

## Anti-nociceptive and Anti-inflammatory Activities of *Crotalaria pallida* Aiton (Fam: Fabaceae) Leaves

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**Abstract:** The present study was designed to evaluate the anti-nociceptive and anti-inflammatory properties of the ethanolic extract of *Crotalaria pallida* Aiton (Family: Fabaceae) leaves based on its traditional use in the treatment of urinary disorders, external application as a poultice to treat painful swelling of joints and to reduce fever. The anti-nociceptive effect was assessed in mice using acetic acid-induced writhing, formalin-induced paw licking and tail immersion assays. Anti-inflammatory activity was evaluated by carrageenan induced paw oedema in rats. In acetic acid-induced writhing test, the extract at different doses (50 mg/kg and 100 mg/kg bw) significantly ( $p < 0.001$ ) and dose-dependently reduced pain by 37.50% and 47.70%, respectively. The extract also significantly inhibited formalin-induced paw licking in mice at both the early and late phases. In the tail immersion test, the extract caused a significant inhibition of pain (64.91% inhibition after 4 hrs) at a dose of 100 mg/kg. *C. pallida* exhibited a considerable inhibition of paw oedema development in the carrageenan-induced oedema test. The findings of the study suggested that *C. pallida* leaves have significant anti-nociceptive and anti-inflammatory effects, confirming the traditional use of this plant in the treatment of various diseases associated with pain and inflammation.

**Key words:** *Crotalaria pallida*, Fabaceae, anti-nociceptive, anti-inflammatory

### Introduction

Hyperalgesia and inflammation are associated with several pathological conditions. Most of synthetic analgesic drugs such as NSAIDs, COX-2 inhibitors and opioids exhibit an extensive range of adverse effects including gastrointestinal disorders, kidney problems, cardiovascular risks (Antman *et al.*, 2007) and opioids show addiction and misuse (Boldrin *et al.*, 2013). Therefore, advent for safe and effective analgesic drugs now-a-days researchers shift into natural products as an alternative.

*Crotalaria pallida* Aiton (Family: Fabaceae), known as Kudug Jhunjhuni (Chakma), Fasygaas (Tanchung), Tha Sim Noi, Rati Aapa (Marma) and Roa Bay (Murang), is frequently distributed and extensively used traditionally by tribal people,

popularly known as "rattle or rattlesnake" due to the sound of its fruits when dry. *Crotalaria* is one of the largest genera in tropical areas. The plant *C. pallida* is an annual erect herb, approximately 1.50 m in height, which grows widely in tropical and subtropical regions of Bangladesh and India. In traditional medicine, *C. pallida* is used to treat urinary problems. A poultice made of the roots is applied to the painful swelling of joints and an extract of the leaves is taken as vermifuge. In Laos, the plant is used to reduce fever. Root extract is taken to cure stomachache and indigestion.

Alkaloids of the plant have an anti-tumouraction against Walker 256 and Sarcoma 180 rat tumours (Neichi *et al.*, 1992). The anti-inflammatory activities of *C. pallida* were evaluated by inhibiting the heat-

induced albumin denaturation and red blood cell membrane stabilisation (Gepdiremen *et al.*, 2005; Govindappa *et al.*, 2011). It also has potent antioxidant (Adedapo *et al.*, 2008; Adesegun *et al.*, 2009; Lai *et al.*, 2009; Zhang and Lin, 2008; Govindappa *et al.*, 2011) and antimicrobial activities (Adedapo *et al.*, 2008; Adesegun *et al.*, 2009; Lai *et al.*, 2009; Kaur and Arora, 2009; Erdemoglu *et al.*, 2007; Govindappa *et al.*, 2011; Umashankar *et al.*, 2014; Singh and Singh, 2003). It contains tannin, flavonoids, terpenoids, phenol and saponins from ethanol extracts (Govindappa *et al.*, 2011; Singh and Singh, 2003). Phytochemical screening of the plant has revealed phenol and alkaloids (Adedapo *et al.*, 2008). Barks of *C. pallida* contain anti-inflammatory constituents like pterocarpanoids, crotafurans (Weng *et al.*, 2003). Seeds contain alkaloids, mucronatine, usaramine, nilgirine, mucronatinine and crotastratine (Neichi *et al.*, 1992). Luteolin, vitexin, its O-xyloside and chrysoeriol-7-rutinoside have also been isolated from seeds. Vitexin, vitexin-4'-O-xyloside and apigenin have been isolated from leaves and stem bark (Nogala-Kalucka *et al.*, 2010). It significantly inhibited the proliferation of HeLa cells in a concentration and time-dependent manner (Umashankar *et al.*, 2015). From other reports of *Crotalaria juncea* L. shows anti-inflammatory and anti-ulcerogenic effect on animal model (Ashok *et al.*, 2006). In view of this evidence from the existing articles *C. pallida* may be used as antioxidant, antimicrobial, anti-inflammatory. There is some evidence of *in vitro* anti-inflammatory tests but no reports on the *in vivo* anti-nociceptive and anti-inflammatory activities of *C. pallida*. Thus, the present study was aimed to investigate the anti-nociceptive and anti-inflammatory activities of ethanolic extracts of *C. pallida* on animal models.

## Materials and Methods

**Plant material:** The leaves of *C. pallida* were collected in September, 2015 from Sylhet hill track, Bangladesh. The plant was identified by Bangladesh National Herbarium, Dhaka, where a voucher

specimen has been deposited (DACB Accession No. 42023). Plants were then washed properly to remove dirty materials and shade-dried for several days with occasional sun drying. These were then dried in an oven for 24 hours at considerably low temperature for better grinding. The dried plants were ground into a coarse powder and preserved in an airtight container against the re-absorption of moisture, oxidation, excessive heat or humidity, growth of moulds and bacteria, and infestation by insects and rodents.

**Extract preparation:** About 500 g of powdered plant was taken in a flat bottom glass container and soaked in ethanol. The container with its content was sealed with aluminium foil and kept at room temperature for a period of 7 days with occasional shaking and stirring. The extract was filtered through a fresh cotton plug followed by Whatmann No.1 filter paper (Bibby RE-200, Sterilin Ltd., and UK). The filtrate obtained was evaporated by rotary evaporator (RE309, Stuart, Japan) at 50 to 60 rpm and at 50°C temperature. It rendered a gummy concentrate of dark brown colour. This gummy concentrate was designated as ethanolic extract of *C. Pallida*.

**Experimental animals:** Swiss Albino mice (25-30 g) and Wister male rat (150-200 g) obtained from the Animal Resource Branch of the International Centre for Diarrhoeal Diseases and Research, Bangladesh (ICDDR, B) were used for the experiment. They were kept in standard environmental conditions (relative humidity 55-65%, relative temperature 23.0±2.0°C and 12 h light: dark cycle) and fed ICDDR; B formulated rodent food and water.

**Drugs and chemical:** Diclofenac Na (MERCK, Mumbai, India), indomethacin (BASF Aktiengesellschaft, Germany), formaldehyde (MERCK, Mumbai, India), carrageenan (Hi Media Laboratories Pvt. Ltd., Mumbai, India) and 0.9% NaCl saline solution (Popular Pharmaceuticals Ltd., Bangladesh) were used. All other reagents were of analytical grade.

**Acute oral toxicity test:** An acute oral toxicity test was carried out in accordance with the

Organisation for Economic Cooperation and Development (OECD) guidelines for testing of chemicals (Ramabadran *et al.*, 1989). Swiss albino mice maintained under standard laboratory conditions being used for the acute toxicity study. A total of three mice from each group received a single oral dose (500, 1000 and 2000 mg/kg bw) of the extract. Animals were fasted overnight prior to administration. After administration of the extracts, food was withheld for a further 3 to 4 hrs. The animals were then individually observed (with special attention during the first 4 h) for possible behavioural changes, allergic reactions (skin rash, itching), eyes and mucous membrane, and mortality for the next 72 hrs.

#### Anti-nociceptive activity

**Acetic acid-induced writhing test:** For the writhing test, the animals were divided into four groups including control (Group I), positive control (Group II) and two test groups (Group III and IV). The control group was treated with 1% tween 80 in saline water at the dose of 10 ml/kg p.o. and the positive control group received indomethacin (standard drug) at the dose of 10 mg/kg orally. The animals of groups III and group IV were treated with plant extracts at two different doses of 50 mg/kg and 100 mg/kg b.w. orally. Thirty minutes after administration of vehicle, standard drug and test samples 0.7% acetic acid is injected intraperitoneally (i.p.) at a dose of 10 ml/kg bw and the intensity of analgesic behaviour was quantified by counting the total number of writhes over a period of 30 minutes. The percentage analgesic activity was calculated as follows:

$$\text{Percentage analgesic activity} = [(N_c - N_t)/N_c] \times 100\%$$

Where  $N_c$  is the average number of writhes of the control group and  $N_t$  is the average number of writhes of the test/positive control group.

**Formalin-induced paw licking test:** The formalin-induced paw licking method was performed according to Gharate and Kasture (2013). For this method, animals were kept in four groups, with 4

mice in each, and were treated in the following manner: group I received vehicle (isotonic saline solution, 0.9%), group II received indomethacin as standard drug at the dose of 10 mg/kg bw and group III and IV received ethanolic extract of *C. pallida* (50 and 100 mg/kg of bw). One hour after the oral administration of vehicle, standard drug and test samples mice received 20  $\mu$ l of 2% formalin in sub plantar region of hind paw and the number of paw licking events was measured in each mouse from 0-5 min and 20-30 min. The number of paw licks in the first 5 min indicates response to neurogenic pain and the number of paw licks in 20-30 min indicates inflammatory pain.

**Tail immersion method:** The tail immersion test was performed to evaluate analgesic activity by Mali *et al.*, (2013). In this test, mice were divided into four groups of 4 mice, each treated orally with vehicle (isotonic saline solution, 0.9%), diclofenac sodium as standard drug (10 mg/kg) and ethanolic extract of *C. pallida* (50 and 100 mg/kg of bw). One hour after administration of the vehicle, standard drug and test samples the tip of the tail was immersed up to 5 cm in hot water maintained at 55°C. Sudden withdrawal of the tail from the hot water was taken as the reaction time. To avoid damage to the tail, a cut-off time of 20 s was maintained. The reaction time was measured at 0, 1, 2, 3, 4 and 5 h.

#### Anti-inflammatory activity

**Carrageenan-induced rat paw oedema test:** The method described by Mali *et al.*, (2013) was used to study acute inflammation. In this experiment, rats were divided into four groups of four mice and were treated orally with vehicle 1 ml/kg (Group I), diclofenac sodium 10 mg/kg (Group II) and ethanolic extract of *C. pallida* (50 and 100 mg/kg of b.w. for Groups III and IV, respectively). One hour after administration of the vehicle, standard drug and plant extract 0.1 ml of 1% w/v of carrageenan suspension in 0.9% normal saline was injected into the sub planter region of the left hind paw of the rat. The paw volume was determined with a micrometre screw gauge at 1, 2, 3, 4 and 5 h after administration of the

drug and the extract. The percentage inhibition in paw volume after administration of the extract was calculated using the following formula:

$$\text{Percentage inhibition in rat paw volume} = (1 - V_t/V_c) \times 100$$

Where,  $V_t$  is the mean paw volume in control group and  $V_c$  is the mean paw volume in test group.

**Statistical analysis:** Statistical analysis was carried out using one-way ANOVA followed by Dunnet's multiple comparisons for analgesic and anti-inflammatory tests. The results obtained were compared with the vehicle control group. P values <0.05, <0.01, <0.001 were considered to be statistically significant.

## Result

**Acute oral toxicity test:** In the acute toxicity assay, no deaths were observed during the 72 hrs period at the doses tested. At these doses, the animals showed no stereotypical symptoms associated with toxicity, such as convulsion, ataxia, diarrhoea or increased diuresis.

**Acetic acid-induced writhing test:** The results of the ethanolic extract of *C. pallida* leaves on acetic acid-induced writhing in mice and percentage inhibition of pain are shown in Table 1. The percentage inhibition of writhing produced by the extracts at the dose of 100 mg/kg bw was 47.70%; that result was statistically significant ( $P < 0.001$ ) compared to the standard drug indomethacin, which showed 70.67% writhing inhibition.

**Formalin-induced paw licking test:** The effects of the ethanol extract of *C. pallida* leaves on formalin induced paw licking in mice and percent of inhibition in both phases are shown in Table 2. In the first phase of the formalin-induced pain model, ethanol extract at 100 and 50 mg/kg bw produced 40.44% and 27.94% inhibition of pain response whilst at the second phase *C. pallida* leaf extract produced 48.15% and 20.37% inhibition of pain response, respectively. It was found that *C. pallida* extract showed an analgesic effect both in the early and late phase of the formalin test which indicates that the extract exerts its analgesic effect through the peripheral and central mechanism.

**Table 1. Effects of ethanolic extract of *C. pallida* leaves on acetic acid-induced test.**

Group	No of writhing	% of Inhibition
Control	35.37 ± 4.59	-
Standard	10.38 ± 3.01***	70.67%
<i>C. pallida</i> (50 mg/kg)	24.25 ± 4.50*	37.5%
<i>C. pallida</i> (100 mg/kg)	18.50 ± 2.76***	47.70%

Values are expressed as mean ± S.E.M. (n=5); significance at \*\*\*p<0.001, \*\*p<0.01, \*p<0.05 as compared to control.

**Table 2. Effects of ethanolic extract of *C. pallida* leaves on formalin-induced test.**

Group	Early Phase	% of inhibition	Late Phase	% of inhibition
	0-5 min.	in early phase	20-30 min.	in late phase
Control	34.00 ± 2.10	-	13.50 ± 2.10	-
Standard	17.00 ± 1.50	50.05%	2.50 ± 1.20	81.48%
<i>C. pallida</i> (50 mg/kg)	24.50 ± 14.15	27.94%	10.75 ± 6.20	20.37%
<i>C. pallida</i> (100 mg/kg)	20.25 ± 11.69	40.44%	7.00 ± 4.04	48.15%

Values are expressed as mean ± S.E.M. (n = 5); significance at \*\*\*p < 0.001, \*\*p < 0.01, \*p < 0.05 as compared to control.

**Tail immersion test:** The effects of the ethanol extract of *C. pallida* leaves on tail immersion test in

mice were significant at the level of  $P < 0.001$  shown in Table 3. In the tail immersion method of analgesic

activity, the ethanolic extract of *C. pallida* exhibited potent activity at 100 and 50 mg/kg b.w. The response time observed was significantly increased when compared to normal control. However, the standard diclofenac sodium was found to have better activity than the extract during the 4h response. The maximum percentage protection after 4h for diclofenac sodium was 78.95% and for 100 mg/kg

dose of the ethanolic extract of *C. pallida* was 64.91%.

**Carrageenan-induced rat paw oedema test:** Carrageenan induced rat paw oedema was reduced considerably at both the doses of 50 and 100 mg/kg b.w. and it was significant ( $P < 0.01$ ) at 3<sup>rd</sup> hour at a dose of 100 mg/kg b.w. The observations are shown in Table 4.

**Table 3. Effects of ethanolic extract of *C. pallida* leaves on tail-immersion method test.**

Treatment	Retention time in (sec)						% of inhibition
	Initial	Time after drug administration					
	0 h	1 h	2 h	3 h	4 h	5 h	
Control	10.75 ± 1.2	11 ± 1.56	9.75 ± 0.75	10.75 ± 1.2	14.25 ± 0.98	10.0 ± 0.94	-
Standard	11.25 ± 0.86*	4.5 ± 1**	4.75 ± 1.9*	4.75 ± 2.1**	3 ± 1.15***	5 ± 1.05*	78.95%
<i>C. pallida</i> (50 mg/kg)	11.75 ± 0.8	5.75 ± 0.98**	7.25 ± 0.55	7 ± 0.47	6.75 ± 0.28***	6.75 ± 0.28*	52.63%
<i>C. pallida</i> (100 mg/kg)	14.75 ± 1.4	7.25 ± 0.72*	5.25 ± 1.2*	4.25 ± 0.86**	5 ± 0.81***	6.75 ± 0.98**	64.91%

Values are expressed as mean ± S.E.M. (n = 5); significance at \*\*\*p < 0.001, \*\*p < 0.01, \*p < 0.05 as compared to control

**Table 4. Effects of ethanolic extract of *C. pallida* leaves on carrageenan-induced rat paw oedema test.**

Treatment	Reaction time in hours				
	Initial	Time after drug administration			
	0 h (mm)	1 h (mm)	2 h (mm)	3 h (mm)	4 h (mm)
Control	17.5 ± 0.71	17.87 ± 0.59	16.37 ± 0.64	16.25 ± 0.5	15.75 ± 0.37
Standard	16.37 ± 0.49	13.5 ± 0.78	10.25 ± 0.61	09.37 ± 0.27***	09.12 ± 0.36
CP50 mg/kg	16.12 ± 0.49	15 ± 0.47	14.62 ± 0.54	13.56 ± 1.2	13.75 ± 1.19
CP100 mg/kg	17.62 ± 0.27	14.5 ± 0.57	11.63 ± 1.03	10.5 ± 1.2**	10.03 ± 0.49

Values are expressed as mean ± S.E.M. (n=5); significance at \*\*\*p < 0.001, \*\*p < 0.01, \*p < 0.05 as compared to control.

## Discussion

Results of the present study showed that the ethanolic extract of *C. pallida* possessed marked antinociceptive and anti-inflammatory effects with a reasonable safety profile. The anti-nociceptive effect of ethanol leaves extract of *C. pallida* was evaluated for the first time in various experimental test models.

Satyanarayana *et al.* (2004) showed that acetic acid induces writhing by stimulating the production of prostaglandins. Standard analgesic drug

Indomethacin has been shown to inhibit prostaglandin synthesis in the brain (Rang *et al.*, 2012; Flower and Vane 1972). Since *C. pallida* therapy antagonized acetic acid-induced writhes, it is possible to suggest that the plant extract may be producing anti-nociceptive activity through manipulation of the prostaglandin system.

The formalin-induced paw licking model comprises of early phase and late phase. The early phase (immediately after injection) seems to be

caused by C-fibre activation due to the peripheral stimulus. The late phase (20 min after formalin injection) appears to depend on the combination of an inflammatory reaction, activation of NMDA and non-NMDA receptors and NO cascade (Davidson and Carlton, 1989) in the peripheral tissue and the functional changes in the dorsal horn of the spinal cord. In our study, the ethanolic extract of *C. pallida* significantly inhibited the late phase of formalin-induced pain.

The tail immersion test is used to determine both centrally acting analgesics (Ramabadran *et al.*, 1989), like Diclofenac Na (Schweizer and Brom, 1985) and peripherally acting analgesics like NSAIDs which inhibit cyclooxygenase in peripheral tissues, thereby interfering with the mechanism of transduction of primary afferent nociceptors (Saad *et al.*, 2014). The results observed for the tail immersion test clearly showed that the ethanolic extract of *C. pallida* possessed a dose-dependent anti-nociceptive activity.

In the present study, the leaf ethanol extract of *C. pallida* considerably reduced carrageenan-induced paw oedema in mice. Diclofenac also antagonised carrageenan-induced paw oedema in mice. Carrageenan-induced inflammatory pain is well known to involve inflammatory mediators like cyclooxygenase products (PGE<sub>2</sub>), leukotrienes histamine, 5-HT and cytokines (Yaksh *et al.*, 2001) which are released as a result of tissue injury. *C. pallida* reduced carrageenan-induced rat paw oedema, suggesting that the plant may have affected inflammatory mediators to produce its anti-inflammatory activity.

### Conclusion

To conclude, the ethanolic extract of *C. pallida* was proven to be a natural secure remedy for the treatment of analgesia and inflammation. Our current findings demonstrated scientific rationale for the folk use of the plant as an analgesic. Interestingly, the ethanolic extract of *C. pallida* exhibited both peripheral as well as a central anti-nociceptive effect which might be attributed to the presence of such active principles, due to which it has proven folk use

in various nervous disorders. Nevertheless, the isolation of pure secondary metabolites from the plant will help us to further understand the mechanism of these activities and in the identification of primary compounds of clinical utility.

### Conflict of Interest

The authors declare no conflicts of interest.

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