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RESEARCH ARTICLE

IMMUNONATURE OF *ECTEINASCIDIA VENUI* MEENAKSHI, 2000 AGAINST EHRLICH ASCITES CARCINOMA

***,[1]Sankaravadivu, S., [2]Jothibai Margret, R. and [3]Meenakshi, V. K.**

[1]Department of Chemistry, V.O. Chidambaram College, Tuticorin
[2]Department of Chemistry, Pope's College, Sawyerpuram, Tuticorin
[3]Department of Zoology, A.P.C. Mahalaxmi College for Women, Tuticorin

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ABSTRACT

In the recent scenario of emerging infectious diseases, studies on immunomodulations as an effective and protective approach have become inevitable. Demand for safe therapeutic agents in the treatment of cancer is on the increase. The immunonature of the ethanol extract of *Ecteinascidia venui* was determined against Ehrlich Ascites Carcinoma (EAC) bearing Swiss albino mice. After tumor inoculation, the extract at the dose of 100, 150 and 200 mg/kg body weight were administered orally and compared with the standard drug Vincristin (80 mg/kg bw). Analysis of Bone marrow cellularity, β-esterase activity, antibody titre, plaque forming cells, GGT, GSH and NO levels were carried out adopting standard procedures. Treatment with the extract increased bone marrow cellularity (26.16×10^6 cells/femur) and β-esterase positive cells (1218/4000 cells). The antibody titer was maximum (216.13±6.84) in Group III on the 15th day of treatment. Plaque forming cells in spleen increased gradually reaching a peak (224.53±2.65) on the 6th day. A reduction in serum Gamma Glutamyl Transpeptidase (GGT), cellular Glutathione (GSH) and restoration of NO levels was observed on the 15th day in treated mice. The results indicate that the extract contains bioactive compounds playing important role in immune response.

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INTRODUCTION

The great majority of chemicals identified as cytotoxic to cancer cells are generally also toxic to normal cells (Kim *et al*., 1996). Nevertheless, the potentiation of host defense mechanisms has been recognized as a possible means of inhibiting tumor growth without harming the host (Ameho *et al*., 1997). Therefore, searching for materials with immunonature from natural herbs, marine organisms and characterizing the immune enhancement effects may have great potential in cancer treatment (Rivera *et al*., 2003). The most important marine source for active agents except sponge is the ascidians commonly found in waters all over the world, along the coasts and deep to the bottoms. Since 1980s, a great variety of bioactive substances were extracted from the ascidians with cytotoxic, antitumor, antivirus, antimicrobial, immune regulatory and biocatalyst activities. Further studies showed that the active chemical compounds in sea squirt mainly included peptides, alkaloids, polyethers, macrolides, terpenes and polysulfides (Hindawi 2014; Zabriskie *et al*., 1989; Kobayashi *et al*., 1991; Davidson *et al*., 1990; Makarieva *et al*., 1995 Ford *et al*., 1997; Ireland *et al*., 1980; Chun *et al*., 1986; Baker *et al*., 2002) among which some have entered into clinical trials as anticancer reagents (Vera *et al*., 2002; Jin *et al*., 2000; Brain 2002). By strengthening organisms' defense and host immune response, cell mediated immunity can improve its overall antitumor ability and thus play an important role in immune antitumor treatment. *Ecteinascidia venui* remains neglected as a potential marine chemical resource. Ecteinascidins, a promising group of bioactive metabolite isolated from the colonial ascidian *Ecteinascidia turbinata* (Herdman), exhibits strong antitumor properties (Waterval *et al*., 2001; Cai 2006; Wagner 1990). Antiproliferative activity of different species of ascidians to human cancer cell lines have been investigated (Ghule *et al*., 2006; Makare *et al*., 2009; Stewart *et al*., 2011; Zitvogel *et al*., 2008a,b; Xua *et al*., 2009)). Many Indian ascidians have been proved to exhibit pharmacological potency (Gopalakrishnan *et al*., 2011a,b; 2012a-d 2013a,b; Meenakshi *et al*., 2012a,b,c, 2013a-e, 2014 a-e; Kohila Subathra Christy *et al*., 2014a,b,c, 2015; Delighta *et al*., 2015a-c; Paripooranaselvi *et al*., 2015a,b; Shanmuga Priya *et al*., 2015a-d, 2016; Stella *et al*., 2013,2015) Review of literature reveals that only chemical investigation of *Ecteinascidia venui* has been carried out so far (Sankaravadivu *et al*., 2013a,b, 2015, 2016a,b,c,d,e). Hence,

Corresponding author: Sankaravadivu, S.,
Department of Chemistry, V.O. Chidambaram College, Tuticorin.

the present study was designed to evaluate its immunonature against EAC.

MATERIALS AND METHODS

Specimen collection and identification

Ecteinascidia venui was collected from the hull of ships during dry docking in the month of May 2013. Epibionts and particles of shell, coral fragments attached to the colony were carefully removed. Identification up to the species level was carried out based on the key to identification of Indian ascidians (Meenakshi 1997). A voucher specimen AS 2247 has been submitted in the ascidian collections of the Museum of the Department of Zoology, A. P. C. Mahalaxmi College for Women, Tuticorin - 628002, Tamilnadu, India.

Systematic position

Ecteinascidia venui belongs to Phylum: Chordata, Subphylum: Urochordata, Class: Ascidiacea, Order: Enterogona, Suborder: Phlebobranchia, Family: Perophoridae, Genus: *Ecteinascidia*, Species: *venui*.

Experimental animals

Swiss albino mice weighing 20-25 g were collected from Central Animal House, Dr. Raja Muthiah Medical College, Annamalai University, Chidambaram, Tamilnadu. The animals were fed with normal mice chow and water ad libitum in air-controlled room with constant 12 hours of dark light schedule, room temperature (24±2 ºC) and 60 - 70 % humidity. The experimental work was done as per the rules and regulations of Animal Ethical Committee, Government of India.

Cells for cytotoxic study

Ehrlich Ascites Carcinoma (EAC) cells were purchased from Adayar Cancer Institute, Chennai, India. The cells were maintained as ascites tumors in Swiss albino mice.

Preparation of powder and extract

Colonies of *Ecteinascidia venui* were dried at 45°C, powdered, soaked overnight in 100 ml 70% ethanol and 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 vivo studies. It was suspended in 1% gum acacia blended with vanillin and administered orally using intra gastric catheter for animal experiments.

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 100, 150 and 200 mg/kg of the extract. Group V was treated with standard drug Vincristin (80 mg). EAC cells (1×10^6 cells/mouse) were injected intraperitoneally for 5 days.

Effect on Bone marrow cellularity and β- esterase activity

24 hours after drug treatment for 5 consecutive days, the experimental animals were sacrificed. From the femur, bone marrow cells were collected made into single cell suspension and the number of cells determined by using haemocytometer. On clear glass slide cells from the above preparation was smeared and stained with Harri's Hematoxylin to determine the nonspecific β- esterase activity (Bancroft *et al*., 1984).

Effect on circulating antibody titer

Five groups of 6 Swiss albino mice each were selected. Group I was immunized with SRBC (0.1 ml, 20 %). Group II, III, IV and V were treated with 100, 150, 200 mg/kg body weight of the extract and Vincristin (80 mg) along with 0.2 ml SRBC for 5 consecutive days. For a period of 30 days blood was collected from caudal vein every 3rd day after drug administration. The estimation of antibody titer (Singh *et al*., 1984) using SRBC as antigen was done using serum separated and heat-inactivated at 56°C for 30 minutes.

Effect on antibody producing cells

After immunization up to 9th day, half of the experimental animals from the above treatment were sacrificed on different days starting from the third day to determine the effect of the extract on the antibody producing cells. The number of plaque forming cells (PFC) was determined by Jerne's plaque assay using processed cell suspension of spleen (Jerne 1963).

Effect on serum Gamma Glutamyl Transpeptidase (GGT) and Nitric oxide (N0) levels

At different time points (5, 10, 15th day), from the experimental animals treated with the extract for 5 consecutive days, blood was collected and the serum was used for the estimation of GGT (Snasz 1976) and NO levels (Green *et al*., 1982).

Effect on cellular Glutathione (GSH) and Nitric Oxide (NO) levels

For the estimation of GSH (Moron *et al*., 1979) and NO (Green *et al*., 1982) $1X10^6$cells/ml were sonicated for 30 seconds from the blood collected at different time points (5, 10,15th day).

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 noted significant increase in bone marrow cellularity ($26.16X10^6$cells/femur) and number of β-esterase positive cells (1218/4000 cells) Table 1. Bone marrow is a site of continued proliferation and turnover of blood cells involved in immune reactivity, rendering it a sensitive target, particularly to cytotoxic drugs (Moron *et al*., 1979). 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 (Pelczar *et al*., 1979)The increase in the number of bone marrow cells and differentiating stem cells with esterase activity in the extract treated animals suggests the presence of factors that bring about immunological response (Manu *et al*., 2009).

Table 1. 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	16.13x10^6±0.18	643±18
II -100	18.32x10^6±0.93	816±27*
III - 150	20.88x10^6±0.81*	1104±37**
IV -200	26.16x10^6±1.31**	1218±43**
V - Vincristin (80)	25.13x10^6±1.16	1193±69

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

Table 2. Effect on Antibody Titer

Days Treated	Antibody Titer				
	Group & Dose (mg/kg bw)				
	Group – I T. Control	Group – II 100	Group – III 150	Group – IV 200	Group – V Vincristin 80
3	19.22±0.05	24.13±0.13	28.11±2.16	26.13±1.93	21.84±0.84
6	48.13±0.09	69.11±1.83	84.16±3.16	72.16±1.39	41.84±3.16
9	78.27±0.98	103.84±2.51	128.29±4.16	113.84±3.38	108.13±4.84
12	159.11±0.24	168.11±3.84	197.24±4.93*	193.86±2.84*	183.80±4.16
15	178.00±0.05	199.33±4.84	216.13±6.84**	208.73±4.84**	198.38±3.84
18	189.65±0.48	131.84±2.31	167.16±3.04	179.31±3.16	183.15±2.16
21	141.84±0.98	113.96±2.14	96.16±2.83	103.84±3.14	138.16±4.93
24	84.33±1.89	78.24±1.33	61.37±0.98	73.18±1.31	118.04±1.04
27	48.08±2.11	27.93±1.08	21.16±1.36	26.84±2.04	50.16±1.43
30	13.84±1.33	11.96±1.31	10.16±1.12	12.16±0.92	18.14±0.92

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

Table 3. Effect on Plaque Forming Cells (PFC)

Days Treated	PFC/10^6 spleen cells				
	Group & Dose (mg/kg bw)				
	Group – I (T. Control)	Group – II 100	Group – III 150	Group –IV 200	Group – V Vincristin 80
3	51.65±1.16	82.16±0.67	103.42±5.41	124.24±1.95	68.27±0.84
4	83.27±1.81	112.67±0.16	123.94±2.84	168.53±2.61	93.64±0.46
5	126.39±2.16	148.13±0.27	142.66±3.06	195.16±1.94	138.15±1.24
6	163.16±3.06	193.15±2.15*	204.15±2.65**	224.53±2.65***	152.66±1.36
7	131.43±2.16	158.15±1.93	164.16±1.65	132.40±2.92	128.13±0.68
8	109.27±2.46	131.63±1.54	136.28±1.83	121.83±2.16	103.69±0.27
9	113.46±1.26	148.15±1.68	142.96±2.15	139.76±3.65	95.46±0.92

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

Table 4. Effects on the Serum GGT and NO levels

Group & Dose (mg/kg bw)	GGT (nmol p-nitroaniline/ml)			NO (μM)		
	5th day	10th day	15th day	5th day	10th day	15th day
I -T. Control	31.65±0.54	81.22±1.51	89.65±1.24	16.31±0.31	30.65±0.21	39.63±0.75
II - 100	23.88±0.27	46.84±1.26*	56.35±1.61*	13.66±0.75	21.63±0.69*	26.22±0.53*
III - 150	18.31±0.36*	29.33±1.04**	39.66±1.21**	9.63±0.35*	14.84±0.36**	19.65±0.23**
IV - 200	15.22±0.27*	24.38±0.67**	32.81±0.67***	7.41±0.34**	11.53±0.46***	14.73±0.69***
V - Vincristin (80)	24.96±0.36	39.22±1.26	47.34±1.29	11.76±0.39	21.66±0.69	26.18±0.65

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

Table 5. Effect on the Cellular GSH and NO levels

Group & Dose (mg/kg bw)	GSH (nmol/mg protein)			NO (μM)		
	5th day	10th day	15th day	5th day	10th day	15th day
I - T. Control	9.22±0.16	21.63±0.92	13.46±0.27	16.34±1.24	18.42±1.03	26.33±1.69
II - 100	7.16±0.21	18.36±0.74	10.22±0.16	13.54±0.93	14.27±0.68	16.22±0.31
III - 150	5.27±0.16	13.42±0.48*	7.43±0.18*	9.65±0.7*	8.54±0.37*	11.83±0.27*
IV - 200	4.39±0.21	9.36±0.27**	4.16±0.21**	6.88±0.39**	5.04±0.21**	7.46±0.18**
V - Vincristin (80)	7.36±0.44	8.93±0.55	5.84±0.24	6.08±0.32	6.18±0.31	7.22±0.27

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

Effect on circulating antibody titer

Table 2 shows the effect of *Ecteinascidia venui* on antibody titer. Antibody titer exhibited a gradual increase from 3rd to 15th day followed by a decrease towards the end of the experiment. The most significant value (216.13±6.84) was observed on 15th day in Group III treated with 150 mg/ kg body weight compared to control (178.00±0.05). This shows treatment with extract stimulates the production of anti SRBC antibody. Increased titer remained for several days indicating sustained immunological activity (Manu *et al*., 2009). Elevation in the circulating antibody titer may indicate the stimulatory effect of the extract on the humoral arm of the immune system (Guruvayoorappan *et al*., 2007).

Effect on plaque forming cells

Effect of the extract on plaque forming cells is shown in Table 3. Plaque forming cells were maximum in the highest dose treated group (224.53±2.65) compared to control (163.16±3.06

PFC/10^6 spleen cells) on the sixth day. Antibody produces plaque forming cells. Hence an increased antibody titer might have caused higher PFC. Humoral immune response activation may be due to increase in plaque forming cells in the spleen (Manu *et al*., 2009).

Effect on serum GGT and NO levels

On the 15th day, GGT in the serum of tumor control was (89.65±1.24 nmol p- nitroaniline /ml) and on treatment with the extract of *Ecteinascidia venui* a significant reduction (32.81±0.67 n mol p- nitroaniline/ml serum) was noted (Table 4). 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. GGT, a glycosylated protein, plays critical roles in antioxidant defense, detoxification and inflammation processes (Zhang *et al*., 2009). 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 catalysis the transfer of gamma glutamyl moieties from glutathione to other amino acids and dipeptides (Meister 1976). Serum NO level was found to be maximum (39.63±0.75µM) on the 15th day of tumor progression in control, whereas in the treated group there was a significant decrease (14.73±0.69). As NO is a lipophilic, highly diffusible and short lived physiological messenger (Lancaster 1987), it regulates a variety of important physiological activities like immune response and apoptosis (Ignarro *et al*., 1987). NO may participate in the induction of tumor cell growth and invasion (Lala *et al*., 2001).The reduction of NO in tumor cells may increase cell death and exert anticancer properties.

Effect on GSH and NO levels

In control, a maximum cellular GSH (21.63±0.92) 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 (4.16±0.21) where as the control showed higher value (13.46±0.27). Earlier reports have shown that GSH, a major non protein thiol is required for the proliferation and metabolism of tumor cells (Manu *et al*., 2009). 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 (Han *et al*., 2009; Chow *et al*., 2007). Serum NO level was found to be maximum (26.33±1.69µM) on the 15th day of tumor progression in control, whereas in the treated group there was a significant decrease (7.46±0.18). The alteration of redox status and transcriptional pattern modifications induced by NO in tumor cells may exert anticancer properties (Muntane *et al*., 2010). The GC-MS analysis of ethanolic extract of *Ecteinascidia venue* (Sankaravadivu *et al*., 2013) showed compounds with antioxidant, cancer preventive and anticancer properties. Studies on the isolation, purification and structure determination of the chemical compounds may lead to a drug molecule.

Conclusion

The above results and analysis using *Ecteinascidia venui* extracts demonstrates its in vivo immunonature against EAC bearing mice, with significant activities against cancer cells. The findings also provide empirical explanation for the use of ascidians to enhance immune systems of cancer patients. Further studies to ascertain the immune nature and active constituents involved have to be worked out.

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