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Study of Antinociceptive, Antipyretic and Neuropharmacological Activities of Leaf Extracts of *Citrus assamensis*

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Abstract

The present study was executed to explore *in vivo* antinociceptive, antipyretic and neuropharmacological activities of different leaf extracts of *Citrus assamensis* in Swiss albino mice. *C. assamensis* leaf extract displayed marked dose dependent antinociceptive potential in the five pain models. All leaf extracts except chloroform (200 mg/kg body weight) extract, produced significant (**p<0.01, ***p<0.001) anti-nociception against thermal induced pain stimuli in mice at various time points of post treatment in hot plate study compared to the control group. In acetic acid induced writhing assay, methanol (100 mg/kg body weight) and chloroform (200 mg/kg body weight) extracts showed significant (*p<0.01) result. In tail immersion test, basal reaction time was found significant (*p<0.01) in case of ethanol (100 mg/kg body weight) and chloroform (200 mg/kg body weight) extracts compared against control. Both the doses of all the extracts exhibited significant (***p<0.001) activity in formalin induced paw licking as well as percent inhibition of glutamate induced writhing study. Both the doses of methanol and ethanol extracts showed significant (*p<0.05) results in decreasing in rectal temperature after 1 hr. Higher doses of all the extracts showed significant (*p<0.05) decrease in duration of immobility in foreed swimming test (FST). The higher dose of ethanol extract (200 mg/kg body weight) significantly (*p<0.05) decreased the rate of movement with time in open field test. The test samples displayed marked antinociceptive potential in all the test procedures and also displayed marked antipyretic and neuropharmacological activities at different test doses.

Key words: Antinociceptive, antipyretic, neuropharmacological activity, *Citrus assamensis*, leaf extracts.

Introduction

Over the past few decades, strategies of drug discovery have been generally focused on an approach based on single target along with the rapid growth in genetics and molecular biology (Lenardao *et al.*, 2016). Herbal medicines derived from plants are being increasingly utilized to treat a wide variety of diseases and clinical disorders, but relatively little knowledge available about their exact effective doses and mode of action (Saieed *et al*., 2006). Now-a-days, in many parts of the world traditional medicine replaces conventional medicine because herbal remedies are cost effective, have minimum side effects with reduced health hazards and are easily available in market as compared to synthetic medicines. Many plant extracts possess *in vitro* and *in vivo* biological activities, which inspire intense research on their use in traditional medicine (Khandaker *et al*., 2016; Das *et al*., 2017a, b; Eshita *et al*., 2017; Hossain *et al*., 2016; Laboni *et at*., 2017a,b; Shahriar *et al*., 2018b).

Citrus genus belongs to the large family, Rutaceae, which contains 130 genera under seven subfamilies (Lucker *et al*., 2002). *Citrus assamensis* locally known as Satkora in Bangladesh, is a small tree, 4.5 to 7.5 meter tall and moderately branched

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and thorny plant which is used as medicine by local tribes of Assam, India. Leaves, flowers, and fruits of *C. assamensis* are used for treating dysentry, indigestion, pimples and intestinal worms (Das *et al*., 2013). Preliminary phytochemical screening revealed that this plant is rich in alkaloid, phytosterol, phenol, tannin, glycoside, saponin and flavonoids. Pharmacological investigations have demonstrated that *C. assamensis* has antibacterial, thrombolytic, membrane stabilizing, anti-inflammatory and antitumor activities (Shahriar *et al*., 2018b).

As part of our continuing studies on *C. assamensis*, the organic extracts of the leaf extracts of *C. assamensis* was evaluated for antinociceptive and antipyretic activities as well as neurophramacological study in mice for the first time.

Materials and Methods

Collection, identification and processing of plant sample: Leaves of *C. assamensis* were collected from Jayantapur, Sylhet, Bangladesh (January, 2014) and the plant was taxonomically identified with the help of the National Herbarium of Bangladesh, Mirpur, Dhaka (DACB; Accession Number- 38759). Leaves were sun dried for seven days. The dried leaves were then ground in coarse powder using high capacity grinding machine (Jaipan Designer Mixer Grinder, India) which was then stored in air-tight container in cool, dark and dry plac.

Extraction procedure: The powdered leaves (30 gm) was successively extracted in a soxhlet extractor at elevated temperature using 500 ml of distilled methanol (40-60)°C which was followed by ethanol and chloroform. After extraction all extracts were kept in refrigerator 4°C for further investigation.

*Experimental animal***:** Swiss albino mice of either sex, 4-5 weeks of age, weighing between 10-24 gm were collected from ICDDR,B, Dhaka. Animals were maintained under standard environmental conditions and free access to feed and water. The animals were acclimatized to laboratory condition for one week prior to experiments.

In vivo antinociceptive study:

Hot plate method: The paws of mice are very sensitive to temperature at 55 ± 0.5 °C, which are not damaging to the skin. The animals were placed on Eddy's hot plate kept at a temperature of 55 ± 0.5°C. A cut off period of 30 sec (Franzotti *et al.*, 2000), was observed to avoid damage to the paw. Reaction time was recorded when animals licked therefore or hind paws, or jumped at 0, 30, 60, 90 and 120 min after oral administration of the samples. The animals of test groups received test samples at the doses of 100 and 200 mg/kg body weight. Positive control group received standard drug diclofenac sodium at the dose of 10 mg/kg body weight and saline water.

Acetic acid induced writhing test: The acetic acid writhing test in mice was conducted as described by Khandaker *et al.* (2016).

Tail immersion test: The tail immersion method was used to evaluate the central mechanism of analgesic activity. Here the painful reactions in animals were produced by thermal stimulus that is by dipping the tip of the tail in hot water. The tail immersion test in mice was conducted as described previously by Barai *et al.* (2015).

Formalin induced nociception: Animals received 20 µl of 2.5% formalin solution (7% formaldehyde) made up in saline and injected intra-planetary in the ventral surface of the right hand paw. Animals were observed from 0 to 5 min (neurogenic phase) and 15-30 min (inflammatory phase) and the time spent licking the injected paw was recorded as indicative of nociception. The animals received methanol, ethanol and chloroform extracts of *C. assamensis* at 100 and 200 mg/kg 1 hr before, with basis of a previous time response curve. Positive control group received standard drug diclofenac sodium at the dose of 10 mg/kg body weight (Santos *et al.*, 1999).

Glutamate induced nociception: A volume of 20 µl of glutamate (10 µmol/paw prepared in saline)

was injected intra-plantarly in the ventral surface of the right hand paw. Animals were observed individually for 15 min after glutamate injection. The amount of time spends in licking the injected paw was indicated as the nociception. The animals were treated with methanol, ethanol and chloroform extracts of *C. assamensis* at 100 and 200 mg/kg 1 hr before glutamate injection. Positive control group received standard drug diclofenac sodium at the dose of 10 mg/kg body weight (Beirith *et al*., 2002).

In vivo antipyretic activity: Antipyretic Activity in mice was performed according to Khandaker *et al*. (2016).

In vivo neuropharmacological study:

Hole cross test: The most consistent behavioral change is a hyper emotional response to novel environmental stimuli. The aim of this study was to characterize the emotional behavior of mice using the hole-board test. The hole-cross test in mice was conducted as described by Subhan *et al*. (2008).

Forced swimming test (FST): Forced swimming test was performed according to Khandaker *et al*. (2016) at the doses of 100 & 200 mg/kg body weight of methanol, ethanol and chloroform extracts respectively.

Open field test (OFT): According to previous work by Eshita *et al.* (2017), open field test was performed to monitor behavioral responses in mice that were placed in a novel and bright arena, at the doses of 100 & 200 mg/kg body weight of methanol, ethanol and chloroform extracts respectively.

Statistical analysis: Data was expressed as mean ± SEM (Standard error of mean). The results were analyzed statistically by ANOVA followed by Dunnet's test. Results with $p<0.05$, $p<0.01$ and $p<0.001$ were considered statistically significant.

Results and Discussion

In vivo antinociceptive study:

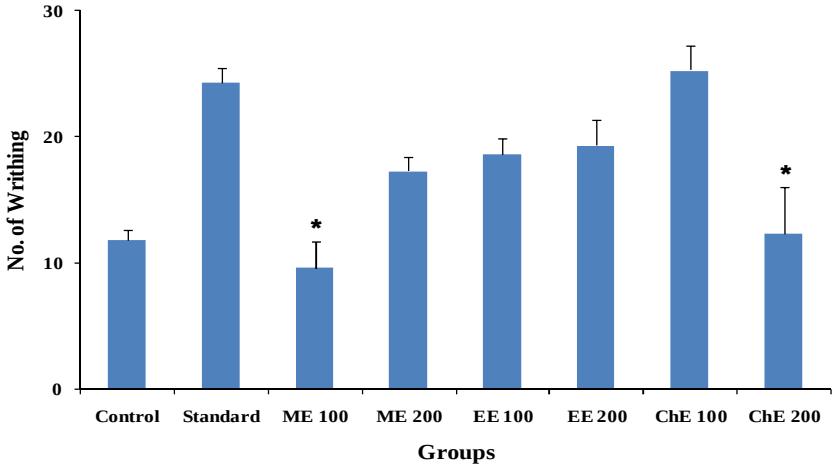
Hot plate test: The hot plate assay is a simple method of the pain reaction in animals by which effectiveness of centrally acting analgesics can be tested by measuring the heat induced pain response. The application of such stimuli evokes a behavioral response which results in the withdrawal of foot which varies inversely with the intensity of the stimulus. In this study, all extracts of the leaves of *C. assamensis* as well as standard diclofenac sodium produced significant (**p<0.01, ***p<0.001) antinociception against thermal induced pain stimuli in mice at various time points of post treatment while compared with the control group (Table 1). From findings of our study, it was apparent that the lower doses of the extracts have more potent central antinociceptive effect.

Table 1. Effect of different leaf extracts of *C. assamensis* on hot plate test.

Treatment	Dose (mg/kg)	Response times (Sec.)				
		0 min	30 min	60 min	90 min	120 min
Control	10 ml/Kg	12.02 ± 0.06	14.27 ± 0.16	15.20 ± 0.11	17.11 ± 0.04	12.40 ± 0.13
Diclofenac Na	10	8.18 ± 0.03***	9.29 ± 0.01***	11.36 ± 0.02***	10.16 ± 0.05***	8.04 ± 0.02***
Methanol extract	100	9.18 ± 0.03***	11.21 ± 0.05***	11.55 ± 0.09***	10.17 ± 0.11***	9.22 ± 0.01***
	200	10.52 ± 0.03***	12.06 ± 0.01**	12.18 ± 0.06***	11.49 ± 0.01***	9.85 ± 0.03***
Ethanol extract	100	10.64 ± 0.02***	12.27 ± 0.04***	13.41 ± 0.14***	11.11 ± 0.03***	9.87 ± 0.04***
	200	12.19 ± 0.07	14.69 ± 0.04	14.97 ± 0.02	16.23 ± 0.04***	14.44 ± 0.07***
Chloroform extract	100	11.57 ± 0.04**	11.83 ± 0.02**	11.98 ± 0.03***	11.66 ± 0.09***	10.99 ± 0.01**
	200	12.02 ± 0.06	14.27 ± 0.16	15.20 ± 0.11	17.11 ± 0.04	12.40 ± 0.13

Values are mean ± SEM (n=6), **(p< 0.01), ***(p< 0.001) significantly different when compared with the corresponding value of control group, done by independent sample *t*-test

Acetic acid induced writhing test: In the acetic acid-induced writhing test, the anti-nociceptive effect represented by writhe reduction and elicited by 100 and 200 mg/kg body weight of *C. assamensis* leaf extract in mice was similar to that of a standard drug, when groups were compared to control (Figure 1). Acetic acid induced abdominal constriction is a standard, simple, and sensitive test for measuring analgesia induced by drugs and chemicals, often advantageous in preclinical investigations of analgesics that represent pain sensation by triggering localized inflammatory response (Khandaker *et al.*, 2016). In the present study methanol and chloroform extracts inhibited no. of writhing compared to standard diclofenac sodium significantly (*p > 0.05).



Values are mean ± SEM (n=6), *(p< 0.05) significantly different when compared with the corresponding value of control group, done by independent sample t-test

Figure 1. Effect of *C. assamensis* leaf extracts on acetic acid induced writhing test.

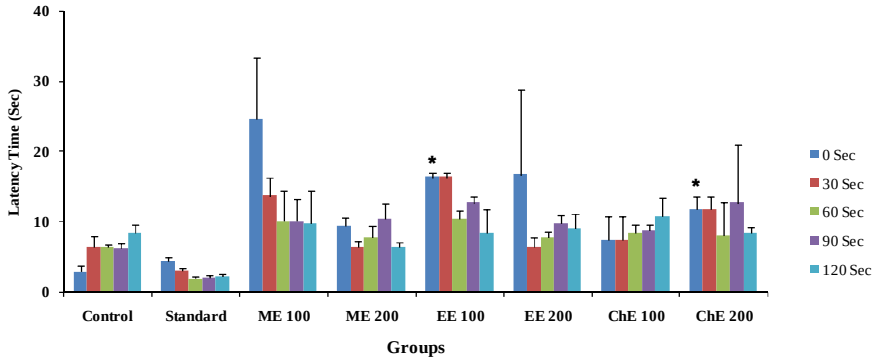
Tail immersion test: In tail immersion method, the heat itself acts as a source of pain whereas the effectiveness of analgesic agents in this pain model is highly correlated with relief of human pain. The different concentrations of methanol, ethanol and chloroform leaf extracts of *C. assemensis* (100 and 200 mg/kg body weight) and diclofenac sodium (50 mg/kg body weight) were administered to mice and observed the basal reaction time in different time intervals (Figure 2). In the present study ethanol and chloroform extracts showed significant (*p<0.05) analgesic activity compared to standard drug. The basal reaction time increased with increasing the concentrations along with increasing the time.

Formalin induced nociception: Formalin induced pain paradigm is well recommended biphasic procedure for the determination of anti-nociceptive activity. The different concentrations of methanol, ethanol and chloroform leaf extracts of *C. assamensis* has significantly (***p<0.001) decreased the paw licking in both neurogenic and inflammatory phases of formalin induced paw liking test in mice in the present investigation compared to the reference drug diclofenac sodium (Table 2). Chloroform extract (200 mg/kg body weight) caused 32.30% and 48.72% inhibition in early phase and late phase, respectively. Considering the inhibitory property of *C. assamensis* on the second phase of formalin, plant extracts might have anti-inflammatory property.

Glutamate induced nociception: In glutamate induced abdominal writhing test, the test groups treated with 100 and 200 mg/kg of body weight methanol, ethanol and chloroform leaf extract of *C. assamensis*, exhibited profound significant inhibition

of the abdominal writhes. Marked activity (36.11%) was recorded with chloroform extract (200 mg/kg body weight), when compared to standard group. The reference standard drug diclofenac sodium produced greater inhibitory effect (61.01%) as compared to the highest dose of chloroform extract (200 mg/kg body weight), depicted in table 2. In present study, we observed that the plant extracts inhibited mechanisms of pain, suggesting that *C. assamensis* contain chemical compound which may act as a narcotic analgesic.

In vivo antipyretic test: The results of anti-pyretic test of *C. assamensis* leaf extracts using Brewer's yeast induced pyrexia in mice have been shown in table 3. In the present study both methanol and ethanol leaf extracts of *C. assamensis* showed a significant (*p<0.05) decrease in rectal temperature at doses 100 and 200 mg/kg after 1 hr when compared with the control. However, no significant decrease in mean temperature was noted by chloroform extract when compared with control. But both doses of chloroform extract showed a progressive decline in mean temperature pattern with the increase in the dose (Table 3). Present study suggests a possibly better blockage of prostaglandins biosynthesis or mimicry of paracetamol action by the active principles in the extract.



Values are mean ± SEM (n=6), *(p< 0.05) significantly different when compared with the corresponding value of control group, done by independent sample t-test

Figure 2. Effect of *C. assamensis* leaf extracts on tail immersion test.

Table 2. Effect of different leaf extracts of *C. assamensis* on formalin and glutamate induced nociception.

Treatment	Dose (mg/kg)	Formalin induced nociception				Glutamate induced nociception
		Licking time (Sec)		% inhibition		% inhibition
		Early phase	Late phase	Early phase	Late phase	
Control	10	160 ± 23.88	65.20 ± 12.50	-	-	-
Diclofenac Na	10	95 ± 12.75	25.79 ± 6.22	40.42 ± 0.25	61.71 ± 0.38	61.01 ± 0.49
Methanol extract	100	150 ± 18.02	60.20 ± 9.42	10.41 ± 0.14***	9.74 ± 0.10***	21.41 ± 0.45***
	200	155 ± 20.20	62.32 ± 15.22	12.38 ± 0.12***	10.40 ± 0.14***	20.38 ± 0.55***
Ethanole	100	148 ± 11.32	58.23 ± 11.42	11.14 ± 0.04***	8.74 ± 0.07***	20.96 ± 0.08***
	200	155 ± 21.23	63.03 ± 8.23	15.55 ± 0.17***	11.48 ± 0.11***	22.10 ± 0.17***
Chloroform extract	100	116.9 ± 10.89	39.32 ± 8.92	29.50 ± 0.14***	43.60 ± 0.11***	31.33 ± 0.23***
	200	118.8±8.87	32.50±5.68	32.30 ± 0.25***	48.72 ± 0.06***	36.11 ± 0.07***

Values are mean ± SEM (n=6), ***(p< 0.001) significantly different when compared with the corresponding value of standard group, done by independent sample *t*-test

Table 3. Effect of leaf extracts of *C. assamensis* in brewer's yeast induced pyrexia in mice.

Treatment	Dose (mg/kg) body weight	Normal temp (°F)	Temp 18 hours after Brewer's yeast inj. (^{0}F)	Temperature after doses (°F)		
				1hr	2hr	3hr
0.9% NaCl	1ml/100gm b.w	97.82 ± 0.12	99.17 ± 0.18	95.62 ± 0.12	95.08 ± 0.01	95.95 ± 0.17
Paracetamol	50	97.14 ± 0.14	98.36 ± 0.08	93 ± 0.22	93.2 ± 0.23	93.78 ± 0.59
Methanol extract	100	95.25 ± 0.11	96.3 ± 0.13	96.2 ± 0.15*	96.5 ± 0.11*	96.42 ± 0.10
	200	96 ± 0.0.14	97.35 ± 0.46	97.37 ± 0.39*	96.62 ± 0.41*	97.45 ± 0.30
Ethanol extract	100	96.1 ± 0.0.58	96.92 ± 0.53	96.35 ± 0.51*	96.8 ± 0.48	96.52 ± 0.47
	200	94.55 ± 0.07	96.12 ± 0.09	95.65 ± 0.09*	94.55 ± 0.13*	95.2 ± 0.12
Chloroform extract	100	93 ± 0.09	94.17 ± 0.14	93.87 ± 0.19	92.8 ± 0.16	93.37 ± 0.13
	200	93.05 ± 0.3	94.42 ± 0.30	94.05 ± 0.28	93.15 ± 0.29	93.82 ± 0.28

Values are mean ± SEM (n=6), * ($p< 0.05$), significantly different when compared with the corresponding value of control group, done by independent sample *t*-test

In vivo neuropharmacological study:

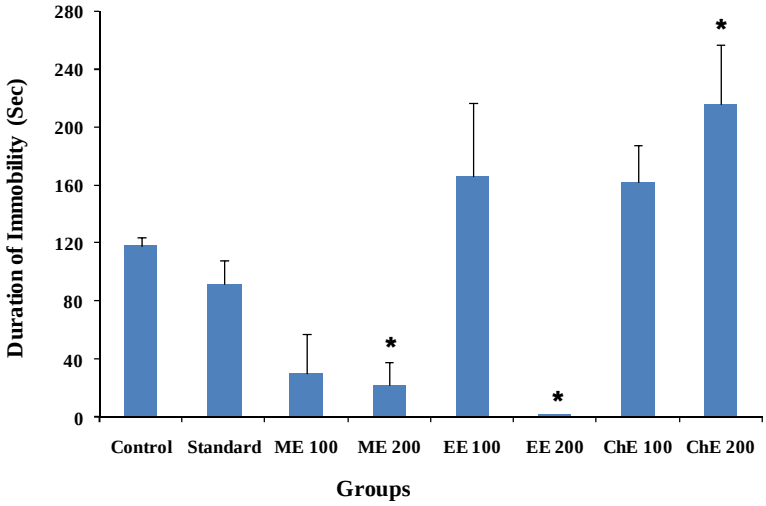
Hole cross test: To acquire evocative results regarding the sedative effects of methanol, ethanol and chloroform leaf extracts of *C. assamensis* by recording spontaneous locomotor activity of mice, hole cross method was implemented. Mice treated with methanol, ethanol and chloroform leaf extracts of *C. assamensis* at two doses (100 mg/kg & 200 mg/kg) showed dose dependent reduction in the locomotor activity which was comparable with standard drug diazepam. In case of control group, negligible variation in number of holes crossed from one chamber to another by mice was observed from 30 to 60 minute whereas groups treated with leaf extract at the above mentioned doses showed significant decrease of movement from their primary value at 90 minute (Table 4). Extracts of *C. assamensis* leaf decreased locomotor activity indicating its CNS depressant activity.

Forced swimming test (FST): Forced swimming test (FST) was performed to evaluate anti-depressant effect of leaf extracts of *C. assemensis* on mice. The main advantages of this procedure lie in its relatively easy operation and fast results. This test can also differentiate between drugs that are not aimed for the treatment of depression such as benzodiazepines, which have been shown to possess anti-anxiety effects. In the present study methanol, ethanol and chloroform extracts (200 mg/kg body weight), showed significant (*$p<0.05$) decrease in duration of immobility compared to control (Figure 3). Among the three extracts and standard, chloroform extract was found to be more effective.

Table 4. Effect of leaf extracts of *C. assamensis* in hole cross test in mice.

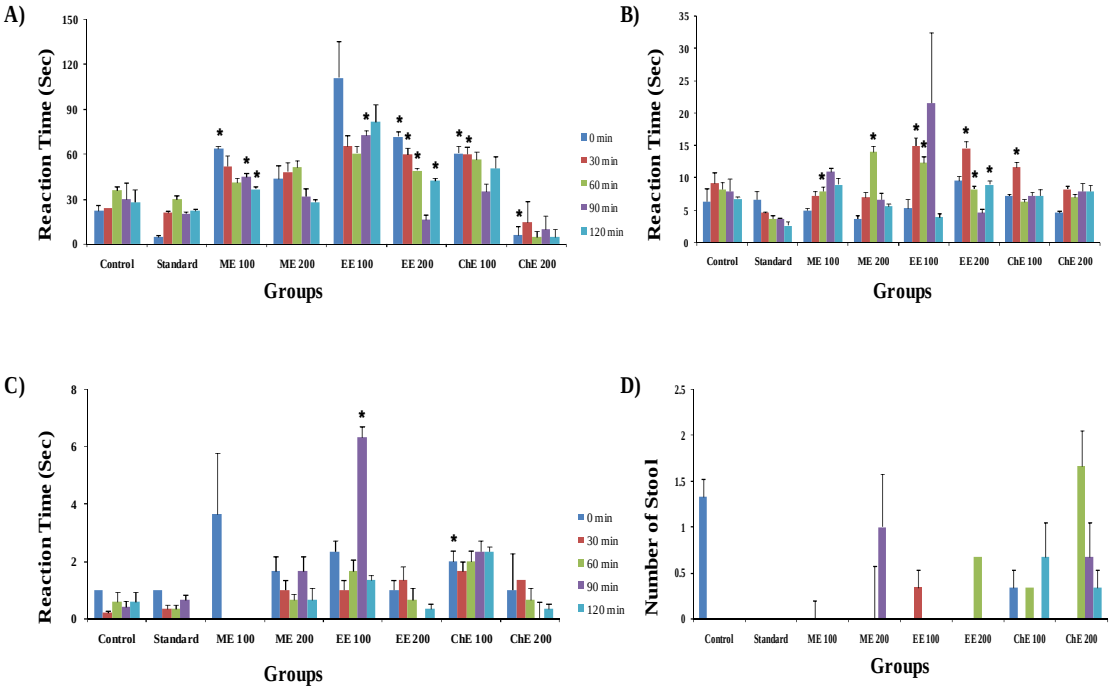
Group	Dose (mg/kg) body weight	Observation			
		0 min	30 min	60 min	90 min
Control	-	18.40 ± 0.39	10.85 ± 0.32	10.45 ± 0.21	6.95 ± 0.66
Standard	50	6.60 ± 0.21	5.60 ± 0.22	8.20 ± 0.21	7.21 ± 0.78
Methanol Extract	100	7.23 ± 0.58	5.32 ± 0.45	8.45 ± 0.45	5.21 ± 0.41
	200	9.31 ± 0.25	7.85 ± 0.58	8.74 ± 0.42	6.54 ± 0.24
Ethanol Extract	100	8.32 ± 0.78	5.42 ± 0.55	9.25 ± 0.21	7.52 ± 0.11
	200	7.51 ± 1.23	6.42 ± 0.21	9.74 ± 0.22	8.25 ± 0.34
Chloroform Extract	100	6.89 ± 0.52	5.10 ± 0.50	7.41 ± 0.21	5.21 ± 0.17
	200	6.65 ± 1.23	4.32 ± 0.52	7.12 ± 0.24	5.23 ± 0.13

Values are mean ± SEM (n = 6)



Values are mean ± SEM (n=6), * ($p < 0.05$) significantly different when compared with the corresponding value of control group, done by independent sample *t*-test

Figure 3. Effect of different leaf extracts of *C. assamensis* in forced swimming test.



Values are mean ± SEM (n=6), * ($p < 0.05$) significantly different when compared with the corresponding value of control group, done by independent sample *t*-test

Figure 4. Graphical representation of the effects of different leaf extracts of *C. assamensis* in open field test (A: movement, B: standing, C: center, D: stool).

Open field test (OFT): The crude methanol, ethanol and chloroform extracts of *C. assamensis* were subjected to assay for open field test following standard protocol and the obtained results were represented in figure 4 (A-D). In the open-field test methanol (100 mg/kg), ethanol (100 and 200 mg/kg) and chloroform (100 mg/kg and 200 mg/kg) extracts showed significant results in case of movement and

standing assay as compared to control group (Figure 4A & 4B). The results of the present investigation indicated that the extracts significantly decreased the locomotor activity as shown by the results of the open field tests. In the present study it was also observed that the extracts decreased the frequency of standing, entrance into center and stool count compared with control and standard. Therefore, the use of *C. assamensis* in folkloric medicine may be due to its CNS action validated by our findings. However, further investigation is necessary to determine the exact phytoconstituents and mechanism of action that are responsible for the biological activities of the extracts of *C. assamensis*.

Conclusion

Biological investigations carried out on *C. assamensis* plant in this study mainly focusing on the leaf of the plant but those are considered to be preliminary. As a result more elaborated research may be necessary to reach concrete conclusion about the finding of the present study. On the basis of above results and available reports, all three leaf extracts of *C. assamensis* showed potent antinociceptive, antipyretic and neuropharmacological activities. However, isolating new bioactive compounds and evolution of their extracts, mode of action and chronic toxicity profile might be the next steps to be followed to eventually find new lead compounds. The plant can be further screened against various diseases in order to find out its unexplored efficacy and can be a potential source of chemically interesting and biologically important drug candidates.

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