International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Index Copernicus Value (2015): 78.96 | Impact Factor (2015): 6.391

Anticancer Property of Simple Ascidian Phallusia Nigra Savigny, 1816 against MDA-MB-231 Cells

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Abstract: ***Background: Breast cancer is one of the most prevalent disease affecting women. Methods: Female adult Swiss albino mice weighing 20-25 g were used to assess anticancer activity of the ethanol extract of the simple ascidian Phallusia nigra against human breast cancer cell lines (MDA-MB-231) by evaluating cytotoxicity, relative organ weight of vital organs, tumor weight, % inhibition, solid tumor volume, median survival time, % increase of lifespan, packed cell volume, viable and non-viable cell count adopting standard procedures. Results: 100% cytotoxicity was obtained at 0.60 mg/ml concentration. Negligible changes were noted in relative organ weight. There was a dose dependent increase in % inhibition of tumor and decrease in tumor weight. A highly significant reduction in tumor volume was noted. Median survival time, % increase of life span, non viable cells increased significantly and packed cell volume, viable cells decreased. Conclusion: In-vivo studies indicate the presence of bioactive compounds in Phallusia nigra with anticancer property against MDA-MB-231.***

Keywords: *Phallusia nigra*, anticancer, MDA-MB-231 cells

1. Introduction

Cancer still remains one of the most serious human health problems and breast cancer is the second most universal cause of deaths in women [1]. One of the treatments used currently is chemotherapy which kills cancer cells along with healthy ones. The major drawback associated with chemotherapy is multidrug resistance to anticancer drugs [2]. Therefore, development of a target specific drug without any side effect to normal cells is an ongoing effort in the field of cancer drug discovery. In this context, natural products from marine organisms especially ascidians rank second with most promising source of drugs for cancer [3]. They are an interesting group of marine sedentary organisms commonly called 'sea squirts' found to occur in Tuticorin coast.

2. Literature Survey

Ecteinascidin-743 (ET-743), a tetrahydroisoquinoline alkaloid originally isolated from the Caribbean tunicate *Ecteinascidia turbinata*, became clinically available in the EU and South Korea under the trade name Yondelis to treat soft tissue sarcoma and relapsed platinum-sensitive ovarian cancer [4],[5]. The *in vitro* growth inhibitory and potential anticancer efficacy of lissoclibadins and lissoclinotoxins isolated from the tropical ascidian *Lissoclinum* cf. *badium* against nine human cancer cell lines including MDA-MB-231 were evaluated [6]. Eusynstyelamide B of ascidian *Eusynstyela latericius* showed cytotoxicity against MDA-MB-231 breast cancer cells by inducing apoptosis [7]. The molecular fraction of the extract of *Phallusia nigra* obtained by ultrafiltration inhibited cell proliferation by decreasing the number of viable cells and DNA synthesis of T47D cells derived from human breast carcinoma [8]. A significant anticancer activity to DLA, EAC, S-180 and HLCA-549 cells was reported with the ethanolic extract of *Phallusia nigra* [9]-[12]. Antitumour effect to DLA and EAC cells using *Ecteinascidia venui* and *Microcosmus exasperatus* had been carried out [13]-[15]. From literature survey, it is evident that only scanty of work against cancer cell lines has been carried out in India. *Phallusia nigra*, an easily available biofoulant, considered as a nuisance of Tuticorin harbour area has been chosen to assess the anticancer activity against MDA-MB-231 bearing mice.

3. Materials and Methods

3.1 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 [16]. A voucher specimen AS 2083 has been submitted to the museum, Department of Zoology, A.P.C. Mahalaxmi College for Women, Tuticorin - 628002.

3.2 Systematic position

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

3.3 Animal material

Phallusia nigra is a simple ascidian with thick leathery envelope (tunic) containing cellulose like material. The tunic encloses a sac-shaped body with separate water entrance and exit called siphons. It is sessile and lives on plankton that it filters from seawater with a mucous net. An adult *Phallusia nigra* measures 10 cm. The tunic is usually velvet black or dark brown and grey in specimens that are younger or living in shaded areas. They occur in shallow sheltered waters attached to hard natural and artificial substrata. *Phallusia nigra* is a hermaphrodite and a broad cast spawner. The larvae can swim for few hours before settlement on a substrate where they metamorphose into sessile adult form (Plate 1).

Plate 1: *Phallusia nigra* Savigny, 1816

3.4 Preparation of Powder and Extract

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

3.5 Experimental Animals

Female 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±3ºC, constant 12 hrs of darkness and 12 hrs light schedules, humidity 60-70%, fed with normal mice chow and water 'ad libitum'. They were kept under fasting for 16 hrs before the experiment. Protocol used in the study for use of mice as an animal model for anticancer was in accordance with the standards of Animal Ethical Committee, Government of India.

3.6 Acute Oral Toxicity Studies

To determine the minimum lethal dose, acute oral toxicity studies were performed as per OECD guidelines 2002 [17]. Three female adult Swiss albino mice were selected and an oral dose of 2000 mg/kg body weight of the ethanolic extract of *Phallusia nigra* was given 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 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 present study.

3.7 Cells for cytotoxic study

MDA-MB-231cells were procured from Adayar Cancer Institute, Chennai, India. The cell line was maintained and propagated in 90% Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. They were cultured up to 70 to 80% confluence as adherent monolayer and maintained at 37°C in a humidified atmosphere of 5% CO2. Cells were trypsinized to harvest after attaining confluence [18].

3.8 *In vitro* cytotoxicity to MDA-MB-231cells

MDA-MB-231cells ($1x10^6$cells) were incubated with various concentrations (0.05, 0.10, 0.20, 0.40 and 0.60 mg/ml) of extract of *Phallusia nigra* in a final volume of 1 ml for 3 hrs at 37° C. The viability of the cells was confirmed by Trypan blue dye exclusion method [19]. The percentage cytotoxicity was calculated using the following formula.

$$\% \text{ Cytotoxicity} = \frac{No.of\ dead\ cells}{No.of\ viable\ cells + No.of\ dead\ cells} x100$$

3.9 Induction of Tumor

Exponentially growing MDA-MB-231 cells were harvested, washed twice with PBS and resuspended in 1:1 PBS/matrigel at a density of $50x10^6$ cells per ml. A suspension of 0.1 ml containing $5x10^6$ cells was injected in the right flank subcutaneously in the recipient mouse [20],[21]. A day of incubation was allowed for multiplication of the cells.

3.10 Experimental protocol

Tumor bearing Adult Swiss albino mice were divided into five groups of six animals (n=6) each. Group I acted as control and was given normal saline. Group II, III, IV, V were treated with ethanolic extract of *Phallusia nigra* at 50, 100, 150 and standard drug Vincristin at 80 mg/kg body weight respectively with 24 hours interval for 9 days. Food and water were withheld 18 hours before sacrificing the animals.

The antitumor effect of the ethanolic extract of *Phallusia nigra* was measured in MDA-MB-231 induced animals with respect to the following parameters:

3.11 Weight of relative organs, tumor and % inhibition

At the end of experimental period of 10 days, vital organs like spleen, thymus, liver, kidney and tumors were removed and weighed upto the nearest mg. Percentage inhibition was calculated by the following formula.

$$\% \text{ inhibition} = \frac{C - T}{C} x100$$

C - Mean weight of tumor in control; T - Mean weight of tumor in experimental group

3.12 Solid tumor volume

The radii of the tumors were measured using Vernier Calipers at 5 days intervals for one month starting with 15th

day and the volume of the tumor was calculated using the formula [22].
Volume = $(\text{width})^2$ x length/2

3.13 Median survival time, percentage increase of life span, packed cell volume, viable and non viable cell count

The effect of the extract on tumor growth was monitored by recording the mortality daily for 30 days and percentage increase in life span (% ILS) was calculated by Geran's method [23].

$$\text{MST} = \frac{\text{Day of first death} + \text{Day of last death}}{2}$$

$$\%\ \text{increase of life span} = \frac{T - C}{C} x100$$

T - MST of treated group; C - MST of control group

3.14 Packed cell volume, viable and non viable cell count

Packed cell volume was measured by microhematocrit method [24]. The cells were stained with Trypan blue (0.4% in normal saline) dye. Those that did not take up the dye were viable and those which took the stain were non viable. The viable and non viable cells were counted using Neubauer chamber [19].

4. Results

Acute oral toxicity study for a period of 24 hours did not show any mortality upto a dose of 2000 mg/kg bw. Hence sub-lethal doses of 50, 100 and 150 mg/kg bw were used for the experiments.

4.1 *In vitro* cytotoxicity to MDA-MB-231cells

Figure - 1 shows the percentage cytotoxicity of the ethanolic extract of *Phallusia nigra* to MDA-MB-231 cells. Ethanolic extract of *Phallusia nigra* was found to be 100% toxic at a concentration of 0.60 mg/ml.

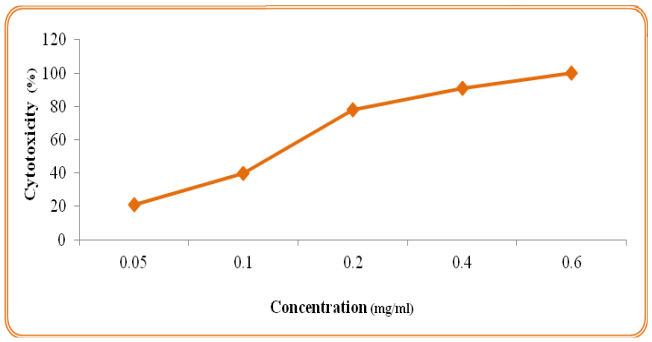

Figure 1: Cytotoxicity to MDA-MB- 231

4.2 Weight of relative organs, tumor and % inhibition

Weight of body and relative organs in MDA-MB-231 tumor bearing mice is shown in Table - 1. The relative organ weight of vital organs such as spleen, liver and kidney recorded a slight increase when compared to control whereas the weight of thymus showed a marginal decrease. The effect of the ethanolic extract of *Phallusia nigra* on the tumor weight and percentage inhibition of tumor is shown in figure - 2&3. The reduction in tumor weight and percentage inhibition registered in the mice treated with highest dose was greater than that of control.

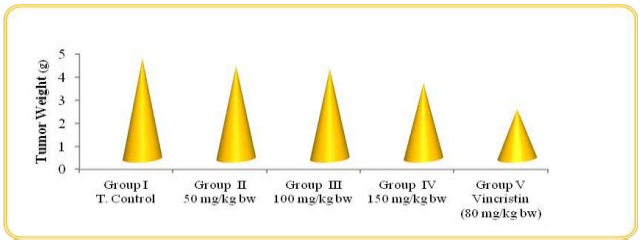

Figure 2: Effect on Tumor Weight

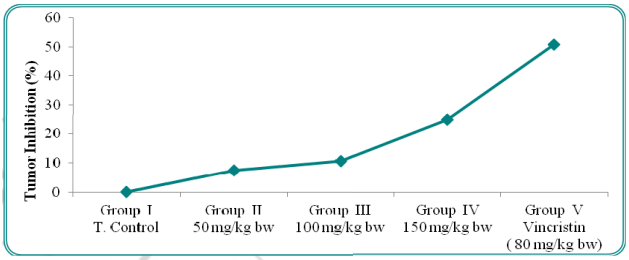

Figure 3: Effect on Tumor Inhibition

Table 1: Effect on relative organ weight

Relative Organ Weight (g/100g bw)					
Group	Body weight (g)	Spleen	Thymus	Liver	Kidney
I	26.14±1.85	0.39±0.01	0.23±0.03	3.11±0.17	2.54±0.02
II	24.56±1.21	0.48±0.02	0.18±0.01	3.54±0.16	2.94±0.02
III	27.21±1.84	0.43±0.02	0.19±0.02	3.16±0.15	3.27±0.02
IV	23.11±1.34	0.46±0.02	0.17±0.03	3.12±0.14	3.88±0.01
V	26.54±1.25	0.43±0.15	0.21±0.02	3.51±0.12	3.64±0.02

Data represented as mean ±SEM, (N=6)

4.3 Solid tumor volume

There was a dose dependent decrease in tumor volume. Figure - 4 shows the effect of the extract on solid tumor volume of MDA-MB-231 bearing mice. A highly significant dose related marked reduction in tumor volume in group IV was evident compared to the control.

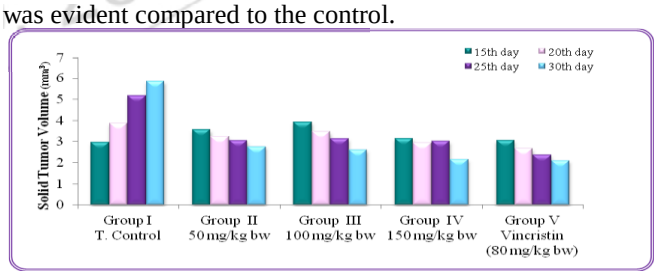

Figure 4: Effect on Solid tumor volume

4.4 Median survival time, Percentage increase of Life Span, Packed cell volume, Viable and Non viable cell count

The effect of *Phallusia nigra* extract on median survival time, increase of life span, packed cell volume is shown in Table - 2. There was a highly significant increase in the mean survival time in a dose dependent manner in group II to IV. The percentage increase of life span recorded for group IV

was significant. Group IV treated with highest dose of the extract showed a significant decrease in packed cell volume. The viable cell count decreased whereas nonviable cell count increased in all the treated groups (Figure - 5).

Table 2: Effect on Median Survival Time, Percentage increase of Life span and Packed cell volume

Group & Dose (mg/kg bw)	Median Survival time (Days)	Increase of life span (%)	Packed cell volume (ml)
I - T. Control	18.50±0.16	-	3.98±0.19
II - 50	20.10±0.24	8.64	3.14±0.22
III - 100	22.15±0.31	19.72	2.65±0.16*
IV - 150	24.60±0.22*	32.97	2.07±0.29**
V - Vincristin (80)	28.90±0.10	56.21	1.84±0.14

Data represented as mean ±SEM, (N=6). Significance between MDA-MB-231 control and extract treated groups.*P <0.05; **p <0.01.

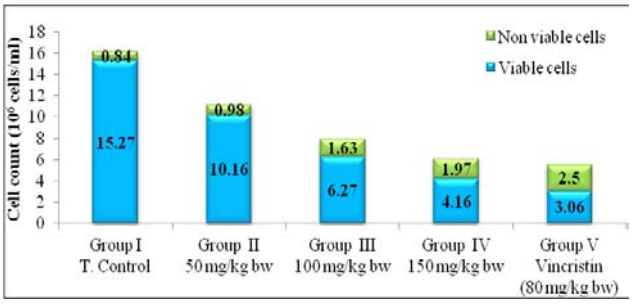

Figure 5: Effect on Viable and Non viable cell count

5. Discussion

Studies on the cytotoxicity of ethanolic extract of *Phallusia nigra* to MDA-MB-231, showed an increase in the percentage of toxicity with increasing concentration and 100% toxicity was observed with 0.60 mg/ml. Eusynstyelamide B of ascidian *Eusynstyela latericius* showed cytotoxicity against MDA-MB-231 breast cancer cells by inducing apoptosis [7]. Biologically active pyridoacridine metabolites - Sebastianines A and B isolated from a Brazilian collection of the ascidian *Cystodytes dellechiajei* exhibited cytotoxic activities towards colon cancer cells [25]. Ascididemnin, a pyridoacridine alkaloid derived from a *Didemnum* sp. brought about cytotoxicity by prompting oxygen - stress related proteins and reactive oxygen species [26]. Cytotoxic studies with the colonal ascidian *Cystodytes dellechiajei* indicated high antiproliferative activity to breast cancer SKBR3 cell lines [27]. Lissoclibadins extracted from the ascidian *Lissoclinum cf badium* revealed most potent inhibitory activity against MDA-MB-231 cells [6]. Ascidians are a rich source of bioactive compounds with cytotoxic properties [28]. The presence of such compounds in the extract might account for the cytotoxicity noted.

In the present study, the weight of the body was stimulated by the extract of *Phallusia nigra* and this may be due to the regular intake of food and growth during the experimental period. The relative weight of vital organs such as spleen, liver and kidney recorded a slight increase when compared to control, whereas the weight of thymus showed a marginal decrease. An increase in the weight of spleen in the treated group can be attributed to their increased activity and production of immunocompetent cells. As the blood supply to the body is filtered by the liver, this is a very common site for breast cancer to spread. Adequate liver function is crucial to detoxify the harsh drugs that are given. The kidneys act as organ maintaining the salt and water balance of the body. Its normal function is essential for homeostasis. In the present study the relative weight of the liver and kidneys did not show any significant change indicating the safety of the extract administered.

There was a decrease in the weight of tumor in the treated groups. The percentage of inhibition of tumor was found to increase in a dose dependent manner. The group treated with the highest dose showed an inhibition less than that of standard drug. Hence, though it is effective in inhibiting the growth of cells, a higher dose might be needed. (6)-gingeral extracted from *Zingiber officinale* has been shown to inhibit cell adhesion, invasion and motility of MDA-MB-231 cells [29]. A similar mechanism might be suggested here also.

Solid tumor volume indicated a decrease from 15th day 30th day when compared to control. The decrease noted was highly significant in group IV. Quercetin, a flavonoid gives protection against chemically induced and spontaneous formation of tumors in animals [30]. Quercetin present in the extract of *Phallusia nigra* may be a reason for the decrease in tumor volume [31]. The reduction in tumor volume might be due to the cytotoxic action of the extract.

The median survival time and percentage of life span increased in a dose dependent manner in all the groups. The reliable criteria for judging the value of any anticancer drug is the prolongation of life span and reduction in solid tumor volume [32]. Cyclooxygenase inhibitor suppresses tumor growth through multiple mechanisms, including antiproliferative, apoptosis and brings about an increase in lifespan. They also added that tumor growth inhibition may be the contributing factor in ovarian cancer xenograft models [33]. The presence of cyclooxygenase inhibitors have been reported from the ascidians *Ciona savignyi* and *Ciona intestinalis* [34],[35]. The increase in life span noted on treatment with the extract of *Phallusia nigra* can be attributed to the same.

A highly significant decrease in packed cell volume was noticed in the treated groups in a dose related way. This decrease is an indication of tumor inhibition. Chemical screening of *Phallusia nigra* extract showed the presence of saponins [31]. Saponins have been found beneficial as they target inhibition of tumor angiogenesis by suppressing its inducer in the epithelial cells of blood vessels and then on adhering, invasion and metastasis [36]. Polyphenolic extract from *Vaccinium macrocarpon* inhibited tumor growth and proliferation of breast tumor cells and hence there was an increase in non viable cells which in turn results in decreased viable cells [37]. The molecular fraction of the extract of *Phallusia nigra* obtained by ultrafiltration inhibited cell proliferation by decreasing the number of viable cells and DNA synthesis of T47D cells derived from human breast carcinoma [8]. The decrease in viable cells noted may be the

result of inhibition of tumor growth and multiplication by the components present in the extract.

6. Conclusion

Administration of the extract of *Phallusia nigra* decreased the weight, volume of the tumor and increased the percentage inhibition. An increase on median survival time, percentage of life span, non viable cells and decrease in packed cell volume, viable cells in tumor bearing mice indicates anticancer property. The ethanolic extract of *Phallusia nigra* contains compounds 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. One or more of these compounds may be responsible for the antioxidant, cancer preventive and anticancer properties observed at present. It is a promising source for isolation of compounds which can be applied in prophylaxis and treatment of cancer [38].

7. Future Scope

A further study on isolation, purification, structure determination and subsequent recognition of the novel mechanism of action of the clinically effective agent is suggested.

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