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Anti cancer activity of ethanolic extract of Leaves of *Plumeria rubra (Linn).*

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Abstract
Despite the great development of organic synthesis, currently 25% of prescribed drugs worldwide are still derived from plant sources, showing that plant species are still an important source of new drugs for diseases that continue to lack a cure, such as cancer[1]. The ethanolic extract of leaves of *Plumeria rubra (Linn)* were evaluated for their anti cancer activity against Ehrlich Ascites Carcinoma (EAC) in Swiss albino mice. The extract of *Plumeria rubra (Linn)* at the dose of 200 mg/kg body and 400 mg/kg body weight were administered orally. Anti tumor efficacy of plant extracts were compared with 5-Fluorouracil (20 mg/kg/day i.p) for 9 days. The anti cancer activity of *Plumeria rubra (Linn)* was examined by determining the tumor volume, tumor cell count, viable tumor cell count, non viable tumor cell count, mean survival time and increase in life span in experimental animal models. Ethanolic extract of *Plumeria rubra (Linn)* increased the life span of EAC treated mice and restored the hematological parameters as compared with the EAC bearing mice. Thus the present study revealed that the ethanolic extract of *Plumeria rubra (Linn)* showed significant anti cancer activity in the tested animal models [2, 3].

Key Words
Plumeria rubra (Linn), ehrlich ascites carcinoma, anticancer activity, 5-Fluorouracil, tumor volume, viable cell count.

Introduction
Plumeria rubra (Linn) (Family: Apocynaceae) is a deciduous tree with thick, widely distributed in common rather moist garden, in lawns and in open plantation. Tree is unusual in appearance. Leaves are alternate, spiral, simple, potiolate, petiole undissected, elliptic or ovate shape, base tapering (narrow attenuate) or oblique, margins entire or undulate, apex acuminate or acute or obtuse. Blade 12 to 18 inches with indumentums, venation pinnate, brachidodrome deciduous in taste. Almost eight species *Plumeria rubra (Linn)* occurs in India. Their leaves are useful in inflammation, rheumatism, abortifacient, bactericide, bronchitis, cathartic, cholera, cold, cough, dropsy, dyspepsia, fever, fungicide, flu, gingivitis, herpes, itch, pectoral, piles, poison, purgative, rheumatism, and stimulant. The present study was carried out to evaluate the *in-vivo* anti tumor activity of ethanolic extract of leaves of *Plumeria rubra (Linn)* against EAC in mice.

Materials and Methods
Collection and Extraction

The leaves of *Plumeria rubra (Linn)* were collected from ABS garden, Salem during the month of July 2009. The species for proposed study was identified by Department of Pharmaceutical Chemistry, Vinayaga Mission's College of Pharmacy & Research Institute, Salem and authenticated as *Plumeria rubra (Linn)* by Dr. P. Jayaraman, Botanist, (PARC) Chennai by specimen no. PARC/2008/211. Preparation of Extract for anti cancer activity was achieved by extracting the powdered leaves of *Plumeria rubra (Linn)* with ethanol using Soxhlet's apparatus. Filtrate is subjected to concentrate under reduced pressure. Residue is dried and used for the *in vivo* Anti cancer activity.

Experimental animals
Swiss albino mice having weight 180-230gm were kept in quarantine for 10 days under standard husbandry conditions (27.3oC, Relative humidity 65 ±10%) for 12 hrs in dark and light cycle respectively and were given standard food and water *ad. libitum*. The study was permitted by the Institution Animal Ethical Committee with Reg. No: Ph.Chem/25/2010/IAEC/VMCP.

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Tumor cells

Ehrlich ascites carcinoma (EAC) cells were obtained from Bhopal Cancer Research Centre, Bhopal (MP), India. EAC cells were maintained *in vivo* in Swiss albino mice by weekly intraperitoneal (i.p.) inoculation of 1×10^6 cells/mouse after every ten days. EAC cells 9 days old were used for the screening of the MEMP.

Acute oral toxicity study

Acute oral toxicity was performed by following OECD guideline – 420 fixed dose procedure for ethanol extract of *Plumeria rubra (Linn)* and it was found that dose increasing up to 2000 mg/kg body wt. shown no toxicity or mortality in experimental rats. The LD_{50} of the ethanol extract of *Plumeria rubra (Linn)* as per OECD guidelines–420 is greater than 2000 mg/kg [4, 5].

Experimental Protocol

Male Swiss albino mice were divided in to five groups of eight animals (n=6) each. The *Test samples* was dissolved in isotonic saline (0.9% NaCl w/v.) solution and used directly in the assay. EAC cells were collected from the donor mouse and were suspended in sterile isotonic saline. The viable EAC cells were counted (Trypan blue indicator) under the microscope and were adjusted at 1×10^6 cells/mL. 0.1 mL of EAC cells per 10g body weight of the animals was injected (i.p.) on day zero (day 0). A day of incubation was allowed for multiplication of the cells. Fourteen doses of the *Test samples* (200 mg and 400 mg/kg, 0.1 mL/10g body weight) and 5-Fluorouracil (20 mg/kg body weight) were injected i.p. from the first day up to the 14th day with 24-h intervals. Control animals received only vehicle (isotonic saline solution). Food and water were withheld 18 h before sacrificing the animals. On day 15, half of the animals (n = 3) in each cage were killed and the remaining animals were kept to observe the life span of the hosts. 5-Fluorouracil (5-FU) at a dose level of 20 mg/kg body weight was used as standard. The anti-tumor activity of the ethanol extract of *Test samples* were measured in EAC animals with respect to the following parameters.

Tumor cell count

The ascitic fluid was taken in a WBC pipette and diluted 100 times. Then a drop of the diluted cell suspension was placed on the Neubauer counting chamber and the number of cells in the 64 small squares was counted.

Viable/non-viable tumor cell count (Tryphan blue dye assay)

The cells were then stained with tryphan blue (0.4% in normal saline) dye. The cells that did not take up the dye were viable and those that took the stain were nonviable. These viable and nonviable cells were counted. Cytotoxicity was assessed by incubating 1 X 10^6 EAC cells in 1 ml phosphate buffer saline with varying concentration (50-1000 µg/ml) of the extract at 37ºC for 3 hour in CO_2 atmosphere. The viability of cells was determined by tryphan blue dye where as visible cells exclude the dye.

Percentage increase life span (% ILS)

Animal were inoculated 1×10^6 cells/mL. 0.1 mL of EAC cells per 10g body weight of the animals was injected (i.p.) on day zero (day 0). A day of incubation was allowed for multiplication of the cells. Fourteen doses of the *Test samples* (200 mg and 400 mg/kg, 0.1 mL/10g body weight) and control group was treated with same volume of saline(0.9% sodium chloride solution) and compared with 5-Fluorouracil (20 mg/kg body weight) were injected i.p. from the first day up to the 9^{th} day with 24-h intervals. The effect of *Test samples* on tumor growth was monitored by recording the mortality daily for a period of 9 days and percentage increase in life span (%ILS) was calculated by following equation.

$$\text{Increase in life span} = T\text{-}C / C \times 100$$

Hematological Parameters

At the end of the experimental period, all mice were by cervical dislocation. Blood was collected from freely flowing tail vein and used for the estimation Hemoglobin (Hb) content, red blood cell count (RBC) and white blood cell count (WBC). WBC differential count was carried out from Leishman stained blood smears [6-09].

Statistical analysis

Results of estimation of biochemical and functional parameters have been reported as mean value ± SEM. The variation in a set of data has been estimated by performing one way analysis of variance (ANOVA). Individual comparisons of group mean values were done using Dunnet's test (Sigma stat 3.5). P values <0.001 were considered statistically significant.

Results and Discussion

From Table 1, it was revealed that administration of ethanol extract of leaves of *Plumeria rubra (Linn)* exhibited an ability to reduction significantly in tumor volume and Table 2, revealed that administration of ethanol extract of leaves of *Plumeria rubra (Linn)* appreciably decreases the viable cell count, while increasing significantly the non viable tumor cell count, compared to EAC control group. From table 3, it was found that the median survival time and percentage of life span in mice was increased in extracts treated groups. From table 4, it revealed that hematological parameters of tumor bearing mice on day 16 were found to be significantly differences compared to the extracts treated groups. In tumor bearing mice it was found that increased in WBC count and decreased in HB content with RBC count. In differential count of WBC, the percent of neutrophils and monocytes increased while the lymphocyte count decreased. The extract treated group at dose of 200 mg/kg and 400 mg/kg body weight restored all the altered hematological parameters to almost near normal. All these results suggest the anticancer nature of the extract, the dose of 400 mg/kg body weight was found to be more potent than 200 mg/kg body weight. However, the standard 5-FU at the dose of 20 mg/kg body weight produced better results in all these parameters. The present investigation was carried out to evaluate the antitumor activity of the ethanol extract of leaves of *Plumeria rubra (Linn)* in EAC bearing mice. The reliable criteria for judging the value of an anticancer drug is the prolongation of the life span and median survival time of animals. In tumor bearing mice, a regular rapid increase in ascitic tumor volume was observed. Ascitic fluid is the direct nutritional source for tumor cells and a rapid increase in ascitic fluid with tumor growth would be a means to meet the nutritional requirement of tumor cells The EAC bearing mice orally administered ethanol extract of leaves of *Plumeria rubra (Linn)* at the dose of 200mg/kg body weight and 400 mg/kg body weight showed no significant change in the average life span compared to animals of the tumor control group. However, increase in packed tumor cell volume, and number of viable tumor cells were found to be significantly less than the tumor control animals, mean while increase significantly in non viable tumor cell count, indicating the anticancer nature of the extracts. It may be concluded that ethanol extract leaves of *Plumeria rubra (Linn)* by decreasing the nutritional fluid volume and arresting the tumor growth increases the life span of EAC bearing mice. The reversal of Hb content, RBC, WBC and differential count of WBC by the ethanol extract treatment towards the normal values clearly indicates that ethanol extract of leaves of *Plumeria rubra (Linn)* possessed protective action on the haemopoietic system. In conclusion, the present study demonstrates that the ethanol extract of leaves of *Plumeria rubra (Linn)* increased the life span of EAC tumor bearing mice and was effective in inhibiting the tumor growth in ascitic tumor models.

Conclusion

The pharmacological studies showed significant anti-cancer properties at the dose of 200mg/kg and 400 mg/kg with ethanolic extract of leaves of *Plumeria rubra (Linn)*. Therefore further studies are required to isolate and characterize the active principles of *Plumeria rubra (Linn)* which can offer enhanced anti-cancer activity and to establish their mechanism of action.

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Table 1: Antitumor activity of ethanol extract of leaves of *Plumeria rubra (Linn)* on tumor volume..

Groups	Dose (mg/kg)	Solid tumor volume			
		15th day	20th day	25th day	30th day
EAC Control	-	03.92±0.057	04.67±0.047	05.92±0.097	06.72±0.107
Plant Extract (200mg/kg))*[Plumeria rubra (Linn)]*	200	03.12±0.072	04.42±0.081 [a]	04.92±0.083 [a]	05.91±.0.054
Plant Extract (400mg/kg)*[Plumeria rubra (Linn)]*	400	02.62±0.042[a]	04.42±0.081 [a]	03.92±0.078	04.71±0.034 [a]

N=6 animal in each group; Values are expressed as mean ± SEM [a]P < 0.01; [b] < 0.05 Vs Control.

Table 2: Antitumor activity of ethanol extract of leaves of *Plumeria rubra (Linn)* on viable tumor cells count and nonviable tumor cells count.

Conc. (µg/ml)	Total cell count	Viable cell count	Dead cell count
50	179	105	74
100	438	192	246
200	471	138	333
400	430	72	358
800	512	36	476
1000	330	9	321

Table 3: Anti-cancer activity of ethanol extract of leaves of *Plumeria rubra (Linn)* on median survival time and percentage increase in life span.

Treatment	EAC + Control	EAC+ Plant extract 200 mg/kg *[Plumeria rubra (Linn)]*	EAC+ Plant extract 400 mg/kg *[Plumeria rubra (Linn)]*	EAC + 5 Fluorouracil 20 mg/kg
Median Survival time (days)	18.0 ± 1.20	26.8 ± 2.4*	31 ± 0.98*	35 ± 2.10*
Percentage increase in Life span (%)	-	44.44	72.22	94.44

Table 4: Anticancer activity of ethanol extract of leaves of *Plumeria rubra (Linn)* on hematological parameters.

Treatment	Hb Content (gm %)	Total RBC (Million /mm^3)	Total WBC (10^3cells/ mm^3)	Differential count		
				Lymphocytes (%)	Neutrophils (%)	Monocytes (%)
EAC Control	8.5 ± 0.56^{a}	2.6 ± 0.06^{a}	14.7 ± 1.32^{c}	30 ± 1.36^{a}	68 ± 2.10^{a}	2 ± 0
Plant Extract (200 mg/kg) ***[Plumeria rubra (Linn)]***	10.2 ± 0.42^{a}	$3.6 \pm 0.28^{b,e}$	10.3 ± 1.24	65 ± 2.12^{d}	33 ± 1.1^{d}	2 ± 0
Plant Extract (400 mg/kg) ***[Plumeria rubra (Linn)]***	12.4 ± 0.62^{d}	4.15 ± 0.16^{d}	9.1 ± 1.10^{f}	70 ± 2.45^{d}	29 ± 1.6^{d}	1 ± 0
EAC+5FU 20 mg/kg	13.5 ± 0.3	4.5 ± 0.17	8.6 ± 1.26	70 ± 1.31	29 ± 1.6	1 ± 0

Values are expressed as mean ± SEM, n = 6 in each group. *P<0.001 compared to EAC control group. $^{a}P < 0.001$; $^{b}P < 0.05$; $^{c}P < 0.01$ vs. Normal; $^{d}P < 0.001$; $^{e}P < 0.01$; $^{f}P < 0.05$ vs. Tumor control. Data were analyzed by one-way ANOVA followed by Tukey multiple comparison test.

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