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Original Research Article

Evaluation of anticancer activity of *Ecteinascidia venui* Meenakshi, 2000 against S-180

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Abstract: Cancer is a complex disease involving cell transformation, dysregulation of apoptosis, proliferation, invasion, angiogenesis and metastasis. Treatment for cancer does not have potent medicine as the currently available drugs are causing side effects in some instances. New drugs with milder side effects are needed to replace and improve existing medicine. In this context, the natural products derived from marine organisms have gained significance. The current study aims at evaluating anticancer activity of *Ecteinascidia venui* against S-180 cell line. The *in vitro* viability of S-180 cells to varying concentrations of the extract was assessed. *In vivo* anticancer activity of the ethanol extract of *Ecteinascidia venui* at doses of 100,150, 200 and vincristin 80 mg/kg body weight against S-180 bearing mice was made by the study of the following parameters: weight of relative organs, tumour, percentage inhibition, median survival time, solid tumor volume, packed cells, viable, non viable cell count, increase in life span and hematology adopting standard methods. The results showed a decrease in tumor weight, volume, packed, viable cells and increase in percentage inhibition, life span, median survival time and non-viable cells. In the groups treated with different doses of extract, the content of Hb, RBC, and Lymphocytes increased whereas WBC, Neutrophils and Eosinophils decreased. The results suggest that the extract of *Ecteinascidia venui* exhibit significant anticancer activity on S-180 bearing mice.
Keywords: S-180, anticancer, hematology, *Ecteinascidia venui*.

INTRODUCTION

Cancer is a class of disease surpassing other illnesses as a principle cause of morbidity and mortality even in developing countries with the changing standard of living and food habit[1]. Surgery, radiotherapy and chemotherapy- the established treatment modalities for various cancers are costly, having serious toxic side effects, residual morbidity and frequent relapse which results in restricted usage. Chemoprevention is a rapidly growing field of oncology which aims to prevent cancer growth using natural or synthetic interventions[2]. The demand for newer, more effective and safer therapeutic agents for the treatment, control and prevention of human cancer has increased. From marine organisms such as ascidians, sponges and soft corals containing symbiotic microorganisms, numerous novel compounds with biological activities have been isolated [3-5]. 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 sarcomas [6]. Various species of ascidians from Indian water has been studied for their toxicity, antimicrobial, antiproliferative, antitumour, immunomodulatory, analgesic, anaesthetic, antipyretic, antiinflammatory, antidiabetic, hepatoprotective, antifertility, wound healing, CNS depressant, cardioprotective and hyperlipidemic activities [7-38]. Review of literature reveals that only chemical investigations and antitumour effect to DLA, EAC cells using *Ecteinascidia venui* has been carried out so far [39-47]. The objective of the present study is to evaluate the anticancer activity of the ethanolic extract of colonial ascidian *Ecteinascidia venui* against S-180 bearing Swiss albino mice.

MATERIALS AND METHODS

Specimen collection and identification

Samples of *Ecteinascidia venui* - a marine sedentary colonial ascidian were collected during dry docking in the month of May 2014. Fouling organisms attached to the colony were removed. They were identified up to

the species level based on the key to identification of Indian ascidians[48]. 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

Adult swiss albino mice weighing 20-25 g were collected from Central Animal House, Dr. Raja Muthiah Medical College, Annamalai University, Chidambaram, Tamilnadu. They were fed with pellet diet, water ad libitum and housed in well ventilated room with 12 hours of dark light cycle, 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.

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 vitro* and *in vivo* studies. It was suspended in 1% gum acacia blended with vanillin and administered orally using intra gastric catheter for animal experiments.

Cells for cytotoxic study and their maintenance

S-180 cells were purchased from Adayar Cancer Institute, Chennai, India. All cells were routinely kept in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin G/ 100 µg/ml streptomycin at 37° C in a humidified 5% atmosphere of 5% CO_2-95% air incubator under standard conditions. To maintain exponential growth, cells were seeded at $1x10^5$ cells/ml every 4 to 5 days[49].

Culture of S-180

The viable S-180 cells were counted (Trypan blue indicator) under the microscope and were adjusted at 1×10^6 cells/mL. 0.1 mL of S-180 cells per 10 g body weight of the animals was injected intraperitoneally on day zero. In the abdominal cavity of mouse, they were cultured for 7-10 days. Along with the peritoneal fluid, the cells were harvested, transferred to PBS (Phosphate Buffered Saline) and centrifuged at 1200 rpm for 10 minutes.

***In vitro* cytotoxic activity to S-180 cells**

Various concentrations (0.05, 0.10, 0.20, 0.40, 0.60, 0.80 and 1.00 mg/ml) of *Ecteinascidia venui* extract were prepared. S-180 cells (1×10^6 cells) were incubated with the extract at 37°C for 3hr in a final volume of 1ml. Trypan blue dye exclusion method was followed for testing the viability of the cells[50].

Induction of tumor

After centrifugation, the separated S-180 cells were floated in PBS, again centrifuged. 0.2 ml of S-180 cells was injected subcutaneously in the left groin of the mice. A day of incubation was allowed for the multiplication of cells.

Experimental protocol

Tumor bearing mice were subdivided into five groups of six each. Group I acted as control and was given normal saline. Group II, III and IV were treated with ethanolic extract of *Ecteinascidia venui* at 100, 150 and 200 mg/kg body weight respectively for 9 days and group V with standard drug Vincristin at 80 mg with 24 hours interval. The extract was blended with 1% gum acacia and vanillin solution and administered intra gastrically. Food and water were with held 18 hours before sacrificing the animals.

Weight of body, relative organs, Tumor, % inhibition

At the end of the experiment, the weight of body was noted. Relative organ weight of vital organs - spleen, thymus, liver and kidney were recorded sacrificing the animals, 24 hours after the last dose, solid tumor was removed and weighed. Percentage inhibition of tumor growth was calculated using the following formula:

% inhibition = (Cw-Tw)/Cw x 100

Cw- Average tumor weight of the control
Tw-Average tumor weight of the experimental group

Measurement of solid tumor Volume

With the remaining set of mice the experiment was continued. A vernier calliper was used to measure the radii of tumors at an interval of 5 days for one month starting with 15th day. The tumor volume was calculated using the formula $V=4/3\ \Pi\ r1^2r^2$, where ‘r1’ and ‘r’ represent the major and minor diameter respectively [51].

Effect on Median survival time, lifespan, packed cell volume, viable and non-viable cell Count Median survival time and percentage increase of life span (% ILS)

Daily mortality was recorded for six weeks by monitoring the effect of the extract on tumor growth. Percentage increase of life span (% ILS) was calculated by the following equation[52].

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

Percentage Increase of lifespan = T-C/C × 100
T-Median survival time of treated group
C- Median survival time of control group

Packed cell volume

Packed cell volume was determined by centrifuging a known volume at 10,000 rpm for 5 minutes in a graduated centrifuge tube by microhematocrit method [53].

Viable and non viable cell count

Trypan blue (0.4% in normal saline) dye was used to stain the cells. The cells which did not take up the dye were counted as viable and those which took the stain as non viable[49].

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 after thirty days. For total count, blood was diluted with Turk's fluid (1:20) so as to lyse all the erythrocytes, and leukocytes were loaded onto the Neubauer haemocytometer. Total white blood cell count was determined using the following formula:

$$\frac{\text{No. of cells counted} \times \text{dilution factor} \times \text{depth factor}}{\text{Area counted}}$$

The differential count of WBC was performed to identify lymphocytes, neutrophils and eosinophils in the blood smear[54].

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.

RESULTS AND DISCUSSION

Cytotoxic activity to S-180 cells

Cytotoxicity of the ethanolic extract of *Ecteinascidia venui* to S-180 cells is given in Table 1. Different concentration of extracts - 0.05, 0.10, 0.20, 0.40, 0.60, 0.80 and 1.0 mg/ml indicated 5, 25, 35, 70, 90 and 100 percent cytotoxicity to S-180 cells respectively. A dose dependent increase in cytotoxicity was observed. Ethanolic extract of *Ecteinascidia venui* was found to be 100% toxic at a concentration of 0.80 mg/ml to S-180 cells.

Effect on Relative Organ Weight

Table 2 shows the effect on weight of body, relative organs and percentage of inhibition in S-180 bearing mice. A comparison of the result indicates a dose dependent reduction in body weight in the treated mice which may be because of the decrease in tumour weight. Relative organ weight of spleen, thymus, liver and kidney showed only negligible change compared to control. Decrease in tumor weight corresponds to increase in percentage of inhibition. Spleen, thymus, liver and kidney are vital organs necessary for the stimulation and production of immune related cells to destroy abnormal cells, by cell mediated immune response. Treatment with the extract did not show any change in these organs, indicating normal functioning of the immune system to overcome the stress induced by tumor growth which may be because of the bioactive components like phenols and flavonoids present acting as antioxidants. GC-MS report of colonial ascidian *Ecteinascidia venui* have shown the presence of compounds like Eicosane, Tetradecyloxirane Nonadecane, Methyl ester of (E,E)-9,12-Octadecadienoic acid, methyl ester of (E)-9-Dodecanoic acid with anticancer and antioxidant activity [40].

Effect on Solid Tumor Volume

The effect on solid tumor volume of S-180 bearing mice is shown in Table 3. On 25th and 30th days, a significant dose dependent decrease in tumor volume was noticed in the experimental groups compared to tumor control. On all the days of the experiment, the tumour volume observed in group IV was nearer to that of the group treated with the standard drug.

Effect on Median Survival Time, Percentage increase of Life Span, Packed cell volume, Viable and Non viable cell count

The results on median survival time, percentage increase of life span, packed cell volume, viable and non viable cell count in S-180 tumor bearing mice is noted in Table 4. There was a highly significant increase in median survival time, life span in a dose dependent manner in experimental groups. Percentage increase in life span is one of the reliable criteria for judging the value of any anticancer drug[55]. Packed cell volume and viable cell count indicated significant decrease with increasing concentration of extract. These results could indicate either a direct cytotoxic effect on tumor cells or an indirect local effect, which may involve macrophage activation and vascular permeability inhibition [55]. Non viable cell count increased in a significant and proportionate manner in the experimental groups. Similar observations have been reported with the ethanolic extract of *Phallusia nigra* and *Microcosmus exasperatus* by earlier workers [10, 16].

Effect on Hematological Parameters

The changes in the hematological parameters in S-180 bearing mice are given in Table 5. In the groups which received the extract, a significant dose dependent increase in Hb percentage, RBC and Lymphocytes were noted. WBC, Neutrophils and Eosinophils indicated proportionate decrease. The hematological parameters

were brought back to that of normal on administration of the extract and the values were almost same as that of the standard. The acceptance criteria for verifying the antitcancer activity of a compound is the determination of circulating RBC and the life span prolongation [56, 57]. The major complication in cancer chemotherapy is reduction in RBC count and myelosupression. Treatment with the extract increased the Hb and RBC content of experimental groups indicating normal hemopoiesis and iron absorption. Earlier reports using the extract of simple ascidians have also given similar results [10,16].

Table 1. Cytotoxicity of ethanolic etract of *Ecteinascidia venui* to S-180

S. No	Concentration (g/ml)	Percentage of Cytotoxicity
1	0.05	5
2	0.10	25
3	0.20	35
4	0.40	70
5	0.60	90
6	0.80	100
7	1.00	-

Table 2. Effect of ethanolic extract of *Ecteinascidia venui* on Relative Organ Weight

Group/ Dose (mg/kg)	Relative Organ Weight (g/100g body wt)						
	Body weight	Spleen	Thymus	Liver	Kidney	Tumour weight (g)	Inhibition %
I - Control	29.33±1.84	0.78±0.011	0.39±0.024	3.54±0.16	2.12±0.25	5.98±1.13	
II - 100	29.84±1.36	0.66±0.024	0.22±0.014	3.52±0.34	2.43±0.31	4.03±0.91	32.60
III - 150	29.31±1.31	0.53±0.019	0.26±0.031	3.89±0.13	2.78±0.27	3.38±0.67	43.47
IV - 200	28.16±1.84	0.58±0.016	0.20±0.018	4.31±0.62	3.24±0.13	2.43±0.32	59.36
V - Vincristin 80	28.22±1.92	0.56±0.016	0.24±0.021	4.31±0.18	3.34±0.63	2.28±0.61	61.87

Data represented as mean±SEM, (N=6).

Table 3. Effect of ethanolic extract of *Ecteinascidia venui* on solid tumor volume

Group/ Dose (mg/kg)	Solid Tumor Volume			
	15th day	20th day	25th day	30th day
I – Control	3.93±0.24	4.80±0.28	5.04±0.21	5.38±0.18
II – 100	3.65±0.37	3.25±0.23	2.90±0.18*	2.35±0.34**
III – 150	4.16±0.28	3.45±0.34**	3.10±0.27**	2.15±0.65**
IV – 200	4.24±0.31	3.45±0.28**	2.93±0.13**	2.04±0.54***
V - Vincristin 80	4.08±0.75	3.26±0.18	3.04±0.22	2.21±0.37

Data represented as mean±SEM, (N=6). Significance between S-180 control Vs extract treated group *P<0.05; **P<0.01; ***P<0.0001

Table 4. Effect of ethanolic extract of *Ecteinascidia venui* on median survival time, life span, packed cell volume and viable and non-viable cell count

Group/Dose (mg/kg)	Median Survival time (Days)	Increase of life span (%)	Packed cell volume	Viable cell count 1x 10^{6} cells/ml	Non-viable tumor cells count 1x 10^{7}cells/ ml
I-Control	16.35±0.16	-	3.68±0.18	16.31±1.28	0.98±0.031
II-100	22.16±0.38*	35.53	3.04±0.24	7.34±0.13*	1.24±0.039*
III-150	26.22±0.67**	60.36	2.18±0.18*	5.28±0.22**	2.13±0.048**
IV-200	29.85±0.32***	82.56	0.98±0.11**	3.04±0.45***	2.98±0.027***
V-Vincristin 80	28.25±0.13	72.78	0.93±0.34	3.12±0.28	3.04±0.035

Data represented as mean±SEM, (N=6). Significance between S-180 control Vs extract treated group *P<0.05; **P<0.01; ***P<0.0001

Table 5. Effect of ethanolic extract of *Ecteinascidia venui* on hematological parameters

Group/ Dose (mg/kg)	Hb (gm%)	RBC (million/mm^3)	WBC 10^3 cells/ mm^3)	Differential count		
				Lymphocytes	Neutrophils	Eosinophils
Normal - Saline	13.45±0.83	4.96±0.56	10.13±0.27	50.64±0.23	42.74±0.18	5.63±0.34
I - Control	8.27±0.31	3.18±0.16	13.84±0.28	41.27±0.65	46.18±0.27	12.55±0.62
II - 100	10.29±0.92	3.45±0.25	11.84±0.16	45.16±0.27	50.22±0.34	4.62±0.13
III – 150	12.81±0.75*	4.15±0.27*	12.84±0.31**	49.27±0.31	45.93±0.28	4.80±0.12
IV - 200	13.16±0.35*	4.91±0.28**	10.28±0.69**	50.11±0.65**	41.84±0.37*	8.05±0.55*
V - Vincristin 80	12.93±0.22	4.24±0.11	10.73±0.36	52.15±0.71	40.22±0.26	7.63±0.36

Data represented as mean±SEM, (N=6). Significance between S-180 control Vs extract treated group $^{*}P<0.05$; $^{**}P<0.01$

CONCLUSION

The present study demonstrates that treatment with ethanolic extract of *Ecteinascidia venui* showed a decrease in tumor volume, packed, viable cells and increase in median survival time, life span and non-viable cells of S-180 tumour bearing mice and was effective in inhibiting the tumour growth. All these parameters suggest that *Ecteinascidia venui* exhibits potential anticancer activities.

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