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Antiproliferative Activity of *Phallusia nigra* Savigny, 1816 Against Dalton's Lymphoma Ascites

Meenakshi VK*, Paripooranaselvi M, Gomathy S and Chamundeswari KP

Department of Zoology, A.P.C. Mahalaxmi College for Women,Tuticorin,Tamilnadu, India.

*Corresponding Author: E-Mail: vkmeenakshi@yahoo.com.

ABSTRACT

The present study was carried out to assess the antiproliferative effect of the ethanol extract of *Phallusia nigra* against Dalton's Lymphoma Ascites (DLA) on adult Swiss Albino mice. The extract was toxic at 0.60 and 0.12 mg/ml to DLA and L 929 cells in culture respectively. After tumor inoculation the extract at the dose of 50, 100 and 150 mg were administered intraperitonially and compared with the standard drug Vincristin (0.08 mg/kg body weight). The results showed significant, dose dependent decrease in tumor volume, packed cell volume, viable cell count, increase in non viable cell count and mean survival time there by increasing the life span of DLA tumor bearing mice by 96.4% in group IV treated with 150 mg/kg body weight. Administration of the extract restored the hematological parameters near to normal when compared to those treated with standard drug.

Key words: *Phallusia nigra*, antiproliferative and DLA

1. INTRODUCTION

Cancer is a malignant disease, characterized by rapid and uncontrolled growth leading to the formation of abnormal cells. It is considered as one of the most common causes of mortality worldwide, affecting people of all ages. More than 60 percent of currently used anticancer agents have been derived from natural sources including plants, marine and microorganisms. Even though bioactive compounds with cytotoxic activities have been isolated from plants, they are exploited for various reasons. Progress made in cancer therapy has not been sufficient to significantly lower annual death rate from most tumor types and there is an urgent need for new strategies in cancer control [1]. The ocean is considered to be a source of potential drugs. Marine organisms such as ascidians, sponges and soft corals containing symbiotic microorganisms are a rich source of bioactive compounds [2,3,4]. One promising group of bioactive metabolite is ecteinascidins, biosynthesized by the colonial ascidian *Ecteinascidia turbinata* (Herdman), which exhibits strong antitumor properties [5,6,7]. Investigations on anticancer properties have been carried out to various cancer cell lines with many species of ascidians [8,9,10,11,12]. Though *Phallusia nigra,* a commonly available ascidian of Tuticorin coast has been subjected to chemical screening [13,14,15], antimicrobial [16] and pharmacological assay [17], antiproliferative activity has not been attempted at all.

2. MATERIALS AND METHODS

2.1. Specimen collection and identification

Samples of *Phallusia nigra* were collected from the under surface of the barges of Tuticorin harbour. Identification up to the species level was carried out based on the key to identification of Indian ascidians [18].

2.1.1. Systematic position

Phallusia nigra belongs to Phylum: Chordata, Subphylum: Urochordata, Class: Ascidiacea, Order: Enterogona, Suborder: Phlebobranchia, Family: Ascidiidae, Genus: *Phallusia,* Species: *nigra*.

2.2. Experimental animals

Adult Swiss Albino mice weighing 20-25 g were obtained from the Breeding section, Central Animal House, Dr. Raja Muthiah Medical College, Annamalai University, Chidambaram, Tamilnadu. The animals were kept in air-controlled room, fed with normal mice chow and water ad libitum. The experiments were conducted according to the rules and regulations of Animal Ethical Committee, Government of India.

2.3. Cells for cytotoxic study

L 929 cells were used for *in vitro* cytotoxicity assessment. These cells were received from the Department of Biotechnology, Division of Animal Tissue and Cell Culture, Tamilnadu Veterinary Science and Animal Husbandry, Chennai. The cells were maintained in Minimum Eagle's medium supplemented with 10% fetal calf serum and antibiotics. Dalton's Lymphoma ascites

(DLA) cells were procured from Adayar Cancer Institute, Chennai, India. Sheep red blood cells (SRBC) were collected from local slaughter house in Alsever's solution.

2.4. Preparation of powder and extract

Samples of *Phallusia nigra* were dried at 45°C and powdered. Ten grams of the powder was soaked over night in 100 ml of 70% ethanol. It was centrifuged at 10,000 rpm at 4°C for 10 minutes; supernatant was collected and evaporated to get a residue, which was used for in vitro studies. For animal experiments, it was suspended in 1% gum acacia blended with vanillin and administered intraperitonially.

2.5. In vitro cytotoxic activity to DLA cells

DLA cells (1×10^6 cells) were incubated with various concentrations (0.05, 0.10, 0.20, 0.40 and 0.60 mg/ml) of extract of *Phallusia nigra* in a final volume of 1ml for 3hr at 37°C. The viability of the cells was confirmed by trypan blue dye exclusion method [19].

2.6. Cytotoxicity to L 929 cells in culture

The cells were seeded in 96 well flat-bottom plates (5000 cells/well) and allowed to adhere for 24hr at 37°C with 5% CO_2 atmosphere. Different concentrations of the ethanolic extract of *Phallusia nigra* (0.01, 0.02, 0.04, 0.06, 0.08, 0.10 and 0.12 mg/ml) were added and incubated further for 48hr. Before four hours of the completion of incubation, 20ml of MTT (5mg/ml) was added [20,21]. Percentage of dead cells was determined using an ELISA plate reader set to record absorbance at 570 nm.

2.7. Experimental protocol

Healthy adult Swiss Albino mice were weighed and divided into five groups of six each. Group I acted as control, Group II, III, and IV received 50,100 and 150 mg/kg of the extract and Group V with standard drug Vincristin (0.08 mg). DLA cells (1×10^6 cells/mouse) were injected intraperitonially for 9 days. On the 10th day body weight of the animal was noted. 24 hr after the last dose of the drug, one set of animals were sacrificed and the weight of the vital organs such as spleen, thymus, liver, kidney and lungs were recorded and expressed as relative organ weights. With the remaining set of mice the experiment was continued. Blood was collected from caudal vein and parameters such as Hb, RBC, WBC and differential count were recorded 30 days after the administration of the extract [22].

2.8. Induction and measurement of solid tumor volume

Tumor was induced by injecting DLA cells (1×10^6 cells/mouse) subcutaneously to the right hind limbs of the animals for five groups. The radii of the tumors were measured using Vernier Calipers at 5 days intervals for one month starting with 15th day and the volume of the tumor was calculated using the formula $V=4/3 \prod r1^2r^2$, where 'r1' and 'r' represent the major and minor diameter respectively [23].

2.9. Effect on Median survival time, lifespan, packed cell volume, viable and non-viable cell count

2.9.1. Median survival time and percentage increase of life span (% ILS)

The effect of the extract on tumor growth was monitored by recording the mortality daily for six weeks and percentage increase in lifespan (% ILS) was calculated by the following equation.

MST % = Median survival time of treated group – Median survival time of control group/ Median survival time of control group × 100

Median survival time (MST) = (Day of first death + Day of last death) / 2

Increase in lifespan = T-C/C × 100

2.9.2. Packed cell volume

The mice were dissected and the ascetic fluid was collected from the peritoneal cavity. The volume was measured by taking it in a graduated centrifuge tube and packed cell volume was determined by centrifuging at 10,000 rpm for 5 minutes.

2.9.3. Viable and non viable cell count

The cells were stained with trypan blue (0.4% in normal saline) dye. Those that did not take up the dye were viable and those which took the stain are non viable. The viable and non viable cells were counted.

2.10. Effect on hematological parameters

Blood was collected from caudal vein of the experimental mice and parameters such as hemoglobin, RBC, WBC and differential count was recorded prior to the extract administration and continued every third day for thirty days.

2.11. Statistical Analysis

Values were expressed as mean ± SEM. The statistical analysis was done by one-way analysis of variance (ANOVA) followed by Dunnett's test. P-values less than 0.05 were considered to be significant.

3. RESULTS AND DISCUSSION

3.1. Cytotoxic activity to DLA and L 929 cells

In the present study the antiproliferative activity of *Phallusia nigra,* an interesting simple ascidian of Tuticorin coast against DLA bearing

Table -1: Cytotoxicity of ethanolic extract of *Phallusia nigra* to DLA and **L** 929 cells

Dalton's Lymphoma Ascities		**L** 929 Cells	
Concentration (g/ml)	Percentage Cytotoxicity	Concentration (g/ml)	Percentage Cytotoxicity
0.05	14	0.01	0
0.10	35	0.02	12
0.20	55	0.04	35
0.40	85	0.06	64
0.60	100	0.08	78
-	-	0.10	92
-	-	0.12	100

Table -2: Effect of *Phallusia nigra* extract on Relative Organ Weight of tumor induced mice

Group	Dose (mg/kg)	Relative Organ Weight (g/100g body weight)					
		Body weight	Spleen	Thymus	Liver	Kidney	Lungs
I	Control	32.98±1.67	0.41±0.021	0.21±0.027	2.97±0.13	1.98±0.023	0.53±0.021
II	50	24.67±1.32*	0.43±0.043	0.19±0.055	2.91±0.25	2.69±0.013*	0.55±0.039
III	100	20.46±0.98**	0.45±0.012	0.13±0.019	3.08±0.33	2.97±0.017**	0.45±0.026
IV	150	17.22±1.12***	0.48±0.021	0.10±0.036	3.23±0.52*	3.21±0.031***	0.56±0.019
V	Vincristin 0.08	19.51±1.89**	0.47±0.017	0.13±0.021	3.19±0.21*	3.13±0.057**	0.52±0.047

Data represented as mean ±SEM, (N=6). Significance between DLA control and extract treated group. * P < 0.05, ** P < 0.01, ***p < 0.001

Table -3: Effect of *Phallusia nigra* extract on solid Tumor volume

Group	Dose (mg/kg)	Solid Tumor Volume			
		15th day	20th day	25th day	30th day
I	Control	3.34±0.12	3.56±0.56	4.06±0.73	4.87±0.45
II	50	3.13±0.32	3.43±0.17	3.61±0.11	3.92±0.28
III	100	3.47±0.83	3.17±0.32	2.76±0.08*	2.57±0.37*
IV	150	3.22±0.45	2.76±0.26*	2.31±0.57*	2.03±0.11**
V	Vincristin 0.08	3.57±0.43	2.69±0.24*	2.13±0.46*	1.96±0.62**

Data represented as mean ±SEM, (N=6). Significance between DLA control and extract treated group. * P < 0.05, ** P < 0.01

Table - 4: Effect of the extract on median survival time, life span, packed cell volume, viable and non-viable cell count

Group	Dose (mg/kg)	Median Survival time (Days)	Increase of life span (%)	Packed cell volume	Viable cells 1X10^6 cells/ml	Non-viable cells 1X10^{6}cells/ml
I	Control	19.46±0.34	-	2.96±0.029	12.67±1.23	0.69±0.014
II	50	24.67±0.87	26.77*	1.37±0.044*	8.93±0.93*	0.97±0.05
III	100	29.43±0.32*	51.23**	1.02±0.073*	5.35±0.42**	1.84±0.97*
IV	150	38.22±0.18**	96.40***	0.99±0.037**	2.03±0.78***	2.14±0.76**
V	Vincristin 0.08	36.64±0.12**	88.28***	0.84±0.022**	2.16±0.56***	2.05±0.66**

Data represented as mean ±SEM, (N=6). Significance between DLA control and extract treated group. * P < 0.05, ** P < 0.01, ***p < 0.001

Table -5: Effect of the extract on hematological parameters in DLA Tumor bearing mice

Group	Dose (mg/kg)	Hb (gm%)	RBC (million/mm³)	WBC ((10³ cells/ mm³)	Differential Count		
					Lymphocytes	Neutrophils	Eosinophils
I	Control	7.49±0.67	2.18±0.23	18.78±1.23	34.56±1.33	54.59±1.42	12.59±1.42
II	50	8.97±0.46	3.12±0.73	13.49±1.12*	37.91±1.33	52.16±1.75	11.98±1.23
III	100	10.78±0.91*	3.14±0.31	10.57±0.76**	50.54±2.37*	42.46±1.32	7.58±1.22*
IV	150	13.56±1.48**	4.23±0.69**	8.56±0.33***	56.83±1.22**	40.57±1.48*	5.96±1.31**
V	Vincristin 0.08	12.98±0.47**	3.97±0.29*	8.61±0.11***	53.21±1.65**	39.86±1.38*	7.67±1.03*

Data represented as mean ±SEM, (N=6). Significance between DLA control and extract treated group. * P < 0.05, ** P < 0.01, ***p < 0.001

mice was investigated. The extract exhibited 100% toxicity at a concentration of 0.60 and 0.12 mg/ml to DLA and L 929 cells respectively indicating higher toxicity to L 929 cells.

3.2. Effect on organ weight

A significant decrease in the body weight of treated groups compared to the untreated control was observed. The value obtained for group IV treated with 150 mg/kg of extract was highly significant. *Phallusia nigra* extract stimulated the weight of the vital organs like spleen, thymus, liver and kidney dose dependent manner indicating the stimulation and production of immune related cells to fight against rapidly proliferating cells. Thymus is a lymphoid organ which plays an important role by activating the humoral and cellular immune system.

3.3. Effect on tumor volume

There was a significant reduction in tumor volume in group III and IV on 20th, 25th, and 30th day compared to the control. Administration of the extract significantly reduced solid tumor volume indicating inhibition in the growth and multiplication of tumor cells which may be due to the decrease in the ascites fluid acting as a direct nutritional source [24] or the presence of compounds inhibiting mitosis, DNA synthesis or replication via enzyme pathways.

3.4. Effect on median survival time, lifespan, packed cell volume, viable and non viable cell count

An increase in median survival time and lifespan can be used as a reliable indicator of the effectiveness of the extract being investigated [25]. The DLA bearing mice treated with the ethanol extract showed significant increase in median survival time and percentage increase in lifespan. The activity was higher in Group IV compared to that of the standard drug indicating a more effective cytotoxic action. The antiproliferative nature of the extract was evident from the significant decrease in the packed cell volume, viable cell count and an increase in the non viable cell count compared to control. These results indicate either a direct cytotoxic effect of the extract on tumor cells or an indirect local effect involving macrophage activation and inhibition of vascular permeability [26].

3.5. Effect on hematological parameters

Administration of *Phallusia nigra* extract increased the Hb, RBC significantly indicating stimulation of the haemopoietic system. Moreover treatment with the extract brought back the total WBC and differential count to normal values. The dose of 150 mg/kg body weight was found to be more potent and produced better results compared to the standard drug in all the parameters studied. The major problems encountered in cancer chemotherapy are myelosuppression and anaemia [27]. The anaemia evident in tumor bearing mice is mainly due to reduction in RBC or haemoglobin percentage which may occur either by iron deficiency, hemolytic or myelopathic conditions [28].

4. CONCLUSION

The results indicated a significant antiproliferative activity to DLA and L 929 cells with the ethanolic extract of *Phallusia nigra* at a dose of 150 mg/kg body weight. The activity was comparatively greater than that observed for the standard drug in all the parameters tested. A preliminary GC-MS studies of the ethanolic extract has shown the presence of compounds like 2-Piperidinone, Benzeneacetamide, Tetradecanoic acid, n-Hexadecanoic acid, 3-pentadecyl-Phenol, (Z,Z,Z)- phenylmethyl ester of 6,9,12-Octadecatrienoic acid, Cholesterol, Cholestan-3-ol, 3-hydroxy-, (3á,17á)- Spiro[androst-5-ene-17,1'-cyclobutan]-2'-one and (Z)- phenylmethyl ester of 9-Octadecenoic acid exhibiting anticancer, cancer preventive and antioxidant activity. A further study on isolation, purification and subsequent recognition of the novel mechanism of action of the clinically effective agent is suggested.

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