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Anti-anaphylactic and Anti-inflammatory Activities of AFPA from the Root Bark of *Plumeria acutifolia* Poir.

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ABSTRACT

In the present study, anti-anaphylactic, anti-inflammatory and membrane stabilizing property of alkaloid rich fraction from the root bark of *Plumeria acutifolia* (AFPA) were investigated in animal models. The anti-anaphylactic activity of AFPA (100, 250 and 500 mg/kg) was studied by using models such as passive cutaneous anaphylaxis, passive paw anaphylaxis and also investigated for its anti-inflammatory activity against carrageenan induced paw edema and cotton pellet granuloma in albino rats. 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 alkaloid rich fraction 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. Anti-anaphylactic activity of AFPA may be possibly due to inhibition of the release of various inflammatory mediators. Anti-inflammatory activity of AFPA may be related to the inhibition of the early phase and late phase of inflammatory events.

Keywords: *Plumeria acutifolia,* Alkaloid, Anti-anaphylactic, Anti-inflammatory

INTRODUCTION

Allergic disorders are in rise every year and are 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]. The number of plants are described in Ayurveda for use in the treatment of allergic disorders, namely psoriasis, eczema, bronchial asthma, etc. Only a few have been studied for their antiallergic activity, which was not studied earlier. On activation, mast cells released immediately 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. 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].

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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* 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, also possess cytotoxic activity and anti-microbial activity. Earlier phytochemical studies on the root bark had shown the presence of iridiods, tannins and alkaloids[5]. One of the largest groups of arsenals produced by plants is the alkaloids. Alkaloids are known by their anti-cholinergic, anti-inflammatory and anti-histamine activities and inhibiting

pro-inflammatory cytokine production and their receptors**.** In addition, alkaloids have been shown to affect many enzyme systems involved in allergic and inflammatory responses such as tyrosine and serine-threonine protein kinases, phospholipases A2, phospholipase C, and lipoxygenase[6]. In continuation of our studies on medicinal plants for their chemical constituents and biological activities, AFPA from the root bark of *Plumeria acutifolia* Poir., was evaluated for anti-anaphylactic, anti-inflammatory and membrane stabilizing activities.

MATERIALS AND METHODS

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, Vels University, for future reference.

Determination of Total Alkaloids

10 gms of powdered crude drug was macerated with 50 ml of 2% acetic acid solution for two days and filtered through muslin cloth. The filtrate was added with ammonia solution, extracted with chloroform, the chloroform layer was collected washed with water, chloroform layer was dried over anhydrous sodium sulphite then the chloroform layer was distilled to get the residue. The amount of residue was weighed to calculate the amount of total alkaloid[7].

Extraction of alkaloid rich fraction

The coarsely powdered root bark of *Plumeria acutifolia* (2 kg) was exhaustively extracted with methanol (5×1 L) at room temperature. The methanol extract (40 g) was acidified (pH 2) with 2M hydrochloric acid and the final volume was adjusted to 400 mL. The aqueous acidic solution of methanol extract 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 alkaloid rich fraction.

Thin layer chromatography

A number of developing solvent systems were tried for fractions showing presences of alkaloid, but the satisfactory resolution was obtained in the solvent systems Toluene: Chloroform: Ethanol (2:2:6) and the plates were dried and detected by spraying with Dragendorffs reagent, a reagent specific for alkaloids.

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 the present study. 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.

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[8]. Mice were divided into six groups. The first 5 groups received oral doses of 1000, 2000, 3000, 4000 and 5000 mg/kg of AFPA. The sixth group received saline (10 ml/kg) orally. Mortality was assessed 24 hours after administration. The animals were also observed for toxic symptoms.

Anti-allergic activity

Preparation of antiserum from rats

The wistar rats of either sex were injected intraperitoneally with 0.2 ml, 10 % egg albumin and 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 1500 rpm. The separated serum was stored at 20 °C until it was used for the experiment.

1. Study on passive cutaneous anaphylaxis[9]

Rats (6 per group) were divided into five groups. The first 3 groups received oral doses of 100, 250 and 500 mg/kg of the AFPA. 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. The AFPA 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 will penetrate in that tissue area. This area of skin was removed after sacrificed. The skin portion was transferred to the solution of 70% acetone for 24hrs. The dye was extract out in the acetone and evans blue dye was measured colorimetrically at 620 nm. The amount of dye penetrate in

the skin area reflects the severity of hypersensitivity reaction.

The % inhibition was calculated by using the formula, (C-T/C)×100

2. Passive paw anaphylaxis[10]

Rats (6 per group) were divided into five groups. The first 3 groups received oral doses of 100, 250 and 500 mg/kg of the AFPA. 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.

Two hours 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 hours 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 minutes by volume displacement method using mercury column plethysmometer. The % inhibition was calculated by using the formula, (C-T/C)×100.

Results were presented in **Table. 2 & 3.**

Anti-inflammatory activity

(a) Acute model[11]

Carrageenan induced rat paw edema*:* 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 100, 250 and 500 mg/kg of the AFPA. 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 after oral administration of the AFPA suspended in 2% carboxy methyl cellulose (100, 250 and 500 mg/kg. p.o.), indomethacin at a dose of 10 mg/kg, p.o. was 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, shown in **Table. 4,** calculated by the formula $(1 - Vt/Vc) \times 100$ where Vt and Vc are the mean paw volume in the treated and controlled groups, respectively.

(b) Chronic model

Cotton pellet granuloma pouch method[12]

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 100, 250 and 500 mg/kg of the AFPA. 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. Drugs were administered orally for 7 consecutive days from the day of cotton pellet implantation. Animals were sacrificed on day 8th and the granuloma was dissected out, dried in an oven at 60° C for 24 hours 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 shown in **Table. 5,** determined using the formula $(1 - Wt/Wc) \times 100$ where Wt – Dry weight of the cotton in test animals and Wc - Dry weight of the cotton in control animals.

Membrane stabilizing activity[13]

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 minutes at 3000 g.

Hypotonic solution-induced rat erythrocyte haemolysis: Membrane stabilizing activity of the AFPA was assessed using hypotonic solution-induced rat erythrocyte haemolysis. The test sample consisted of stock erythrocyte (RBC) suspension (0.50 ml) mixed with 5 ml of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffered saline (pH 7.4) containing the AFPA (100, 250 and 500 µg/ml) or indomethacin (100, 250 and 500 µg/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 3000 g and the absorbance of the supernatant was measured at 540 nm. The percentage inhibition of haemolysis or membrane stabilization were presented in **Table. 6,** calculated by the formula $100 \times (OD_1 - OD_2/OD_1)$

Where, OD_1 = *Optical density of hypotonic-buffered saline solution alone*

OD_2 = *Optical density of test sample in hypotonic solution*

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.

RESULTS

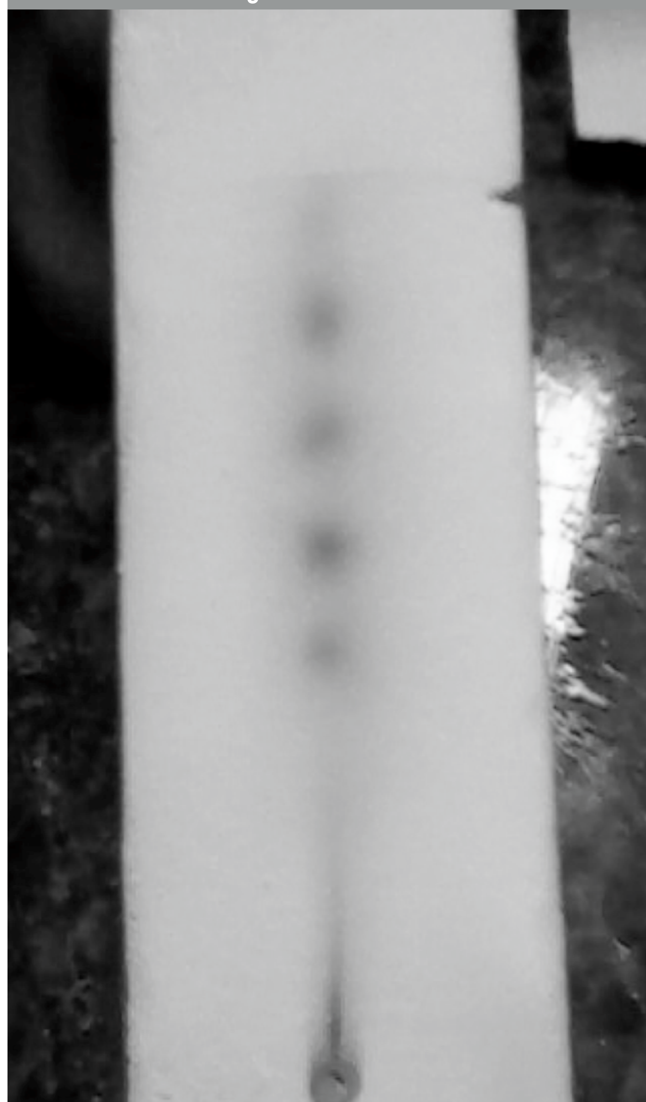
TLC

In the present study, AFPA was isolated from the root bark of *Plumeria acutifolia* (5.4 g), showing positive result for test of alkaloid. TLC findings were in agreement with the data of qualitative chemical tests. The AFPA showed well marked 4 spots, shown in **Fig. 1** and **Table. 1**. The phytoconstituent total alkaloid content was estimated using standard procedure. The amount of total alkaloids was found to be 6.2 % w/w

Acute toxicity studies

Acute toxicity study showed that the AFPA possessed high safety profile as no death was observed at oral doses of 1000-5000 mg/kg in mice.

Fig. 1: TLC of AFPA



Anti-anaphylactic activity

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

Anti-inflammatory activity

In acute model of inflammation (carrageenan induced), paw edema volume is inhibited by the AFPA (100, 250 and 500 mg/kg p.o) dose-dependently. As shown in **Table. 4**, the AFPA (500 mg/kg) reduced the edema swellings by 75.45 % as compared with 90.24 % reduction produced by indomethacin (10 mg/kg, p.o) at 3rd hour of carrageenan administration. In chronic model of inflammation, AFPA at a dose of 100, 250 and 500 mg/kg exhibited significant ($P<0.01$) reduction in granuloma weight by 37, 43, 49 % respectively. These results were comparable with that the standard drug. **(Table. 5)**

Effect on erythrocyte membrane stability

The AFPA (at concentration range of 0.100-0.500 mg/ml) significantly protect the rat erythrocyte membrane against lysis induced by hypotonic solution. At a concentration of 0.500 mg/ml, the extract produced 46.68±0.90 % inhibition of RBC haemolysis as compared with 61.16±0.80 % produced by indomethacin **(Table. 6).**

DISCUSSION

The present study was undertaken for the evaluation of anti-anaphylactic activity and anti-inflammatory property of AFPA from the root bark of *Plumeria acutifolia*. The anti-anaphylactic activity was done using passive cutaneous anaphylaxis for evaluation of AFPA on immediate hypersensitivity reaction. Mediators like leukotriene, prostaglandins, PAF and cytokines are reported to be responsible for the immediate hypersensitivity reaction, but it was observed that enhanced vascular permeability and leukocyte infiltration at the sites of allergen challenge. In passive cutaneous anaphylaxis 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 AFPA (100, 250 and 500 mg/kg) than the control animals. This activity can partly be due to inhibition of leukotriene synthesis. Passive paw anaphylaxis is another in vivo model for IgE-mediated immediate hypersensitivity

Table 1: Thin layer chromatography of AFPA

Test extract	Solvent system	Number of spots	Rf values	Detecting agent
AFPA	Toluene: chloroform: ethanol (2 : 2: 6)	4	0.875 0.775 0.625 0.176	Dragendorffs reagent

Table 2: Study of AFPA on passive cutaneous anaphylaxis (PCA)

S.No	Group	Dose(mg/kg)	Amt of Dye Leaked(mcg/site)	% Inhibition
1	Control	-	1.21±0.01**[a]	-
2	AFPA	100	0.51±0.02**[a]	58 %±1.36**[a]
		250	0.36±0.04[a]	70 %±0.45**
		500	0.26±0.02**[a]	79 %±1.13**[a]
3	Ketotifen standard	5	0.11±0.01**[a]	91 %±0.98**

Values are mean ± SEM of 6 parallel measurements.
All the values are significant **P< 0.01 when compared against control.
All the values are significant a P< 0.01 when compared against standard

Table 3: Study of AFPA on p assive paw anaphylaxis (PPA)

S. No	Group	Dose(mg/kg)	Paw Volume (mm) %	Inhibition
1	Control	-	1.59±0.04** a	-
2	AFPA	100	0.55±0.08** a	65.4±0.46** a
		250	0.43±0.04** a	72.9±2.44** a
		500	0.31±0.06** a	80.5±0.22** a
3	Indomethacin (Standard)	5	0.14±0.02** a	91.1±1.50**

Values are mean ± SEM of 6 parallel measurements.
All the values are significant **P< 0.01 when compared against control.
All the values are significant a P< 0.01 when compared against standard

Table 4: Anti-inflammatory activity of AFPA on Carrageenan induced paw edema model

Groups	Dose (mg/ kg)	Initial Paw Volume (Mm/Hg)	Paw Volume		% Inhibition	
			3 H (Mm/Hg)	5 H (Mm/Mm)	3 H	5 H
Control	1.2	2.6	5.4±0.023**[a]	6.4±0.019**[a]	-	-
AFPA	100	2.5	1.1±0.032**[a]	1.1±0.063**[a]	51.72±1.21**[a]	63.15±0.64**[a]
	250	2.5	1.4±0.021**[a]	1.4±0.047**[a]	62.07±0.86**[a]	71.05±1.98**[a]
	500	2.5	1.6±0.028**[a]	1.7±0.051**[a]	66.9± 2.18**[a]	75.45±1.26**[a]
Indomethacin (Standard)	5	2.5	1.8±0.052**[a]	2.1±0.010**[a]	77.42±1.29**[a]	90.24±1.57**[a]

Values are mean ± SEM of 6 parallel measurements.
All the values are significant **P< 0.01 when compared against control.
All the values are significant a P< 0.01 when compared against standard

Table 5: Anti-inflammatory activity of AFPA on Cotton pellet granuloma pouch model

S.No	Group	Dose(mg/kg)	Wt. of Dry Cotton Pellet (mg) %	Inhibition
1	Control	-	47.70±6.80	-
2	AFPA	100	30.18±3.56**	37
		250	27.24±2.25**	43
	500	24.15±2.05**	49	3
	Indomethacin (Standard)	10	22.20±3.20**	53

Values are mean ± SEM of 6 parallel measurements.
All the values are significant **P< 0.01 when compared against control.

Table 6: Effect of the AFPA on rat erythrocyte haemolysis induced by hypotonic solution

Concentration	AFPA	Indomethacin
100 µg/ml	36.21 ±0.40	41.5±1.29**
250 µg/ml	41.05 ±0.030	50.8±1.65**
500 µg/ml	46.68 ±0.90	61.16±0.80**

Values are mean ± SEM of 6 parallel measurements.
All the values are significant **P< 0.01 when compared against control.
All the values are significant a P< 0.01 when compared against standard

reactions[14]. In both these models a prominent inhibitory effect of alkaloids is suggestive of its antianaphylactic activity.

The results of the study showed that the AFPA possesses anti-inflammatory property, as it significantly inhibited oedema induced by carrageenan, granuloma tissue formation in rats and offer significant protection of the erythrocyte against lysis induced by hypotonic solution.

The inflammatory condition induced by carrageenan 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 edema[15]. 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[16,17]. Administration of AFPA (100, 250 and 500 $mgkg^{-1}$ bw) appear to be effective in inhibiting the dry weight of the cotton pellet that was almost near to that of indomethacin. These data support the hypothesis of the greater effect of the AFPA 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[18]. The AFPA demonstrate significant membrane stabilizing property (**Table. 6**), which suggests that its anti-inflammatory activity observed in this study, may be related to the inhibition of the late phase of inflammatory events.

Thus, the results of the present study demonstrated that the AFPA has significant anti-anaphylactic and anti-inflammatory activities. However a more extensive study is necessary on the isolation and structural characterization of major alkaloid from the root bark of *Plumeria acutifolia* and to throw light for the future development of phyto-medicine with anti-anaphylactic and anti-inflammatory properties.

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