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European Journal of Applied Engineering and Scientific Research, 2013, 2 (1):20-24
(http://scholarsresearchlibrary.com/archive.html)

ISSN: 2278 – 0041

Immunomodulatory activity of ethanol extract of *Phallusia nigra* Savigny 1816, against Dalton's Lymphoma Ascites

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ABSTRACT

Immunomodulatory activity of the ethanol extract of Phallusia nigra was evaluated against Dalton's Lymphoma Ascites (DLA) cells on Swiss Albino mice. After tumor inoculation the extract at the dose of 50, 100 and 150 mg/kg body weight were injected intraperitoneally and compared with the standard drug Vincristin (0.08 mg/kg body weight). Administration of the extract increased bone marrow cellularity (27.38×10^6 cells/femur) and β-esterase positive cells (1375/4000 cells). The antibody titer was maximum (247.68±3.19) in Group IV on the 12th day of treatment. Plaque forming cells in spleen increased gradually reaching a peak (169.93×10^6) on the 6th day. A reduction in the serum Gamma Glutamyl Transpeptidase (GGT), cellular Glutathione (GSH) and restoration of NO levels was observed on the 15th day in tumor bearing mice. The results indicate that the extract contains bioactive compounds playing important role in immune response.

Key words: *Phallusia nigra*, immunomodulatory, DLA cells

INTRODUCTION

Cancer is an uncontrolled proliferation of cells resulting in millions of death worldwide. Plant derived natural products like flavonoids, terpenes, alkaloids [1,2,3] 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 [4]. Immunomodulators are well known for their antitumor activity. Polysaccharides are very proficient immunomodulators activating the immune system [5,6]. There are several medicinal plants that are considered to possess immunomodulatory properties augmenting specific cellular and humoral immune response [7]. From marine organisms numerous novel compounds with biological activities have been isolated. Compounds derived from various species of ascidians like Wakayin [8] from *Clavelina* species, ascididemnin [9] from *Didemnum* sp., 2-Bromoleptoclinides [10] from *Leptoclinides*, Cystodytin A,B and C [11] from *Cystodytes dellechiajei*, Varamines A and B [12] from *Lissoclinum various*, Diplamine [13] from genus *Diplosoma*, Eudistomidins B,C and D [14] from *Eudistoma glaucus*, Grossularines [15] from *Dendrodoa grossularia* and Bistramides A,B,C,D [16] from *Lissoclinum bistratum* are known to possess anticancer properties. As ascidians are available in plenty along the Tuticorin coast an attempt has been made to assess their immunomodulatory effect against DLA tumor bearing mice.

MATERIALS AND METHODS

Specimen collection and identification: Samples of *Phallusia nigra* were collected from Tuticorin coast and identified up to the species level using key to identification of Indian ascidians [17].

Systematic position: *Phallusia nigra* comes under Phylum: Chordata, Subphylum: Urochordata, Class: Ascidiacea, Order: Enterogona, Suborder: Phlebobranchia, Family: Ascidiidae, Genus: *Phallusia*, Species: *Phallusia nigra*.

Experimental animals: Healthy Adult Swiss Albino mice weighing 20-25 g were procured and maintained in well ventilated room, fed with normal mice chow and water ad libitum. The experiments were conducted as per the rules and regulations of Animal Ethical Committee, Government of India.

Tumor cells: Dalton's Lymphoma ascites (DLA) cells were obtained from Adayar Cancer Institute, Chennai, India. Sheep red blood cells (SRBC) were collected from local slaughter house in Alsever's solution

Extraction: Samples of *Phallusia nigra* were dried at 45^{o}C and powdered. Ten grams of the powder was soaked overnight in 100 ml 70% ethanol and centrifuged at 10,000 rpm at 4^{o}C for 10 minutes. The Supernatant was collected, evaporated and suspended in 1% gum acacia, blended with vanillin and administered intraperitoneally.

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. Group V was treated with standard drug Vincristin (0.08 mg). DLA cells (1×10^{6} cells/mouse) were injected intraperitoneally for 5 days.

Effect on Bone marrow cellularity and β- esterase activity: Experimental animals treated for 5 consecutive days were sacrificed 24 hours after drug Treatment, the bone marrow cells were collected from the femur, made into single cell suspension and the number of cells determined by using haemocytometer. Bone marrow cells from the above preparation was smeared on clear glass slide and stained with Harri's Hematoxylin to determine the non-specific β- esterase activity by the azodye coupling method [18].

Effect on circulating antibody titer: Swiss Albino mice were divided into five groups of 6 animals each. Group I was immunized with SRBC (0.1 ml, 20 %). Group II, III, IV were treated with 50, 100, 150 mg/kg body weight of the extract and Group V with Vincristin (0.08 mg) along with 0.2 ml SRBC for 5 consecutive days. Blood was collected from caudal vein every 3rd day after drug administration and continued for a period of 30 days. Serum was separated, heat-inactivated at 56°c for 30 minutes and used for the estimation of antibody titer [19] using SRBC as antigen.

Effect on antibody producing cells: To determine the effect of the extract on the 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 9th day. Spleen was processed to single cell suspension and the number of plaque forming cells (PFC) was determined by the Jerne's plaque assay [20].

Effect on serum Gamma Glutamyl Transpeptidase (GGT) and Nitric oxide (N0) **levels:** Blood was collected from experimental animals treated with the extract for 5 consecutive days at different time points (5,10, 15th day) and the serum was used for the estimation of GGT [21] and NO [22] levels.

Effect on cellular Glutathione (GSH) and Nitric Oxide (NO) levels: Blood was collected at different time points (5, 10,15th day) and the cells (1X10 cells/ml) were sonicated for 30 seconds and used for the estimation of GSH [23] and NO [22].

Statistical Analysis: The results are expressed as mean ± SEM and 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

Effect on Bone marrow cellularity and β- Esterase activity: Extract treated animals showed significant increase in bone marrow cellularity ($27.38X10^{6}$cells/femur). The number of β-esterase positive cells increased significantly (1375/4000 cells) given in Table 1. Thymus is a lymphoid organ which plays an important role in immunomodulations by activating the humoral and cellular immune system. 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 in the extract treated animals suggests *Phallusia nigra* has factors that bring about immunological response [24].

Effect on circulating antibody titer: Administration of the extract increased the antibody titer in a dose dependent manner (Table 2). The most significant value (247.68±3.19) was observed on 12th day in Group IV treated with 150 mg/ kg body weight compared to control with 148.22±5.57 on the same day. This shows treatment with extract stimulates the production of anti SRBC antibody. Increased titer remained for several days indicating there is a sustained immunological activity [24]. Increase in the circulating antibody titer may indicate the stimulatory effect of the extract on the humoral arm of the immune system [6].

Effect on plaque forming cells: The effect of extract on the number of plaque forming cells is shown in Table 3. The maximum number of plaque forming cells in the treated group (169.93 PFC/10^6 spleen cells) was observed on the sixth day where as the control animals had a maximum of 128.56 PFC/10^6 spleen cells. Antibody produces plaque forming cells. Hence an increased antibody titer might have caused higher PFC. The increase in plaque forming cells in the spleen may be due to the activation of humoral immune response [24].

Effect on serum GGT and NO levels: On the 15thday, an elevated level of GGT in the serum of control tumor bearing mice (103.55+2.01nmol p- nitroaniline /ml serum was observed. There was a significant reduction (31.88+0.85 n mol p- nitroaniline/ml serum) after the administration of the extract of *Phallusia nigra* (Table 4). The serum NO level was found to be maximum (46.23+0.54µm) on the 15th day of tumor progression, whereas in the treated group there was a significant reduction (27.18±0.43). GGT is an important enzyme required in the maintenance of the steady state concentration of glutalhione both inside the cells and in the extra cellular fluids. GGT, a glycosylated protein, plays critical roles in anti oxidant defense, detoxification and inflammation processes [25]. The increased GGT level may indicate an adaptive response upon exposure to oxidative stress. Administration of extract was found to reduce the serum gamma glutamyl transpeptidase (GGT), that catalyses the transfer of gamma glutamyl moieties from glutathione to other amino acids and dipeptides [26]. As NO is a lipophilic, highly diffusible and short lived physiological messenger [27], it regulates a variety of important physiological responses like immune response and apoptosis [28]. NO may participate in the induction of tumor cell growth and invasion [29].The reduction of NO in tumor cells may increase cell death and exert antineoplastic properties.

Effect on GSH and NO levels: In the control a maximum cellular GSH (10.54±0.56) was observed on the 10th day of tumor growth (Table 5). On the 15th day, in group IV the level of GSH was restored to normal (6.88±0.23) where as the control showed higher value (8.67±0.21). Earlier reports have shown that GSH, a major non protein thiol is required for the proliferation and metabolism of tumor cells [6]. A significant reduction of GSH in group IV is indicative of the antiproliferative nature of the extract. Moreover 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 [30,31]. The alteration of redox status and transcriptional pattern modifications induced by NO in tumor cells may exert anticancer properties [32].

TABLE 1: Effect on bone marrow cellularity and β-esterase activity in DLA tumor bearing mice

Treatment	Bone marrow cellularity (10^6cells/femur)	β-Esterase activity (β-esterase positive cells /4000 cells)
Group I	18.56±0.58	795± 43
Group II	19.44±0.32	932± 21
Group III	23.76±0.48*	1176± 43*
Group IV	27.38±0.55**	1375± 53**
Group V	25.22±0.97**	1215± 34**

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

TABLE 2: Effect on antibody titer in DLA tumor bearing mice

Days Treated	Antibody titer				
	Group I	Group II	Group III	Group IV	Group V (Vincristin)
3	15.56± 0.14	18.34± 0.56	14.98±1.21	20.54 1.55	17.45± 1.34
6	26.87±1.11	31.67 ±1.45	69.28 ±1.87*	98.69± 3.72*	89.11± 2.92*
9	67.34± 3.45	64.22± 2.12	136.91 2.93*	168.67 2.64**	149.47±2.78*
12	148.22 5.57	114.54±2.78	162.80±2.63**	247.68±3.19***	211.64 ±.34***
15	94.89±3.78	71.85± 1.96	83.89± 2.34	131.67 ±2.94	103.73±2.66
18	43.64± 2.97	41.48± 2.17	39.03 ±1.98	64.77±2.83	53.45±2.63
21	40.87± 2.73	32.56 ±1.21	28.46± 1.66	41.33± 2.62	37.19±2.94
24	28.66± 2.56	23.45±1.38	19.29± 1.81	27.29±2.38	23.55±1.78
27	19.67 ±1.45	17.73± 1.05	13.45±0.93	16.59± 1.52	17.29±1.34
30	13.27± 1.44	15.98 ±1.65	11.31 ±1.31	13.67± 1.31	16.58±1.93

*Each value is ±SEM of 6 animals, Significance between tumor induced control vs drug treated group * P < 0.05, ** P < 0.01, *** p<0.001.*

TABLE 3: Effect on plaque forming cells (PFC)

Days Treated	PFC/10^6 spleen cells				
	Group I (Control)	Group II	Group III	Group IV	Group V (Vincristin)
3	47.34± 2.13	76.39± 2.65	82.92±2.17	102.93±2.34	57.84± 1.27
4	64.78±2.78	97.28 ±2.11	113.58 ±1.96	128.48±1.66	81.07± 1.36
5	90.34±2.78	118.72± 3.02	136.79 ±2.53	146.94± 2.79	103.47±1.66
6	128.56± 3.65	153.84± 2.55	159.47± 2.58	169.93± 2.16*	132.54± 1.48
7	112.73± 2.13	141.66 ±3.54	138.94± 2.59	150.44± 2.41	112.72 ±1.89
8	94.28± 2.15	114.59± 2.16	123.66± 2.04	128.78±2.04	98.04±1.57
9	65.88± 2.87	93.48± 2.72	101.38± 1.93	113.50± 2.41	73.64 ±1.21

*Each value is ±SEM of 6 animals Significance betwee n tumor induced control vs Drug treated group * P < 0.05.*

TABLE 4: Effect on the serum GGT and no levels of DLA-bearing mice

Group	Dose (mg/ml)	GGT (nmol p-nitroaniline/ml)			NO (µM)		
		5^{th} day	10^{th} day	15^{th} day	5^{th} day	10^{th} day	15^{th} day
Group I	T.Control	38.85± 1.03	84.83± 1.69	103.55±2.01	20.78±0.46	31.75±0.43	46.23± 0.54
Group II	50	22.43± 0.23*	66.78± 0.38*	53.83± 1.45*	17.29±0.12	26.07±0.98	38.71± 0.53
Group III	100	18.67± 0.56**	48.86± 0.53*	46.98± 0.94**	13.58±0.79*	19.56±0.67*	31.97± 0.67
Group IV	150	16.75± 0.08***	40.28± 0.47*	31.88± 0.85***	10.17±0.46*	18.60±0.11**	27.18± 0.43*
Group V	Vincristin	23.48± 0.27*	37.59± 0.36**	53.78± 0.46*	18.99±0.26	25.33±0.17*	36.91± 0.43*

*Each value is ±SEM of 6 animals Significance betwee n tumor induced control vs drug treated group * P < 0.05, ** P < 0.01, *** p<0.001.*

TABLE 5: Effect on the cellular GSH and no levels of DLA cells at different stages of tumor growth in vivo

Group	Dose (mg/ml)	GST (nmol/mg protein)			NO (µM)		
		5^{th} day	10^{th} day	15^{th} day	5^{th} day	10^{th} day	15^{th} day
Group I	T.Control	5.34±0.07	10.54±0.56	8.67±0.21	6.35±0.65	10.34±0.13	12.07±0.56
Group II	50	5.97 ±0.43	7.56± 0.41*	6.48±0.05	5.23 ±.04	6.57±0.23	7.88± 0.62*
Group III	100	7.04 ±0.22*	8.67 ±0.28	5.93±0.08	4.81±0.12	4.06±0.44**	6.20± 0.14
Group IV	150	7.93± 0.34*	8.23± 0.39*	6.88±0.23	5.93±0.71*	4.91±0.76**	5.89± 0.23
Group V	Vincristin	7.79± 0.72*	8.93± 0.45	7.56±0.53	8.94±0.63	7.99± 0.45	6.48± 0.31

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

CONCLUSION

Administration of the extract of *Phallusia nigra* increased 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 on the 15^{th} day in DLA 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* [33] showed compounds with antioxidant, cancer preventive and anticancer properties like 2-Piperidinone, Benzeneacetamide, Tetradecanoic acid, n-Hexadecanoic acid, Phenol 3-pentadecyl, (Z,Z,Z)- phenylmethyl ester of 6,9,12-Octadecatrienoic acid, (z)-phenylmethyl ester of 9-Octadecenoic acid, Cholesterol,Cholestan-3-ol and 3-hydroxy-,(3a,17a)-Spiro [androst-5-ene-17,l'-cyclobutan]-2'-one. Further studies on the isolation, purification and structure determination are needed to come to any definite conclusion on the compound responsible for and the mechanism of action.

Acknowledgement
The authors express their sincere thanks to the UGC, New Delhi for financial assistance and to Dr. R.Smpathraj, Director, Samsun Clinical Research Laboratory, Tirupur for animal studies.

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