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Original Research Article

Antitumour Activity of Methanolic Extract of *Plumeria alba* L. Leaves Against Dalton Lymphoma Ascites in Mice

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Abstract

Purpose: To evaluate *Plumeria alba* leaves for antitumor activity against Ehrlich ascites carcinoma (EAC) and Dalton lymphoma ascites (DLA) bearing Swiss albino mice.

Method: The antitumour activity of the methanolic extract of *Plumeria alba* leaves (MPA) was evaluated against EAC and DLA using *in-vitro* cytotoxic and mean survival time, a decrease in the tumour volume and viable cell count in the DLA tumour hosts. The animal was observed for improvement in the haematological parameters (e.g., haemoglobin content, red and white blood cells count, and differential cell count) following MPA treatment of the tumour bearing mice.

Results: MPA was found to be cytotoxic in the *in-vitro* model. Intraperitoneal administration of MPA increased the survival time, dead cell count haematological parameters and solid tumour mass was also significantly reduced.

Conclusion: MPA possesses significant antitumour activity.

Keywords: Antitumour, cytotoxicity *Plumeria alba* L.

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Introduction

The alternative systems of medicine like ayurvedha, siddha, unnani and other tribal folklore medicines have significantly contributed to the health care of the population of India. Today, these systems are not only complementary but also competitive in the treatment of various diseases. Initially, the materials employed in these traditional medicines were almost of botanical origin. Several chemotherapeutic agents have been developed in the modern system of medicine as a result of screening of the medicinal plants in various parts of the world. The isolation of biologically active alkaloids such as atropine, quinine, serpentine, reserpine, narcotine, caffeine, nicotine etc are the result of the initial leads obtained from the traditional system of medicine.¹

The plant *Plumeria alba* L (Family Apocynaceae) has been used in the Indian system of medicines for various ailments. The milky sap of the stem and leaf is applied to skin diseases such as herpes, scabies and ulcers.² Its bark is used as plaster over hard tumours^{3,4}, the seeds in hemostasis while the latex is used as purgative, cardiotoxic, diuretic and hypotensive.^{5,6} Evaluation of the antitumour activity of the plant leaves has not been reported. However, various plumeria species have been evaluated using different cellines, composed of murine lymphocytic leukemia and a number of human cancer cell types (breast, colon fibrosarcoma, lung melanoma)⁷.

This present study was carried out to evaluate the *in vivo* anti-tumour activity of methanolic extract of the leaves of *Plumeria alba* (MPA) against Ehrlich ascites

carcinoma (EAC) and Dalton lymphoma ascites (DLA) in mice.

Materials and Methods

Collection and Extraction

The leaves of *Plumeria alba* were collected in Puducherry in June 2006 and authenticated by a botanist, Dr P Jayaraman, at the Plant Anatomy Research Centre (PARC), Chennai, Tamil Nadu, India. The leaves were air dried, pulverized and the powder was treated with petroleum ether for dewaxing and removal of chlorophyll. Then the coarse powder of the leaves (2 kg) was extracted with n-hexane, chloroform, ethyl acetate and methanol successively in an aspirator bottle at room temperature. The plant material was soaked in the solvent for 72 hr. Nearly 80% of the solvent was removed by distillation on a water bath at atmospheric pressure and the last traces were removed under reduced pressure using rotary evaporator. The methanol extract was completely dried and used for the *in vitro* and *in vivo* cytotoxic activities.

Tumour Cell lines

Ehrlich ascites carcinoma (EAC) and Dalton lymphoma ascites (DLA) cells were obtained under the courtesy of Amala Cancer Research Center, Thrissur, India, They were maintained by weekly intra-peritoneal inoculation of 10^6 cells/per mouse.

Animals

Male adult Swiss Albino mice (20-25 gm) were procured from Animal Experimental Laboratory of Madras Medical College, Chennai, Tamil Nadu and used throughout the study. They were housed in microlon boxes in a controlled environment (temperature 25 ± 2 °C) and 12 hr dark/light cycle) with standard laboratory diet (Sai Durga feeds and Foods, Bangalore) and water *ad libitum*. The study was conducted after obtaining Institutional animal ethical

committee's clearance (10/243/August 2006). As per the standard practice, the mice were segregated based on their gender and quarantined for 15 days before the commencement of the experiment. They were fed on healthy diet and maintained in hygienic environment in our animal house.

Determination of in vitro cytotoxic activity using DLA and EAC cell lines

Dalton lymphoma ascites (DLA) and Ehrlich ascites carcinoma (EAC) cells were aspirated from the peritoneal cavity of mice and washed three times in phosphate buffer saline. One million cells were incubated with different concentrations of the extract (10, 25, 50, 100 and 200 µg/ml) in a total volume of 1.0 ml for 3 hr at 37°C. The viability of the cells was then determined using trypan blue exclusion method.⁸

In-vivo antitumor activity model using DLA cell lines

(i) Effect of methanolic extract of Plumeria alba (MPA) on survival time

Animals were inoculated with 1×10^6 cells per mouse on day 0 and treatment with MPA, 24 hr after inoculation, at a dose of 200 mg/kg and 400 mg/kg/day i.p. The control group was treated with the same volume of 0.9% sodium chloride solution. All the treatments were continued for 10 days. The mean survival time (MST) of each group consisting of 6 mice was noted and the antitumor efficacy of MPA was compared with that of 5-fluorouracil (5fu) (20mg/kg, i.p.).⁹ The MST of the control group was subtracted from the MST of the treated group and expressed as a percentage of the MST of the control group.

Effect of methanolic extract of Plumeria alba on Hematological parameters

In order to determine the effect of MPA on the haematological parameters of DLA bearing mice comparison was made amongst four groups (n = 6) of mice on the

14th day after transplantation. The four groups comprised of (1) tumour bearing mice, (2 and 3) tumour bearing mice treated with MPA (200 mg/kg and 400 mg/kg body weight), and (4) normal mice. Blood was drawn from each mouse from tail vein under sterile condition and the red blood cell (RBC) count, haemoglobin content (Hb)¹⁰ and white blood cell (WBC) count¹¹ were determined using cell diluting fluids and a haemocytometer. Differential cell (DC) count was carried out using Leishman stained blood smears.¹²

Effect of methanolic extract of Plumeria alba on solid tumor

Mice (n = 6) were divided into 3 groups and tumor cells (1×10^6 cells/mice) were injected into the right hind limb (thigh) of all the animals, intramuscularly. Group 1 animal was tumor control group, while groups 2 and 3 received MPA (200 and 400 mg/kg intraperitoneally, respectively) for 10 continuous days. The dose was selected based on toxicity studies which showed no toxicity up to 2000mg/kg (p.o.). Tumor mass was measured from the 7th day of tumor induction. The measurement was carried out every 6th day for a period of 48 days¹³ and the volume of tumor mass was calculated using the formula

$$V = 4/3 \pi r_1^2 r_2.$$

where r_1 and r_2 are the radii of the tumor from two directions.

Effect of methanolic extract of Plumeria alba on dead cell count

Mice (n = 6) were divided into 3 groups. Group 1 animals were tumor control animals while groups 2 and 3 received MPA (200 and 400 mg/kg). Ascitic fluid (0.1 ml) was aseptically withdrawn on 10th and 20th day post tumor inoculation using a 1.0 ml syringe and diluted with 50 mg/ml solution of trypan blue up to 1.0 ml mark in the syringe. It was mixed well and kept aside for 2 min. Then a drop of the resulting solution was kept on the

clean glass slide, made into a smear, air dried and then examined under a microscope. The number of (stained) dead cells randomly in every 200 cells were counted.¹⁴ The results were recorded as percent protection against tumor growth calculated as the difference between the number of dead cells in treated and untreated animals expressed as a percentage of the number of dead cells in untreated (control) animals

Statistical methods

All values are expressed as mean \pm SEM. The data were statistically analysed using one-way analysis of variance. At 95% confidence interval, 2-tailed p values less than 0.05 were considered considered to be significant.

Results

Cytotoxicity of *Plumeria alba* towards DLA and ECA cell lines

The methanolic leaf extract of *Plumeria alba* was found to be cytotoxic towards Daltons lymphoma ascites and Ehrlich ascites carcinoma cells only at higher concentration (Table 1). At concentration of 100 μ g and 200 μ g it produced 100% cell death. The

Table 1: Effect of methanolic extract of *Plumeria alba* leaves (MPA) on cytotoxicity of Dalton lymphoma ascites (DLA) cell lines and Ehrlich ascites carcinoma (EAC) cell lines

Concentration/ml	% cytotoxicity DLA cells	% cytotoxicity EAC cells
Control	8	7
10 μ l	19	18
25 μ l	24	20
50 μ l	35	32
100 μ l	100	100
200 μ l	100	100

methanolic leaf extract produced a concentration dependent cytotoxic effect to DLA and EAC cells.

Effect of MPA on survival time

The effect of MPA on the survival of tumour bearing mice showed the MST for the control group to be 23 days, while it was 38 \pm 1.74 days for MPA I (200 mg/kg i.p.), 40 \pm 1.36 days for MPA II (400 mg/kg/day i.p.) and possible control group (standard : 5-fluorouracil (5fu, 20mg/ kg i. p. per day) found to be 42 \pm 1.35 days, respectively. The increase in the lifespan of tumour bearing mice treated with MPA and 5fu was found to be 65.2%, (p < 0.05), 73.9% (p < 0.05) and 82.6% (p < 0.001), respectively as compared to the control group (Table 2).

Table 2: Effect of methanolic extract of *Plumeria alba* (MPA) leaves treatment on the survival of tumour bearing mice

Treatment	Mean survival days	Increase in lifespan (%)
Tumour control	23 \pm 1.10	-
5fu (20 mg/kg ip)	42 \pm 1.35*	82.60
MPA (200mg/kg ip)	38 \pm 1.74**	65.21
MPA (400mg/kg ip)	40 \pm 1.36**	73.9

*p < 0.001 and **p < 0.05 compared with control: n = 6 animals in each group: 5fu =

Effect of MPA on haematological parameters

Haematological parameters of tumour bearing mice on the 15th day showed significant changes when compared with the normal mice (Table 3). A fall in haemoglobin

level in control group and MPA treated groups have been observed nearly close to the normal group. RBC count showed a modest change but WBC counts significantly increased ($p < 0.001$). Treatment with MPA could not normalise the WBC count ; on the other hand, the but the differential counts were found to be similar to that of tumour bearing mice.

Effect of MPA on solid tumour induced by DLA cells with simultaneous drug treatment

Significant reduction in tumour was also found in DLA induced solid tumour animals

treated with two different concentrations of MPA extract. On 22nd day onwards the significance was seen which continued up to the end of the experiment. On 34th day the mean tumour volume of the control animals was $1.29 \pm 0.61 \text{ mm}^3$, which was significantly higher compared to treated groups 200mg/kg ($p < 0.05$) $0.71 \pm 0.07 \text{ mm}^3$, $0.14 \pm 0.2 \text{ mm}^3$ and 400mg/kg ($p < 0.001$) respectively. There was significant decrease in tumour volume in treated groups on other days as well (Table 4).

Dead cell count

The results of the dead cell count indicate that the group treated with higher dose

Table 3: Effect of methanolic extract of *Plumeria alba* (MPA) on haematological parameters

Treatment	Hb (g%)	RBC $10^6/\text{mm}^3$	WBC/ mm^3	Lymphocytes	Neutrophils	Eosinophils	Mono-cytes	Basophils
	Normal mice	11.5±4.9	10.86±0.96	5000-7000	74±3.52	25±1.7	2	2
Tumour bearing mice (Control)	3.45±0.61	5.2±0.76	43,850±1.86	58±5.58	41±3.2	1	1	1
MPA Treated tumor bearing mice (200mg/kg ip)	9.2±0.36*	8.84±0.03*	17,533±1.33*	66±3.42*	24±1.23	1	1	1
MPA Treated tumor bearing mice (400mg/kg ip)	10.6±0.40**	9.92±0.22**	26,583±1.96**	70±4.56**	29±1.94	1	1	1

* $p < 0.05$ and ** $p < 0.001$ compared with control; $n = 6$ animals in each group; days of drug treatment = 10. Values are expressed as mean \pm SEM

Table 4: Effect of methanolic extract of *Plumeria alba* leaves (MPA) extract on solid tumour induced by Dalton lymphoma ascites cells

Treatment	Tumor volume mm^3						
	10 th day	16 th day	22 nd day	28 th day	34 th day	40 th day	46 th day
Control	0.27±0.07	0.37±0.06	0.71±0.34	1.38±0.98	1.29±0.61	3.66±1.23	4.74±1.25
200 mg/kg	0.18±0.05	0.17±0.08	0.2±0.14*	0.51±0.46*	0.71±0.7*	0.95±0.96*	1.71±1.89*
400 mg/kg	0.24±0.11	0.15±0.14	0.12±0.14**	0.1±0.1**	0.14±0.2**	0.16±0.26**	0.22±0.41**

* $p < 0.001$ and ** $p < 0.05$ compared with control; $n = 6$ animals in each group; 5fu = 5-fluoro-uracil; days of drug treatment = 10

(400mg/kg body wt) to possess more anti-tumour activity as compared to lower dose (200mg/kg body weight). However, on 20th day, post tumour inoculation, the dead cell count in the both treated groups were found to be more than that observed on 10th day post tumour inoculation (Table 5).

that MPA possess protective action on the haematopoietic system. Further, analysis of haematological parameters showed minimum toxic effects in mice treated with MPAs. In DLA bearing mice, blood parameters were restored to normal by MPA administration.

Table 5: Effect of methanolic extract of *Plumeria alba* leaves (MPA) on dead cell count of Dalton Lymphoma Ascites (DLA) in mice

S/N	Group	(%) dead cells		% protection against tumour	
		10 th day	20 th day	10 th day	20 th day
1.	Control	25	35	-	-
2.	5fu (standard)	35	49	40	40
3.	MPA 200 mg/kg	32	48	28	37.14
4.	MPA 400 mg/kg	38	54	52	54.29

5fu = 5-fluoro-uracil; MPA = methanolic extract of *Plumeria alba* leaves

Discussion

The results of the present study clearly demonstrate the tumour inhibitory activity of MPA against DLA strain. The reliable criteria for evaluating an anticancer drug are prolongation of lifespan of the animal and decrease in WBC count of blood. Our results show an increase in lifespan accompanied by a reduction in WBC count in MPA treated mice. It had significant effect in increasing the life span of ascities tumour bearing animals and also found to reduce the solid tumour in animal models. These results clearly demonstrate the antitumour effect of MPA against DLA. In cancer chemotherapy the major problems are of myelosuppression and anaemia^{15,16} the anaemia encountered in tumour bearing mice is mainly due to reduction in RBC and HB% and this may occur either due to iron deficiency or due to hemolytic or myelopathic conditions.¹⁷

Treatment with MPA restored the hemoglobin content, RBC and WBC cell count to normal near values. This indicates

A regular and rapid increase in ascetic fluid volume was observed in DLA bearing mice. Ascetic fluid is the direct nutritional source for tumour growth; it meets the nutritional requirements of tumor cells. MPA treatment decreased the volume of solid tumour and viable cancer cell count, and increased the lifespan. It may be suggested that MPA decreases the nutritional fluid volume and thereby arrests tumour growth and increases the life span. There was a reduction in solid tumour volume in mice treated with MPA ($p < 0.001$).

Preliminary phytochemical screening indicated the presence of triterpenoids, flavonoids and steroids in MPA. Flavonoids have been shown to possess anti-mutagenic and anti-malignant effect.¹⁸ The cytotoxic and antitumour properties of the extracts may be due to these compounds.

Conclusion

The results of the present study has shown that MPA can significantly prolong the life span, reduce tumour volume and improve the haematological parameters of the host. Further research work to is needed to

establish the exact antitumour mechanism of action of MPA.

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