose 8.95 seconds at 60 minutes which was higher than the control group. However, the standard drug morphine showed reaction time of 16.20 seconds at 60 minutes (Table 4).

Table 4 — Analgesic activity of PEHE in tail flick model

Treatment groups	Dose (mg/kg)	Reaction time (secs)				
		0 min	30 min	60 min	90 min	120 min
Control(p.o.)	-	$5.22\pm0.16_A^a$	$5.56\pm0.22_A^a$	$5.88\pm0.17_A^a$	$5.71\pm0.22_A^a$	$5.81\pm0.20_A^a$
PEHE(p.o.)	50	$3.68\pm0.68_A^b$	$3.85\pm0.41_A^b$	$4.27\pm0.39_B^b$	$4.32\pm0.57_B^a$	$5.92\pm0.77_C^a$
	100	$6.16\pm0.58_A^c$	$6.55\pm0.66_A^c$	$7.65\pm0.46_B^c$	$8.13\pm0.49_C^b$	$6.40\pm00.42_D^b$
	200	$6.00\pm0.44_A^c$	$6.48\pm0.39_A^c$	$8.95\pm1.42_B^d$	$7.93\pm0.53_C^c$	$7.08\pm0.42_C^c$
Morphine (i.p.)	1.5	$3.31\pm0.22_A^b$	$6.10\pm0.23_A^c$	$16.20\pm0.71_B^e$	$17.90\pm0.48_C^e$	$19.90\pm0.34_D^d$

Values are given as mean ± SE of 6 animals

Means bearing the different superscript in rows and different subscript differ significantly P< 0.01.

Discussion

The present study revealed that the hydroethanol extract of *Plantago erosa* exhibit potent anti-nociceptive and analgesic activity. Acetic acid induced writhing response in mice is not only simple and reliable but also affords rapid evaluation of peripheral type of analgesic action [16]. Pain sensation in acetic acid induced writhing method is elicited by triggering localized inflammatory response resulting release of free arachidonic acid from tissue phospholipids via cyclo oxygenase (COX) and prostaglandin biosynthesis [17, 18]. Acetic acid induced writhing is associated with increased level of PGE_2 and $PGF_{2\alpha}$ in peritoneal fluids as well as lipoxygenase products [19]. The increase in prostaglandin levels within the peritoneal cavity then enhances inflammatory pain by increasing capillary permeability [20]. PEHE causes reduction in the number of writhing in a dose dependent manner in the acetic acid induced writhing model which indicates that PEHE might act through peripheral mechanism. Further, PEHE might inhibit the prostaglandin synthesis along with reduced production of PGE_2 and $PGF_{2\alpha}$ and decreased inflammatory response due to the active anti-nociceptive principles present therein.

The formalin induced paw licking comprises of early phase and late phase. The early phase (immediately after injection) seems to be caused by C-fiber activation due to the peripheral stimulus. The late phase (starting approximately 20 minutes after formalin injection) appears to depend on the combination of an inflammatory reaction, activation of NMDA and non-NMDA receptors and NO cascade [21] in the peripheral tissue and the functional changes in the dorsal horn of the spinal cord [22]. PEHE completely abolishes the early phase at 100 and 200mg/kg p.o. which indicate that PEHE might cause complete inactivation of C- fiber in the early phase. In late phase, PEHE decreased the reaction time dose dependently which is indicative of partial inactivation of NMDA and non- NMDA receptors.

The hot plate test measures the complex response to a non-inflammatory, acute nociceptive input and is one of the models normally used for studying central nociceptive activity [23]. It is an established fact that any agent that causes prolongation of the hot plate latency using this test must be acting centrally [24]. PEHE showed dose and time dependent activity in this test. Prolongation of the reaction time in hot plate test signifies that PEHE acts through central mechanism.

Tail flick model is used to evaluate analgesic agents acting through central nervous system. Tail flick test is very useful for discriminating between centrally acting morphine like analgesics and non-opiate analgesics giving positive response for former. There is thick band of 5-HT_3 receptors on capsacin sensitive primary efferent terminals in superficial dorsal horn and 5-HT_3 receptor have major role to play in the transmission of painful stimuli. The role of 5-HT_3 is reported to be pronociceptive in the spinal cord. 5-HT_3 enhances the spinal cord response to the peripheral stimuli and stimulation of 5-HT_3 receptors in the spinal cord cause release of GABA that may cause inhibition of nociceptive signal. From the study, it might be assumed that PEHE might inhibit 5-HT_3 receptors which in turn reduce the GABA release demonstrating PEHE acts through central mechanism also.

Phytochemical screening of PEHE showed presence of steroids, triterpenes, tannins and diterpenes. Analgesic property in *Scoparia dulcis* was attributed to the phytoconstituents, Glutionl, a triterpene [25, 26] and Scoparinol, a diterpenes [27] isolated from the plant extract that showed analgesia by peripherally acting mechanism similar to the non-steroidal anti-inflammatory agents, like indomethacin and diclofenac sodium. In another study, the bark extract of *Taxus baccata* was shown to exhibit analgesic activity which was attributed to the presence of steroids [28]. Hence from these studies it can be concluded that presence of similar phytoconstituents might also be responsible for its anti-noceptive and analgesic activity of PEHE. In both the models it is evident that PEHE might be acting through central and peripheral mechanism as well. Further study is going on order to understand the precise mechanism with purified fractions of the extract for in depth pharmacological studies, which will throw light on the mode of action of the plant extract.

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