ISSN 2348-313X (Print)
International Journal of Life Sciences Research ISSN 2348-3148 (online)
Vol. 6, Issue 3, pp: (389-394), Month: July - September 2018, Available at: www.researchpublish.com

IMMUNOMODULATORY ACTIVITIES OF TUNICATE-*PHALLUSIA NIGRA* SAVIGNY, 1816 ON MDA-MB-231 CELL LINES

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***Abstract*: Breast cancer in women is a major health burden worldwide. By evaluating the bone marrow cellularity, β-esterase activity, antibody titer, plaque forming cells (PFC), serum GGT-NO levels and cellular GSH-NO levels the ethanolic extract of *Phallusia nigra* was assessed for immunomodulatory activity against MDA-MB-231 bearing adult Swiss albino mice. A significant, dose dependent increase in bone marrow cellularity, β-esterase activity, decrease in serum GGT-NO and cellular GSH-NO was observed. Antibody titer and PFC was maximum on 15th and 6th day of treatment respectively in group IV treated with 150 mg/kg body weight of the extract. The bioactive 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 reported as per the previous GC-MS analysis of ethanolic extract of *Phallusia nigra* might have modulated the immune system.**

***Keywords*: immunomodulatory, *Phallusia nigra*, MDA-MB-231.**

I. INTRODUCTION

Breast cancer is an extremely aggressive and lethal type of cancer due to its ability to rapidly metastasize [1]. It is the second most universal cause of cancer deaths in women and its invasion and metastasis are the primary causes of treatment failure and death [2], [3]. Chemotherapy and radiation cause severe adverse effects, such as bone marrow suppression resulting in cytopoenia, and subsequent devastation of the immune response. Radiotherapy is a standard treatment for many localized solid cancers and it may increase the invasive activity of cancer cells and potentially distant metastasis. Role of lysosome exocytosis was evaluated on invasive activity of human - MDA-MB-231 and murine - 4T1 breast cancer cell lines upon radiation [5]. The existing treatment protocol for cancer produces several side effects. 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. Bioactive peptides with novel structures have also been shown in marine sedentary ascidians [6]. Sac-like sea squirts inhabiting the sea floor produce complex anti-tumor compounds which are hundreds to thousands of times more influential than any cancer concoction now in use. Literature survey shows that immunomodulatory work on tunicates in India is very few. A significant immunomodulatory activity to DLA, EAC, S-180, HLCA-549 and MDA-MB-231cells was obtained with the ethanolic extract of *Phallusia nigra* [7-15]. As tunicates are abundant along the Tuticorin coast an attempt has been made to assess their immunomodulatory aspect to MDA-MB-231.

II. MATERIALS AND METHODS

A. Collection of animal material:

Samples of *Phallusia nigra* were collected from the under surface of barges of Tuticorin harbour. Identification up to species level was carried out based on 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 - 628 002, Tamilnadu, India.

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B. Systematic position:

Phallusia nigra belongs to the phylum - Chordata, subphylum - Tunicata, class: - Ascidiacea, order - Enterogona and family - Ascidiidae.

C. Preparation of powder and extract:

Animals were dried at 45^{o} C and powdered. Ten gram of 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 evaporated to get a residue which was re-suspended in 1% gum acacia blended with vanillin and administered orally at different concentrations for *in vivo* animal experiments.

D. Experimental animals:

20-25 g adult Swiss albino mice were received 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 ^{o} C, constant 12 hrs of darkness, 12 hrs light schedules, humidity 60-70%, fed with normal mice chow and water 'ad libitum'. They were kept under fasting 16 hrs before the commencement of 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.

E. Cells for Immunomodulatory assays:

MDA-MB-231 cells were procured from Adayar Cancer Institute, Chennai, India and maintained in RPMI-1640 medium supplemented with 10% heat-inactivated calf serum, 100 U/ml penicillin G, 100 U/ml streptomycin at pH 7.4 in a Water Jacketed CO_2 incubator with a humidified atmosphere of 5% CO_2 at 37^{o} C. Sheep red blood cells (SRBC) were collected from local slaughter house in Alsever's solution. As per the standard procedures suggested by Aravind *et al*., 2012., Bancroft and Cook, 1984; Singh *et al*., 1984; Jerne and Nordin, 1 963; Szasz *et al*., 1976; Green *et al*., 1982 and Akerboom and Sies, 1981 Bone marrow cellularity, β-esterase activity, antibody titer, plaque forming cells, serum GGT, NO, cellular GSH and NO was assessed [17-23].

F. Statistical analysis

Values are expressed as mean ± SEM. The statistical analysis was done by one-way analysis of variance (ANOVA) compared to control followed by Dunnett's test. p values less than 0.05 were considered to be significant.

III. RESULTS AND DISCUSSION

A. Effect on Bone Marrow Cellularity and β-Esterase Activity:

Table - 1 shows the effect *Phallusia nigra* on bone marrow cellularity and β-Esterase activity. A dose dependent significant increase was noted in Bone marrow cellularity and β- Esterase activity in the experimental mice. Bone marrow produces the cellular elements of blood including platelets, red blood cells and white blood cells. A dose related increase in the bone marrow cells and β-Esterase activity denotes the efficacy of *Phallusia nigra* extract. Miller, 1968 suggested that 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 [24].

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	13.84±0.39	647.24±19.32
II - 50	14.17±0.24	688.59±17.34
III - 100	16.66±0.17*	715.70±20.37*
IV - 150	19.84±0.26**	926.80±18.55**
V - Vincristin (80)	21.44±0.18	1015.15±20.07

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

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B. Effect on Antibody Titer:

Effect of *Phallusia nigra* extract on antibody titer is expressed in Figure - 1. Observations on the antibody titer from 3rd to 30th day in treated groups showed an increase up to the 15th day. From 18th day onwards a gradual decrease of antibody was noted. The antibody titer was maximum on the 15th day (236.59±3.25) in group IV. Wharton, 1951 stated that under favourable conditions of growth, carcinomas also are capable of modifying the antibody status of the host. Bone marrow synthesizes antibody during primary response.

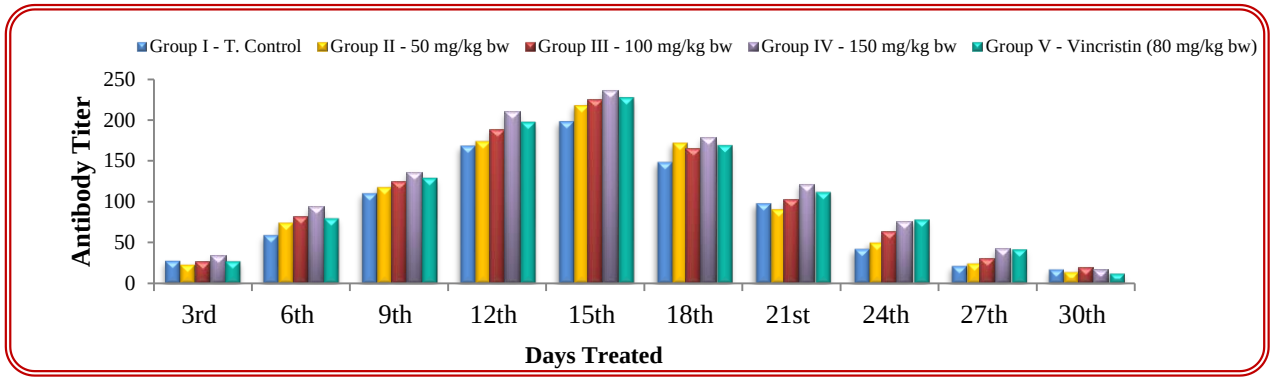

Fig 1: Effect on Antibody Titer

C. Effect on Plaque Forming Cells:

Figure - 2 depicts the effect of *Phallusia nigra* on PFC which increased gradually from 3rd day and was maximum on 6th day (193.67±1.93) in group IV. Starting from the 8th day, decrease in the level of antibody was noted. Same trend was noted to standard drug also.

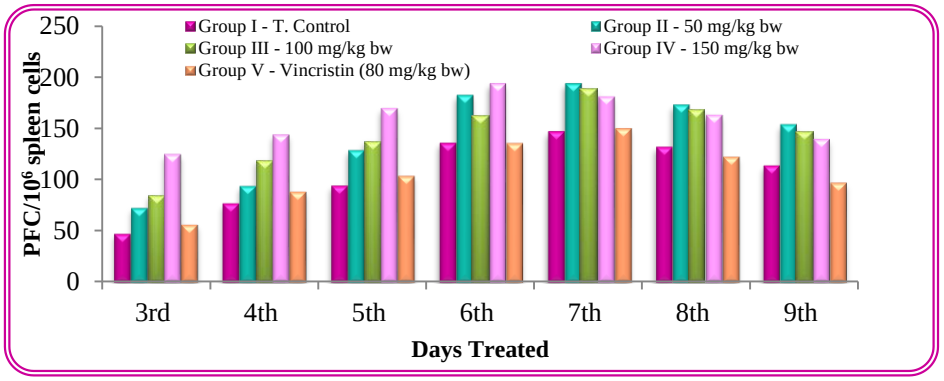

Fig 2: Effect on Plaque Forming Cells

D. Effect on Serum GGT and NO levels

Effect of the ethanolic extract of *Phallusia nigra* on serum GGT and NO is shown in Table - 2. In the treated groups serum GGT and NO level decreased dose dependently. GGT plays critical roles in antioxidant defense. Brown, 1980; Hirano *et al*., 1989 suggested that flavonoids have been found to possess antimutagenic and antimalignant effect. Elevated serum GGT activity has been generally considered as a marker of diseases [25], [26]. Decreased GGT level might indicate the antitumor activity of the drug.

Table 2: Effect on the Serum GGT and NO level

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	34.54±0.14	87.13±1.53	101.55±1.84	16.91±0.84	29.54±0.13	32.54±0.75
II - 50	21.56±0.51	52.14±0.73	59.14±0.16*	13.16±0.34	23.69±0.64	30.11±0.34
III - 100	19.27±0.73*	43.99±0.84*	52.93±0.84**	11.43±0.15*	19.33±0.73*	21.68±0.77*
IV - 150	15.21±0.13*	37.93±0.94**	39.53±0.88***	9.33±0.84*	16.59±0.54**	17.63±0.65**
V - Vincristin (80)	20.68±0.28	31.91±0.56	47.88±0.91	11.36±0.16	19.27±0.52	28.10±0.73

Data represented as mean ±SEM, (N=6). Significance between MDA-MB-231 control and extract treated groups.

*p <0.05; **p <0.01; ***p <0.001.

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E. Effect on Cellular GSH and NO levels:

Effect of ethanolic extract of *Phallusia nigra* on cellular GSH and NO is given in Table - 3. From 5th to 10th day, cellular GSH level in group IV and V showed an increase and then a decrease. Jagatheesh *et al*., 2010 noted that GSH acts on multiple levels of the defense system and the thiol group of GSH participates in the protection against deleterious effects of reactive oxygen species evolved during biological imbalance as well as cancerous conditions [27]. GSH strengthens the immune system by producing T cells. GSH is a detoxifier which cleans the cells, flushes waste and toxins. According to Lancaster, 1997 Nitric oxide is a lipophilic, highly diffusible and short lived physiological messenger [28]. NO regulates immune response and apoptosis. Lala and Chakraborty, 2001 stated that NO may participate in the induction of tumor cell growth and invasion [29]. Melino *et al*., 1997; Pervin *et al*., 2001 and Jaiswal *et al*., 2001 stated that NO may influence the carcinogenesis process by alteration of cell cycle check point; apoptosis and DNA repair [30-32]. Dose dependent decrease in the level of NO in the treated groups may indicate their role in immune response and apoptosis.

Table 3: Effect on the Cellular GSH and NO level

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	8.93±0.19	17.22±0.87	11.26±0.28	10.68±0.24	14.53±0.92	18.61±0.93
II - 50	8.11±0.73	15.43±0.18	9.33±0.14	7.84±0.18	11.44±0.84	13.91±0.93
III - 100	7.46±0.74*	13.84±0.39*	7.84±0.64*	6.24±0.18*	7.54±0.12*	10.11±0.63*
IV - 150	6.34±0.66*	10.92±0.16**	5.88±0.36**	4.84±0.38**	4.93±0.74***	7.13±0.39**
V - Vincristin (80)	7.86±0.72	8.13±0.84	5.14±0.15	5.16±0.19	5.11±0.67	5.18±0.54

Data represented as mean ±SEM, (N=6). Significance between MDA-MB-231 control and extract treated groups.

*p <0.05; **p <0.01; ***p <0.001.

IV. CONCLUSION

In the present study, the ethanolic extract of *Phallusia nigra* stimulated the Immune system thereby producing significant and dose related increase in bone marrow cellularity and β-esterase activity whereas a decrease in serum GGT-NO, cellular GSH-NO in a dose dependent manner against MDA-MB-231 cells. GC-MS analysis of ethanolic extract of *Phallusia nigra* by Meenakshi *et al*., 2012 has shown the presence of 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 with antioxidant, cancer preventive and anticancer properties [33]. Further studies on isolation, purification and structure determination are needed to conclude on the compound responsible for and the mechanism of action.

ACKNOWLEDGEMENT

The authors express their deep sense of gratitude to Dr. R. Sampath Raj Ph.D., Dr Samsun Immuno Clinical laboratory for providing facilities to conduct the experiments.

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