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THE ANTINOCICEPTIVE ACTIVITY AND POSSIBLE MECHANISMS OF CHLOROFORM EXTRACT FROM *RICINUS COMMUNIS* LINN. LEAVES IN MICE

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ABSTRACT

The leaves of Ricinus communis has been used as a folk medicine for the treatment of liver disorders and inflammations in India. Aim of the present study was to investigate the antinociceptive activity and possible mechanism of chloroform extract of the leaves of *Ricinus communis* L. (RCLC) in mice using chemical and thermal methods of nociception. The results revealed the presence of alkaloids, terpenoids, flavonoids, fatty acids, coumarins, glycosides. Acute toxicity studies showed RCLC extract was found to be safe up to 2000 mg/kg dose with no sign of allergic reactions and mortality. The antinociceptive potential of RCLC extract was found dose-dependent and peak effect observed at 100 mg/kg, i.p. ($p< 0.001$, one way ANOVA followed by Tukey-Kramer as *posthoc* test) as compared to control in all animal models. The antinociceptive effect of RCLC extract in the formalin and hot plate test was significantly ($p< 0.001$) attenuated by pretreatment with either naloxone. It may be concluded from the results RCLC extract exhibited dose-dependent antinociceptive activity modulated via peripheral as well as central opioid receptors.

Key words: *Ricinus communis*; Antinociception; Opioid receptors.

INTRODUCTION

Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage or is described in terms of such damage. Pain is classified in several ways, but pain is primarily differentiated into acute and chronic pain (Ozkay UD and Can OD, 2013). It is most common motivating factor to seek medical attention. Although adequate pain relief is achieved with the currently available analgesic agents like opioids or NASAIDs, some of their serious side effects are major limitations to their routine use in therapy (Vidyalakshmi K *et al*., 2010). As alternatives to these drugs, a number of plants and plant-derived compounds that are well tolerated are being sought as new medicines for pain (Olonode ET *et al*., 2015).

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Ricinus communis L. (RC) i.e. castor oil plant, documented as traditional contraceptive, belongs to Euphorbiaceae family (Ross IA, 2001). It is terrestrial, flowering, robust perennial shrub with cosmopolitan distribution. Phytosterols, proteins, fatty acids, coumarins, phenolic compound (Williamson EM, 2002), flavonoids (Byamukama R *et al*., 2008), alkaloids (Kang SS *et al.*, 1985), terpenoid and tocopherol-related compounds (Tan QG *et al.*, 2009) have already been isolated from different parts of this plant. In India RC is traditionally used in inflammations and liver disorder (Kirtikar KR and Basu BA, 1991). The plant has been reported to be anti-inflammatory, analgesic (Darmanin S *et al*., 2009) anticancer (Shokeen P *et al*., 2008), antidiabetic, contraceptive (Nath S *et al*., 2013), antioxidant (Gupta MK *et al.*, 2006; Singh PP *et al*., 2009) and hepatoprotective (Prince ES *et al.*, 2011).

Previously some studies have established the analgesic potential of crude extracts of RC roots and leaves. In the present study we have investigated involvement of opioid receptors, in antinociceptive action of chloroform extract of *Ricinus communis* (RCLC) using chemical and thermal stimuli of nociception in mice.

MATERIAL AND METHODS

Plant material and extraction

For the present study, the leaves of RC were collected from the different localities of Sangamner, Ahmednagar, India (coordinates 19.5700° N, 74.2200° E), in the month of January and February 2013 and identified by Dr. K. J. Salunke from the Department of Botany, Padmashri Vikhe Patil College of Arts, Science and Commerce, Pravaranagar, India. A voucher specimen (V. No. HD-401) was deposited at the herbarium of this department.

The leaves of RC were shade dried (1.0 Kg), grinded to coarse powder and subjected to Soxhlet extraction with chloroform for 48 h. The extract was concentrated under reduced pressure using rotary evaporator (Heidolph Labrota 4000 Efficient, Germany). The RCLC extract obtained was dark brown colour and the percentage yield was 8.93 % (w/w).

Animals

Swiss albino mice weighing 20-25 g of either sex were used for the tests. The animals were housed in room with controlled temperature (25 ±1 °C), humidity (45–65%) and light (12 h light/12 h dark cycle, light on at 07:00 a.m.) and fed with rodent pellet diet (Nutivet Life Sciences, Pune) and water *ad libitum*. The animals were acclimatized to laboratory environment for at least 48 h before the experiments. The experimental protocols were approved by the Institutional Animal Ethical Committee, Amrutvahini College of Pharmacy, Sangamner, India (CPCSEA/AVCOP/01/2014).

Drugs and treatments

The solvents used in this study were of analytical grade (Merck, India), the chemicals acetylsalicylic acid (Ranbaxy Laboratories Ltd, Baddi, Solan), formalin, acetic acid, (Merck, India), morphine sulphate, naloxone hydrochloride, (Sigma Chemicals Co., St. Louis, USA) were used. Normal saline containing 0.5 % Tween 80 was used as a control in all studies and the RCLC extract (25, 50 and 100 mg/kg) used in studies was prepared in normal saline containing 0.5 % Tween 80. Drugs were freshly prepared on the day of each experiment and administered intraperitoneally (i.p.) in a volume of 10 mL/kg body weight.

Acute toxicity study

The test was performed (Bruce RD, 1985) with slight modifications. Swiss albino mice were divided into desired groups containing five animals in each. The RCLC extract was administered to the animals orally at the doses of 500, 1000 and 2000 mg/kg. The animals were then allowed to take food and water *ad libitum* and observed for next 72 h to check any abnormal behaviors, allergic symptoms and mortality induced by RCLC. During the observation period, animals behaviors like scratching and rubbing around the nose and head, irritability or aggression, hypersensitivity to touch and cyanosis around mouth and tail and puffiness around the eyes and mouth were considered as the indications of allergic symptoms.

Phytochemical screening

Phytochemical screening was carried out on the RCLC extract using chemical tests to detect the presence of chemical constituents as detailed in the literature (Evans WC, 2002).

Antinociceptive activity

Acetic acid-induced abdominal writhing

Acetic acid-induced abdominal writhing was carried out according to the previously described method [19]. The RCLC extract (25, 50 and 100 mg/kg, i.p.), morphine (5 mg/kg, i.p.), acetyl salicylic acid (200 mg/kg, i.p.) or vehicle (0.5 % Tween 80 in saline, 10 mL/kg, i.p.) were administered to the mice 30 min before i.p. injection of acetic acid (0.9% in saline solution, 10 mL/kg). The writhing response consists of contraction of the abdominal muscle together with a stretching of the hind limbs. The number of writhes was counted for 15 min.

Formalin-induced paw licking

The method employed was similar to that described previously (Rabelo AS *et al.*, 2013; Venancio AM *et al.*, 2011) with slight modifications. Briefly, twenty microlitres of 1% formalin was injected subcutaneously into the right hind paw of mice. The time (in seconds) spent in licking and biting responses of the injected paw was taken as an indicator of nociception response. The responses were measured for 5 min (first phase) and 15-30 min (second phase) after the injection of formalin. RCLC extract (25, 50 and 100 mg/kg, i.p.), morphine (5 mg/kg, i.p.) or vehicle (0.5 % Tween 80 in saline, 10 mL/kg, i.p.) were administered 30 min before the injection of formalin. Naloxone (5 mg/kg, i.p.) was administered 15 min prior to the administration of RCLC (100 mg/kg) and morphine (5 mg/kg. i.p.).

Hot-plate test

The hot plate test measured response latencies according to the method described by (Pires JM *et al.*, 2009) with slight modifications. Briefly, the animals were placed on hot-plate maintained at 55±1°C. The time between the placement of the animal on the hot-plate and

the occurrence of either the licking of the hind paws, shaking or jump off from the surface was recorded as response latency. Mice with base line latencies of more than 10 seconds were eliminated from the study 24 h previously. The cut-off time for the hot plate latencies was set at 30s. Animals were treated intraperitoneally with RCLC extract (25, 50 and 100 mg/kg, i.p.), morphine (5 mg/kg, i.p.) or vehicle (0.5 % Tween 80 in saline, 10 mL/kg, i.p.) was administered 30 min before the experiments. Naloxone (5 mg/kg, i.p.) was administered 15 min prior to administration of RCLC extract (100 mg/kg) and morphine (5 mg/kg). The latency time of animals were observed at 0, 30, 60, 90 and 120 min.

Statistical analysis

The obtained data were analyzed using InStat Graph Pad Prism software program Version 6.0 and expressed as mean±SEM. The statistical differences between the groups were calculated by the application of an analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test *P*-values less than 0.05 ($P< 0.05$) were considered as the significance level.

RESULTS

Phytochemical screening revealed the presence of alkaloids, flavonoids, glycosides, tannins, triterpenes and fatty acid.

Acute toxicity study

No visible adverse reaction, obvious behavioral changes and mortality were observed up to 72 h when the mice were treated orally using dose of RCLC 500-2000 mg/kg body weight (data is not shown).

Effect of the crude extract of RC on acetic acid induced writhing in mice

The extract RCLC (25-100 mg/kg) produced a significant dose-dependent reduction in the number of writhes with highest effect at a dose of 100 mg/kg ($p<0.01$, q= 26.21, 72.77 % inhibition). The effect of RCLC was comparable with acetylsalicylic acid (200 mg/kg; $p<0.01$, q= 29.45, 81.74 % inhibition) and significantly different from control group. The reference drug morphine (5 mg/kg) showed significant reduction in number of writhes ($p<0.01$, q= 32.80, 91.03 % inhibition) as shown in Table 1.

Effect of crude extract of RC on formalin paw-licking in mice

The formalin test which persists for several minutes, and from which the animal cannot escape, represents a nociceptive stimulus of tonic and moderate character. In the first phase, injections of formalin into the sub-plantar tissue of the right hind paw of control mice. The extract RCLC (25, 50 and 100 mg/kg) produced a significant dose-dependent inhibition of nociceptive reaction with peak effect at dose 100 mg/kg ($p<0.001$, q= 11.32, 51.79 % inhibition). The effect morphine in first phase was significantly different ($p<0.001$, q= 19.17, 87.76 % inhibition). In the second phase (inflammatory phase) the extract RCLC showed dose dependant reduction in paw licking response, with the peak effect (72.34 % inhibition) produced at 100 mg/kg ($p<0.001$, q= 20.02) as compared to control group. Morphine produced a significant inhibition of 86.38 % which was comparable with the extract at 100 mg/kg ($p<0.001$, q= 7.85). Pretreatment with naloxone (5 mg/kg, i.p) 15 min prior to administration of morphine (5 mg/kg) or RCLC (100 mg/kg) significantly ($p<0.001$) reversed the antinociceptive effects in both phases (as shown in Table 2).

Effect of crude extract of RC on the hot plate test in mice

The extract RCLC (25-100 mg/kg, i.p.) increased latency reaction in hot plate. All the doses administered dose-dependently increased the latency time in hot plate when compared to control. The significant effect was found at 100 mg/kg ($p<0.001$, q= 6.63) as compared to control. Pretreatment with naloxone (5 mg/kg) reversed the analgesia induced by the RCLC extract at 100 mg/kg significantly at all time point (as shown in Table 3).

Table 1. Effect of chloroform extract of *Ricinus communis* on the acetic acid-induced abdominal writhing in mice

Treatment	Number of writhes	Percent inhibition
Control (0.5 % Tween 80 in saline, 10 mL/kg, i.p.)	50.17 ± 1.47	-
RCLC (25 mg/kg, i.p.)	42.50 ± 1.60*	15.28
RCLC (50 mg/kg, i.p.)	29.83 ± 1.62**	40.54
RCLC (100 mg/kg, i.p.)	13.66 ± 1.54**	72.77
Morp (5 mg/kg, i.p.)	4.50 ± 0.76**	91.03
ASA (200 mg/kg, i.p.)	9.16± 1.13**	81.74

Results are expressed as mean ±SEM. *$p < 0.01$ and **$p < 0.01$ as compared to control group (One-way ANOVA followed by Tukey-Kramer multiple comparison as the *post hoc* test, n=6).RCLC= chloroform extract of *Ricinus communis*, Morp= Morphine, ASA= Acetyl salicylic acid.

Table 2. Effect of chloroform extract of *Ricinus communis* on the formalin induced paw licking in mice.

Treatment	Licking time (in seconds)		Percentage inhibition	
	1st phase	2nd phase	1st phase	2nd phase
Control (0.5 % Tween 80 in saline, 10 mL/kg, i.p.)	55.6 ± 2.87	70.5±3.59	-	-
RCLC (25 mg/kg, i.p.)	45.5± 2.46#	51.3± 3.52*#	18.16	27.23
RCLC (50 mg/kg, i.p.)	36.3± 2.99*#	43.5± 2.81*#	34.71	38.29
RCLC (100 mg/kg, i.p.)	26.8± 2.07*#	19.5± 1.78*	51.79	72.34
Morp (5 mg/kg, i.p.)	6.8± 0.94*	9.6± 1.58*	87.76	86.38
Nal (5 mg/kg, i.p) + Morp (5 mg/kg, i.p.)	52.3± 2.77#	59.5± 2.32#	5.93	15.60
Nal (5 mg/kg, i.p.) + RCLC (100 mg/kg, i.p.)	51.8± 2.3#	44.1± 2.22*#	6.83	37.44

Results are expressed as mean ±SEM. *$p < 0.001$ as compared to control group; and #$p < 0.001$ as compared to Morp group (One-way ANOVA followed by Tukey-Kramer multiple comparison as the *post hoc* test, n=6). RCLC= chloroform extract of *Ricinus communis*, Morp= Morphine, Nal= Naloxone.

Table 3. Effect of chloroform extract of *Ricinus communis* on the hot-plate test in mice

Treatment	Latency time (in seconds)				
	0 min	30 min	60 min	90 min	120 min
Control (0.5 % Tween 80 in saline, 10 mL/kg, i.p.)	5.93±0.38	6.19 ± 0.46	5.90 ± 0.45	5.63 ± 0.62	6.10 ± 0.64
RCLC (25 mg/kg, i.p.)	5.76±0.36	6.98 ± 0.24#	7.75 ± 0.44#	8.02 ± 0.45#	7.67 ± 0.50#
RCLC (50 mg/kg, i.p.)	5.30±0.49	7.69 ± 0.31#	9.23 ± 0.31#	9.50 ± 0.48*#	8.64 ± 0.46#
RCLC (100 mg/kg, i.p.)	5.82±0.61	9.24 ± 0.71*#	10.72 ±0.60**#	10.89±1.03***#	9.13 ± 0.82#
Morp (5 mg/kg, i.p.)	5.41±0.43	17.27±0.83***	19.62±1.42***	19.76 ±1.16***	18.11±0.76***
Nal (5 mg/kg, i.p) + Morp (5 mg/kg, i.p.)	6.47±0.78	7.03± 0.63#	6.94 ± 0.73#	6.68 ± 0.69#	7.07 ± 1.28#
Nal (5 mg/kg, i.p.) + RCLC (100 mg/kg, i.p.)	5.97±0.85	7.04 ± 0.82#	7.75 ± 1.13#	7.19 ± 0.83#	7.11 ± 0.73#

Results are expressed as mean ±SEM. *P<0.05, **p<0.01 and ***p<0.001 as compared with control group; #p<0.001 as compared to morphine group (One-way ANOVA followed by Tukey-Kramer multiple comparison as the *post hoc* test, n=6). RCLC= chloroform extract of *Ricinus communis*, Morp= Morphine, Nal= Naloxone.

DISCUSSION

Plants show diverse biological actions because of the existence of various types of phytoconstituents. It is compelling evidence that many chronic diseases such as inflammatory disorders; rheumatism, diabetes, cardiovascular and many neurodegenerative disorders are treated by the plants or the active principles in traditional medicine that can be a new therapeutic source for the treatment of chronic disorders.

Acute toxicity studies are usually carried out to determine the dose that will cause death or serious toxic manifestations when administered singly or severally at few doses in order to establish doses that should be used in subsequent studies (Wanda MH, 2002). The result obtained suggests that the plant extract is non-toxic as no mortality was recorded and in accordance with Pingale.

The present study was intended to broaden our understanding of the strength of RCLC extract in alleviating pain and to reveal the mechanisms underlying the antinociceptive potential. We determined nociception in three different animal models including chemical nociception in acetic acid induced abdominal writhing, formalin induced paw licking and thermal nociceptive threshold on the hot plate in order to evaluate analgesic effect of RCLC extract in mice.

The visceral pain model is frequently used as a screening tool for the evaluation of anti-nociceptive properties of new agents (De Souza MM *et al*., 2009). An i.p. injection of acetic acid, as a chemical noxious agent, causes a response characterized by contraction of the abdominal muscles accompanying an extension of the hind limbs and elongation of the body (Park SH *et al.*, 2012). The i.p. irritation triggers the release of several mediators such as neurotransmitters and neuromodulators, kinins, histamine, acetylcholine, substance P, and prostaglandins. These mediators increase vascular permeability, reduce the threshold of the nociception, and stimulate the nociceptive neurons sensitive to NSAIDs and/or opioids (García MD *et al.*, 2004; Nguemfo EL *et al.*, 2007). In this study, RCLC significantly decreased the number of abdominal constrictions induced by acetic acid injections and protected the animals from writhing (Table 1).

In the formalin test, the subcutaneous administration of formalin induced a pain-related behavior of licking and biting of the injected paw in two

distinct phases. As we expected the early phase of the nociceptive response peaked 0 to 5 min after formalin injection and the late phase 15 to 30 min after formalin injection, representing the neurogenic and inflammatory pain responses, respectively. The activity of analgesics is different in the neurogenic and inflammatory phases. Centrally acting drugs (e.g. opioids) inhibit both phases in similar doses, whereas non-opiate analgesic which has a central and peripheral site of action (e.g. NSAIDs) produces an antinociceptive effect in both phases of the formalin test, but particularly pronounced in the second phase, where pain is inhibited by lower doses than those necessary to inhibit pain in the first phase (Tjølsen A *et al.,* 1992; Shibata M *et al.,* 1989). This general truth was also observed in our study for morphine. The results of RCLC extract (100 mg/kg) have revealed that, it was effective in both phases of the formalin test, with a greater potency in the inflammatory phase, which indicated that these compounds are similar to non-opiate drugs (Mogilski S *et al.,* 2015).

To evaluate the possible central antinociceptive effects of RCLC extract, the hot-plate test was adopted. RCLC at doses of 25, 50 and 100 mg/kg significantly increased latencies in the hot-plate model compared with the control group, and these doses produced more intense effects on pain relief. These results indicate that RCLC might exert its influence on pain relief through the central nervous system. The hot-plate test was used to evaluate central pain at the supraspinal and spinal levels [31-33] in which C-, Aδ type I-, and Aδ type II-sensitive fibers play a role in this model (Pietrovski EF *et al.,* 2006).

Opioid receptors are widely distributed throughout the central and peripheral nervous system, and these receptors play an important role in pain (Lopes LS *et al.,* 2009; Quock RM *et al.,* 1999). Therefore, we performed study using opioid receptor antagonist naloxone to clarify the opioid receptors that are involved in the observed activity. Antinociceptive effect of morphine and RCLC was reversed by pretreatment of naloxone (5 mg/kg, i.p.) in the formalin and hot-plate test.

Overall, the results suggested participation of peripheral as well as central opioid receptors in the antinociceptive activity of RCLC extract. Various flavonoids, both glycosides and aglycones were previously reported having potent anti-inflammatory and antinociceptive activity (Kupeli E, Yesilad E *et al.,* 2007; Kaur R *et al.,* 2005; Thirugnanasambantham P *et al.,* 1985; Muthiah NS *et al.,* 1983) in other plant species.

CONCLUSION

In conclusion, the present study demonstrated the dose-dependent analgesic activity of RCLC extract in the animal models of chemical nociception induced by acetic acid, formalin and in nociception induced by thermal stimuli, and further suggested that analgesic activity of RCLC extract might be related to peripheral as well as central involvement of opioid receptors, which merited further studies regarding the precise site and the mechanism of action.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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