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IMMUNOSTIMULATING ACTIVITIES OF *PHALLUSIA NIGRA* SAVIGNY, 1816 ON SARCOMA-180 TUMOR-BEARING MICE

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ABSTRACT

Ascidians or tunicates are loaded with secondary metabolites as chemical defence. *Phallusia nigra* is a simple ascidian common along the Tuticorin coast. Since its report from India only a few work on chemical screening and pharmacology has been attempted. The present study was carried out to assess the immunostimulating activities of ethanolic extract of *Phallusia nigra* on S-180 tumor bearing Swiss albino mice. Standard methodology has been adopted to assess the various parameters. The results showed highly significant and dose related increase in quantitative hemolysis of sheep red blood cells, lymphocyte proliferation, percentage NK cytotoxic activity, phagocytosis rate, bone marrow cellularity, β-Esterase activity, antibody titer and plaque forming cells. Serum GGT, NO and cellular GSH, NO levels decreased significantly. The results obtained for group IV treated with highest dose of the extract was near to the group treated with the standard drug vincristin.

Keywords: *Phallusia nigra*, S-180, Immunostimulating, Hemolysis, Lymphocyte proliferation NK cytotoxic activity.

INTRODUCTION

Tumor is treated by chemotherapy and radiation which cause severe adverse effects, such as bone marrow suppression resulting in cytopenia, and subsequent devastation of the immune responses [1]. Immunomodulators are well known for their antitumor activity. Polysaccharides are very proficient immunomodulators activating the immune system [2, 3]. The aim of immunoadjuvant therapy is to stimulate the innate and adaptive immune systems to overcome the immunosuppressive situation in cancer which is usually the side effect of the conventional modes of cancer treatments, such as surgery, radio or chemotherapy. There are several medicinal plants that are considered to possess immunomodulatory properties augmenting specific cellular and humoral immune response [4]. Plant derived natural products like flavonoids, terpenes, alkaloids have received considerable attention in recent years due to their cytotoxic and cancer chemo preventive effects which is exhibited through immune potentiating of immune effector cells [5-8]. Marine organisms especially ascidians are rich source of secondary metabolites with immense curative properties. Many species of ascidians have been reported from Indian waters [9-11]. Among these, *Phallusia nigra* is a continuous breeder, hardy, fast growing, and most commonly available simple ascidian of Tuticorin coast. Though this species has been evaluated for its larvicidal, antimicrobial, pharmacological and antitumor potential no prior attempt has been taken to study the immunostimulating activity against S-180- cells [12-22]. Hence a preliminary attempt has been made.

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 [23]. A voucher specimen AS 2083 has been submitted to the museum, Department of Zoology, A.P.C. Mahalaxmi College for Women, Tuticorin - 628002.

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Systematic position

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

Preparation of powder and extract

The animal was dried at 45^{o} 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^{o} 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.

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, at a temperature of 22±3^{0} C, with constant 12 hrs of darkness and 12 hrs light schedules, humidity 60-70%, fed with normal mice chow and water 'ad libitum'. Selected mice were put to fasting for 16 hrs before the experiment. Protocol used in the study for the use of mice as an animal model for anticancer was in accordance with the standards of the Animal Ethical Committee, Government of India.

Acute oral toxicity studies

To determine the minimum lethal dose, acute oral toxicity studies were performed as per OECD guidelines 2002 [24]. Adult Swiss albino mice of either sex weighing 20-25 g were used. Three animals were selected and an oral dose of 2000 mg/kg body weight of the ethanolic extract of *Phallusia nigra* was given orally using intra gastric catheter to overnight fasted mice. They were observed continuously for any gross behavioral changes and toxic manifestations like hypersensitivity, grooming, convulsions, sedation, hypothermia and mortality during the first three hours. The experiment was repeated with the same dose of the extract for 7 more days. There after the animals were continuously monitored at regular intervals for fourteen days. Sub-lethal doses of 50, 100 and 150 mg/kg bw were used for the following experiments.

Cells for cytotoxic study

S-180 cells were procured from Adayar Cancer Institute, Chennai, India. Sheep red blood cells (SRBC) were collected from local slaughter house in Alsever's solution. S-180 cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated calf serum, 100 U/ml penicillin G and 100 U/ml streptomycin, pH 7.4 in a Water Jacketed CO_2 incubator with a humidified atmosphere of 5% CO_2 at 37^{o} C.

Immune Function

The micro hemolytic test was performed in 96 well 'V' bottom micro titer plates by following the standard method [25]. The relative viability of lymphocytes isolated from the blood using ficoll-hypaque gradient centrifugation was examined by measuring the amount of purple formazan formed by MTT assay [3].

Bone marrow cellularity and β-Esterase activity

After 24 hours of drug treatment, the experimental animals treated for 5 consecutive days were sacrificed. The bone cells collected from the femur were made into single cell suspension and the number counted by haemocytometer and expressed as million cells/femur [3]. A smear of cells from the above preparation stained with Harri's hematoxylin was used to determine the non-specific β-Esterase activity by azodye coupling method [26].

Circulating antibody titer

Swiss Albino mice divided into five groups of 6 animals each were immunized with SRBC (0.1 ml, 20 %). Group I received normal saline whereas group II, III and IV were treated with different dose of extract and group V with Vincristin for 5 consecutive days. During the experimental duration of 30 days blood was collected from caudal vein at an interval of three days. Serum was separated, heat inactivated at 56°c for 30 minutes and used for the estimation of antibody titer using SRBC as antigen [27].

Antibody producing cells

Half of the experimental animals from the above treatment were sacrificed on different days starting from the third day after immunization up to the 9th day to determine the effect of the extract on the antibody producing cells. Spleen was processed to single cell suspension and the number of plaque forming cells (PFC) was studied by the Jerne's plaque assay [28].

Serum Gamma Glutamyl Transpeptidase (GGT) and Nitric Oxide (NO) levels

Blood was collected from experimental animals on 5th, 10th and 15th day and the serum was used for the estimation of GGT and NO levels [29,30].

Determination of cellular Glutathione (GSH) and Nitric Oxide (NO) levels

Blood was collected on the 5th, 10th and 15th day; the cells (1X10^{6} cells/ml) were sonicated for 30 seconds and used for the estimation of GSH and NO [31,30].

Statistical Analysis

Values are 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 0.5 were considered to be significant.

RESULTS

Effect on Immune Function

A significant increase on the quantitative hemolysis of SRBC (123.85±10.16), lymphocyte proliferation (4950±1150), NK cytotoxic activity (51.84±0.69) and phagocytosis rate (35.65±1.35) in the extract administered groups. The values obtained for group V was highly significant and group III and IV was significant compared to that of control (Table 1).

Effect on Bone Marrow Cellularity and β-Esterase activity

Dose related significant increase in the number of bone marrow cells in the experimental groups (21.46±0.27) compared to group I (14.37±0.54). The bone marrow cellularity observed for group IV and V was highly significant. There was a decrease in the number of β-Esterase positive cells in tumor control (704.10±23.50) while in the experimental mice (1113.65±19.27) a significant increase in a dose dependent manner was noted (Table 2).

Effect on Antibody Titer

An increase in the level of antibody was noted in group III from the 3rd to the 15th day (191.55±1.76), after that a gradual decline in the titer was registered as days proceed (Figure 1). The titer was maximum and very highly significant on the 15th day (230.37±2.14) in group IV showing a gradual increase from 3rd day. Starting from 18th day a decrease in the level of antibody was noted. In the group treated with Vincristin also a similar increase in the level of antibody was observed from 3rd to the 15th day (213.38±1.94).

Effect on Plaque Forming Cells

Figure - 2 indicates the effect of the ethanolic extract of *Phallusia nigra* on plaque forming cells in S-180 tumor bearing mice. The PFC was maximum and very highly significant on the 6th day (193.63±2.91) in group IV showing a gradual increase from the 3rd day. Starting from the 7th day, decrease in the level was noted. The group which was treated with the standard drug also showed an increase in the PFC count from the 3rd to the 6th day.

Effect on Serum GGT and NO levels

The group treated with 150 mg/kg bw extract showed a highly significant decrease (52.66±0.61) in the level of GGT on the 15th day (Table - 3). A dose related decrease (24.84±0.89) in the level of NO was evident in the groups treated with the extract in comparison to the tumor control.

Effect on Cellular GSH and NO levels

Cellular GSH level was high in the tumor control (8.31±0.22) whereas in the treated groups there was a dose related decrease (5.83±0.14). On the 10th day of experiment the decrease (15.27±0.39) observed in group IV treated with highest dose was highly significant where as the group treated with Vincristin was significant (Table - 4).

Table 1. 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*

Each Value is ±SEM of 6 animals. Significance between S-180 control and extract treated groups. *p <0.05; **p <0.01; ***p <0.001.

Table 2. Effect on Bone Marrow Cellularity and β-Esterase Activity

Group & Dose (mg/kg bw)	Bone marrow cellularity (10^6cells/femur)	β-Esterase activity (β-esterase positive cells /4000 cells)
I – T. Control	14.37±0.54	704.10±23.50
II - 50	16.33±0.13	743.30±18.30
III - 100	18.68±0.38*	914.50±13.35*
IV - 150	21.46±0.27**	1113.65±19.27***
V - Vincristin (80)	20.11±0.13**	1094.15±14.30**

Each Value is ±SEM of 6 animals. Significance between S-180 control and extract treated groups. *p <0.05; **p <0.01; ***p <0.001.

Table 3. Effect on the Serum GGT and NO levels

Group & Dose (mg/ml bw)	GGT (nmol p-nitroaniline/ml)			NO (µM)		
	5th day	10th day	15th day	5th day	10th day	15th day
I - T. Control	34.63±0.61	81.64±1.65	102.54±1.24	20.56±0.93	40.88±0.93	51.12±1.13
II - 50	27.63±0.19*	70.54±0.81	58.66±0.91*	18.16±0.74	31.83±1.61	42.63±1.56
III - 100	20.56±0.14**	43.65±0.54*	56.11±0.74*	15.11±0.69*	19.66±0.56**	24.84±0.89*
IV - 150	16.55±0.54**	39.15±0.64**	52.66±0.61**	13.16±0.18**	17.28±0.14**	21.93±0.65**
V - Vincristin (80)	29.61±0.84*	46.16±1.24*	63.19±1.04	15.22±0.16*	20.16±0.35**	28.16±1.14*

Each Value is ± SEM of 6 animals. Significance between S-180 control and extract treated groups. *p < 0.05; **p < 0.01; ***p <0.001.

Table 4. Effect on the Cellular GSH and NO levels

Group & Dose (mg/ml bw)	GSH (nmol/mg protein)			NO (µM)		
	5th day	10th day	15th day	5th day	10th day	15th day
I - T. Control	8.31±0.22	18.14±0.91	13.67±0.56	9.34±0.54	13.34±0.36	15.06±0.74
II - 50	7.13±0.17	17.21±0.39	11.93±0.16	7.23±0.76	8.17±0.64	9.34±0.43
III - 100	6.33±0.54	15.27±0.39	8.16±0.74*	6.11±0.52*	6.12±0.37*	7.23±0.23*
IV - 150	5.83±0.14*	8.54±0.54**	5.09±0.16**	5.32±0.27**	5.33±0.76**	6.24±0.47**
V – Vincristin (80)	6.05±0.16*	10.11±0.27*	5.81±0.14**	7.64±0.24	6.63±0.18*	6.75±0.32*

Each Value is ±SEM of 6 animals. Significance between S-180 control and extract treated groups. *p < 0.05; **p < 0.01.

Fig 1. Effect on Antibody Titer

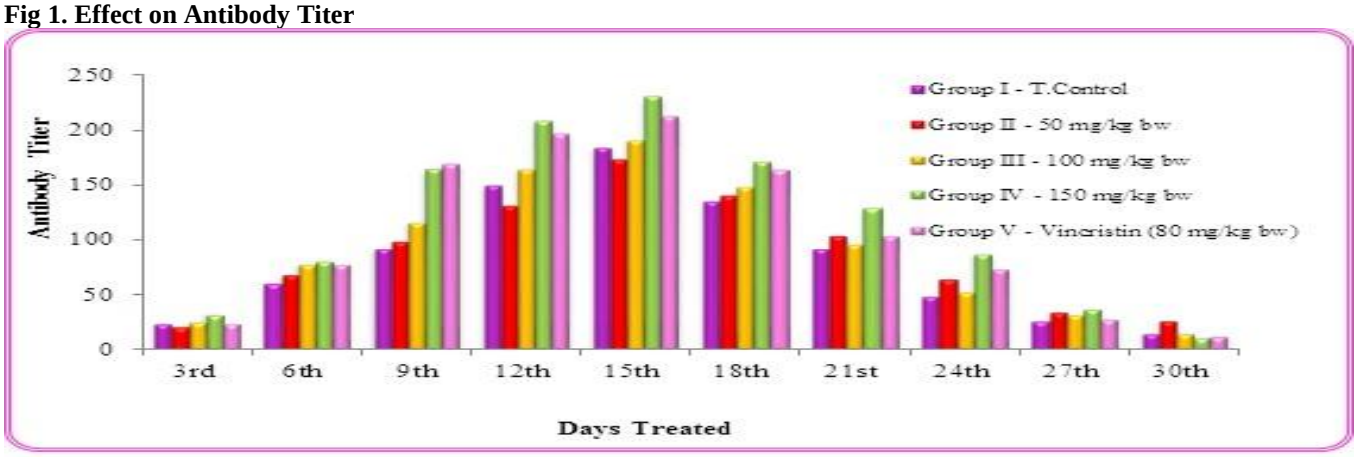
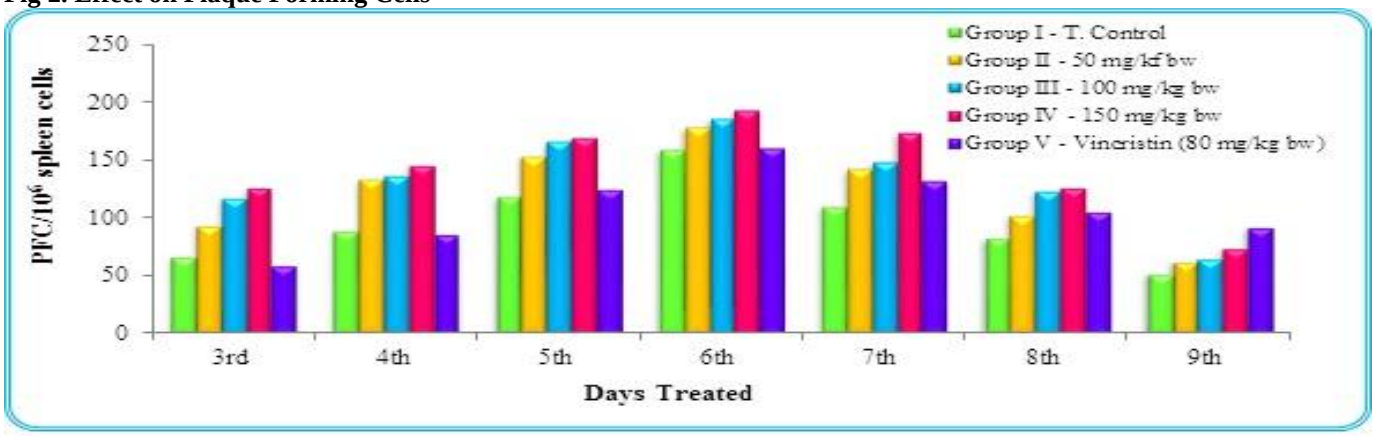


Fig 2. Effect on Plaque Forming Cells



DISCUSSION

Noni fruit juice is 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. In the presence of Noni polysaccharide, macrophages produce NO, several cytokines including interleukin-l, tumor necrosis factor and IL- 12 which stimulate the immune system. The increasing interferon-gamma production stimulates macrophages, NK cells and cytotoxic T cells toward killing tumor cells [32]. It is suggested that the polysaccharides present in the test of *Phallusia nigra* also might play a similar role in stimulating immune function. NK cells are a type of lymphocytes that form part of the first line of innate defense against cancer cells and virus infected cells [33]. 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 a patient's cellular immune response *in vitro* and can also be used to test the antitumor activities of possible drugs [34]. 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. 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 [35]. Elicitation of an effective T and B cell immunity can be seen by the stimulation of lymphocyte proliferation response [36]. 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.

The count of bone marrow nucleated cells is an index which directly reflects hematopoiesis. A large number of bone marrow nucleated cells represent a large number of immature blood cells, which indicates good bone marrow hematopoiesis [37,38]. The count of peripheral white blood cells directly reflects the condition of blood cells and indirectly reflects bone marrow hematopoiesis. Bone marrow is a site of continued proliferation and turnover of blood cells and is a source of cells involved in immune reactivity [39]. A high degree of cell proliferation renders bone marrow a sensitive target, particularly to cytotoxic drugs. The extract was found to increase bone marrow cells and β-Esterase activity significantly. This may indicate an enhancement of the differentiation of stem cells. The increase in the number of bone marrow cells and differentiating stem cells with esterase activity were responsible for immunological response [40]. The same could be attributed to the present findings on treatment with the extract.

Treatment with the extract stimulated the production of anti SRBC antibody, which was maximum on the 15^{th} day in group IV [40]. The increased titer remained for several days indicating there is a sustained immunological activity. An increase in the circulating antibody titer may indicate the stimulatory effect of the extract on the humoral arm of the immune system [2]. Bone marrow is generally considered to be a primary lymphoid organ, since among its progeny are lymphocytes which are of major importance for the immunological capacity of other lymphoid organs [41]. It is capable of antibody synthesis and may be a major source of immunoglobulins [42-44].

During primary response a great number of PFC appears in the spleen and it contains the majority until 9 days after injection [45]. Plaque Forming Cells are responsible for the production of antibody. In the present observation maximum PFC was on the 6^{th} day. The increase in plaque forming cells in the spleen may be due to the activation of humoral immune response [40]. Spleen plays multiple supporting roles in the body. It participates in the creation of blood cells and also helps to filter the blood and fight infection as part of the immune system. It also helps to control the amount of blood circulating through the body. Old red blood cells are recycled and platelets, white blood cells are stored in the spleen.

GGT is an important enzyme required in the maintenance of the steady state concentration of glutathione both inside the cells and in the extra cellular fluids. The serum GGT concentration was higher in the tumor control compared to the treated groups on all the days of observation. GGT, a glycosylated protein, plays critical role in anti oxidant defense, detoxification and inflammation processes [46]. An increased GGT level noted in the tumor control may indicate an adaptive response upon exposure to oxidative stress. Administration of extract was found to reduce the serum GGT, that catalysis the transfer of gamma glutamyl moieties from glutathione to other amino acids and dipeptides [47].

A reduction in NO level was observed during the days of study on treatment with the extract of *Phallusia nigra*. NO is a lipophilic, highly diffusible and short lived physiological messenger which regulates a variety of important physiological processes like immune response and apoptosis [48,49]. NO may participate in the induction of tumor cell growth and invasion [50]. The reduction of NO in tumor cells may increase cell death and exert antineoplastic properties. This might be the reason for the recovery and increase in life span of treated mice.

Glutathione (GSH), a potent inhibitor of the neoplastic process, plays an important role in the

endogenous antioxidant system. It is found in particularly high concentration in the liver and is known to have a key function in the protective process. Excessive production of free radicals results in oxidative stress, which leads to damage to macromolecules, for example, lipid peroxidation *in vivo* [51]. GSH, a major non protein thiol is required for the proliferation and metabolism of tumor cells [2]. A significant reduction of GSH in group IV is indicative of the antiproliferative nature of the extract.

GSH is the master antioxidant which strengthens the immune system by producing T cells and changing the level of reactive oxygen species in isolated cells grown in laboratory which may play a role in reducing cancer development [52,53]. The alteration of redox status and transcriptional pattern modifications induced by NO in tumor cells may exert anticancer properties [54]. A significant reduction in the level of NO was noted in group IV on all the days of study suggesting the same mechanism of action.

CONCLUSION

Administration of the extract of *Phallusia nigra* increased quantitative hemolysis of sheep red blood cells, lymphocyte proliferation, percentage NK cytotoxic activity, phagocytosis rate, bone marrow cellularity, β-esterase positive cells, antibody titer and the plaque forming cells in spleen. A reduction in the serum Gamma Glutamyl Transpeptidase (GGT), cellular Glutathione (GSH) and restoration of NO levels was observed in S-180 tumor bearing mice. The activity was comparatively greater than that observed for the standard drug in all the parameters tested. The GC-MS analysis of ethanolic extract of *Phallusia nigra* [55] showed compounds with antioxidant, cancer preventive and anticancer properties. Studies on the isolation, purification and structure determination of the chemical compounds may lead a drug molecule.

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