



Original Research Article

Immunomodulatory activity of *Phallusia nigra* Savigny, 1816 against S-180

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A B S T R A C T

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Immunomodulatory activity of ethanolic extract of simple ascidian *Phallusia nigra* was determined against sarcoma 180 (S-180) cells. For 0.60 mg/ml concentration, 100% toxicity was observed. The extract administration at 50, 100 150 and standard drug Vincristin at 80 mg/kg body weight to different groups showed significant reduction in weight of body. The relative organ weight did not register any significant change. The results indicated that extract inhibited tumor growth by 52.69% compared to 47.12% in the standard drug treated group. The extract also significantly increased immune functions by increasing quantitative hemolysis of sheep RBC, lymphocyte proliferation, NK cytotoxicity and phagocytosis rate. The immunomodulatory effect was dose dependent. It is suggested that bioactive compounds present in *Phallusia nigra* modulates immune functions and hence may play a vital role in cancer prevention and treatment.

Introduction

Cancer is a broad group of various diseases, all involving unregulated cell growth and division. Chemo and radio therapy given to cancer patients suppress the immune functions and results in several side effects. Hence there is an urgent need for the development of a drug which can enhance the immune system. Number of natural products is being used as therapeutic agents. The first natural product derived from marine sources to enter clinical trials is Didemnin B (Rinehart *et al.*, 1990). Ascidiaceans which are marine sedentary organisms have been largely studied since it has been shown

that many extracted compounds display potent antitumoral and antiviral activities. ET-743 is a novel chemical compound isolated from the tunicate *Ecteinascidia turbinata* which exhibit potent *in vitro* and *in vivo* antitumor activity in three chemo-sensitive human tumour xenografts-MEXF 989, LXFL 529 and HOC22 (Rinehart *et al.*, 1990; Jimeno *et al.*, 1996; Cragg *et al.*, 1997). The extracts of *Cystodytes dellechiaiei* showed remarkably high antiproliferative activity in human lung carcinoma A-549, colon adenocarcinoma H-116, pancreatic adenocarcinoma PSN - 1 and breast

carcinoma SKBR3 cell lines (Garcia *et al.*, 2007). Isogranulatimide from *Didemnum granulatum*, Aplidin from *Aplidium albicans*, clavaminols A-F from *Clavelina phlegraea*, aplidinone A from *Aplidium conicum*, Cephalostatin from *Cephalodiscus gilchristi*, ritterazine G from *Ritterella tokioka*, Styelin D from *Styela clava*, Lissoclinamides from *Lissoclinum patella*, Tamandarins A and B from the family *Didemnidae* and 7-Oxostaurosporine from *Eudistoma vannamei* showed antitumor activity against various cell lines (Berlinck *et al.*, 1998; Fernandez, 2002; Aiello *et al.*, 2007; Aiello *et al.*, 2010; Wojtkielewicz *et al.*, 2003; Taylor *et al.*, 2000; Schmitz *et al.*, 1993; Vervoort *et al.*, 2000 and Jimenez *et al.*, 2012).

A significant antiproliferative and immunomodulatory activity to DLA and EAC cells was obtained with the ethanolic extract of *Phallusia nigra* (Meenakshi *et al.*, 2012a,b, 2013). Earlier studies have shown that *Phallusia nigra* is abundant throughout the year from Tuticorin coast but research on immunomodulatory aspect to S-180 cell lines is lacking.

Materials and Methods

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 (Meenakshi, 1997). A voucher specimen AS 2083 has been submitted to the museum, Department of Zoology, A.P.C. Mahalaxmi College for Women, Tuticorin - 628002, Tamilnadu, India.

Systematic position

Phylum: Chordata; Subphylum: Urochordata; Class: Ascidiacea; Order: Enterogona; Suborder: Phlebobranchia; Family: Ascidiidae; Genus: *Phallusia*; Species: *nigra*

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.

Preparation of powder and extract

The animal was dried at 45° C and powdered. Ten grams of the powder was soaked overnight in 100 ml of 70 percent ethanol and filtered. The filtrate was centrifuged at 10,000 rpm at 4° C for 10 minutes. The supernatant was collected and evaporated to get a residue, which was used for *in vitro* studies. For *in vivo* animal experiments it was resuspended in 1% gum acacia blended with vanillin and administered orally at different concentrations.

In vitro cytotoxic activity

S-180 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 in a final volume of 1ml for 3hr at 37° C. After incubation the viability of the cells was confirmed by trypan blue dye exclusion method (Ebada *et al.*, 2010).

Effect of *Phallusia nigra* extract on S-180 bearing mice

The effect of the extract was determined by evaluating cytotoxicity, weight of body, relative organs, tumor, percentage of tumor inhibition and immune function. S-180 cells were procured from Adayar Cancer Institute, Chennai, India and maintained in RPMI-1640 medium supplemented with 10% heat-inactivated calf serum, 100 U/ml penicillin G, 100 U/ml streptomycin, pH 7.4 in a Water Jacketed CO₂ incubator with a humidified atmosphere of 5% CO₂ at 37° C.

Experimental protocol

Adult Swiss albino mice were divided into five groups of six animals each. Tumor was induced by injecting 0.1 ml of 1×10⁶ S-180 cells per 10 gram body weight of animals intraperitoneally on day zero. A day of incubation was allowed for multiplication of cells. Group I acted as control and was given normal saline. Group II, III and IV and V were treated with ethanolic extract of *Phallusia nigra* at 50, 100, 150 and standard drug Vincristin at 80 mg/kg bw respectively for 9 days. The extract was blended with 1% gum acacia and vanillin solution and administered intra gastrically. On the 10th day, weight of body, vital organs and tumor was noted.

Determination of Humoral immune function

Quantitative hemolysis of sheep red blood cells assay

The micro hemolytic test was performed in 96 well 'V' bottom micro titer plates. Different rows were selected for sheep blood. Serial two fold dilutions of the crude extract were made in 100 mL of

normal saline. This process was repeated upto the last well. Then RBC was added to all the wells in the ratio 100:1. Appropriate controls were included in the test. To the 1% RBC suspension normal saline was added, which served as negative control. The plate was gently shaken and then allowed to stand for two hours at room temperature and the results were recorded. Uniform red colour suspension in the wells was considered as positive hemolysis and a button formation in the bottom of these wells was considered as lack of hemolysis. Reciprocal of the highest dilution of the crude extract was taken as 1 Hemolytic Unit (Thirunavukkarasu *et al.*, 2011).

Determination of cellular immune function

Lymphocyte proliferation

Lymphocytes isolated from the blood using ficoll-hypaque gradient centrifugation were cultured in RPMI-1640 containing 10% FCS and 20 µL of 5 mg/mL mitogen phytohemagglutinin in 96-well microtitre plates at 37° C in a 5% CO₂ atmosphere for 0 hr and 72 hrs. The relative viability of lymphocytes was examined by measuring the amount of purple formazan formed by MTT assay. The experiments were done in triplicate and absorbance was read at 570 nm (Aravind *et al.*, 2012).

Percentage cell viability - $T/C \times 100$;
where T - Test OD; C - Control OD

NK cell cytotoxicity

The function of NK cells is to identify and kill foreign and abnormal cells. Identification of the number and activity of these cells gives us an idea of the strength of this killing capacity. The

activity of these cells in the body is assessed by measuring their activity in the laboratory. Natural Killer cells are separated from blood and are cultured with fixed number of selected cancer cell line and different concentration of extract. After two to four hours of culturing the NK cells with the target cells, a special DNA dye called propidium iodide is added to the culture. This dye is taken up by the DNA of the cells that have been killed. The living cells do not take up the dye. The cell suspensions are then put into the flow cytometer and the percentage of dead to live cells at the different dilutions determined (Sachs *et al.*, 1999).

Determination of non specific immune function

Phagocytic activity

Phagocytosis in the immune system is a major mechanism used to remove pathogens and cell debris. The measurement of phagocytosis activity of immune cells indicates the strength of innate immune system in the host. Peritoneal fluid of mice was collected by injecting 5 mL of DMEM Hams F-12 medium. For *in vitro* assays peritoneal exudate cell suspension was used as a source of macrophages. The number of macrophages in fluid was counted using Neubauer chamber. 1×10^6 cells/ mL was incubated in a humidified atmosphere of 5% CO₂ in air at 37° C for 2 hours to allow adherence of cells. Non-adherent cells were removed by washing 3 times with phosphate buffer saline. The cells were cultured in DMEM Hams F-12 medium and incubated in a humidified atmosphere of 5% CO₂ for 18 h at 37° C. After incubation 100µl of 1×10^6 S-180 cells/ml were added and incubated for 1 hour at 37° C. The cells were washed with

PBS. One mL of 1% tannic acid solution was added and kept for 1 minute, washed with PBS, air dried, stained with May Grunwald stain freshly diluted with Giemsa buffer (1:2) for 2 minutes, washed with PBS and dried. The percent phagocytosis was calculated by counting the number of S - 180 cells internalized per 100 macrophages.

Statistical Analysis

Values are expressed as mean \pm 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.

Results and Discussion

Cytotoxic activity to S-180 cells

Administration of the extract at a concentration of 0.05, 0.10, 0.20, 0.40 and 0.60 mg/ml produced 10, 35, 48, 89 and 100 percent cytotoxicity to S-180 cells respectively (Table 1). A dose dependent increase in percentage cytotoxicity was observed. This can be attributed to the presence of bioactive compounds in the extract and an increase in its level with the increase in concentration. Ascidian derived natural products have yielded promising drug leads among which ET-743 from *Ecteinascidia turbinata* was approved as a drug with the trade name Yondelis against refractory soft-tissue Sarcoma (Ebada *et al.*, 2010). Flavonoids produce several biological effects and have been shown to have antitumor actions causing the inactivation of carcinogen, cell cycle arrest, induction of apoptosis, inhibition of angiogenesis, antioxidation and the reversal of multidrug resistance or a combination of these mechanisms (Shen *et al.*, 2003 and Ren *et*

al., 2003). Naringenin, one of the most abundant flavonoids of citrus fruits were found to inhibit tumor growth in S-180 implanted mice (Kanno, 2005). Presence of flavonoids has been reported in *Phallusia nigra* and this might be a reason for the significant suppression of tumor growth (Gopalakrishnan *et al.*, 2013). The results indicate a dose dependent decrease in body weight of the treated groups (Table 2). The weight change noted in relative organs was negligible. The reduction in tumor weight in the treated groups was dose related. In the groups IV and V which received the extract and standard drug very highly significant decrease was recorded. *Phallusia nigra* extract stimulated the relative weight of vital organs like spleen, liver and kidney in a dose dependent manner indicating the stimulation and production of immune related cells to fight against rapidly proliferating cells. Zuojinwan comprising Chinese medicinal herbs *Coptis chinensis* and *Evodia rutaecarpa* promote immune function in S-180 model by increasing white pulp of the spleen which could be a reason for the increase in spleen weight (Wang *et al.*, 2009). Similar observation was recorded on treatment in the present study. The presence of tumors in the human body or in experimental animals is known to affect the functions of many vital organs, especially the liver, even when the site of the tumor does not interfere directly with organ function (DeWys, 1982). The same may apply to the changes noticed on the weight of vital organs. An increase in the percentage of inhibition of tumor growth in a dose dependent way in the groups which received the extract of *Phallusia nigra* was noted. The inhibition registered for group IV was greater than that noted for group treated with standard drug indicating the

effective role of the extract in controlling tumor growth.

Effect on Immune Function

The quantitative hemolysis (HC₅₀) of sheep red blood cells increased significantly in a dose dependent manner when compared to control (Table 3). Lymphocyte proliferation, NK cytotoxic activity and the percentage of phagocytosis increased significantly in the experimental groups with the increase in concentration of the extract. Studies on humoral, cellular and nonspecific immune function by quantitative hemolysis of SRBC, lymphocyte proliferation, NK cytotoxic activity and phagocytosis rate showed a significant increase in the extract administered groups. Humoral immune response is initiated by the production of antibody by B cells. Processing of antigen and immunization is carried out by other immune specific cells.

Neutralization of toxin produced by carcinogens is brought about by antigen-antibody complex which gives protection to the body. Noni fruit juice has been shown to be one of the powerful antitumor immunostimulators of plant food origin which can indirectly kill the cancer cells via activation of the cellular immune system involving macrophages, natural killer cells and T cells (Furusawa, 2003). In the presence of Noni polysaccharide, macrophages produce NO and several cytokines including interleukin-1 (IL-1), tumor necrosis factor (TNF) and IL-12 which stimulate the immune system. The increasing interferon-gamma (IFN- γ) production stimulates macrophages, NK cells and cytotoxic T cells toward killing tumor cells. It is suggested that the polysaccharides present in the test of

Table.1 Cytotoxicity of ethanolic extract of *Phallusia nigra* to S-180

S. No	Concentration (mg/ml)	Percentage of Cytotoxicity
1	0.05	10
2	0.10	35
3	0.20	48
4	0.40	89
5	0.60	100

Table.2 Effect on Relative Organ Weight

Group & Dose (mg/kg bw)	Relative Organ Weight (g/100g bw)							
	Body weight		Spleen	Thymus	Liver	Kidney	Tumor weight (g)	Inhibition (%)
	Before	After						
I - T. Control	20.38±1.59	28.15±1.13	0.51±0.012	0.34±0.014	3.28±0.13	2.04±0.19	5.39±1.04	-
II - 50	20.90±1.22	28.48±1.06	0.59±0.013	0.24±0.017*	3.68±0.17	2.54±0.48	4.34±0.74	19.48
III - 100	21.30±1.68	27.15±1.13	0.81±0.027**	0.21±0.033*	3.96±0.34	2.98±0.34	3.65±0.89**	32.28
IV - 150	20.55±1.36	26.90±1.55	0.97±0.028** *	0.18±0.011* *	4.28±0.18*	3.16±0.29*	2.55±0.92***	52.69
V - Vincristin (80)	21.66±1.38	29.63±1.45	0.83±0.022**	0.21±0.28*	4.12±0.13*	3.24±0.13*	2.85±0.90***	47.12

Data represented as mean ±SEM, (N=6). Significance between S-180 control and extract treated groups. *p <0.05; **p <0.01; ***p < 0.001.

Table.3 Effect on Immune Function

Group & Dose (mg/kg bw)	Quantitative hemolysis of sheep red blood cells (HC ₅₀)	Lymphocyte proliferation (cpm)	NK cytotoxic activity (%)	Phagocytosis rate (%)
I - T. Control	38.56±3.54	2719±1010	37.66±0.83	21.60±1.32
II - 50	102.65±10.18*	3460±1815*	36.33±0.64	32.50±1.24
III - 100	119.84±07.13*	4290±1005**	48.94±0.88*	34.15±1.35*
IV - 150	123.85±10.16**	4950±1150***	51.84±0.69**	35.65±1.35**
V - Vincristin (80)	148.71±11.85***	4315±1150**	46.10±0.94*	33.15±1.15*

Data represented as mean ±SEM, (N=6). Significance between S-180 control and extract treated groups. *p <0.05; **p <0.01; ***p <0.001.

sedentary *Phallusia nigra* also might play a similar role in stimulating immune function.

Cell mediated immune defense is mediated specifically by T cells. In addition to killing the tumor cells directly, T cells can produce many lymphocyte factors consisting of macrophage mobile factor, lymphotoxin transfer factor and interferon. Such factors could promote the proliferation and differentiation of immune cells, macrophage phagocytosis and the capacity of killing target cells, so that they play a role in preventing tumor (Kim *et al.*, 2001). The ability to elicit an effective T and B cell immunity can be seen by the stimulation of lymphocyte proliferation response (Marciani *et al.*, 2000). Administration of the extract of *Phallusia nigra* also showed an increase in lymphocyte proliferation which can be considered as an indication of the activation of cellular and humoral immunity.

NK cells are a type of lymphocytes that form part of the first line of innate defense against cancer cells and virus infected cells (Moretta *et al.*, 2001). With spontaneous cell mediated cytotoxicity, they are functionally similar to cytotoxic T lymphocytes. The killing by NK cells is non specific, and they do not need to recognize antigen/MHC on the target cell. They can react against and destroy target cell without prior sensitization. NK cell activity assay is a routine method for analysis of cellular immune response *in vitro* and can also be used to test the antitumor activities of possible drugs (Zhang *et al.*, 2005). In the present investigation an increase in NK cytotoxic activity on treatment with the extract of *Phallusia nigra* can be taken as an

evidence of the activation of inborn natural immunity against the proliferating tumor cells. In the tumor control a reduction in the percentage of phagocytosis rate was noted whereas on treatment with the extract, there was a significant increase. This observation is supported by the increased production of lymphocytes and NK cytotoxic activity which is an indication of the stimulation of cell mediated immunity.

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-, (3 \acute{a} ,17 \acute{a})-Spiro[androst-5-ene-17,1'-cyclobutan]-2'-one and (Z)- Phenylmethyl ester of 9-Octadecenoic acid exhibiting anticancer, cancer preventive and antioxidant activity (Meenakshi, 2012c). Further detailed work on isolation, purification and structure determination using spectroscopic methods is suggested to conclude the compounds responsible and mechanism of action.

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