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Antinociceptive and anti-inflammatory activity of betulin isolated from *Clerodendrum phlomidis* in mice and rats

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ABSTRACT

The compound isolated from *Clerodendrum phlomidis* L. belonging to the family of Verbenaceae and the isolated active ingredient (botulin) wasevaluated for its antinociceptive, and anti-inflammatory activity in mice and rats respectively. Analgesic activity was studied by using acetic acid-induced mouse withering test and eddy's hot plate method in mice. The 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. The betulin showed a dose dependent significant reduction of the number of writhes ($P<0.05$) with 100 mg.kg-1 body weight dose giving the highest reduction. Betulin showed an significant reductionin the hot plate reaction time ($P<0.001$). In the carrageenan induced paw edema, a dose dependent significant inhibition was observed ($P<0.001$) between the 2nd and 5th 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 betulinhas 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.

Keywords: Antinociceptive, Anti-inflammatory, Isolated compound Betulin. Carrageenan, Morphin and Indomethacin.

1. INTRODUCTION

Inflammation is a complex pathophysiological process mediated by a variety of signaling molecules produced by leukocytes [1], especially macrophages [2-4] mast cells [5] 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 [6]. 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 immune modulation of essentially granulocytes, macrophages, and natural killer cellsand complement factors [7].

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 [8-10]. The leaves of the plant are used in inflammation. The decoction of the roots and leaves of the herb is used in rheumatism, antimicrobial [11], nervous diseases, convalescence of measles, piles, chronic bronchitis [12] etc. The present investigation was undertaken to study the antinociceptive, and anti-inflammatory activities of isolated compound Betulin from *C.phlomidis* in acute and chronic inflammations.

2. MATERIALS AND METHODS

2.1. 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 Vatharayanthattu, Cuddalore (Dist), Tamilnadu, India. The leaves were dried under shade, segregated, pulverized by a mechanical grinder and passed through 40 mesh sieves.

2.2. Preparation of extracts

The powdered leaves (500 g) were successively extracted with ethanol (70-80 ℃) 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.

2.3. Animals

The animals for the present study were procured after ethical clearance from the Institutional Animal Ethical Committee (IAEC) in Annamalai University, Annamalainagar, 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 (150-200g) were used for testing anti-inflammatory activity. The albino mice (20-25g) 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 conditionsof temperature (23 ± 1 °C), relative humidity(55 ± 1%), 12 hrs light and dark cycles andfed with standard pellet diet, and tap water *adlibitum.*

2.4. 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.

2.5. Preliminary phytochemical and compound isolation

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 [13]. The compound was isolated by column chromatography and TLC and these fractions subjected to characterized by using IR, Mass, NMR.

2.6. Antinociceptive activity

2.6.1. Acetic acid writhing reflex

This was performed according to [14]. Albino mice (six per group) were injected intra peritoneally with 0.6% acetic acid at the dose of 10 ml.kg-1. The isolated compound Betulin(100mg.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.

2.6.2. Hot plate method

The albino mice (20-25 g) were divided into four 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 and IV were administered with betulin at 100, 200 mg.kg-1 body weight orally. The hot plate was maintained at 55 ± 5 °C [15]. The response was recorded at different time intervals such as 0, 60,90,120,150 and 180 after administration of normal saline, standard drug and betulin to the corresponding animal group.

2.6.3. Carrageenan-induced paw edema

The isolated compound betulin from *C.phlomidis* (100, 200mg.kg-1)in inflammation was tested using a method described by Winter *et al*. [14]. Rats of either sex were divided into fourgroups of six animals each. The animals ingroup I were administered saline 5 ml.kg-1body weight. The animals in group II were administered with caragenan(0.1 ml) in hind paw. The animals in group III were administered indomethacine (10 mg.kg-1 bodyweight) orally and the animals in group IV and V were administered betulin at 100 and 200 mg.kg-1body weight orally 30 min before the subplanter injection of edematogenic agent. The administration of betulin, saline and drugs were 30 min prior to the injection of 0.1 ml of 1% carrageenan in saline into the subplanter region of the right hind paw of each rat [15]. 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 5 hrs.

2.6.4. 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 $p<0.05$.

3. RESULTS

3.1. Acute toxicity

The isolated compound betulin from *C. phlomidis* didn't show any mortality and toxicity even at highest dose of 2000 mg.kg-1 body weight employed from that we have fixed the dose 1/20th for the present work. The present research study was carried out using different doses of betulin such as 100 and 200 mg.kg-1 body weight for antinociceptive and anti-inflammatory study.

3.2. Antinociceptive effect

3.2.1. Acetic acid writhing reflex

Similarly for Betulin significantly reduced writhings and stretching induced by 0.6% acetic acid at doses of 10 ml. kg$^{-1.}$ and this effect was dose dependent. The percentage of writhings and stretchings was 22.66 at the dose of 100 mg.kg^{-1}body weight. The % reduction in activity observed with standard drug acetyl salicylic acid was 74.11 at a dose of 100 mg.kg^{-1}.The percentage inhibition was significant ($p<0.01$) at the dose of botulin (100 mg.kg^{-1}). The results are shown figure 1.

The betulin at the doses of 100 and 200 mg.kg^{-1} body weight showed significant reduction in antinociceptive activity at different time interval such as 60, 90, 120, 150 and 180 and the results are shown in figure 2.

3.2.2. Carrageenan- induced paw edema

The anti-inflammatory effect of betulin inhibited the inflammation induced by carrageenan and the results are shown in Fig.3. The inhibitory effect produced by the betulin and it was observed at the 3rd hour and the effect was maximum and lasted till 6th hour. The inhibitory effect of the betulin and indomethacin was significantly ($p<0.01$) at a dose level of 100 and 200 mg.kg^{-1} and standard drug indomethacin.

4. Discussion

Acetic acid induced writhing in mice caused due to visceral pain and more attention of screening analgesic drugs [16]. The isolated compound betulin showed significant analgesic activity compared to STD drug. In acetic acid induced writhing method Pain sensation is formed by triggering localized inflammatory response of releasing of free arachidonic acid from tissue phospholipid [17] through cyclooxygenase (COX), and prostaglandin biosynthesis [18]. In addition to these the acetic acid induced writhing has been associated with increased level of PGE2 and PGF2α in peritoneal fluids as well as lipoxygenase products [19].

Pain sensation in acetic acid induced writhing method is elicited by triggering localized inflammatory response resulting release of free arachidonic acid from tissue phospholipid [17] via cyclooxygenase (COX), and prostaglandin biosynthesis [18], other than that the acetic acid induced writhing has been associated with increased level of PGE2 and PGF2α in peritoneal fluids as well as lipoxygenase products [19]. The prostaglandin levels were increased within the peritoneal cavity then enhances inflammatory pain by increasing capillary permeability [20]. This method was found to be effective to evaluate peripherally. The significant pain reduction of betulin might be due to the presence of analgesic principles acting with the prostaglandin pathways. It was found that the observed analgesia inbetulin [21] and was isolated from the plant extract through a peripherally acting mechanism similar to the non-steroidal anti-inflammatory agents, such as aspirin and indomethacin.

The isolated compounds betulin and standard (10 mg/kg) also presented a longer latency time than the control group in the hot plate test in a dose related manner. The hot plat method is selective model for central analgesia. The hot plat test measures the complex response to a non-inflammatory and acute nociceptive [22]. Therefore, the active principle betulin is acting through centrally. Again, narcotic analgesics inhibit both central and peripheral mechanism of pain, while NSAIDs inhibit only peripheral pain [23]. The isolated compound produce both type of pain sensation. The analgesic effect of the isolated compounds produce in both the models suggests that they have been acting through central and peripheral mechanism [22].

The isolated compound betulinat the dose level of 100, 200 mg.kg^{-1} was evaluated for its anti-inflammatory activity in acute animal model using rats. The induction of paw edema by carrageenan is an biphasic event [24]. Inflammation involves three distinct phases of mediator release, observed during the first hour is attributed to the release of histamine and serotonin [25]. The first phase is observed during the first hour is attributed to the release of histamine and serotonin. In the second phase is due to the release of prostaglandins, protease and lysosome and the third one by granuloma formation. We

observed that at the given dose 100, 200 betulinpossess the significant inhibition against carrageenan induced paw edema in rats. This anti-inflammatory effect may be due to the presence of flavonoids and terpenes. It has been reported that a number of flavonoids and terpenes possess anti-inflammatory [26] and analgesic [27] activities. Flavonoids are known to inhibit the enzyme prostaglandin synthesis, more specifically the endoperoxidase [28] and reported to produce anti-inflammatory and analgesic effects [29].

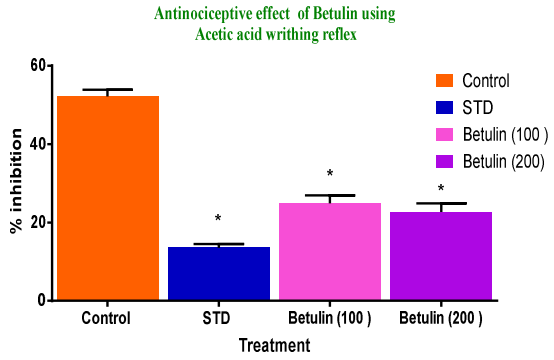

Figure - 1: The antinociceptive effect betulin.

Figure 1 the antinociceptive effect betulin(100 and 200 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. Eachvalue represents mean ± S.E.M, n=6. The statistical analysis was carried out using one way ANOVA method, where * P <0.5.

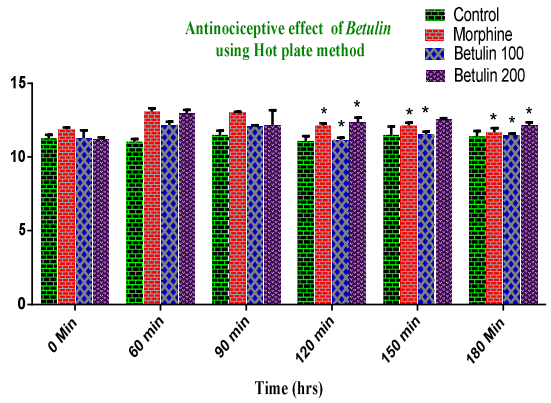

Figure - 2: the antinociceptive effect of betulin.

Figure 2 the antinociceptive effect of betulin(100 and 200 mg.kg-1body weight) tested by hot plate method using mice. Morphine (10 mg.kg-1) was used as astandard drug. The control animal was given normal saline (5 ml.kg-1). The antinociceptive effect was tested at different timeinterval such as 0, 1, 2, 3, 4, and 5 hrs. Each `value represents mean ± S.E.M, n=6. The statistical analysis was carried outusing one way ANOVA method, where *p < 0.001.

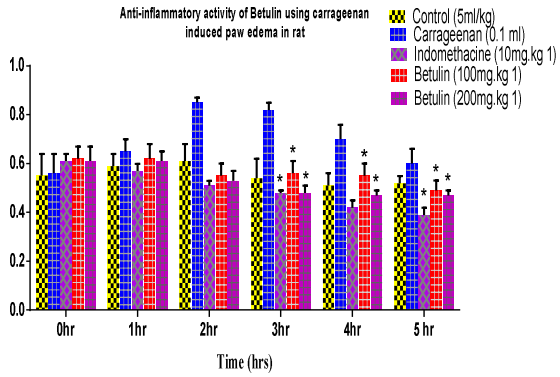

Figure - 3: The anti-inflammatory effect of betulin.

Figure 3 The anti-inflammatory effect of betulin(100 and 200mg.kg-1 body weight) against carrageenan-induced paw edema in rat. Indomethacine (10 mg.kg-1 bodyweight) 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 and 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.

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