



ANTINOCICEPTIVE AND ANTI-INFLAMMATORY ACTIVITIES OF CROTON SPARSIFLORUS MORONG LEAVES IN ANIMAL MODELS

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ABSTRACT

A depth study was carried out to find out the potential of chloroform extract of *Croton (C) sparsiflorus morong*. (Family-Euphorbiaceae) leaves on antinociceptive, behavioral study and anti-inflammatory effects using various animal models. The dried, powdered leaves of, *C. Sparsiflorus Morong* were extracted successively with petroleum ether (60-80°C) and chloroform in soxhlet apparatus. The chloroform extract (yield 5.60% w/w with respect to dry powdered plant material) was selected for all experimental procedure. Two models were put to investigate the effects of nociception, by the tail immersion and hot plate method on Swiss albino mice and anti-inflammatory effect were studied by employing the carrageenan induced rat paw edema test in adult Wistar albino rats. Behavioral activity was carried out by elevated plus maze method in Swiss albino mice. Results shows that the CSCE has significant antinociceptive effect ($P < 0.001$) at the dose levels of 100, 200 and 400 mg/kg, orally in mice and also produced remarkable anti-inflammatory effect ($P < 0.001$) at the same dose levels used in the rats. Behavioral study of the CSCE has no differential anxiolysis effect when used orally. It concludes that, CSCE possessed remarkable antinociceptive effect and anti-inflammatory effect but no anxiolytic effect on animal models.

KEY WORDS: Croton Sparsiflorus Morong Leaves; Chloroform extract; Antinociceptive; Anti-Inflammatory; Behavioral study

INTRODUCTION

Croton Sparsiflorus Morong belongs to the plant family *Euphorbiaceae* is a small annual herb, growing up to 1-2 ft tall. Alternately arranged leaves, 3-5 cm long, are lance-shaped, with a toothed margin. Small white flowers are borne in 3-8 cm long racemes at the end of branches. Flowers have 5 sepals and 5 petals and numerous long stamens protruding out. Fruit is a 5 mm oblong capsule, with a warty surface. It is grown abundantly in the rural areas of Malda, West Bengal. It is sufficiently found in the Coastal east Orissa of India. The plant is well known under vernaculars as 'BanTulasi' in Hindi, 'Banamaricho' in Oriya¹.

The powdered leaves are useful in controlling high blood pressure, and for the treatment of skin diseases and cuts and wounds²⁻⁴. This plant contains main chemical constituents like glycosides saponins, tannins, flavanoids, terpenoids, alkaloids^{5,6}. These antimicrobial activities against bacteria and fungi confirm the presence of broad spectrum antibiotic compounds in the leaves of this species⁷. The literature quercitol reveals that the *C Sparsiflorus Morong* leaves are used as antiseptic and antidote also²⁻⁴ and most of the phytoconstituents were isolated from leaves of *C Sparsiflorus Morong*. Hence, the leaves of this plant have been used for all pharmacological activities. We found limitations of opioid and NSAID therapy and hence it has become necessary to continuing search for new analgesics and there is no scientific proof in traditional treatment of this plant in some painful and inflammatory conditions. Hence, an endeavor has been made to establish the scientific validity to explore the possible antinociceptive, anti-inflammatory and also behavioral study of the crude chloroform extract of *C Sparsiflorus Morong* leaves in animal models.

MATERIALS AND METHODS

Plant Material

Croton Sparsiflorus Morong leaves were collected during the month of September from the rural belt of Chapada village, Jagatsinghpur Dist, Orissa India, identified and authenticated by Prof. S. K. Dash, HOD, PG Department of Bioscience, College of Pharmaceutical Sciences, Mohuda; comparing

with the voucher specimen (CSML-I) present in the herbarium, has been kept in the laboratory for future references. The collected plants were washed and air-dried under the shade,

cut into tiny pieces, powdered by a automatic grinder and passed through 40-mesh sieve and stored in a closed vessel for future use.

Preparation of Croton Sparsiflorus Morong Chloroform Extract

The dried, powdered leaves of *Croton Sparsiflorus Morong* (1 kg) were extracted successively with 1,200 ml of petroleum ether (60 - 80°C) and 1200ml of chloroform in soxhlet apparatus by following standard TAPPI test Method. A dark greenish black coloured petroleum ether extract was obtained. The same powdered leaves (marc), after proper drying, were extracted with chloroform (5 hr) to produce a greenish brown semisolid mass. The extractions were carried out until the solvents became colourless. These extracts were again dried and concentrated by evaporating the solvent completely under vacuum at the range of boiling points of solvent (Chloroform at 62°C) using rotatory evaporator (Jain Scientific glass works, DTC 201, Ambala cantt, India). The chloroform extract (yield 5.60 % w/w with respect to dry powdered plant material) was selected for all experimental procedure. The chemical constituents of the extract was identified by qualitative analysis and established by the thin layer chromatography (i.e. R_f values). CSCE was prepared an emulsion by triturating the accurately weighed quantity of the extract with 0.025% w/v of carboxyl methyl cellulose (CMC) used for the study. All extractive solvents are of analytical grade reagents (AR).

Preparation of Drugs

Tramadol (Contramal, Nicholas Piramal India Limited, Mumbai.) was dissolved in 0.025% w/v of CMC. Diclofenac sodium (Diclomax, Torrent Pharmaceutical Pvt. Ltd., Ahmedabad, India) and carrageenan (Sigma Chemicals Company, St. Louis, MO, USA) were used for the Anti-inflammatory study. The standard drug diazepam (Calmpose, Ranbaxy Lab, India) was

used for behavioral study. CSCE and standard drugs were prepared by suspending them in 0.025% w/v CMC at definite concentrations separately for all pharmacological studies.

Preliminary Phytochemical Analysis

The CSCE was subjected to preliminary phytochemical screening for finding of major chemical groups. In each case test 10% w/v solution of the extract in chloroform was used and unless otherwise mentioned in individual test ⁸.

Experimental Animals

Adult wister albino rats and Swiss albino mice of either sex weighing between 180 - 220 g and 18 - 22 g were used respectively for the experiments, obtained from M/s Ghosh & Ghosh enterprises, Kolkata, India and were housed in standard polypropylene cages at room temperature of 30 ± 2°C and 60 - 65% relative humidity and had free access to food and water *ad libitum*.

The animals were used for the experiment after becoming acclimatization for a period of one week. All procedures described were reviewed and approved by the Institutional Animals Ethical Committee (CPCSEA approval no. 1018/c/06/CPCSEA) of Royal College of pharmacy and Health Sciences, Berhampur, Orissa.

Acute Toxicity Analysis

Toxicity study of the CSCE was carried out to find out, how safe is this extract for the therapeutic use. The LD₅₀ value of CSCE was derived by the method ⁹. The maximum non-lethal dose was found to be 4,000 mg/kg body weight, orally. The 0.025% CMC was used as a vehicle and showed no mortality. The determination of acute toxicity by adopting fixed dose guideline of CPCSEA and 1/10th of LD₅₀ cut off values ^{8,10} of the extracts were taken as screening dose. i.e. 100, 200, 400 mg/kg for subsequent studies.

Antinociceptive Activity

Antinociceptive activity of the CSCE was tested by using the Experimental models of Tail immersion method and Hot plate method. In the tail immersion method, the tail of mouse was immersed to a constant level (5 cm) in a water bath maintained at 55 ± 0.5°C. The time to flick the tail from water (reaction time) was recorded. As per ¹¹, a maximum immersion time of 30 s. is to be maintained to prevent thermal injury to the animals. The reaction time was measured 30 min before test and reference standard. A significant increase in reaction time compared with control was considered a positive analgesic response. The Hot plate test was carried out using an UGO Basile hot plate apparatus (Socrel model D-S37, Italy). The hot plate test was used to measure latency time by the method ¹². As described by ¹³, the temperature of the hot plate was maintained at 55 ± 0.5°C to assess the thermal-induced antinociceptive activity. Animals were placed into Perspex cylinder on the heated surface and the time between placement and licking of the paws or jumping was recorded as response latency. After 16 h. fasted mice were divided into five groups of six mice in each. Group-I, served as a control, received 0.025% w/v CMC, 10 ml/kg, orally, Group-II to IV, animals received CSCE at dose of 100, 200 and 400 mg/kg, orally and Group-V, animals were treated with tramadol (50 mg/kg, orally) as a positive-control. Cut off time for the response was set at 60 s to avoid tissue damage to the mice paws ¹⁴. After the determination of baseline response latencies, hot-plate latencies were re-determined at 30min, 60min, and 90min after oral administration of tested drugs and positive-control in this experiment. The pain inhibition percentage was

calculated ^{15,16} according to the following formula. % of (PIP) = $\frac{\text{Latency (test)} - \text{Latency (control)} \times 100}{\text{Latency (control)}}$.

Anti-Inflammatory Assay

Anti-inflammatory activity was performed by using the carrageenan-induced edema in rat paw according to the technique ^{17,18}. After 16 h, the fasted rats were divided into five groups of six each. Group-I, served as a control, received 0.025% w/v CMC at the dose level of 10 ml/kg, orally, Group-II to IV, animals received CSCE at dose of 100, 200 and 400 mg/kg, orally, Group-V, animals were treated with standard drug diclofenac sodium at the dose level of 10mg/kg, orally. Acute inflammation was induced by carrageenan in sub planter side of the right hind paw in rats. The paw was marked with ink at the level of the lateral malleolus and dipped in Perpex cell up to this mark. The measurement of the paw volume was carried out by means of Ugo Basile Plethysmograph model 7150, before and after 4 h after carrageenan injection ¹⁹.

Percentage inhibition of edema was calculated using formula ²⁰. % Pain Inhibition = $\frac{(1 - V_t/V_c) \times 100}{V_t - V_c}$. Where, V_t = Increase in paw volume in drug treated rats. V_c = Increase in paw volume in control group treated rats.

Behavioral Analysis

Behavioral analysis of the animals was performed by Elevated plus maze method (EPM). The EPM apparatus consisted of two open arms (30 × 5 cm) and two closed arms (30 × 5 × 20 cm) emanating from a common central platform (5 × 5 cm). The two pairs of equal arms were opposite to each other. The entire apparatus was elevated to a height of 50 cm above the floor level. The animals received the treatment as per the schedule. 15 min. before the start of session. At the beginning of the session, a mouse was placed at the centre of the maze, its head facing the closed arm and permitted to explore the maze for 5 min. The time spent in the open arm, percent entries in the open and closed arms and total entries were recorded. An entry was defined as the presence of all four paws in the arm ²¹. The EPM was carefully wiped with 10% ethanol after each trial to eradicate the possible bias due to the odor of the previous animals ²². Group-I, served as control, received 0.025% w/v CMC, 10ml/kg, orally, Group-II to IV, animals received CSCE at dose of 100, 200 and 400 mg/kg and Group-IV, animals were treated with standard drug diazepam at the dose level of 4 mg/kg, orally and the average time spent in both open and closed arm in each group of the mice were recorded.

Statistical Analysis

The results were presented as Mean ± S.E.M. and statistical significance (P < 0.001) between treated and a control group was evaluated by paired t-test ²³.

RESULTS

Preliminary Phytochemical Analysis

Results of different chemical tests on the chloroform extract of *C. Sparsiflorus* showed the presence of phytoconstituents viz., steroids, terpenoids, flavonoids, saponins and tannins.

Antinociceptive Activity

The effects of the CSCE were used to investigate the antinociceptive effects in animal models by adopting two methods of tail immersion test and hot plate test are shown in Tables 1 & 2 respectively. The extract produced about 66 and 73% of PIP in test animals in case of tail immersion method at the dose of 200 and 400 mg/kg after every one-hour intervals. The results were found to be statistically significant

($P < 0.001$) antinociceptive effects and were comparable to the standard drug tramadol, which showed 70% of PIP at the dose of 50 mg/kg ($P < 0.001$). The centrally acting analgesics generally elevate the pain threshold of mice towards heat. CSCE significantly ($P < 0.001$) increased the reaction time of animals towards the thermal source in a dose-dependent manner. In hot plate test CSCE showed a pain inhibition percentage (PIP) of 61.76% and 65.33%, respectively whereas tramadol showed a greater PIP of 68.29% at 90 min after treatment.

Anti-Inflammatory Activity

Indigenous drug systems can be a source of variety of new drugs, which can provide relief in inflammation but their claimed reputation has to be verified on scientific basis. The present investigation revealed that the anti-inflammatory activity of *C. Sparsiflorus* on carrageenan induced paw edema in rats is shown in Table 3. These results indicate that, CSCE showed significant reduction ($P < 0.001$) in edema volume at oral dose of 100, 200 and 400 mg/kg of body weight, which is comparable to the standard drug diclofenac sodium at the dose of 10 mg/kg in acute inflammatory model.

Behavioral Study By EPM

In EPM, the behaviour, which was absorbed that, confirmed the anxiolytic activity of diazepam as reported. The effect of the CSCE on behavioral study by EPM in mice was depicted in Table 4. It administration of diazepam 4 mg/kg, i.p. dose produce significant anxiolytic effect indicated by increase in the open arm entries, time spent in open arm. However, there was no significant anxiolysis effect or impairment in behavioral of the animals observed with CSCE at the dose levels of 100, 200 and 400 mg/kg of body weight when administered orally.

DISCUSSION

In acute toxicity study, oral administration of CSCE did not produce any mortality in mice upto a dose level of 4 g/kg. This may be due to broad non-toxic range of the plant, where the plant extract showed a high LD_{50} and relatively safety. The antinociceptive effect of CSCE was investigated by two well-established assay procedures. The antinociceptive action of all the tested compounds was clearly evident by a dose dependent reduction on tail immersion test and hot plate test are shown in Tables 1 & 2 respectively. These methods for investigating antinociceptive were selected such that centrally mediated effects were investigated. Even though the present day armamentarium is rich in potent analgesic agents, the search for novel and safe analgesic drugs continues and vigorously pursued in many parts of world. The reasons are very obvious; the most potent opiate group of analgesics is associated with many undesirable side effects and also carries a potential for drug addiction. The other prominent groups of analgesics viz. NSAID are notorious for their ulcerogenic²⁴ and nephrotoxic potential²⁵. In this regard, it is interesting to note that many flavonoids isolated from various plants exhibited potent analgesics and anti-inflammatory action²⁶⁻²⁸. It is also believed that those flavonoids ability to influence the said activities occur through modulation of the pro-inflammatory gene expression, such as inducible NO synthase and

cyclooxygenase-2²⁹. Due to these valid reasons, the plant *C. Sparsiflorus* was explored for its antinociceptive and anti-inflammatory activities. The CSCE at the doses of 100, 200 and 400 mg/kg, p.o. tested was shown to possess antinociceptive activity in tail immersion method. It has been assumed that thermally motivated and tonic tests elicit the selective stimulation of A δ and C fibers, respectively³⁰, it is tempting to propose that CSCE or its metabolites may interfere with the transmission of both fibers or with a common pathway, such as spinal and thalamic pathways. The hot-plate test was selected to investigate central analgesic activity, because it had several advantages, particularly the sensitivity to strong analgesics and limited tissue damage. Hence, the hot plate method was employed to verify if the extract could show any central analgesic effect, as the test is specifies analgesic test³¹. It was demonstrated that the CSCE at dose of 100, 200 and 400 mg/kg, p.o. widely used acute inflammatory model for studying anti-inflammatory agent The CSCE were found to be statistically significant ($P < 0.001$) antinociceptive effects and were comparable to the standard drug tramadol at the dose of 50 mg/kg. Edema represents the early phase of inflammation in carrageenan induced paw edema and is the simplest and most widely used acute inflammatory model for studying anti-inflammatory agent. The development of carrageenan-induced edema is believed to be biphasic of which the first phase is mediated by release of histamine, serotonin and kinine in the first hour after injection of carrageenan and the second phase is related to release of prostaglandin like substances in 2 - 3 h.³²⁻³⁴. The CSCE showed significant anti-inflammatory activity at 4 h against carrageenan injection suggesting that the extract predominantly inhibits the release of prostaglandin like substances from phlogenic stimuli. There are reports that flavonoid possesses anti-inflammatory activity²⁶⁻²⁸ and some of them also act as phospholipase inhibitors³⁵⁻³⁶. Also, there are few reports on the experimental models, the non selective antagonist of opiod receptors apparently acts by antagonizing the action of endogenous opiods involved in pain or stress³⁷. In the present study, the maximum anti-inflammatory effect of CSCE may be attributed to presence of flavonoids as evident by preliminary phytochemical investigations. From the results it could be concluded that the extracts exhibit anti-nociceptive activity by central as well as peripheral mechanism(s). The behavioral study of the animals was evaluated by EPM. The EPM test is based on a premise where the exposure to an EPM evoked an approach avoidance conflict that was considerably stronger than that evoked by the exposure to an enclosed arm^{20,38}. The decrease in aversion to the open arm is the result of an anxiolytic effect expressed by the increase time spent and entries in the open arm. Most of the sedatives and hypnotics drugs were implied by the method of EPM. Generally sedatives and hypnotics suppress cerebral activity. They also depress the CNS beginning with the cerebral cortex and descending with increasing dose to the medullary centers causing medullary paralysis³⁹. It was reported that the administration of diazepam 4 mg/kg, i.p. dose produce significant anxiolytic effect⁴⁰ indicated by increase in the open arm entries, time spent in open arm and closed arm. The control group and the

dose levels of 100, 200 and 400 mg/kg of body weight of CSCE was not produce significant anxiolysis effect when compare to standard drug. Finally, it concluded that the CSCE possess remarkable antinociceptive, anti-inflammatory activity but no anxiolytic activity. However, more detailed phytochemical studies are necessary to identify the active principles and exact mechanisms of action.

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Table 1: Antinociceptive Activity of Croton Sparsiflorus Morong By Tail Immersion Method

Group	Dose (mg/kg)	Pretreatment	Post treatment			
			1h	2h	3h	4h
(Group-I) Control (0.025% CMC)	10 ml	1.6 ± 1.06	1.6 ± 1.11	1.6 ± 1.11	1.6 ± 1.11	1.6 ± 1.11
(Group-II) CSCE	100mg / kg	1.6 ± 1.51	1.69 ± 1.01 (5.32%)	1.82 ± 1.0 (12.08%)	1.84 ± 1.0 (13.04%)	4.18 ± 1.02 (61.72%)
(Group-III) CSCE	200 mg / kg	1.8 ± 0.7	4.7 ± 0.9 ^a (65.96%)	6.3 ± 1.5 ^a (74.60%)	6.4 ± 1.21 ^a (75.00%)	5.2 ± 1.12 ^a (69.23%)
(Group-IV) CSCE	400 MG / KG	1.9 ± 1.00	6.1 ± 1.05 (73.77%)	6.3 ± 1.12 ^a (74.60%)	6.2 ± 1.1 ^a (74.19%)	6.9 ± 1.21 ^a (76.81%)
(Group-V) Tramadol + control (Positive control)	50 mg / kg	1.8 ± 1.1	5.3 ± 1.12 (69.81%)	10.7 ± 1.0 (85.04%)	12.0 ± 1.0 (86.66%)	14.0 ± 1.01 (88.57%)

Results expressed as mean ± S.E.M. ^aP < 0.001 significantly different from control ; Paired t - test (n = 6).
Figures in the parentheses indicate % of (PIP) in mice.

Table 2 : Antinociceptive Activity of Croton Sparsiflorus Morong Leaves By Hot Plate Method

Treatment	Dose (mg/kg)	Paw ticking time in seconds				Paw jumping time in seconds			
		0	30	60	90	0	30	60	90
(Group-I) control (0.025% CMC)	10 ml	3.0 ± 0.1	2.8 ± 0.3	2.7 ± 0.2	2.9 ± 0.1	3.0 ± 1.0	2.8 ± 0.8	2.8 ± 0.6	2.6 ± 0.4
(Group-II) CSCE	100 mg / kg	3.6 ± 0.30	3.56 ± 0.2 ^b (21.35%)	4.5 ± 0.1 ^b (40.00%)	5.0 ± 0.12 ^b (42.00%)	3.5 ± 0.3	6.2 ± 0.1 ^b (54.84%)	6.5 ± 0.2 ^b (56.92%)	6.7 ± 0.4 ^b (61.19%)
(Group-III) CSCE	200 mg/kg	3.6 ± 0.12	4.8 ± 0.15 (41.67%)	4.55 ± 0.1 ^b (40.66%)	5.6 ± 0.12 ^b (48.21%)	3.7 ± 0.3	6.6 ± 0.4 ^b (57.58%)	6.7 ± 0.5 ^b (58.21%)	6.8 ± 0.6 ^a (61.76%)
(Group-IV) CSCE	400 mg/kg	3.9 ± 0.12	4.5 ± 0.2 (37.78%)	4.7 ± 0.8 (42.55%)	5.1 ± 0.5 ^a (43.14%)	4.0 ± 0.6	5.8 ± 0.7 (51.72%)	5.9 ± 0.12 (52.54%)	7.5 ± 0.5 ^a (65.33%)
(Group-I) control + Tramadol	50 mg/kg	3.4 ± 0.2	4.6 ± 0.3 (39.13%)	5.5 ± 0.12 ^a (50.91%)	6.02 ± 0.15 ^a (51.83%)	3.5 ± 0.1	6.56 ± 0.3 ^a (57.32%)	7.5 ± 0.8 ^a (62.67%)	8.2 ± 0.3 ^a (68.29%)

Results expressed as mean ± S.E.M. ^bP < 0.05 , ^aP < 0.001 significantly different from control ; Paired t - test (n = 6).
Figures in the parentheses indicate % of (PIP) in mice.

Table 3 : Anti-Inflammatory Activity of Croton Sparsiflorus Morong Leaves on Carrageenan Induced Paw Edema in Albino Rats

Treatment	Dose (mg/kg)	Percentage of inhibition of paw edema after carrageen injection			
		1h	2h	3h	4h
(Group-I) Control (0.025% CMC)	10mg / kg	13.16 ± 1.98	35.31 ± 4.04	41.23 ± 4.06	41.04 ± 5.14
(Group-II) CSCE	100mg / kg	30.94 ± 2.78 ^a	70.85 ± 9.61 ^a	100.74 ± 8.69 ^b	110.59 ± 10.38 ^b
(Group-III) CSCE	200 mg / kg	24.70 ± 4.20 ^a	46.30 ± 1.01 ^a	70.62 ± 7.79 ^a	80.04 ± 4.56 ^a
(Group-IV) CSCE	400 mg / kg	19.69 ± 0.706 ^a	40.04 ± 0.70 ^a	62.90 ± 7.606 ^a	69.78 ± 4.25 ^a
(Group-V) Diclofenac sodium	10 mg / kg	44.31 ± 2.45 ^a	99.79 ± 3.63 ^a	130.00 ± 2.53 ^a	134.72 ± 5.35 ^a

Results expressed as mean ± S.E.M. ^bP < 0.05 , ^aP < 0.001 significantly different from control ; Paired t - test (n = 6).

Table 4 : Behavioral Study of Croton Sparsiflorus Morong Leaves By Elevated Plus Maze (EPM) in Mice

Treatment	Dose (mg/kg)	Number of arm entry		Time spend in arms(seconds)	
		open	closed	open	closed
(Group-I) Control (0.025% CMC)	10ml	2.1 ± 2.1	4.4 ± 2.4	48.44 ± 1.99	251.61 ± 1.91
(Group-II) CSCE	100mg / kg	1.0 ± 1.15	1.38 ± 1.98	24.0 ± 2.14	228.0 ± 1.98
(Group-III) CSCE	200 mg / kg	1.7 ± 2.0	2.2 ± 2.18	24.0 ± 2.28	275.0 ± 2.02
(Group-IV) CSCE	400 mg / kg	1.6 ± 2.18	2.0 ± 2.17	27.4 ± 2.03	274.7 ± 2.12
(Group-V) Control + Diazepam	4 mg / kg i.p.	5.8 ± 2.14	1.2 ± 2.01	268.0 ± 1.89	31.0 ± 1.97

Administration of the diazepam 4mg / kg , i.p. dose produce significant anxiolytic effect compared to that of the control group.
However, there was no significant anxiolysis effect in CSCE when administered orally.

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