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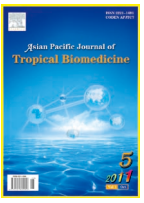

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Anti-anaphylactic and anti-inflammatory activities of a bioactive alkaloid from the root bark of *Plumeria acutifolia* Poir

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ABSTRACT

Objective: To investigate the anti-anaphylactic, anti-inflammatory and membrane stabilizing properties of plumerianine (compound 1) isolated from the root bark of *Plumeria acutifolia* Poir. **Methods:** The anti-anaphylactic activity of compound 1 (10, 25 and 50 mg/kg) was studied by using models such as passive cutaneous anaphylaxis, passive paw anaphylaxis and its anti-inflammatory activity against carrageenin induced paw edema and cotton pellet granuloma in albino rats was also investigated using ketotifen and indomethacin as reference drugs. **Results:** A dose-dependent beneficial effect was observed on leakage of evans blue dye in skin challenged with antigen and on paw anaphylaxis induced by antiserum. The compound 1 also exhibited significant ($P<0.01$) inhibition of rat paw edema and granuloma tissue formation, including significant protection of RBC against the haemolytic effect of hypotonic solution, an indication of membrane-stabilizing activity. **Conclusions:** Anti-anaphylactic activity of compound 1 may be possibly due to inhibition of the release of various inflammatory mediators. Anti-inflammatory activity of compound may be related to the inhibition of the early phase and late phase of inflammatory events.

1. Introduction

Allergic disorders are in rise every year and stated as an endemic disease of the 21st century. Some of the allergic disorders, which may be caused by an allergen originating from immune system, environment, and by genes, are,asthma, eczema, hay fever, anaphylaxis, autoimmune disorders [1]. A number of plants are described in Ayurveda for use in the treatment of allergic disorders, namely psoriasis, eczema, bronchial asthma, *etc*. But only a few have been studied for their antiallergic activity. On activation, mast cells immediately released the preformed and the de novo synthesized mediators such as histamine, proteases, leukotrienes, prostaglandins, and cytokines[2]. As a consequence, the acute reactions such as vasodilation, increased vascular permeability, and bronchoconstriction were induced. In addition, allergic responses also trigger the influx and activation of a variety of inflammatory cells including eosinophils and lymphocytes. Rapidly released mediators and numerous cytokines produced by mast cells are strongly believed to induce and sustain these responses, which may contribute to chronic inflammation.

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Inflammation is a normal protective response to tissue injury caused by physical trauma, noxious chemicals or microbiological agents. Inflammation is body's response to inactivate or destroy the invading organisms, remove irritants and set stage for tissue repair[3]. Inflammation is triggered by the release of chemical mediators from the injured tissues and migrating cells. The specific chemical mediators vary with the type of inflammatory process and include amines such as histamine, serotonin, lipids such as prostaglandins and small peptides such as kinins[4].

Conventional anti-inflammatory drugs such as steroidal anti-inflammatory drugs (SAID) and nonsteroidal anti-inflammatory drugs (NSAID) are used in the treatment of most of the acute and chronic pain and inflammatory disorders including rheumatoid arthritis. However, long-term use of these agents may produce serious adverse effects. Thus, it is worth developing new plant-derived anti-inflammatory agents with fewer adverse effects. *Plumeria acutifolia* (*P. acutifolia*) is one such plant, which is reputed to have numerous applications in traditional medicine. The plant has been mentioned in ancient literature as anti-inflammatory, ant-allergic, diuretic, carminative, laxative, anti-ulcer, and useful in treating leprosy and ascites, and also possesses cytotoxic activity and anti-microbial activity[5]. Earlier phytochemical studies on the root bark had shown the presence of iridiods, tannins and alkaloids[6]. In continuation of our studies on medicinal

plants for their chemical constituents and biological activities, we isolated plumerianine (compound 1) from the root bark of *P. acutifolia* Poir. In the present paper we report the anti-anaphylactic, anti-inflammatory and membrane stabilizing activities of the isolated compound.

2. Materials and Methods

2.1. Plant materials

The plant specimen for the proposed study was collected from Melmaruvattur Chennai, Tamil Nadu. It was identified and authenticated by Dr. P Jayaraman, Director, Plant Anatomy Research Center, (PARC) Tambaram, Chennai. A voucher specimen (accession No. 31238) was deposited in the Herbarium for future reference.

2.2. Extraction and isolation of alkaloid

The coarsely powdered root bark of *P. acutifolia* (2 kg) were exhaustively extracted with methanol (5×1 L) at room temperature. The methanol extract (40 g) was acidified (pH 2) with 2 M hydrochloric acid and the final volume was adjusted to 400 mL. The aqueous acidic solution was then extracted with ethyl acetate (3×200 mL) to remove neutral components. After removal of neutral components the aqueous layer was then made alkaline (pH 9) with 30% ammonium hydroxide solution and repeatedly extracted with chloroform (3×300 mL). The combined extracts were evaporated under vacumm to yield the crude alkaloid (5.4 g). The crude alkaloid was chromatographed over silica gel (60-120 mesh size) and eluted with solvents of increasing polarity *viz.*, hexane, chloroform and methanol. A total of 150 fractions, 25 mL each, were sampled and their homogeneity determined by TLC, using solvent system (4:4:12) toluene, chloroform, ethanol.

Compound 1 (320 mg) was isolated from chloroform (60%), methanol (40%) fraction as yellow amorphous powder that gave positive test with the Dragendorff reagent, R_f 0.874. The compound 1 was characterized and identified by analyzing its spectral data. The 1H NMR spectrum showed the presence of protons between 7.4 and 8.6 ppm, due to the presence of an aromatic moiety or double bonds. The two protons with a chemical shift higher than 8 ppm suggested a pyridine ring, unsubstituted in both positions ortho of the nitrogen atom. This was in agreement with the ^{13}C NMR spectra, where two CH signals at 150.85 and 145.65 ppm were present. In addition, the typical ^{13}C signals for an α,β unsaturated spirolactone moiety were observed. Other structural features, evident from ^{1}H and 13C NMR spectra, were a $-CH_2-CH_2-$ moiety, and a $CH_3-CH-OH$ moiety. Complete assignments based on the two-dimensional NMR spectra were listed in Table 1. The ESI-mass spectrum recorded in the positive ion mode showed an $[M+H]^+$ peak at *m/z* = 232 and an $[M+Na]^+$ peak at *m/z* = 254, which were in agreement with the expected molecular weight of 231 ($C_{13}H_{13}NO_3$).

2.3. Animals

Wistar rats (150-200 g) and Swiss albino mice (18-25 g) of either sex obtained from the Laboratory Animals Center, Vels University, were used for various studies. They were kept in a well-ventilated environment, had free access to food and water ad libitum and kept in the laboratory environment (12 h dark/12 h light cycle) for seven days for acclimatization. Animals were fasted overnight and weighed before the experiment.

2.4. Acute toxicity study

Acute toxicity study-up and down procedure was carried out as per the guidelines by Organization for Economic Co-operation and Development (OECD) 423[7]. Mice (6/group) were divided into six groups. The first 5 groups received oral doses of 100, 200, 300, 400 and 500 mg/kg of isolated compound 1. The sixth group received saline (10 mL/kg) orally. Mortality was assessed 24 h after administration. The animals were also observed for toxic symptoms and mortality was determined 24 h after treatment.

2.5. Anti-allergic activity

2.5.1. Study on passive cutaneous anaphylaxis[8]

The Wistar rats of either sex were injected intraperitoneally with 0.2 mL, 10% egg albumin, 0.2 mL of bordetella pertusis vaccine on day 1, 3, and 5. After 21 days of first immunization, blood was collected from orbital plexus under light ether anesthesia. The blood was allowed to clot and serum was separated by centrifugation at 1 500 rpm. The separated serum was stored at 20 ℃ until it was used for the experiment.

Rats (6 per group) were divided into five groups. The first 3 groups received oral doses of 10, 25 and 50 mg/kg of the compound 1. The 4th and 5th groups were treated orally with ketotifen (5 mg/kg *p.o.*) as a reference drug and saline (10 mL/kg) as control, respectively.

The anti ovalbumin serum was injected intradermally on the dorsal skin of the animal. Drug/extract was administered to animal according to their group for three consecutive days from the day of sensitization. After treatment, 1 mL of 0.5% evans blue solution containing 20 mg of egg albumin was injected intravenously through tail vein. Because of antigen-antibody reaction there was increased vascular permeability and dye would penetrate that tissue area. This area of skin was removed after sacrificed. The skin portion was transferred to the solution of 70% acetone for 24 h. The dye was extract out in the acetone and evans blue dye was measured colorimetrically at 620 nm. The amount of dye penetrating in the skin area reflects the severity of hypersensitivity reaction. The % inhibition was calculated by using the formula: (C-T/C)×100.

2.5.2. Study on passive paw anaphylaxis[9]

The Wistar rats of either sex were injected intraperitoneally with 0.2 mL, 10% egg albumin, 0.2 mL of bordetella pertusis vaccine on day 1, 3, and 5. After 21 days of first immunization, blood was collected from orbital plexus under light ether anesthesia. The blood was allowed to clot and serum was separated by centrifugation at 1 500 rpm. The separated serum was stored at 20 ℃ until it was used for the experiment.

Rats (6 per group) were divided into five groups. The first 3 groups received oral doses of 10, 25 and 50 mg/kg of the compound 1. The 4th and 5th groups were treated orally with indomethacin (10 mg/kg) as a reference drug and saline (10

mL/kg) as control, respectively. The animals were dosed for seven consecutive days.

2 h after last dose of drug administration (on seventh day), rats were passively sensitized into left hind paw with 0.1 mL of the undiluted serum. The contralateral paw received an equal volume of saline. 24 h after sensitization, the rats were challenged in the left hind paw with 10 mg of egg albumin in 0.1 mL saline. The hind paw volume was measured after 30 min by volume displacement method using mercury column plethysmometer. The % inhibition was calculated by using the formula: (C-T/C)×100.

2.6. Anti-inflammatory activity

2.6.1. Acute model[10]

The anti-inflammatory activity was measured by using carrageenan-induced rat paw edema model. Rats (6 per group) were divided into five groups. The first 3 groups received oral doses of 10, 25 and 50 mg/kg of the compound 1. The 4th and 5th groups were treated orally with indomethacin (10 mg/kg) as a reference drug and saline (10 mL/kg) as control, respectively. Acute inflammation was produced by subplantar injection of 0.1 mL of 1% suspension of carrageenan in normal saline, in the right hind paw of the rats. 1 h later, they were oral administrated of the test compound 1 (10, 25 and 50 mg/kg. *p.o.*) and indomethacin at a dose of 10 mg/kg, *p.o.* used as the standard anti-inflammatory drug. The paw volume was measured plethysmometrically at 1, 2, and 3 h after the carrageenan injection. Results were expressed as percentage of inhibition of edema and calculated by the formula (1-Vt /Vc) × 100 where Vt and Vc are the mean paw volume in the treated and controlled groups, respectively.

2.6.2.Chronic model[11]

Chronic inflammation was induced by cotton pellet granuloma. Rats (6 per group) were divided into five groups. The first 3 groups received oral doses of 10, 25 and 50 mg/kg of the compound 1. The 4th and 5th groups were treated orally with indomethacin (10 mg/kg) as a reference drug and saline (10 mL/kg) as control respectively. Autoclaved cotton pellet (50±1) mg was implanted subcutaneously by making incision in the axilla and groin region of each rat under ether anesthesia. Extracts/Drugs were administered orally for 7 consecutive days from the day of cotton pellet implantation. Animals were sacrificed on the 8th day and the granuloma was dissected out, dried in an oven at 60 ℃ for 24 h and weighed. The increment in the dry weight of the pellet was taken as a measure of granuloma formation. The percentage of inhibition of granuloma was determined using the formula: (1-Wt /Wc) × 100. Here Wt means dry weight of the cotton in test animals and Wc means dry weight of the cotton in control animals.

2.7. Membrane stabilizing activity[12]

2.7.1. Preparation of erythrocyte suspension

Whole blood was obtained with heparinized syringes from rats through cardiac puncture. The blood was washed three times with isotonic buffered solution (154 mM NaCl) in 10 mM sodium phosphate buffer (pH 7.4). The blood was centrifuged each time for 10 min at 3 000 rpm.

2.7.2. Hypotonic solution-induced rat erythrocyte haemolysis

Membrane stabilizing activity of the extract was assessed using hypotonic solution-induced rat erythrocyte haemolysis. The test sample consisted of stock erythrocyte (RBC) suspension (0.5 mL) mixed with 5 mL of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffered saline (pH 7.4) containing the compound 1 (10, 25 and 50 mg/mL) or indomethacin (0.1 mg/mL). The control sample consisted of 0.5 mL of RBC mixed with hypotonic-buffered saline solution alone. The mixtures were incubated for 10 min at room temperature and centrifuged for 10 min at 3 000 rpm and the absorbance of the supernatant was measured at 540 nm. The percentage inhibition of haemolysis or membrane stabilization was calculated by the formula: 100 × (OD1-OD2/OD1).

Here, OD1 = Optical density of hypotonic-buffered saline solution alone;

OD_2 = Optical density of test sample in hypotonic solution.

2.8. Statistical analysis

The results were analyzed for statistical significance using one-way analysis of variance (ANOVA) followed by Dunnett's test. Values with $P<0.05$ were considered significant.

3. Results

3.1. Plumerianine

Compound 1 was isolated as amorphous yellow powder and showed a molecular ion peak at *m/z* = 232 ($[M^+H]^+$) and 254 ($[M^+Na]^+$), which were in agreement with the expected molecular weight of 231 ($C_{13}H_{13}NO_3$). 1H-NMR and ^{13}C-NMR spectra of compound showed characteristics of a lupine alkaloid plumerianine (Figure 1).

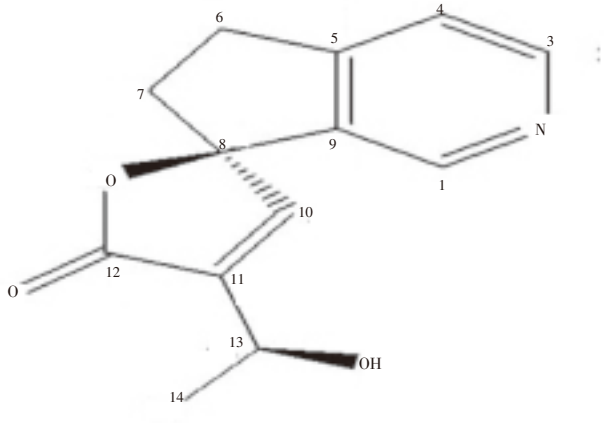

Figure 1. Structure of plumerianine.
{(R)-4′-[(S)-1 hydroxy ethyl)-5,6-dihydro-5′H-spiro [cyclopenta [c] pyridine-7,2′furan]-5′-one}, [α]^{25}D: +18 (c 0.18, CHCl3); ^{1}H- NMR and 13C -NMR: given in Table 1; ESI-MS: *m/z* = 254 ($[M^+Na]^+$), 232 ($[M^+H]^+$).

3.2. Acute toxicity studies

Acute toxicity study showed that the compound 1 possessed high safety profile as no death was observed at

oral doses of 100–500 mg/kg in mice.

3.3. Anti-anaphylactic activity

In the study on passive cutaneous anaphylaxis model, compound 1 produced a significant dose dependent decrease in the amount of evans blue dye leaked at site when compared with control. Standard drug also produced significant decrease in the amount of evans blue dye leaked at site. In passive paw anaphylaxis model, compound 1 produced a significant dose dependent decrease in the paw volume induced by antiserum (Figure 2 and 3).

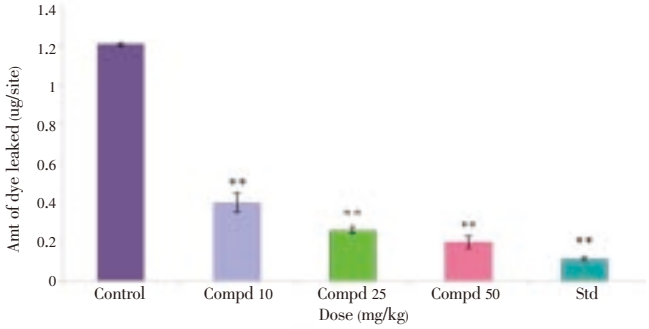

Figure 2. Study of compound 1 on passive cutaneous anaphylaxis. Std: ketotifen at 5 mg/kg p.o. as reference drug. Values are mean ±SEM of 6 parallel measurements. Statistical significant test for comparison was done by ANOVA, followed by Dunnet's '*t*' test (*n*=6). All the values are significant. ***P*<0.01 when compared against control.

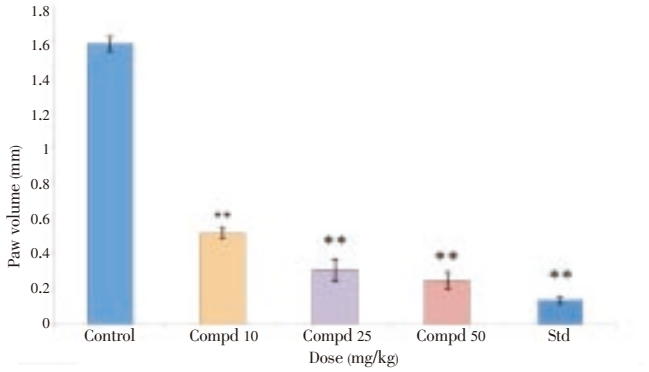

Figure 3. Study of compound 1 on passive paw anaphylaxis. Std: indomethacin at 10 mg/kg as reference drug. Values are mean ±SEM of 6 parallel measurements. Statistical significant test for comparison was done by ANOVA, followed by Dunnet's '*t*' test (*n*=6). All the values are significant. ***P*<0.01 when compared against control.

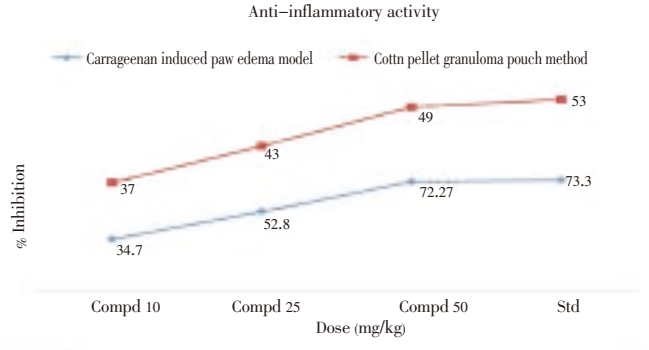

Figure 4. Effects of compound 1 on carrageenan induced paw edema and cotton pellet granuloma pouch method.

3.4. Anti-inflammatory activity

In acute model of inflammation (carrageenan induced), paw edema volume was inhibited by the compound 1 (10, 25 and 50 mg/kg p.o.) dose-dependently. As shown in Figure 4, the compound 1 (50 mg/kg) reduced the oedema swellings by 72.27% as compared with 73.33% reduction produced by indomethacin (10 mg/kg, p.o.) at 3rd h of carrageenan administration. In chronic model of inflammation, compound 1 at a dose of 10, 25 and 50 mg/kg exhibited significant (P<0.01) reduction in granuloma weight by 37%, 43%, 49%, respectively. These results were comparable with that of the standard drug (Figure 4).

3.5. Effect on erythrocyte membrane stability

The compound 1 (at concentration range of 0.10–0.50 mg/mL) significantly protect the rat erythrocyte membrane against lysis induced by hypotonic solution. At a concentration of 0.50 mg/mL, the extract produced 47.22% inhibition of RBC haemolysis as compared with 50% produced by indomethacin (Figure 5).

Table 1
^{1}H (400 MHz) and 13C NMR (100 MHz) assignments of plumerianine.

Carbon No	^{13}C NMR [δ (ppm)]		^{1}H NMR [δ (ppm), mult., J (Hz)]	
	Major	Minor		
1	145.65	145.53	8.22, s	8.27, s
3	150.85	150.89	8.49, d, 5.0	8.49, d, 5.0
4	122.44	122.44	7.47, m*	7.47, m*
5	157.03	157.03	–	–
6	31.09	31.12	3.25, m/	3.25, m/
			3.15,m/	3.15,m/
7	36.76	36.76	2.62,m/	2.62,m/
			2.42,m	2.42,m
8	94.90	94.85	–	–
9	137.84	137.91	–	–
10	150.15	150.15	7.47, m*	7.47, m*
11	139.88	139.90	–	–
12	172.85	172.91	–	–
13	63.73	63.61	4.65, m	4.65, m
14	22.44	22.26	1.49,d,6.6	1.45,d,6.6

* Overlapping signals.

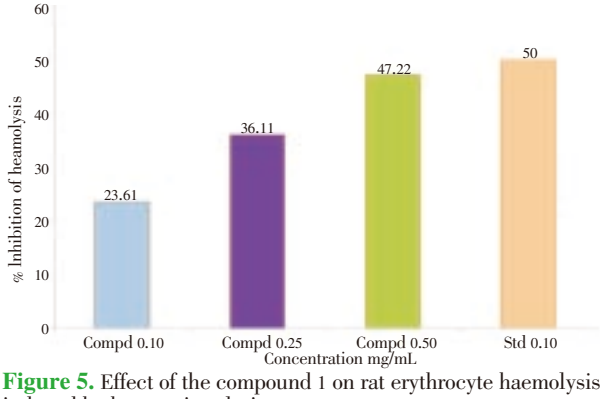

Figure 5. Effect of the compound 1 on rat erythrocyte haemolysis induced by hypotonic solution.
Std: indomethacin.

4. Discussion

The present study was undertaken for the evaluation of anti-anaphylactic activity and anti-inflammatory property of a compound isolated from the root bark of *P. acutifolia*. The anti-anaphylactic activity was done using passive cutaneous anaphylaxis for evaluation of compound plumerianine on immediate hypersensitivity reaction. Mediators like leukotriene, prostaglandins, PAF and cytokines are reported to be responsible for the immediate hypersensitivity reaction. And enhanced vascular permeability and leukocyte infiltration at the sites of allergen challenge were observed. In our model antiovalbumin serum obtained from sensitized rats was injected to the rats. The enhanced vascular permeability was estimated by evans blue dye. The leakage of dye was significantly less in the rats treated with compound plumerianine (10, 25 and 50 mg/kg) than the control animals. This activity can partly due to inhibition of leukotriene synthesis. Passive paw anaphylaxis is another *in vivo* model for IgE-mediated immediate hypersensitivity reactions[13]. A prominent inhibitory effect of plumerianine is suggestive of its antianaphylactic activity in both models.

The results of the study showed that the compound plumerianine possessed anti-inflammatory property, as it significantly inhibited oedema induced by carrageenin, granuloma tissue formation in rats. And it also showed significant protection of the erythrocyte against lysis induced by hypotonic solution.

The inflammatory condition induced by carrageenin involves step-wise release of vasoactive substances such as histamine, bradykinin and serotonin in the early phase and prostaglandins in the acute late phase. These chemical substances produced increase in vascular permeability, thereby promoting accumulation of fluid in tissues that accounts for the oedema[14]. The cotton pellet method is widely used to evaluate the transudative and proliferative components of the chronic inflammation. The dry weight of the pellets correlates with the amount of the granulomatous tissue[15,16]. Administration of compound 1 (10, 25 and 50 mg/kg bw) appear to be effective in inhibiting the dry weight of the cotton pellet that was almost comparable to that of indomethacin (Figure 4). These data support the hypothesis of the greater effect of the compound 1 on the inflammation in rats. This effect may be due to the cellular migration to injured sites and accumulation of collagen and mucopolysaccharides.

The compounds with membrane-stabilizing properties are well known for their ability to interfere with the early phase of inflammatory reactions, namely the prevention of the release of phospholipases that trigger the formation of inflammatory mediators[17]. The compound 1 demonstrate significant membrane stabilizing property, which suggests that its anti-inflammatory activity observed in this study, may be related to the inhibition of the late phase of inflammatory events, namely the release of chemical mediators.

Thus, the results of the present study demonstrate that the alkaloid plumerianine has significant anti-anaphylactic and anti-inflammatory activities. However a more extensive study is necessary to determine the exact mechanism(s) of action.

Conflict of interest statement

We declare that we have no conflict of interest.

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