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Study of analgesic, antipyretic and anti-inflammatory activities of the leaves of *Thunbergia coccinea* Wall.

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Abstract
In this study, the analgesic, antipyretic and anti-inflammatory effects of the methanolic extract obtained from the leaves of *Thunbergia coccinea* Wall were investigated. Analgesic effect of MTC was evaluated by acetic acid –induced writhing, hot plate and tail flick method. Antipyretic activity of MTC was evaluated by yeast –induced hyperpyrexia in rats. The anti-inflammatory activity was studied in carrageenan induced paw oedema. Acute toxicity in animal models at different doses was also evaluated. MTC has been found to be safe up to a dose of 2 g/kg when administered orally to mice. A significant analgesic effect has been observed in rats when tested by chemical as well as thermal methods using acetic acid induced hot plate method and tail flick test. In the case of its effect on pyresis, a significant reduced fever greater than 200mg/kg within 2h on yeast induced hyperthermia in rats was observed. The result showed that the writhes, tail flick, pyrexia and paw volume in experimental rats reduced significantly ($p<0.05$) as compared to that of control and hot plate test showed significant licking effects in rats. These results clearly indicate the methanolic extract of *Thunbergia coccinea* could be a potential source for using as analgesic, antipyretic and anti-inflammatory agent and provide the scientific basis for the folkloric use of the plant in treating inflammation, fever and pain.

Keywords: Anti-inflammatory; Analgesic; Antipyretic; Acute toxicity; *Thunbergia coccinea* Wall., Acanthaceae

INTRODUCTION

Medicinal plants will continue to provide a source of generating novel drug compounds and as phyto-medicine for the treatment of disease [1]. It is estimated that, plant materials are present in, or have provided the models for 50% Western drugs [2]. The primary benefit of using plant –derived medicine is that they are relatively safer than synthetic alternatives, offering profound therapeutic benefits and more affordable treatments. Plants with analgesic, antipyretic and anti-inflammatory properties have been of immense ethnomedicinal uses to mankind. The search for better alternative analgesic, antipyretic and anti-inflammatory drugs from the bounties of our vegetation is thus a worthwhile venture.

Thunbergia coccinea Wall. belonging to family Acanthaceae is a tropical jungle flowering vine, pendant scarlet flowers from late autumn and through the winter originally introduced from Nepal. The flowers are cooked as a vegetable, juice of the plant is applied to cuts and wounds and the root is chewed to treat boils. The vine is used for binding. In other cases, the stem is tied round the neck of cattle to expel the sore-worms. Juice of the leaves is also used for diabetes, eye diseases and new cuts [3] . The leaves are also good for fodder and leaf paste along with little honey is given in cough and hot fermented leaf is applied in muscular sprain [4].

However, survey of literature indicates that there have been no published reports on analgesic, antipyretic and anti-inflammatory activities of this plant. Keeping this in view, the present study was undertaken to investigate the analgesic, antipyretic and anti-inflammatory potential of the methanol leaves extract of *Thunbergia coccinea* in experimental animal models. The results of the present study are given in this communication.

MATERIALS AND METHODS

Plant material

Leaves of *Thunbergia coccinea* were collected from Maulpheng area, Aizawl, Mizoram during July 2009. The plant material was identified by Botanical Survey of India, Shillong and the voucher specimen (BSI/EC/TECH/2009/55, Dated 11th June, 2009. The specimen was deposited in the herbarium of the Department of Pharmacy, Regional Institute of Paramedical and Nursing Sciences (RIPANS), Aizawl, Mizoram for future references. The collected fresh leaves of the plant were cleaned, washed with distilled water, dried in ventilated room under shade for three weeks, pulverized using grinder and passed through 40-mesh sieve to get fine powder.

Extraction of the plant material

The air- dried and powdered leaves (2.0 kg) were extracted at room temperature successively by soxhlet apparatus using petroleum ether (60-80^{0}C b.pt) followed by chloroform and methanol. The extraction was carried out exhaustively and the solvents were recovered by distillation under reduced pressure using rotary vacuum evaporator to obtain crude petroleum ether extract (98 g,

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4 %; w/w), chloroform extract (300 g, 12 %; w/w) and methanol extract (250 g, 10 %; w/w). For pharmacological studies, the methanol extract was used.

Experimental animals

Adult wistar albino rats (120-200 g) and Swiss albino mice (20-30 g) of either sex maintained in the Animal Experimental Laboratory of Department of Pharmacy, RIPANS were selected for the experiments. The animals were grouped and housed in polyacrylic cages (38 x 23 x 10 cm), maintained under standard laboratory conditions (25 ± 2° C) with dark/light cycle (12/12hour). They were allowed free access to standard dry pellet diet (M/S Hindustan Lever Limited, Kolkata, India) and water *ad libitum.* The animals were acclimatized to laboratory conditions for 10 days before commencement of the experiment. The minimum number of animals and duration of observation required to obtain consistent data were employed. "Principles of laboratory animal care" (NIH publication number 85-23, revised 1985) guidelines were followed.

Drugs and reagents

Carrageenan, morphine sulphate (Sd-Fine Chemicals, Mumbai), carboxy methyl cellulose (LOBA Chemmie. Pvt Ltd, Mumbai), aspirin tablets, diclofenac sodium tablets, paracetamol tablets (Torent Pharmaceutical Ltd, Ahmedabad), acetic acid (Merck) and brewer's yeast (Hindustan Lever limited, Kolkata) were used. Carboxy methyl cellulose (0.5% in distilled water, 10 ml/kg, p.o.) was used as control in all studies while the dose levels of the MTC (200 and 400 mg/kg, i.p.) suspended in 0.5 % CMC were employed for the test groups. Diclofenac sodium (40 mg/kg, i.p.), Morphine sulphate (5 mg/kg, s.c), s paracetamol (33 mg/kg, i.p.) were used as reference drugs.

Acute toxicity

Swiss albino mice were divided into five groups, each containing six animals. MTC was administered orally at doses ranging from 5 mg to 2 g/kg following a standard method [5, 6] and Organisation for Economic Co-operation and Development (OECD) guidelines [7] . Animals were individually and continuously observed for 4 h to detect changes in the autonomic or behavioural responses and then monitored for any mortality for the following 14 days. A group of animals treated with the vehicle (0.5 % CMC) served as control. Based on the results of preliminary acute oral toxicity testing, the doses of 200 and 400 mg/kg were chosen for further experiments.

Evaluation of Analgesic activity
Acetic acid-induced writhing test

Analgesic activity was evaluated on the acetic acid- induced writhing according to Koster et al. [8]. Mice are divided in four different groups of six mice each. The two groups received saline (control, 5ml/kg) and morphine sulphate (5mg/kg), b.w. (i.p.) was administered, while the remaining two groups received the extracts (200 and 400 mg/kg), b.w. (i.p.) was administered. After 30 mins later 1 % v/v of acetic acid (1 ml/100 gm) b.w. was injected intraperitoneally. The number of writhing i.e. a response consisting of abdominal contractions and hind limbs stretching were counted for 20 minutes beginning from 5 min. after the acetic acid injection. A significant reduction in the number of writhing compared to the control animals was considered as an analgesic response.
Percentage analgesic activity

$$= \frac{N - N^t}{N} \text{ X100}$$

where "N" is the average number of stretching of control per group and N^t is the average number of stretching of test per group.

Hot plate method

The hot plate latency assay was carried out according to the method of Eddy et al. [9] . The rats used for this study were divided into four groups-two groups received saline (control, 5ml/kg) and morphine sulphate (5 mg/kg), while the remaining two groups received the extracts (200 and 400 mg/kg), b.w. (i.p.) .The extracts, saline or morphine sulphate were administered by i.p. route to the animals after 12 h of fasting. The animals each were placed on a hot plate maintained at 55ºC, 30 mins after administration of extracts, saline or morphine. The time taken for the rats to respond to the thermal stimulus (usually by jumping) was noted as the latency (in seconds). The mean of the latency for each group was determined. The effects of the extracts, morphine and saline were also determined after 60, 90,120 &150 min of administration of rats.

Tail flick test

For this the method of Gray et al. [10] was used. The tail flick was evoked by a source of radiant heat, which was focus on the dorsal surface of the tail. Adult healthy rats were examined for latency to withdraw their tails from a noxious thermal stimulus using a tail-flick meter (Instrument model no. Ugo Basile 7140, Italy). Each rat was tested twice before the administration of MTC and the reaction times were averaged to obtain a baseline. The intensity of heat stimulus was adjusted to achieve a mean tail-flick latency of 3-4 s in control animals. The selected animals were divided into four groups, each group consisting of six rats. First group for the control, second (positive control group) for reference drugs viz morphine sulphate and the remaining two groups for MTC (doses of 200, and 400 mg/kg). Each rat was then tested 30, 60, 90, 120, and 150 min after the administration of 200 and 400 mg/kg i.p. Control rats received 0.9% w/v of saline solution. Morphine sulphate (5 mg/kg, i.p) was administered as a positive control. Treatments were terminated if the animal did not respond within 15 sec in order to avoid tissue damage.

Evaluation of Antipyretic activity
Yeast-induced pyrexia

Rats were divided into four groups of six rats each. The normal body temperature of each rat was measured rectally and recorded. Pyrexia was induced by injecting the yeast suspension by subcutaneous route of administration in hind limbs of the rats. The rats were acclimatized to remain quite in a restraint cage. A thermometer coated with the lubricant was inserted 3-4 cm deep into the rectum and fastened to the tail by adhesive tape. The temperature was measured on a thermometer (60 sec). After measuring the basal rectal temperature, animals were given a subcutaneous injection of 10 ml/kg body weight of (w/v) yeast suspended in 0.5% (w/v) Carboxy methylcellulose solution. Rats

were then returned to their housing cages. After 18 h of yeast injection, the animals were again restrained in individual cages for the recording of their rectal temperature as described previously.

Drug administration

After 18 h of yeast injection the Normal saline solution (0.9 % Nacl, w/v) was administered orally to the control group I. The group II of animals received the standard drug Paracetamol (33 mg/kg body weight) i.p. and the extracts of METC were administered intraperitoneally at doses of 200 and 400 mg/kg body weight to groups III- IV animals respectively. Rats were restrained for recording rectal temperature at the 18 h, immediately before extracts, normal saline or paracetamol administration, and again at one hour intervals up to the 22 h after yeast injection.

Evaluation of Anti-inflammatory activity
Carrageenan-induced rat paw edema

The carrageenan induced hind paw edema model was used for determination of the anti-inflammatory activity [11] . Adult healthy wistar rats weighing 120-200 g, deprived of food overnight were divided into four groups, each group consisting of six rats. First group for the control, second group (positive control group) for reference drug and another two groups for MTC. 0.1 ml of 1%w/v Carragenan was injected into the right paw of each rat under the subplantar aponeurosis. The test groups of rats were administered intraperitoneally with (200 and 400 mg/kg), ½ h before carragenan injection. At the same time the control group received 5 ml /kg of 0.9%w/v saline solution and the reference group received 40 mg/kg Diclofenac sodium (i.p.). The paw volume was measured up to 150 mins after the injection using a plethysmograph by dipping the foot in the mercury bath of the plethysmograph apparatus up to the anatomical hairline on lateral malleolus [12] and compared with the control animals. The inhibitory activity was calculated according to the following formula [13] .

Percentage inhibition

$$= 100 - \frac{\text{(Oedema volume in the treated) x100}}{\text{Oedema volume in the control}}$$

Statistical analysis

Results of the study were expressed as mean ± S.E.M. All the statistical analysis has been done by one-way ANOVA followed by the Dunett t test. P<0.01 was considered significant in all cases.

RESULTS
Oral acute toxicity test

The MTC when orally administered in the dose range of 5, 50, 100, 300 and 2000 mg/kg to mice, did not produce any significant change in the autonomic or behavioural responses during the observation period. No mortality was observed up to the 14 th day of monitoring.

Acetic acid-induced writhing test

The results presented in Table-1 showed that the MTC at the doses of 200 and 400 mg/kg exhibited significant (p<0.01) inhibition of the writhing when compared to that of standard drug (Morhine sulphate, 5mg/kg, i.p.)

Hot plate method

The results of the hot plate test showed that intra peritoneal route administration of the MTC at the doses of 200 and 400 mg/kg produced significant increase in the reaction time when compared to that of standard drug (Morphine sulphate, 5 mg/kg, i.p.) and control groups (Table-2). .

Tail flick test

The results of the tail flick test showed that i.p. administration of the MTC at the doses of 200 and 400 mg/kg produced significant increase in the tail flick latencies when compared to that of standard drug (Morphine sulphate, 5 mg/kg, i.p. and control group (Table-3).

Yeast-induced pyrexia

The methanolic extract of Thunbergia coccinea show significant (p<0.01) antipyretic activity at both 200 and 400 mg/kg. Hence, it is statistically significant as compared to control (Table

Carrageenan-induced rat paw oedema

There was a gradual increase in edema paw volume of rats in the control group. However, in the test groups, the MTC showed a significant reduction in the edema paw volume. A dose related manner inhibition of hind paw edema was observed. Diclofenac sodium as a reference drug (40 mg/kg, i.p.) produced a significant inhibitory effect comparable to the tested MTC (Table-5)

Table 1. Effect of the methanolic extract of. *Thunbergia coccinea* on acetic acid induced writhing in mice

Treatment %	Dose (mg/kg)	No. of writhes per 30 mins	Inhibition
Control	-	64.66 ± 0.42	-
Morphine sulphate	5	13.0 ± 0.36 **	79.89
MTC	200	54.16 ± 0.47 *	16.23
MTC	400	35.33 ± 033**	45.36

Values are expressed in mean ± SEM (n=6). ***P*<0.01 compared with control; **P*<0.05 compared with control .

Table 2. Effect of the methanolic leaves extract of *Thunbergia coccinea* on hot plate method in rats

Treatment	Dose (mg/kg)	Reaction time (s)				
		30 min	60 min	90 min	120 min	150 min
Control	-	3.1±0.03	3.05±0.02	3.06±0.03	3.01±0.03	2.88±0.04
Morphine	5	8.11±0.04	8.13±0.02	8.11±0.04	7.83±0.07	7.20±0.14
MTC	200	4.23±0.07	4.45±0.05	4.55±0.06**	4.50±0.06**	4.13±0.04*
MTC	400	4.73±0.04	4.86±0.06	5.16±0.11*	5.06±0.11**	7.20±0.14*

Values are expressed as mean ± SEM (n=6). ***P*<0.01 compared with control **P*<0.05 compared with control.

Table 3. Effect of the methanolic extract of *Thunbergia coccinea* .on tail flick method in rats

Treatment	Dose (mg/kg)	Predrug reaction time in sec	Average tail withdrawing time in sec				
			30 min	60 min	90 min	120 min	150 min
Control	-	3.06±0.03	2.93±0.07	3.0±0.02	3.01±0.04	3.01±0.03	3.01±0.03
Morphine	5	3.06±0.03	10.5±0.14	10.8±0.10	10.9±0.06	10.8±0.13	10.5±0.08
MTC	200	3.1±0.03	4.5±0.06*	4.8±0.08*	5.2±0.09**	5.4±0.06**	4.6±0.16*
MTC	400	3.1±0.04	5.2±0.07	5.35±0.07*	6.0±0.06**	6.2±0.06**	6.1±0.08*

Values are expressed as mean ± SEM (n=6). ***P*<0.01 compared with control **P*<0.05 compared with control.

Table 4.Effect of the methanolic extract of *Thunbergia coccinea* Wall on yeast induced pyrexia in rats.

Treatment	Dose (mg/kg)	Rectal temperature °C before and after treatment					
		Normal	18h	1h	2h	3h	4h
Control	-	37.5±0.13	38.4±0.10	38.5±0.03	38.5±0.04	38.4±0.11	38.5±0.04
Paracetamol	33	38.5±0.08	37.5±0.13**	37.5±0.09**	37.5±0.07	37.6±0.06**	37.7±0.08**
MTC	200	37.5±0.11	38.3±0.13	37.5±0.06**	37.5±0.03*	37.5±0.06**	37.6±0.08**
MTC	400	37.4±0.11	38.6±0.05	37.7±0.06**	37.5±0.06**	37.4±0.04*	37.4±0.08*

Values are expressed as mean ± SEM (n=6). ***P*<0.01 compared with control **P*<0.05 compared with control.

Table 5. .Effect of the methanolic leaves extract of *Thunbergia coccinea* on carrageenan induced paw edema in rats

Treatment	Dose (mg/kg)	% Inflammation ± SEM at (hours)				
		1h	2h	3h	4h	5h
Control	-	0.72±0.009	0.75±0.004	0.82±0.015	0.85±0.01	0.73±0.01
Diclofenac	40	0.22±0.008**	0.25±0.003**	0.25±0.002**	0.25±0.002**	0.22±0.006**
MTC	200	0.58±0.03	0.58±0.02	0.51±0.01**	0.49±0.01**	0.54±0.02**
MTC	400	0.59±0.01	0.58±0.02	0.56±0.03**	0.57±0.02**	0.57±0.02

Values are expressed as mean ± SEM (n=6). **P<0.01 compared with control, *P<0.05 compared with control.

DISCUSSION

In view of the increasing popular consumption of medicinal plants as alternative therapy, it is necessary to conduct research to support the therapeutic claims and also to ensure that the plants are indeed safe for human consumption [14, 15]. Investigation of acute toxicity is the first step in any toxicological investigation of an unknown substance. The index for acute toxicity is LD_{50}. Historically the LD_{50} was determined with high degree of precision and used to compare the toxicities of compounds relative to their therapeutic doses.

The present research finding has clearly met the objective of the study. From the present study, the MTC did not show mortality up to 2000 mg/kg orally during the observation period as per OECD guidelines, therefore according to the chemical labelling and classification of acute systemic toxicity, based on oral LD_{50} values, which were recommended by OECD, the crude MTC was assigned to class 5 (LD_{50} > 2000 mg/kg), which was termed as the lowest toxicity class [7, 16].

The method for testing analgesic activity was selected such that both centrally and peripherally mediated effects were investigated. The MTC (200 and 400 mg/kg), administered i.p. significantly inhibited the acetic acid-induced writhings in rats. These writhings are related to the increase in the peritoneal fluid level of PG E_2 and $PGF_{2\alpha}$ [17]. This acetic acid-induced writhings method is not only simple and reliable but also affords rapid evaluation of peripheral type of analgesic action. In this test, the animals react with characteristic stretching behaviour, which is called writhing. The abdominal constriction is related to the sensitization of nociceptive receptors to prostaglandins. The results strongly suggested that the mechanism of action of the MTC may be linked partly to lipo-oxygenases and /or cyclo-oxygenase..

Hot plate method and tail flick method has been found to be suitable for the evaluation of centrally but not of peripherally acting analgesics. The increase in the latency may be due to the possible partial opioid agonistic effect of the extracts [18] . Therefore, the result of this study proved the uses of this plant in folklore medicine for the management of pain

Fever may be result of infection of tissue damage inflammation, graft rejection or other disease states. Antipyretics are drugs which reduce elevated body temperature. Regulation of body temperature requires a delicate balance between production and loss of heat and the hypothalamus regulates the set point at which body temperature is maintained. In fever this set point is elevated and a drug like paracetamol does not influence body temperature when it is elevated by factors such as exercise or increase in ambient temperature [19] . The present results showed that the MTC possessed a significant antipyretic effect in yeast-provoked elevation of body temperature in rats and its effect is comparable to that of paracetamol. Therefore, the result of this study proved the uses of

this plant in folklore medicine for the treatment of fever.

Carrageenan induced inflammation has been reported to be a useful model for screening of clinically effective anti-inflammatory agents [20]. Edema formation due to carrageenan in rat is a biphasic event. The initial phase (1-2 h) of edema is attributed to the release of histamine and serotonin and the second phase of edema is due to release of prostaglandins and mediated by bradykinin, leukotrienes and polymorphonuclear cells Further, it has been demonstrated that the second phase is sensitive to the most clinically effective anti-inflammatory drugs [21, 22] . Some of anti-inflammatory drugs strongly inhibit the second phase of the carrageenan-induced edema. However, some anti-inflammatory drugs are effective against both phases [23]. Hence, it is likely that the MTC might elicits its anti-inflammatory activity by inhibiting synthesis and release of prostaglandins, proteases and lysosomal enzymes like nonsteroidal anti-inflammatory drugs [24] .

During the repair process of inflammation, there is proliferation of macrophages, neutrophils, fibroblasts and multiplication of small vessels, which are the basic sources of forming a highly vascularised reddish mass termed granular tissue [25]. There are documented reports that lysosomal enzymes play an important role in the development of acute and chronic inflammation [26, 27].

In the present experiments, the MTC treatment (200 and 400 mg/kg) prevented the increased serum marker enzymes in acute inflammatory condition, thereby suggesting its membrane stabilizing potential. Most of the anti-inflammatory drugs exert their beneficial effect either by inhibiting the release of lysosomal enzymes or by stabilizing lysosomal membrane [28] . Furthermore, it was observed that membrane stabilizing effect of extracts can be correlated to its anti-inflammatory activity. Myeloperoxidase (MPO) is an enzyme present in neutrophils, monocytes and macrophages at a much lesser concentration and has been demonstrated that, the level of MPO activity is directly proportional to neutrophils concentration in the inflamed tissue [29]. Hence, the measurement of the MPO activity has been considered a sensitive index of chemotaxis and neutrophils infiltration into the inflammation site. Pre-treatment with these extracts significantly decreased MPO activity in oedematous tissue. The extent of inhibition of MPO is well correlated with the reduction of edema formation. The significant reduction as well as inhibitory effect of the extracts on the carrageenan-induced edema paw volume is also an indication the anti-inflammatory potentials of the plants. Therefore, the result of this study supports the uses of this plant in folklore medicine for the management of acute inflammation.

Thus the results of the present study provide support to the traditional usage of *Thunbergia coccinea* Wall. for the management of pain, inflammation and fever. This may provide an insight for further isolation of the phytochemical constituents from this plant for discovering novel drugs.

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