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Antinociceptive, antipyretic and anti-inflammatory effects of *Clerodendrum phlomidis* in mice and rats

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ABSTRACT

The ethanolic extract of *Clerodendrum phlomidis* L. belonging to the family of Verbenaceae was evaluated for its antinociceptive, antipyretic and anti-inflammatory activity in mice and rats respectively. Analgesic activity was studied by using acetic acid-induced mouse withering test, hot water tail immersion method and eddy's hot plate method in mice. The antipyretic activity was evaluated against yeast induced pyroxia in rat and anti-inflammatory activity was evaluated by carrageenan-induced hind paw edema and its probable mechanism evaluated in rats. The preliminary phytochemical screening and acute toxicity studies were carried out. *C. phlomidis* extract showed a dose dependent significant reduction of the number of writhes ($P<0.001$) with 800 $mg.kg^{-1}$ body weight dose giving the highest reduction. The extract showed an insignificant elongation of the hot plate reaction time ($P<0.05$) and it produced a significant reduction in hot-water tail immersion method. *C. phlomidis* extract showed significant reduction in brewer's yeast induced hyperthermia in rats. In the carragenan induced paw edema, a dose dependent significant inhibition was observed ($P<0.001$) between the 2^{nd} and 5^{th} hr. Preliminary phytochemical screening of the extracts showed that the carbohydrates, proteins, amino acids, phytosterols, alkaloids, fats, fixed oils, flavonoids and gums were present in the plant extract. It is clear that the ethanolic extract of *C. phlomodis* has significant analgesic and anti-inflammatory activity. Inhibition of the synthesis of prostaglandins and other inflammatory mediators probably account for the analgesic and anti-inflammatory properties.

Key words: *C. phlomidis*, antinociceptive, antipyretic, anti-inflammatory, arthritis.

INTRODUCTION

Inflammation is a complex patho-physiological process mediated by a variety of signaling molecules produced by leukocytes (Safayhi et al., 1997), especially macrophages (Feldmann et al., 1996; Maini and Taylor, 2000; Kalpan et al., 2002), mast cells (Chen et al., 2000) as well as by the activation of complement factors, which bring about edema formation as a result of extravasations of fluid, proteins and accumulation of leukocytes at the inflammatory site (White, 1999). Various non-steroidal anti-inflammatory drugs (NSAID) are widely used clinically for inflammation and rheumatoid arthritis. However, despite their great number, their therapeutic efficacy seems to be hampered by a number of undesired and often serious side effects. Therefore, it is desirable to find less toxic alternative anti-inflammatory and antinociceptive drugs. Some medicinal plants might be candidates for such alternatives. Indian medicinal plants are a rich source of bioactive substances, which are claimed to induce para-immunity, the non specific immunomodulation of essentially granulo-cytes, macrophages, and natural killer cells and complement factors (Sainis et al., 1997).

Clerodendrum phlomidis L. (Family: Verbenaceae) is found in some parts of south India and widely distributed in waste lands. It is a large bush (or) small tree, reaching 9 m height with more or less pubescent leaves and branches. They grow in mesic habitats with moderate rainfall and mild temperatures (Watsan and Malone 1977; Kaplan, et al., 1987; Ali et al., 1995). The leaves of the plant are used in inflammation. The decoction of the roots and leaves of the herb is used in rheumatism, antimicrobial, (Ahmed et al., 1993), nervous diseases, convalescence of measles, piles, chronic bronchitis (Shan, 1982) etc.

The present investigation was undertaken to study the antinociceptive, antipyretic and anti-inflammatory activities of the ethanolic extract of the leaves of *C. phlomidis* in acute and chronic inflammations.

MATERIALS AND METHODS

Plant material

Taxonomic identification of the plant was made from Rapinat Herbarium, St. Joseph's college of arts and sciences, Trichy, Tamilnadu, India (Voucher specimen number RH/CP/24A). Whole fresh plant leaves of *C. phlomidis* were collected from Jeyankondam, Perambalur (Dist), Tamilnadu, India. The leaves were dried under shade, segregated, pulverized by a mechanical grinder and passed through 40 mesh sieves.

Preparation of extracts

The powdered leaves (500 g) were successively extracted with ethanol (70-80 ºC) for 24 hrs by continuous hot percolation method using soxhlet apparatus. The fraction was separated from the solvent by distillation under reduced pressure to yield 5.6% w/w solid mass that was stored in a refrigerator and used for further studies.

Animals

The animals for the present study were procured after ethical clearance from the Institutional Animal Ethical Committee (IAEC) in Annamalai University, Annamalai nagar, Tamilnadu, India. The animal experiments were carried out according to Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) rules. Inbred Wistar rats (150-200 g) were used for testing antipyretic and anti inflammatory activity. The albino mice (20-25 g) were used for testing antinociceptive activity. The animals were housed at the central animal house (Rajah Muthiah Medical College and Hospital, Annamalai University, Tamilnadu, India) under standard conditions of temperature (23 ± 1 °C), relative humidity (55 ± 1%), 12 hrs light and dark cycles and fed with standard pellet diet, and tap water *ad libitum.*

Drugs and chemicals

All the drugs used in this study were of pharmaceutical grade. Carrageenan was supplied by Sigma chemicals, indomethacine and aspirin were the gift samples from Cadila Pharmaceuticals, Ahamedabad, India. Morphine was supplied by Rajah Muthiah Medical College and Hospital, Annamalai University, Tamilnadu, India.

Preliminary phytochemical tests

The leaf extract of *C. phlomidis* was subjected to preliminary phytochemical screening, for various active phytochemical constituents such as carbohydrates, steroids, proteins, flavonoids, amino acids, fat, fixed oil, gum and mucilage (Trease and Evans, 1983).

Acute toxicity studies

Acute toxicity studies were performed (Ecobichon, 1997) according to OECD-423 guidelines (acute toxic class method). Albino mice (n=3) of either sex selected by random sampling technique were employed in this study. The animals were fasted for 4 hrs with free access to water only, after which the extracts were administered orally at the dose level of 5 $mg.kg^{-1}$ body weight by gastric intubation and observed for 14 days. If mortality was observed in two out of three animals, then the dose administered was considered as a toxic dose. If the mortality was observed only in one mouse out of three animals, then the same dose was repeated again to confirm the toxic effect. If mortality was not observed, the procedure was then repeated with higher dose such as 50, 300 and 2000 $mg.kg^{-1}$ body weight.

Antinociceptive activity

Acetic acid writhing reflex

This was performed according to Gaertner et al. (1999). Albino mice (six per group) were injected intraperitoneally with 0.6% acetic acid at the dose of 10 ml.kg^{-1}. The extract (200, 400 and 800 mg.kg^{-1}), aspirin (100 mg.kg^{-1} subcutaneously) and distilled water were orally administered 30 min before the treatment with acetic acid. The writhings induced by the acid, consisting of abdominal constrictions and hind limbs stretchings were counted.

Hot plate method

The albino mice (20-25 g) were divided into six groups of six animals each. They were initially subjected to 16 hrs fasting and basal reaction time was noted, before the administration of standard drug or test extract (licking of the paws or jumping response) was done at 0 and 10 min interval. The average of the two readings was obtained as the initial reaction time. The reaction time followed by the administration of the extract or drugs. The animals in group I were administered normal saline (5 ml.kg^{-1} body weight) orally. The animals in group II (10 mg.kg^{-1} body weight) treated with morphine sub-cutaneously and the animals in group III, IV, and V were administered the ethanolic extract of *C. phlomidis* at 200 and 400 and 800 mg.kg^{-1} body weight orally. The hot plate was maintained at 55 ± 5 °C (Lanhers et al., 1991). The response was recorded at different time intervals such as 0, 1, 2, 3, 4, 5 and 6 hrs after administration of normal saline, standard drug and test extract to the corresponding animal group.

Tail - immersion method

The albino mice (20-25 g) were divided into six groups of six animals each. They were initially subjected to 16 hrs fasting and basal reaction time was noted before the administration of standard drug or test extract. The animals in group I were administered normal saline (5 ml.kg^{-1} body weight) orally. The animals in group II were administered the standard drug morphine (10 mg.kg^{-1} body weight) subcutaneously and the animals in group III, IV and V were administered the ethanolic extract of *C. phlomidis* at 200 and 400 and 800 mg.kg^{-1} body weight orally. The test involved immersing the extremity (3 cm) of the rat's tail in a water bath containing water at a temperature of 55 ± 2 °C. Within a few seconds, the rat reacted by withdrawing its tail and the response was recorded at different time intervals such as 0, 1, 2, 3, 4, 5 and 6 hrs after administration of normal saline, standard drug and test extract to the corresponding animal groups. The withdrawal of tail from hot water was recorded. Normally the cut of period will be 10 seconds i.e. time of no response was put at 10 sec (Asongalem et al., 2004).

Yeast induced hyperthermia in rats

Hyperthermia was induced in rats as described by Teotino et al. (1963). The rats were injected subcutaneously with 20% aqueous suspension of brewer's yeast at a dose of 10 ml.kg^{-1} body weight of animal and the rectal temperatures were recorded initially and at 18 hrs. The animals with an elevation of body temperature at 1 ºC were used for testing the effect of test extract, standard drug and saline control. The animals in group I were administered normal saline (5 ml.kg^{-1} body weight) orally. The animals in group II were administered the standard drug aspirin (400 mg.kg^{-1} body weight) orally and the animals in group III, IV and V were administered the ethanolic extract of *C. phlomidis* at 200, 400 and 800 mg.kg^{-1} body weight orally after 18 hrs of yeast injection when the temperature increases at its peak. The body temperature was measured at 1 hr intervals up to 3 hrs after administration of plant extracts (Brune and Alpermann, 1983).

Carrageenan-induced paw edema

The effect of ethanolic extract of *C. phlomidis* in inflammation was tested using a method described by Winter et al. (1962). Rats of either sex were divided into five groups of six animals each. The animals in group I were administered saline 5 ml.kg^{-1} body weight. The animals in group II were administered indomethacine (10 mg.kg^{-1} body weight) orally and the animals in group III, IV and V were administered the ethanolic extract of *C. phlomidis* at 200, 400 and 800 mg.kg^{-1} body weight orally 30 min before the subplanter injection of edematogenic agent. The administration of extracts, saline and drugs were 30 min prior to the injection of 0.1

ml of 1% carrageenan in saline into the sub-planter region of the right hind paw of each rat (Lanhers et al., 1991). The paw volume of the injected animal was measured using a plethysmograph (Ugo Basile, Italy) before and every 1 hr after the injection up to 6 hrs.

Statistical analysis

Data were presented as a mean ± S.E.M. Stastical difference between control and treated groups were tested by one way ANOVA followed by student's test. The differences were considered significant at $p<0.05$.

RESULTS

Acute toxicity

The leaf extract of *C. phlomidis* didn't show any mortality and toxicity even at highest dose of 2000 $mg.kg^{-1}$ body weight employed. The present research study was carried out using different doses of ethanolic extract of *C. phlomidis* such as 200, 400 and 800 $mg.kg^{-1}$ body weight for antinociceptive, antipyretic and anti-inflammatory study.

Antinociceptive effect

Acetic acid writhing reflex

C. phlomidis significantly reduced writhings and stretchings induced by 0.6% acetic acid at a dose of 10 ml kg^{-1}. The significant productive effect was dose dependent with 33.4% ($p<0.001$) reduction observed from 200 $mg.kg^{-1}$, 40.5% ($p<0.001$) reduction observed from 400 $mg.kg^{-1}$ and 34.1% reduction observed from 800 $mg.kg^{-1}$. Aspirin (100 $mg.kg^{-1}$) had only 23.2% ($p<0.001$). The results are shown in figure 1.

Tail-immersion method

The ethanolic extract of *C. phlomidis* at the doses of 200, 400 and 800 $mg.kg^{-1}$ body weight showed significant increase in antinociceptive activity at different time interval such as 0, 1, 2, 3, 4, 5 and 6 hrs, when compared to the effect produced by normal saline (5 $ml.kg^{-1}$ body weight). The antinociceptive activity produced by the extract of *C. phlomidis* is comparable with the effect produced by standard drug morphine. The results are shown in figure 2. The hot plate method values were insignificant (data not shown).

Yeast-induced hyperthermia in rats

The ethanolic extract of *C. phlomidis* at the doses of 200, 400 and 800 $mg.kg^{-1}$ body weights showed significant reductions in the rectal temperature of hyperthermic rats. The decrease in rectal temperature still existed when assessment was made 2 hrs and 3 hrs after test drug administration and efficacy was comparable to that of aspirin at a dose of 400 $mg.kg^{-1}$body weight. The results are shown in figure 3.

Carrageenan- induced paw edema

The paw volumes and percentages of inhibition by the extract and standard drugs are shown in figure 3. A maximum volume of edema, 1.64 ± 0.07 ml (59.22%) was obtained in 5 hrs. Rats pretreated with *C. phlomidis* significantly decreased the carrageenan-induced edema 30 min post dosing at all dose levels used. The extract was more potent at 200 $mg.kg^{-1}$as an anti-inflammatory agent than at 400 $mg.kg^{-1}$. The percentage inhibition for 200 $mg.kg^{-1}$ranged from 34.38–62.50% compared to 400 $mg.kg^{-1}$ (16.98–40.63%) within 5 hr. At 800 $mg.kg^{-1}$ (26.23%, $P<0.001$), the extract and indomethacin (40.68, $P<0.001$) attained their maximal protective effects within 5 hrs. Both the extract and indomethacin reduced the swellings. The results are shown in figure 4.

DISCUSSION

Induction of inflammation by carrageenan involves 3 distinct phases of mediator release. The first phase involves the release of histamine and serotonin and last between the first to the second hr, the second phase is the release of kinins lasting from the second to the third hr while the third phase involves the release of prostaglandins and lasts from the third to the fifth hr (Surender and Mafumdar, 1995). Thus, it can be inferred

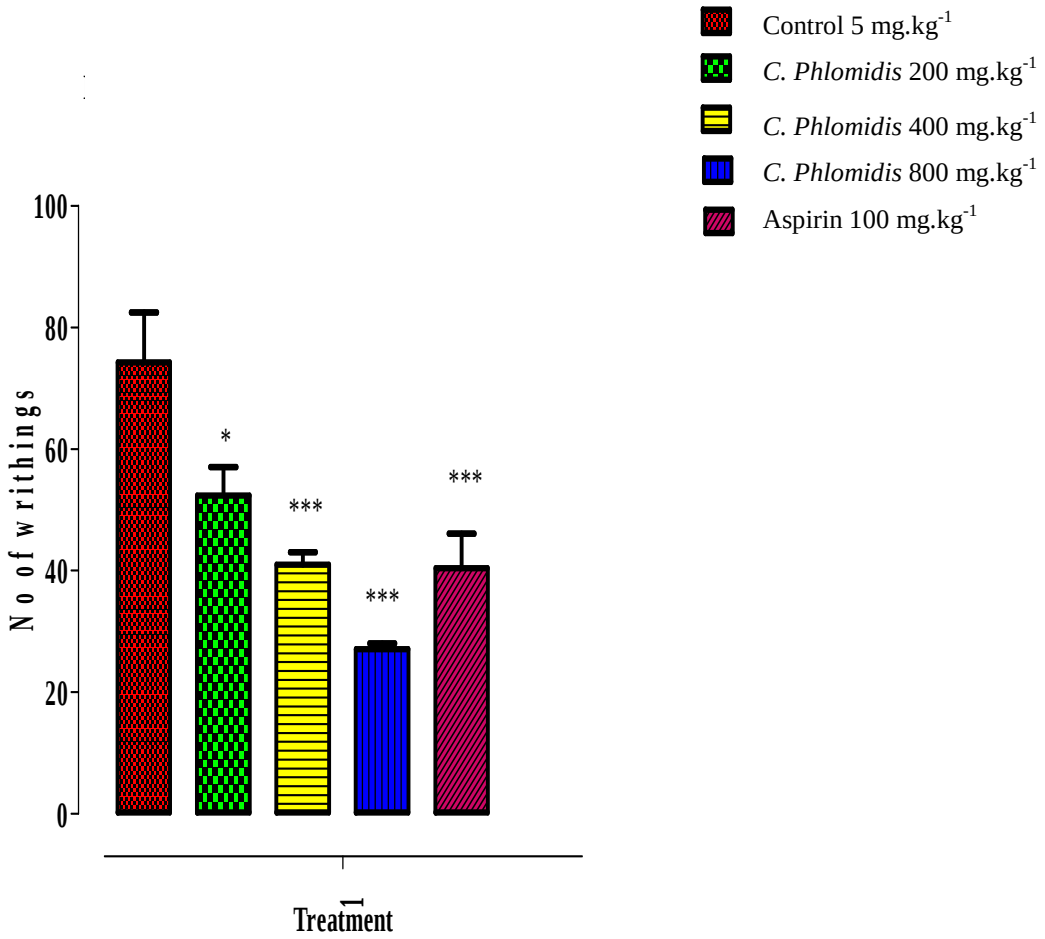

Figure 1: The antinociceptive effect of ethanolic extract of *C. Phlomidis* (200, 400 and 800 mg.kg^{-1} body weight), tested by acetic acid writhing test using mice. Aspirin (100 mg.kg^{-1}) was used as a standard drug. The control animal was given normal saline (5 ml.kg^{-1}). The antinociceptive effect was counted within 30 min. Each value represents mean ± S.E.M, n=6. The statistical analysis was carried out using one way ANOVA method, where *** P < 0.001.

that the mechanism through which the extract elicits its effects is via the inhibition of the synthesis of kinins and prostaglandins, since the extract was effective at these phases of mediator release. The extract's anti-inflammatory effect was dose dependent with 400 m/kg body weight dose giving the highest percentage inhibition. When compared with the standard reference drug, indomethacin (10 mg/kg) the percentage inhibition produced by the extract was 98% at the second hr, 94.8% at the third hr and 85% of that of indomethacin at the fifth hr (results not shown). Its effects were, however, higher at the 30th min, first and fourth hr as the percentage inhibition produced by the extract was 156.8, 112.6 and 105.6% that of indomethacin, respectively (result not shown). This data thus provides pharmacological basis for the use of this plant in the treatment of rheumatism and other ailments in which inflammation is implicated (Watt and Breyer-Bradwijk, 1962).

Inhibition of acetic acid-induced writhing in mice suggests that the analgesic effect of the extracts may be peripherally mediated via the inhibition of the synthesis and release of prostaglandins (Koster et al., 1959). Writhes can be described as a wave of constriction and elongation passing gradually along the abdominal wall with twisting of the trunk and extension of the hind limbs in mice. This is due to the nociceptive property of

Table 1: Inhibitory effects of the extract and indomethacine on carrageenan-induced paw oedema.

Treatment	Dosage (mg/kg)	Percentage inhibition					
		1hr	2	3	4	5	6
Indomethacine	10	21.87**	21.74**	40.68***	22.41**	39.34***	33.96**
Extract	200	62.50****	50.0***	42.44***	43.01***	54.10***	47.17***
Extract	400	28.12**	21.74**	18.64**	20.69**	39.34***	16.98*
Extract	800	9.38*	10.87*	18.64**	13.79*	26.23**	24.53**

Values are mean percentage inhibitions of oedema in both the extract and indomethacin treated groups (n=6 per group). *P < 0.05. ** P < 0.01. *** P < 0.001. **** P < 0.0001.

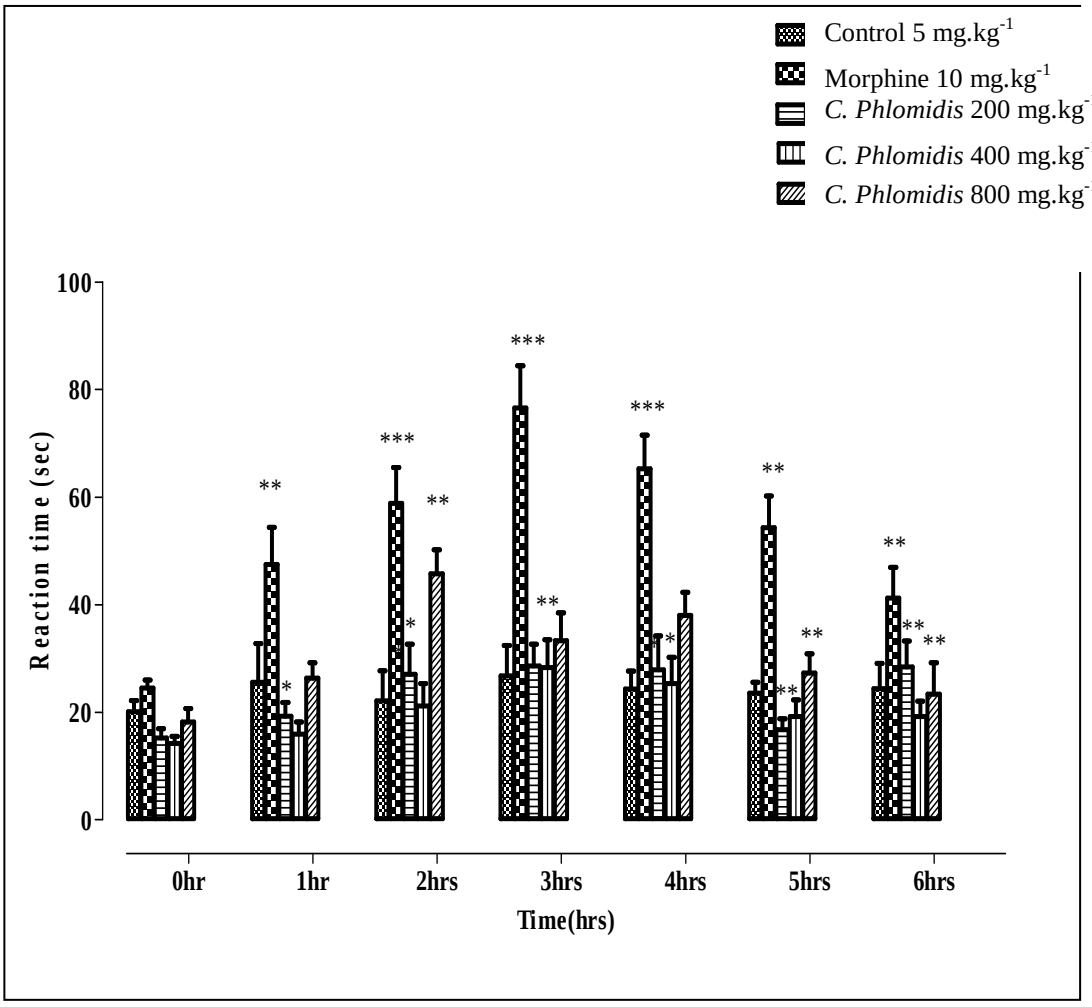

Figure 2: The antinociceptive effect of ethanolic extract of *C. phlomodis* (200, 400 and 800 mg.kg^{-1} body weight), tested by hot water tail-immersion method using mice. Morphine (10 mg.kg^{-1}) was used as a standard drug. The control animal was given normal saline (5 ml.kg^{-1}). The antinociceptive effect was tested at different time interval such as 0, 1, 2, 3, 4, 5 and 6 hrs. Each `value represents mean ± S.E.M, n=6. The statistical analysis was carried out using one way ANOVA method, where ***p < 0.001.

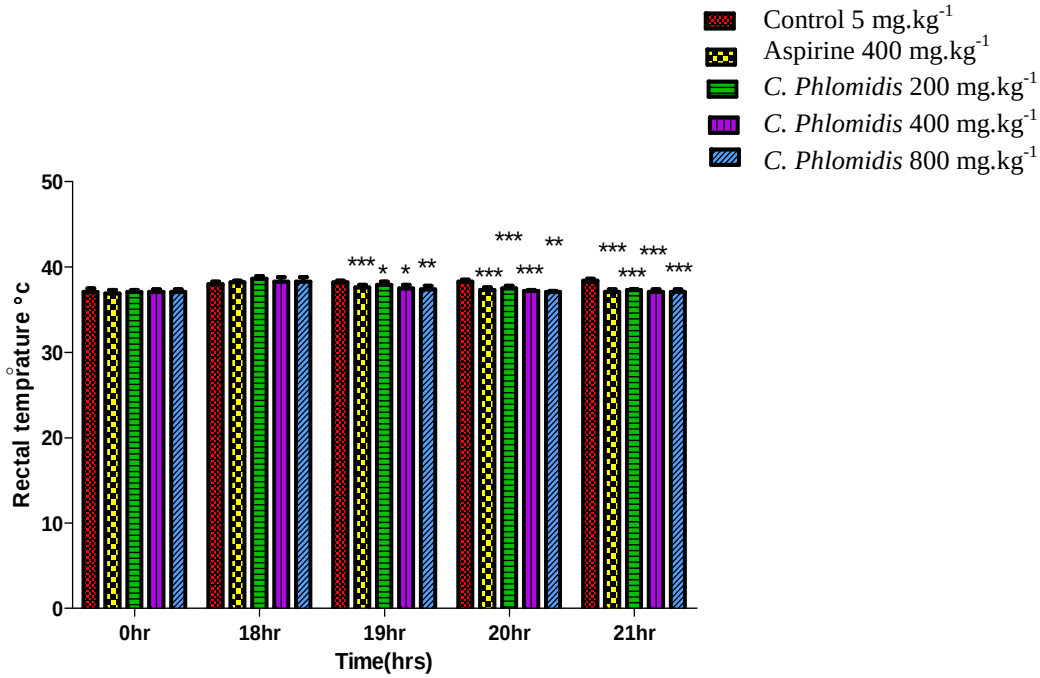

Figure 3: The antipyretic effect of ethanolic extract of *C. phlomidis* (200, 400 and 800 mg.kg^{-1} body weight), tested by brewer's yeast induced pyroxia in rats. Aspirin (400 mg.kg^{-1} body weight) was used as a standard drug. The control animal was given normal saline (5 ml.kg^{-1} body weight). The antipyretic effect was tested at different time interval such as 0 hr, 18 hrs, 19 hrs, 20 hrs and 21 hrs. Each value represents mean ± S.E.M, n=6. The statistical analysis was carried out using one way ANOVA method, where ***P < 0.001.

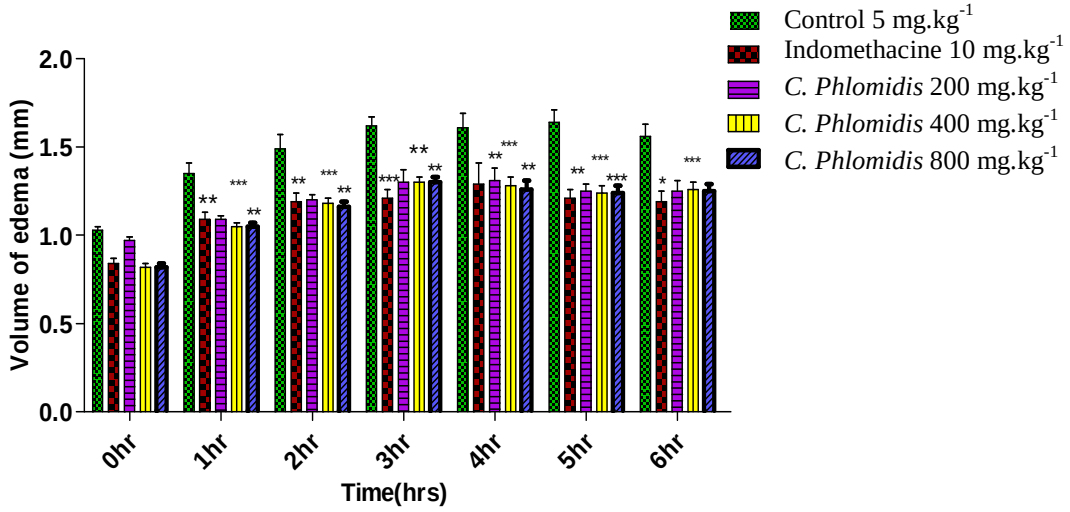

Figure 4: The anti-inflammatory effect of ethanolic extract of *C. phlomidis* (200, 400 and 800 mg.kg^{-1} body weight) against carrageenan-induced paw edema in rat. Indomethacine (10 mg.kg^{-1} body weight) was used as a standard drug. The control animal was given normal saline (5 mg.kg^{-1} body weight). The anti-inflammatory effect was tested at different time interval such as 1, 2, 3, 4, 5and 6 hrs. Each value represents mean ± S.E.M, n=6. The statistical analysis was carried out using one way ANOVA method, where ***P < 0.001.

acetic acid (Surender and Mafumdar, 1995). The percentage of inhibition, clearly shown in Table 1, also indicates that the extract at 400 and 800 $mg.kg^{-1}$ produced a higher inhibition when compared to aspirin (100 $mg.kg^{-1}$), a known standard analgesic drug. The inhibitory effect was 139 and 124% of the effect produced by aspirin at 200 and 400 mg doses, respectively (result not shown).The extract failed to increase mice reaction time on hot plate. The difference between the mean reaction time of the *C. phlomidis* treated groups and the control group was not statistically significant at all doses tested. Its effect was not comparable to morphine, which had a mean reaction time of above 2 min, which was the cut off point ($p<0.0001$).The analgesic effect of the leaf extract of *C. phlomodis* has not been previously reported and the mechanism by which it occurs is most likely via the inhibition of prostaglandin synthesis as indicated by the inhibition of acetic acid-induced mouse writhing. The ethanolic extract of *C. phlomidis* produces significant reduction on tail-immersion method. Also, it is known that centrally acting analgesic drugs elevate the pain threshold of mice towards heat and pressure (Adeyemi et al., 2003). From the above findings, the extract failed to raise the pain threshold on the hot plate, which indicates that it might not be centrally acting. The extract of *C. phlomidis* produces significant reduction on tail-immersion method and it seems to possess analgesic properties, which are mediated via peripheral inhibitory mechanisms.

The antipyretic activity of the extract was expected since from the anti-inflammatory and analgesic tests, the extract was consistently shown to act peripherally on inflammatory mediators especially prostaglandins. The blockade of phase 2 of formalin test was typical of substances, which antagonize cyclo-oxygenase - an enzyme which produces prostaglandins responsible for the genesis of fever (Brune and alpermann, 1983).

Conclusion

The ethanolic extract of *Clerodendrum phlomidis* has anti-inflammatory, peripheral analgesic and antipyretic properties on acute and possibly chronic inflammatory processes. The claim made by tradipractitioners (Adjanohoun et al., 1996) that *Clerodendrum phlomidis* can be used to treat various pains and inflammatory diseases is founded.

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