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Phytochemicals, Central Nervous System and Analgesic Activity of Ethyl Acetate Extract of *Casearia vareca* Roxb. Leaves

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The present study involves the preliminary investigation of phytochemical constituents, acute toxicity study and to evaluate depressant effects on central nervous system of the ethyl acetate extract (EACV) of leaves of *Casearia vareca* Roxb. (*Flacourtiaceae*) in Swiss albino mice. The *Casearia vareca* leaves powder was defatted with petroleum ether and then extracted with ethyl acetate in Soxhlet apparatus. The vacuum dried ethyl acetate leaves extract of *Casearia vareca* (EACV) obtained was subjected to various phytochemical as well as pharmacological screening. The ethyl acetate extract at the doses of 100, 200 and 400 mg/kg body weight was subjected to evaluation of central nervous system and analgesic activities in experimental animal models. Central nervous system activity was evaluated by the observation of behavioural profiles such as awareness and alertness, touch response, righting reflex, pinna reflex, grip strength, pain response, sound response, corneal reflex and spontaneous motor activity in mice at various doses. Analgesic activity was evaluated by the chemical (acetic acid induced writhing response), thermal (hot plate, tail immersion) and mechanical (tail clip) method. The extract exhibited the central nervous system depressant activity at 200 and 400 mg/kg per oral (p.o.) route and the activities were well comparable with the standard drug, diazepam (5 mg/kg, i.p.) The analgesic activity of the extract was evaluated using acetic acid induced writhing response, hot plate, tail immersion and tail clip method. The extract showed analgesic activity that was comparable with standard drug, acetyl salicylic acid and morphine. The data were verified as statistically significant by using one way analysis of variance (ANOVA).

Keywords: *Casearia vareca*, Spontaneous motor activity, Analgesic, Writhing, Hot plate, Tail immersion.

INTRODUCTION

A large number of medicinal plants throughout the world are attributed with having diverse medicinal properties as they contain different classes of phytochemicals. Screening of herbs has become a potential source of compounds of high therapeutic value. Ethnopharmacological studies have become increasingly invaluable in the development of health care. The green pharmaceuticals have now received extraordinary attention and popularity. A study reveals that about three quarters of world population relies on plants or plant extracts for healthcare [1]. The traditional uses of plants by the different tribes may be regarded as the basic material for scientific documentation studies. Therefore, in the last few years, traditional knowledge of plants came into focus for research work and for development of other value added products [2].

Casearia vareca Roxb. (*Flacourtiaceae*) is a popular and socioculturaly recognized plant in Assamese community, which is commonly known as *Chhagladoi.* The plant is distributed all over the North East India and up to 3000 ft, in the Hills [3]. The young leaves and shoots are edible and cooked as vegetable by the Assamese [4]. The fruits are rubbed into a paste and given to people suffering from worms, while the juice of the fruits is dropped into the ear when attacked by ticks [3]. The plant is mentioned in the Dictionary of Indian Folk Medicine and Ethnobotany and use for helmentic, earache, fever, giddin, headache, vermifuse and as anticancer [5]. Assamese use this plant leaves traditionally to treat various diseases, like as to treat cut injuries, burns, abscess, pain and in worm infestation. The plant is also considered to be effective in the treatment of diarrhoea and dysentery. A botanical description of the plant is recorded in Flora of Assam [3].

However, there is no scientific report for phytochemical constituents, central nervous system and analgesic activity of the leafy extracts of *Casearia vareca.* This prompted us to investigate the phytochemical constituents, central nervous system and analgesic activity of the ethyl acetate leaves extract of *Casearia vareca* Roxb.

EXPERIMENTAL

Casearia vareca Roxb. plant was collected in the month of June 2009, from Naharkatia, Dibrugarh, India. The

taxonomical identification and authentication of the plant was done at the Botanical Survey of India (BSI), Eastern circle, Shillong, India. The voucher specimen (No. BSI/EC/Identification/2009/289, dated: 20/07/2009) was deposited in our herbarium for future reference. The collected plant leaves were cleaned, shade dried, coarsely powdered (sieve no. 40) and stored in air tight container for further use.

Preparation of plant extract: The *Casearia vareca* leaves powder was defatted with petroleum ether. It was then extracted in Soxhlet apparatus using ethyl acetate as solvent. The solvent was allowed to evaporate in a rotary vacuum evaporator. The vacuum dried ethyl acetate leaves extract of *Casearia vareca* (EACV) obtained was subjected to various phytochemical as well as pharmacological screening.

Phytochemical investigation: Phytochemical screening of ethyl acetate leaves extract of *Casearia vareca* was carried out using standard procedures [6].

Laboratory animals: Swiss albino mice with a weight between 20 and 25 g (both sex) were used. The animals were kept under standard laboratory conditions (25 ± 5 °C, 40-70 % RH, 12 h light/dark cycle) and had access to standard diet and water *ad libitum.* The animals were acclimatized for a period of 14 days prior to performing the experiments. The experiments were carried out during the light period between 08.00-16.00 h. The experimental protocol was approved by Institutional Animal Ethics Committee (IAEC) of Department of Pharmaceutical Sciences, Dibrugarh University, Assam, India. Protocol approval No. IAEC/DU/02, dt.-04/05/2012. Animal studies were performed as per regulations and in accordance to the guidelines of Committee for the purpose of control, surveillance on experiments on animals (CPCSEA).

Acute toxicity study: Acute toxicity study of the prepared ethyl acetate leaves extract was carried out according to the Organization for Economic Co-operation and Development (OECD) Guidelines-423 [7]. Three animals were used for each step. The animals were fasted for 4 h, but allowed free access to water throughout. The dose level to be used as the starting dose was selected from one of four fixed levels, *i.e.*, 5, 50, 300 and 2000 mg/kg body weight p.o. As per the OECD recommendations, the starting dose level should be that which is most likely to produce mortality in some of the dosed animals; and when there is no information available on a substance to be tested in this regard; for animal welfare reasons, recommended starting dose is 300 mg/kg body weight.

Pharmacological activity

General behavioural profiles: Swiss albino mice were divided into 5 groups and each group contained 6 mice. Ethyl acetate leaves extract of *Casearia vareca* (EACV) was administered to the second, third and fourth group of animals at the dose of 100, 200, 400 mg/kg, p.o. respectively. While the first and last group were administered 0.5 % carboxy methyl cellulose (CMC) suspension (10 mL/kg, p.o.) as a vehicle control and diazepam (4 mg/kg, i.p.) as a standard drug control. The animals were under observation for their behavioural changes, if any, at 30 min intervals in the first 1 h and at the hourly intervals for the next 4 h for the following parameters [8,9].

Awareness and alertness: A mouse was placed in a bell jar and signs of awareness, alertness or stupor were observed. Also visual placing, stereotypy and passivity were observed. It usually shows a moderate degree or inquisitive behaviour [10].

Touch response: The touch response was recorded by touching the mice with a forceps or pencil at various parts of the body *i.e.* on the side of the neck, on the abdomen and on the groin [11].

Righting reflex: A mice was placed gently on its back on an undulated surface made of white iron at 30 °C. If the animal remains on its back for 30 s, it was considered as loss of righting reflex [12].

Pinna reflex: It was tested by touching the center of pinna with a hair or other fine instrument. The withdrawal of pinna from the irritating hair was considered to be positive response [10].

Grip strength: It was measured by allowing the animal to grasp a pencil in the horizontal position and noting the time taken by the animal to drop the pencil [10].

Pain response: The pain response was graded when a small artery clamp was attached to base of the tail and response was noted [11].

Sound response: Albino mice normally utter no sound, so that vocalization may indicate a noxious stimulus.

Corneal reflex: A stiff hair touched to the cornea, causes the animal to close the eyelids [12].

Spontaneous motor activity: Spontaneous motor activity (locomotor activity) can be easily measured using an Actophotometer which operates on photoelectric cells, connected in circuit with a counter. When a beam of light falling on the photocell is cut off by the animal a number is recorded in counter. For the test the animals were individually placed in a Actophotometer and locomotor activity scores were recorded for 10 min duration [13].

Analgesic activity: The analgesic activity of ethyl acetate leaves extract of *Casearia vareca* (EACV) was studied by the chemical (acetic acid induced writhing response), thermal (hot plate, tail immersion) and mechanical (tail clip) method [14].

Acetic acid induced writhing response: The activity of ethyl acetate leaves extract of *Casearia vareca* on acetic acid induced writhings was studied according to Koster *et al.* [15]. The extract at the dose of 100, 200 and 400 mg/kg, were orally administered to the mice 1 h before i.p. injection of 0.6 % (v/v) aqueous acetic acid, at a dose of 10 mL/kg. 0.5 % w/v CMC suspension (10 mL/kg p.o.) was used as a control treatment while the reference group received 150 mg/kg (p.o.) of acetyl salicylic acid (ASA) as a standard. Writhing (a syndrome characterized by a wave of contraction of the abdominal musculature followed by extension of hind limbs) that occurred between 5 and 15 min after acetic acid administration were counted. A reduction in the writhing number as compared to the control group was considered as evidence for the analgesic, which was expressed as percent inhibition of writhing.

Thermal stimulus by Eddy's hot plate method: The hot plate test to measure the latency response was performed according to the method described by Eddy and Leimbach [16]. Five groups of mice were selected for the study. One group received 0.5 % CMC suspension as control. Reference group received standard drug morphine (5 mg/kg, s.c.). Remaining different groups of mice received test extract (100, 200, 400

mg/kg p.o.). Mice were screened by placing them on a hot plate maintained at 55 ± 0.2 °C and recorded the reaction time in seconds for licking of hind paw or jumping. The mice which reacted within 15 s and which did not show large variation when tested on four separated occasions were selected for studies. The reaction time in seconds was recorded for licking of hind paw or jumping after drug administration at 0, 30, 60, 90 and 120 min. A cut-off time of 30 s was used in order to prevent paw tissue damage.

Tail immersion method: Mice were assigned to five groups of six animals each. A control group received 0.5 % CMC suspension (10 mL/kg, p.o.) while the extract (100, 200, 400 mg/kg p.o.) was given to the rest test groups. Morphine (2 mg/kg, s.c.) was administered to the reference group. The analgesic activity was evaluated 1 h after administration of the drugs. The tail (up to 5 cm) of the animal was dipped in a pot of water maintained at 55 ± 0.5 °C. The time in seconds to withdraw the tail out of the water was taken as the reaction time [17].

Tail clip method: All the mice were screened by applying a metal artery clip to the base of the tail with its jaw sheathed with thin rubber tubing. The pressure exerted by the clip was so adjusted that it was just sufficient to make all control mice respond. The animal that did not attempt to dislodge the clip within 10 s were not used for the experiment. Selected mice were divided into five gropes of six animals each. A control group received 0.5 % w/v CMC suspension (10 mL/kg p.o.). The extract (100, 200, 400 mg/kg p.o.) was given to the test groups, while the reference group received morphine (2 mg/kg, s.c.) as a standard. Analgesic activity was evaluated 1 h after per oral administration of test drugs. A tail clip was applied and a positive analgesic response was indicated if there was no attempt to dislodge the clip within 10 s in any of the four consecutive trials after a time period of 2 min [17].

Statistical analysis: Data obtained were expressed as mean ± standard error mean (SEM). The data were analyzed using analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Statistically, a p-value of less than 0.05 was considered to be significant.

RESULTS AND DISCUSSION

Phytochemical studies: Table-1 shows the phytochemicals detected in ethyl acetate leaves extract of *Casearia vareca* (EACV). The phytochemical screening gave positive tests for alkaloid, carbohydrate, glycosides, phenolic compound and tannins, flavonoid, proteins and amino acids, fats and oils and coumarin, while the tests for saponins, steroids, gum and mucilage and volatile oils were negative for the extract.

TABLE-1
PHYTOCHEMICAL CONSTITUENTS OF ETHYL ACETATE LEAVE EXTRACT OF *Casearia vareca* Roxb.

Phytochemicals	Ethyl acetate extract of *Casearia vareca*
Alkaloids	Present
Carbohydrates	Present
Glycosides	Present
Phenolic compounds and tannins	Present
Flavonoids	Present
Saponins	Absent
Proteins and amino acids	Present
Steroids	Absent
Gum and mucilage	Absent
Fats and oils	Present
Volatile oils	Absent
Coumarin	Present

Acute toxicity study: From the acute toxicity study it was concluded that no mortality of mice was observed up to the dose of 2000 mg/kg for the ethyl acetate leaves extract. Hence the extract can be considered as non-toxic.

General behavioural profiles: The results obtained from different experiments are presented in Table-2. The result indicated that the ethyl acetate extract of *Casearia vareca* leaves influence the general behavioural profiles, as evidenced in the awareness, touch response and pain response. It was noted that the extract, at the entire doses produced slight to moderate depression relating to the behavioural profiles when compared to control (CMC, 10 mL/kg). However, the standard drug diazepam (5 mg/kg) caused a significant depression of all these responses.

Spontaneous motor activity: Treatment with the ethyl acetate extract of *Casearia vareca* produced a significant reduction in spontaneous motor activity in tested mice. The mean locomotor activity counts in the animals treated with vehicle control (CMC, 10 mL/kg) was 208.2 ± 2.971, but in animals treated with 100, 200 and 400 mg/kg of the extract, the counts were 186.7 ± 4.958, 136.3 ± 7.704 and 111.3 ± 3.169 respectively. However, the standard drug diazepam (5 mg/kg) caused a significant decrease (91.33 ± 1.430) in locomotor activity as compared to vehicle control (Table-3).

Analgesic activity

Acetic acid induced writhing response: The ethyl acetate extract of *Casearia vareca* leaves effectively reduced the number of abdominal muscle contractions induced by 0.6 % acetic acid solution. The EACV had a dose dependent protection ranging from 10.80 % at 100 mg/kg to 31.88 % at 400 mg/kg against vehicle treated control group ($P < 0.05$) in chemically

TABLE-2
EFFECT OF *Casearia vareca* Roxb. ON BEHAVIOURAL PROFILE IN MICE

Group/Treatment	Awareness response	Touch response	Righting reflex	Pinna strength	Grip strength	Sound response	Pain response	Corneal reflex
CMC (10 mL/kg, p.o.)	0	0	0	0	0	0	0	0
EACV-1 (100 mg/kg, p.o.)	1+	1+	0	0	0	0	1+	0
EACV-2 (200 mg/kg, p.o.)	2+	2+	0	0	0	0	2+	0
EACV-3 (400 mg/kg, p.o.)	2+	2+	0	0	0	0	2+	0
Diazepam (4 mg/kg, i.p.)	4+	4+	4+	4+	4+	4+	4+	4+

Key for scoring: 0, no effect (normal); 1+, slight depression; 2+, moderate depression; 3+, strong depression; 4+, very strong depression. EACV: Ethyl acetate extract of *Casearia vareca* Leave.

TABLE-3
EFFECT OF *Casearia vareca* Roxb. ON SPONTANEOUS MOTOR ACTIVITY IN MICE

Group/ Treatment	Dose (mg/kg)	Total locomotor activity counts, Mean ± SEM	Decrease in locomotor activity (%)
Control	10 mL/kg	208.2 ± 2.971	–
EACV-1	100	186.7 ± 4.958**	10.32
EACV-2	200	136.3 ± 7.704****	34.53
EACV-3	400	111.3 ± 3.169****	46.54
Diazepam	4	91.33 ± 1.430****	56.13

*$p < 0.0001$ as compared to control group. Values are mean ± SEM (n = 6). Data analyzed by ANOVA followed by Dunnett's test. EACV: Ethyl acetate extract of *Casearia vareca* leave.

induced algesia (Table-4). The standard drug acetyl salicylic acid (150 mg/kg) markedly protected against abdominal pain (80.53 %) compared with control group ($P < 0.05$).

TABLE-4
ANALGESIC EFFECT OF *Casearia vareca* Roxb. ON ACETIC ACID-INDUCED WRITHING IN MICE

Group/ Treatment	Dose (mg/kg)	Number of writhings in 10 min Mean ± SEM	Inhibition in writhings (%)
Control	10 mL/kg	30.83 ± 0.477	–
EACV-1	100	27.50 ± 0.562*	10.80
EACV-2	200	22.00 ± 1.095***	28.64
EACV-3	400	21.00 ± 0.577***	31.88
ASA	150	06.00 ± 0.577****	80.53

*$p < 0.05$ as compared to control group. Values are mean ± SEM (n = 6). Data represent mean ± SEM (n = 6). Data analyzed by ANOVA followed by Dunnett's test. EACV: Ethyl acetate extract of *Casearia vareca* leave.

Thermal stimulus by Eddy's hot plate method: The analgesic activity of ethyl acetate extract of *Casearia vareca* leaves increased the response latency in hot plate test which was significant. The effect of the extract was dose as well as time dependent. Almost all the doses used, ethyl acetate extract was most effective at 100, 200 and 400 mg/kg at 90 min as comparable as the control drug, which was highly significant ($p < 0.05$). The analgesic activity of ethyl acetate extract *Casearia vareca* leaves was well comparable with the standard drug, morphine as shown in Table-5.

Tail immersion method: In tail immersion method the ethyl acetate extract of *Casearia vareca* leaves at 200 and 400 mg/kg showed significant % increase in reaction time of 69.31 and 216.68 respectively. The standard drug morphine (246.78 %) showed better increase in reaction time than that of the *Casearia vareca* extract as shown in Table-6.

TABLE-6
ANALGESIC EFFECT OF *Casearia vareca* Roxb. ON TAIL IMMERSION RESPONSE IN MICE

Group/ Treatment	Dose (mg/kg)	Reaction time (s) Mean ± SEM	Increase in reaction time (%)
Control	10 mL/kg	2.565 ± 0.016	–
EACV-1	100	2.867 ± 0.127	11.71
EACV-2	200	4.343 ± 0.182***	69.31
EACV-3	400	8.123 ± 0.067***	216.68
Morphine	2	8.895 ± 0.0589***	246.78

*$p < 0.05$ as compared to control group. Values are mean ± SEM (n = 6). Data analyzed by ANOVA followed by Dunnett's test. EACV: Ethyl acetate extract of *Casearia vareca* leave.

Tail clip method: The analgesic activity of ethyl acetate extract of *Casearia vareca* leaves in tail clip method was dose dependent. All the test doses used showed significant % increase in reaction time with peak effect (181.99 %) produced at the highest dose of 400 mg/kg. The analgesic activity of the extract was very close to the reference drug, morphine (197.76 %) as shown in Table-7.

TABLE-7
ANALGESIC EFFECT OF *Casearia vareca* Roxb. ON TAIL CLIP METHOD IN MICE

Group/ Treatment	Dose (mg/kg)	Reaction time (s) Mean ± SEM	Increase in reaction time (%)
Control	10 mL/kg	3.093 ± 0.019	–
EACV-1	100	4.642 ± 0.351***	50.08
EACV-2	200	6.580 ± 0.345***	112.62
EACV-3	400	8.722 ± 0.102***	181.99
Morphine	2	9.210 ± 0.127***	197.76

*$p < 0.05$ as compared to control group. Values are mean ± SEM (n = 6). Data represent mean ± SEM (n=6). Data analyzed by ANOVA followed by Dunnett's test. EACV: Ethyl acetate extract of *Casearia vareca* leave.

Inhibition of the touch response, righting reflex and grip strength is probably produced due to a pronounced central nervous system depressant action [18]. Reduction of pinna reflex and awareness may be due to synapses block of the afferent pathway or due to overall central nervous system depressant action [19,20]. In this study, the mechanism whereby ethyl acetate extract of *Casearia vareca* depressed awareness, touch responses and pain responses may be due to synapses block of the efferent pathway or by overall central nervous system depressant action.

Spontaneous motor activity test is a quite sensitive and relatively specific to all major classes of central nervous system depressants [21]. Locomotor activity is considered as an index

TABLE-5
EFFECT OF *Casearia vareca* Roxb. ON PAIN INDUCED BY HOT PLATE IN MICE

Group/Treatment	Dose (mg/kg)	Hind paw lick latency time (min)				
		0	30	60	90	120
Control	10 mL/kg	5.25 ± 0.01	5.36 ± 0.00	5.56 ± 0.02	5.76 ± 0.02	5.53 ± 0.11
EACV-1	100	5.20 ± 0.03	5.52 ± 0.07	6.65 ± 0.05****	7.75 ± 0.02****	6.86 ± 0.02****
EACV-2	200	5.21 ± 0.04	5.70 ± 0.11	8.75 ± 0.07****	11.26 ± 0.21****	9.85 ± 0.10****
EACV-3	400	5.15 ± 0.04	5.70 ± 0.03	11.54 ± 0.15****	15.92 ± 0.07****	15.18 ± 0.22****
Morphine	5	5.17 ± 0.03	19.96 ± 0.29****	24.33 ± 0.29****	17.06 ± 0.40****	13.65 ± 0.40****

*$p < 0.05$ as compared to control group. Values are mean ± SEM (n = 6). Data analyzed by two-way ANOVA followed by Dunnett's multiple comparisons test. EACV: Ethyl acetate extract of *Casearia vareca* Leave.

of alertness and a decrease leads to sedation as a result of reduced excitability of the central nervous system [22]. Decrease on locomotion reveals depression effect on central nervous system [23]. The central nervous system depressant activity of ethyl acetate extract of *Casearia vareca* leaves may be due to the increase in the concentration of γ-amino butyric acid (GABA) in brains [24].

Although, the abdominal constriction response induced by acetic acid is a very sensitive procedure that enables the detection of peripheral anti-nociceptive activity of compounds using animal protocols, which is not a specific model [25,26]. This model involves different nociceptive mechanisms, such as sympathetic system (biogenic amines release), cyclo-oxygenases and their metabolites [27] and opioid mechanisms [28]. Thus, other tests such as the hotplate, tail immersion and tail clip tests need to be carried out before any conclusion can be made regarding the mechanism of anti-nociceptive activity of the EACV. The thermal (hot-plate and tail immersion test) method specifically reflexes the involvement of central anti-nociceptive mechanism of extracts/compounds as only the centrally acting drugs were capable of affecting this test [29,30]. Mechanical (tail clip test) method is based on the fact that centrally acting analgesic drugs elevate the pain threshold of rodents towards pressure [31]. Centrally acting agents are known to activate the release of endogenous peptide by periaqueductal gray matters (PAG), which are carrying to the spinal cord to inhibit the pain muscle transmission within the dorsal horn [32]. Based on our present results, several suggestions could be made regarding the mechanisms of anti-nociceptive activity of EACV. The extract exhibited an opioid-mediated anti-nociceptive effect at the peripheral and central levels (based on the EACV ability to inhibit the abdominals constriction and increase in reaction time in both thermal and mechanical stimulus).

Different types of flavonoids and neuroactive steroids, alkaloids, triterpenoids, saponins and flavonoids have been reported to possess central nervous system depression activity [33-35]. It was also reported that the inhibition of pain could arise not only from the presence of opioids and/or opiodiomimetics but could also arise from the presence of saponins, alkaloids, tannins, glycosides, triterpenoids, catechins, phenolic constituents and also steroidal constituents [36-38]. So, it may be due to the similar type of constituents present in the ethyl acetate leaves extract of *Casearia vareca* Roxb. which exhibited the analgesic activity.

In the present study, phytochemicals, central nervous system and analgesic activity of ethyl acetate leaves extract of *Casearia vareca* (EACV) were investigated. The experimental findings in this study suggest that the ethyl acetate leaves extract of *Casearia vareca* Roxb. (*Flacourtiaceae*) posses central nervous system depressant and analgesic activity. From this investigation, we can conclude that the ethyl acetate extract of *Casearia vareca* leaves may be a centrally acting narcotic analgesic, acting through both peripheral and central mechanism of pain (responded to all the methods used). The central nervous system depressant and analgesic activity may be due to the presence of phytochemicals like alkaloids, phenols and tannins, flavonoids, saponins and glycosides.

Conclusion

The results obtained justify the use of the plant extract in traditional medicine for the treatment of central nervous system and painful conditions. An activity guided fractionation of the extract is ongoing to isolate, identify, characterize and elucidate the structure of the phytochemicals responsible for the observed significant pharmacological activities in this study. Further research would be of interest to explain the exact mechanism of the pharmacological effects.

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