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Research Article

ANTICANCER ACTIVITY OF ETHANOL EXTRACTS OF LEAF AND BARK OF *NARINGI CRENULATA* (ROXB) NICOLSON AGAINST EHRLICH ASCITES CARCINOMA

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ABSTRACT

The present study aims to evaluate the antitumor activity of ethanol extracts of leaf and bark of *Naringi crenulata* (Roxb) Nicolson. (NCL and NCB) on Ehrlich Ascites Carcinoma (EAC) model in Swiss Albino mice. Evaluation of the antitumor effect of ethanol extracts of NCL, NCB on tumor growth and hosts survival time was made by the study of the following parameters: tumor volume, viable and non viable cell count and life span of the host. The results showed decrease in tumor volume, and cell viability. Hematological studies revealed that, the Hb content decreased in EAC treated mice, whereas it was induced by the natural tested materials and showed an increase in Hb near to normal levels. The results suggested that the extracts of leaf and bark of *Naringi crenulata* exhibited significant antitumor activity on EAC bearing mice.

Keywords: Ehrlich ascites carcinoma, antitumor, hematological studies, *Naringi crenulata*.

INTRODUCTION

Medicinal plants used as remedies for human diseases contain chemical components of therapeutic value[1] that produce physiological action on the human body. 'Cancer' is a pathological state involving uncontrolled proliferation of tumor cells. Though, it is one of the most dangerous diseases in humans and presently there is a considerable scientific discovery of new anticancer agents from natural products[2]. The inhibition of 'ascites' tumor cells by garlic extracts was investigated[3], soyabean seed extracts showed antitumor activity due to the presence of trypsin inhibitor[4]. Numerous cancer research studies have been conducted using traditional medicinal plants in an effort to discover new therapeutic agents that lack the toxic side effects associated with current chemotherapeutic agents. Hence, a major portion of the current pharmacological research is involved with the anticancer drug design customized to fit new molecular targets[5].

The plant '*Naringi crenulata*' (Roxb.) Nicolson belongs to Rutaceae family, commonly known as 'bilvaparni', 'vilvaparni in Sanskrit', 'nagavilvam' and mahavilvam in Tamil, has been used as folk medicine, in curing vomiting, dysentery, colic disorder etc. However, in spite of traditional use, pharmacology of its aerial parts has not yet been explored scientifically. So far no reports are available in anticancer activity of this plant. The present investigation was carried out to evaluate the anticancer activity of the ethanol extracts of leaf and bark of *Naringi crenulata* against *in vivo* Ehrlich Ascites Carcinoma (EAC) tumor model.

MATERIALS AND METHODS

Collection

The leaves and bark of *Naringi crenulata* (Roxb.) Nicolson were collected in the month of February and March, 2010, from the Agasthiarmalai Biosphere Reserve, Western Ghats, Tamil Nadu. The plant specimen were identified with the help of local flora and authenticated in Botanical survey of India, Southern Circle, Coimbatore, Tamil Nadu, India. A voucher specimen was deposited in Ethnopharmacology Unit, Research Department of Botany, V.O.Chidambaram College, Tuticorin, Tamil Nadu.

Preparation of plant extract for anticancer activity

The leaves and bark of *Naringi crenulata* were cut into small pieces, washed dried at room temperature; the dried leaves and bark were powdered in a Wiley mill. Hundred grams of powdered leaf and bark were separately packed in a Soxhlet apparatus and extracted with ethanol. The ethanol extracts were concentrated in a rotary evaporator. The concentrated ethanol extracts of leaf and bark were used for anticancer activity.

Animals

Healthy male adult Swiss Albino rats (20-25gm) were used for the study. The animals were housed in microlon boxes in a controlled environment (temperature 25 ± 2^0c) and 12 hr dark/eight cycle) with standard laboratory diet (Sai Durga feeds and foods, Bangalore) and water *ad libitum*. The mice well 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.

Tumor Cells

Ehrlich Ascites Carcinoma (EAC) cells were obtained from Division of Oncology Department of Biotechnology, Tamil Nadu, Veterinary and Animal Husbandary, Chennai, Tamil Nadu, India. The EAC cells were maintained *in vivo* in Swiss albino mice by weekly intra peritoneal (i.p) inoculation of 10^6 cells / mouse after every ten days. EAC cells 9 days old were used for the screening of the anticancer activity.

Acute oral toxicity study

Acute oral toxicity was performed by following OECD guideline - 420 fixed dose procedure for ethanol leaf and bark extract of *Naringi crenulata* and it was found that dose increasing up to 2000 mg / kg body weight, shown no toxicity or mortality in experimental rats. The LD_{50} of ethanol extracts of leaf and bark of *Naringi crenulata* as per OECD guidelines-420 is greater than 2000 mg/kg [6, 7].

Antitumor activity

Healthy Swiss albino mice were divided into six groups of six animals (n=6) each. The test samples were dissolved in isotonic saline (0.9% NaCl W/V) 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 X 10^6 cells / ml. 0.1 ml of EAC cells per 10g body weight of the animals were injected (i.p) to each mouse of each group except normal saline group (Group I). This was taken as Day 0. A day of incubation was allowed for multiplication of the cells. Fourteen doses of both the leaf and bark test samples (200mg and 400mg/kg, 0.1ml/10g body weight) (Groups II, III, IV, V) and 5-Fluorouracil (20mg/kg body weight) were injected i.p from the first day up to 14^{th} day with 24h intervals. Control animals received only vehicle (isotonic saline solution) food and water were with held 18 h before sacrificing the animals. On day 15, half of the animals (n=3) in each case were sacrificed and the remaining animals were kept to observe the life span study of the tumor 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 extracts of two different test samples leaf and bark (NCL & NCB) were measured in EAC animals with respect to the following parameters.

Tumor growth response

The effect of ethanol extracts of NCL and NCB on tumor growth and hosts survival time were examined by studying the following parameters such as tumor volume, tumor cell count, viable tumor cell count, non viable tumor cell count, median survival time and increase in life span.

Determination of Tumor volume

The mice were dissected and the ascitic fluid was collected from the peritoneal cavity. The volume was measured by taking it in a graduated centrifuge tube. Packed cell volume was determined by centrifuging the ascitic fluid at 1000 rpm for 5min.

Determination of 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.

Estimation of viable and non viable tumor cell count (Tryphan blue dye assay)
The cells were then stained with tryphan blue (0.4% normal saline) dye. The cells that did not take up the dye were viable and those that took the stain were non viable. These viable and non viable cells were counted.
Cytotoxicity was assessed by incubating 1 x 10^6 EAC cells in 1ml phosphate buffer saline with varying concentration (50 – 1000) µg/ml) of the extract at 37^0c for 3 hours in CO_2 atomosphere. The viability of cells was determined by tryphan blue dye where as visible cells exclude the dye.

Percentage increase of life span (% ILS)
Animals were inoculated (1 x 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/kg 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 (20mg / kg body weight) were injected i.p from the first day upto the 9^{th} day with 24 h intervals. The effect of ethanol extracts of leaf and bark of *Naringi crenulata* 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 from the following equation.

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

Body Weight
Body weights of the experimental mice were recorded both in the treated and control group at the beginning of the experiment (zero day) and sequentially on every 5^{th} day during the treatment period.

Hematological studies
At the end of the experimental period, all mice were sacrificed by cervical dislocation. Blood was collected from freely flowing tail vein and used for the estimation of Hemoglobin content (Hb), Red blood cell count (RBC) and White blood cell count (WBC). WBC differential count was carried out from Leishman stained blood smears[8-12].

STATISTICAL ANALYSIS
The data were analyzed using student's t- test statistical methods. For the statistical tests, *p* values of less than 0.01 and 0.05 was taken as significant.

RESULTS
In acute toxicity study, ethanol extracts of NCL and NCB did not show any toxic effect up to the dose of 2000 mg /kg body weight, accordingly 200 mg/kg and 400 mg/ kg body weight were taken as low and high dose of NCL and NCB for the experiment. The present investigation indicates that ethanol extracts of NCL and NCB showed significant antitumor activity in EAC bearing mice.
In the case of tumor growth response study, treatment with ethanol extracts of NCL and NCB showed significant reduction in tumor volume (Table 1). Table 2 depicts the effect of ethanol extracts of NCL and NCB on prolongation of life span. It also revealed that there was increase in mean survival time. Table 3 revealed that the administration of ethanol extracts of NCL and NCB appreciably decreases the viable cell count compared to EAC control group. Non viable cell count was significantly higher with increase in dosage of extracts. Table 4 showed that hematological parameters of tumor bearing mice on day 16 were found to be significantly different as compared to the extracts treated groups. In tumor bearing mice, it was found that there was increase in WBC count, and decrease in Hb content and RBC count. In differential count of WBC, the percent of neutrophils and monocytes increased while the lymphocyte count decreased in the EAC control group. Treatment with NCL at the dose 200 mg/kg and 400 mg/kg significantly ($p<0.05$ and $p<0.01$ respectively) increased the Hb content and RBC count ($p<0.05$) to more or less normal levels. The total WBC counts and protein were found to be increased significantly in the EAC control group when compared with normal group. Adminstration of NCL (200 mg/kg and 400mg/kg) and NCB (200mg/kg and 400mg/kg) in EAC bearing mice significantly ($p<0.05$ and $p<0.01$) reduced the WBC count and protein at the dose of 400mg/kg reduced significantly ($p<0.01$) as compared with the EAC control. Treatment with NCL and NCB extracts at different doses changed these altered parameters more or less to the normal values.

DISCUSSION
The present study was carried out to investigate the antitumor potential of NCL, NCB against EAC bearing mice. EAC (Ehrlich

Ascites Carcinoma) is a very rapidly growing carcinoma with very aggressive behaviour[13]. It is able to grow in all most all strains of mice. The Ehrlich Ascitic tumor implantation induces *per se* local inflammatory reaction, with increasing vascular permeability, which results in an intense edema formation, cellular migration, and a progressive ascitic fluid formation and accumulation[14]. The ascitic fluid is essential for tumor growth, since it constitutes a direct nutritional source for tumor cells[15]. In EAC bearing mice, a regular rapid increase in ascites tumor volume was noted. NCL, NCB treatment significantly reduced tumor volume probably by lowering the ascetic nutritional fluid volume. Further, the tumor volume and the number of viable EAC cells in peritoneum significantly lower in mice treated with NCL, NCB when compared to the tumor control group. These results could indicate either a direct cytotoxic effect of NCL, NCB on tumor cells or an indirect local effect, which may involve macrophage activation and vascular permeability inhibition.

Prolongation of life span of the animals is an important criterion for judging the value of any anticancer drug[16]. A decrease in tumor volume and viable cell count as mentioned above finally reduced the tumor burden and enhance the life span of EAC bearing mice.

In Chemotherapy treatment, the major problems that are being encountered are of myelosupression and anemia[17,18]. The anemia encountered in tumor mice is mainly due to reduction in RBC or hemoglobin percentage and this may occur either due to iron deficiency or the hemolytic or myelopathic conditions[19]. Treatment with NCL and NCB brought back the heamoglobin content; RBC and WBC count more or less to normal levels. This indicates that the plant extracts possess protective action on the hemopoietic system.

The present study demonstrates that the ethanol extracts of NCL and NCB increase the life span of EAC tumor bearing mice, reduce tumor volume and improve the hematological parameters. All the above parameters suggest that the ethanol extracts of NCL and NCB exhibits antitumor activities. In conclusion, the present pharmacological studies showed significant anticancer properties of ethanol extracts of leaf and bark of *Naringi crenulata*, at the dose level of 400 mg/kg. Further, the isolation of the compounds responsible for anticancer activity has to be taken up which may result in a modern drug from this plant.

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Table 1: Antitumor activity of bark and leaf ethanol extracts of *Naringi crenulata* on solid tumor volume

Groups	Dose (mg/kg)	Solid Tumor Volume			
		15th day	20th day	25th day	30th day
Group I	Saline	3.56±0.023	4.96±0.067	6.04±0.034	6.98±0.012
Group II	200	2.89±0.034	3.86±0.022	5.12±0.056	5.23±0.031
Group III	400	2.03±0.015	3.12±0.062*	4.38±0.031*	4.16±0.047**
Group IV	200	2.91±0.043	3.90±0.032	5.67±0.047	5.91±0.033
Group V	400	2.12±0.073	3.08±0.056*	4.71±0.043*	5.12±0.04*
Group VI	20	2.09±0.032	3.11±0.054	4.21±0.055	5.18±0.036

Each value is SEM of 6 animals. Significance between tumor induced control vs drug treated group * P < 0.05; ** P < 0.01;

Table 2: Antitumor activity of bark and leaf ethanol extracts of *Naringi crenulata* on the survival term, life span

Parameters	Experimental Groups					
	EAC Control + Saline	EAC + NCL		EAC + NCB		EAC + 5 Fu drug
	0.9%	200 (mg/kg)	400 (mg/kg)	200 (mg/kg)	400 (mg/kg)	20 (mg/kg)
Survival time (Days)	19.3±1.20	21.50±1.01	38.45±1.23*	22.70±1.86	25.30±1.28*	39.54±1.37*
Increase of life span (%)	-	11.37	99.20	17.61	31.08	104.87

Each value is SEM of 6 animals. Significance between tumor induced control vs drug treated group *p<0.05; **p<0.01

Table 3: Antitumor activity of bark and leaf ethanol extracts of *Naringi crenulata* on viable tumor cells count and nonviable tumor cells count [Short term Cytotoxicity of *Naringi crenulata* (Tryphan Blue Assay)]

Conc. (µg/ml) NCL/NCB	Leaf			Bark		
	Total cell count	Viable cell count	Non viable cell count	Total cell count	Viable cell count	Non viable cell count
50	226.23±11.23	194.56±4.65	3.11±1.34	218.76±5.87	186.57±7.96	26.14±2.1
100	294.12±12.56	136.85±5.14	154.67±3.56	245.67±4.59	173.37±6.41	76.76±3.67
200	362.59±10.67	112.33±3.56	258.96±6.78	319.56±6.67	165.67±4.56	158.56±6.45
400	425.76±13.32	95.64±2.63	324.78±7.67	384.36±8.16	133.96±4.66	254.77±7.68
800	524.96±12.43	35.35±3.31	493.55±8.66	354.33±7.85	116.56±4.87	246.65±6.89
1000	585.24±13.54	13.67±3.96	576.78±8.99	489.12±8.89	98.56±3.78	393.68±7.14

Each value is SEM of 3 replicates

Table 4: Anticancer activity of NCL and NCB extracts of hematological parameters in EAC Tumor bearing mice

Parameter	Hb (gm%)	RBC (million/mm^3)	WBC (10^3 cells / mm^3)	Proteins (gm%)	Differential count		
					Lymphocytes	Neutrophils	Monocytes
Group I	7.45±0.59	3.21±0.11	13.54±2.15	11.56±2.14	33.34±2.58	57.43±2.53	9.46±1.04
Group II	10.54±0.86*	3.68±0.59	9.58±1.54*	10.32±0.96	46.78±2.34	49.45±1.33	5.03±1.22**
Group III	11.98±0.76**	4.04±0.43*	8.57±0.47**	8.98±1.23**	59.54±3.85*	34.66±2.14*	7.21±1.88
Group IV	12.05±0.34	4.56±0.42*	9.45±0.79*	10.77±0.72	48.32±2.12	46.58±2.79	5.89±1.34
Group V	12.56±0.43**	4.87±0.55*	8.12±0.34**	8.96±0.32**	51.67±0.68*	39.25±1.32*	9.53±1.37
Group VI	12.96±0.64*	4.68±0.32*	8.13±0.35*	7.60±0.61*	65.44±2.38	28.51±0.57**	6.48±1.93*

Each value is SEM of 6 animals. Significance between tumor induced control vs drug treated group * $p < 0.05$; ** $p < 0.01$

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