ETHANOLIC EXTRACT OF *PHALLUSIA NIGRA* SAVIGNY, 1816 INDUCES IMMUNOMODULATIONS IN HLCA-549 BEARING MICE

*M. Paripoorana Selvi and V. K. Meenakshi*

Department of Zoology, A.P.C. Mahalaxmi College for Women, Tuticorin, Tamilnadu, India.

**ABSTRACT**

The immunomodulatory activity of the ethanolic extract of *Phallusia nigra* was assessed against HLCA-549 bearing adult Swiss albino mice by evaluating the immune function, bone marrow cellularity, β-esterase activity, antibody titer, plaque forming cells (PFC), serum GGT-NO levels and cellular GSH-NO levels. The results showed a significant and dose dependent increase in hemolysis of SRBC, lymphocyte proliferation, NK cytotoxic activity, phagocytosis rate, bone marrow cellularity, β-esterase activity and decrease in serum GGT-NO levels and cellular GSH-NO levels. Antibody titer was maximum on 15th day and PFC reached its peak on 6th day of treatment in group IV treated with 150 mg/kg body weight of the extract. It is suggested that bioactive compounds present in the extract might have modulated the immune system.

**KEYWORDS:** immunomodulatory activity, *Phallusia nigra*, HLCA-549.

**INTRODUCTION**

Lung cancer mainly occurs in older people including both men and women. Radiotherapy along with chemotherapy has been used with curative intent in patients who are not eligible for surgery, but cause severe adverse effects such as bone marrow suppression resulting in cytopoenia, and subsequent devastation of immune responses.[1] Multiple new chemotherapeutic agents have been developed and some are in clinical trials.[2,3] Although some of them have produced promising results, their therapeutic spectrum is narrow which induced scientists to search for compounds derived from natural sources. 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. Immunomodulators are well known for their
antitumor activity. The aim of immunoadjuvant therapy is to stimulate the innate and adaptive immune systems to overcome the immunosuppressive situation in cancer which is usually the side effect of conventional modes of cancer treatments.\textsuperscript{[4,5]} Many marine sedentary organisms produce components with unique structural pattern, for their chemical defence. Ascidians rank second with most promising source of drugs.\textsuperscript{[6]} According to survey of Indian literature, very little immunomodulatory work on ascidians has been carried out on DLA, EAC and S-180 cells with the ethanolic extract of Phallusia nigra.\textsuperscript{[7-10]} Based on the easy availability of Phallusia nigra from Tuticorin harbour, it has been decided to assess the immunomodulatory effect against HLCA-549 bearing mice.

**MATERIALS AND METHODS**

**Specimen collection and identification**

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.\textsuperscript{[11]} 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.

**Systematic position**

Phylum: Chordata; Subphylum: Urochordata; Class: Asciidiacea; Order: Enterogona; Suborder: Phlebobranchia; Family: Asciidiidae; Genus: Phallusia; Species: nigra.

**Animal material**

*Phallusia nigra* (Plate-1) is a simple ascidian covered by a thick black envelope (tunic) which contains cellulose like material. An adult *Phallusia nigra* may be 10 cm long. The sac-shaped body has two siphons for water entrance and exit. It is a marine, sessile, filter feeding animal.

![Plate -1 Phallusia nigra Savigny, 1816](image-url)
Preparation of powder and extract
The animal was dried at 45º 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 4º C for 10 minutes. The supernatant was collected and evaporated to get a residue, which was used for in vitro studies. For in vivo animal experiments, it was re-suspended in 1% gum acacia blended with vanillin and administered orally at different concentrations.

Experimental animals
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±3º 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.

Acute oral toxicity studies
To determine the minimum lethal dose, acute oral toxicity studies were performed as per OECD guidelines 2002.[12] Adult Swiss albino mice of either sex weighing 20-25 g were used. Three animals were selected and a dose of 2000 mg/kg bw of ethanolic extract of Phallusia nigra was given orally 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 same dose of extract for 7 more days. Thereafter 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 following experiments.

Cells for cytotoxic study
HLCA-549 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 CO₂ incubator with a humidified atmosphere of 5% CO₂ at 37º C. Sheep red blood cells (SRBC) were collected from local slaughter house in Alsever’s solution.
Immunomodulatory assays
Hemolytic assay, lymphocyte proliferation rate, bone marrow cellularity, β-esterase activity, antibody titer, plaque forming cells, serum GGT, NO, cellular GSH and NO as per the standard procedures suggested by Thirunavukkarasu et al., 2011; Aravind et al., 2012; Bancroft and Cook, 1984; Singh et al., 1984; Jerne and Nordin, 1963; Szasz et al., 1976; Green et al., 1982 and Akerboom and Sies, 1981.[13,4,14-19]

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.

Effect on Morphology and Histopathology
Tumors of HLCA-549 bearing mice treated with ethanolic extract of Phallusia nigra at different concentrations were fixed in 10% buffered neutral formalin separately. A thin slice of the tumor tissue was stained with hematoxylin, eosin and observed under inverted light microscope for morphological and histopathological changes.[20]

RESULTS
Effect on Immune Function
Table - 1: Effect on immune function

<table>
<thead>
<tr>
<th>Group &amp; Dose (mg/kg bw)</th>
<th>Quantitative hemolysis of sheep red blood cells (HC50)</th>
<th>Lymphocyte proliferation (cpm)</th>
<th>NK cytotoxic activity (%)</th>
<th>Phagocytosis rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I - Control</td>
<td>29.56±2.14</td>
<td>2410±694</td>
<td>30.61±1.04</td>
<td>19.55±1.28</td>
</tr>
<tr>
<td>II - 50</td>
<td>109.14±3.16*</td>
<td>4126±850</td>
<td>34.17±1.22</td>
<td>26.45±1.19</td>
</tr>
<tr>
<td>III - 100</td>
<td>134.85±4.91***</td>
<td>5050±960**</td>
<td>40.24±1.84*</td>
<td>31.80±1.13*</td>
</tr>
<tr>
<td>IV - 150</td>
<td>159.16±4.14***</td>
<td>5860±1015**</td>
<td>46.39±1.05**</td>
<td>38.40±1.08**</td>
</tr>
<tr>
<td>V – Vincristin (80)</td>
<td>163.36±3.27***</td>
<td>6110±950**</td>
<td>42.56±1.32*</td>
<td>36.84±1.27**</td>
</tr>
</tbody>
</table>

Data represented as mean ±SEM, (N=6). Significance between HLCA-549 control and extract treated groups.*p <0.05; **p <0.01; ***p <0.001.

Table - 1 shows the effect of ethanolic extract of Phallusia nigra on immune function of HLCA-549 bearing mice. The quantitative hemolysis (HC50) of sheep red blood cells increased proportionately in the extract treated groups compared to control. Rate of lymphocyte proliferation elevated significantly with the increase in concentration of extract. Percentage NK cytotoxic activity registered an upward trend in the experimental groups.
(34.17±1.22, 40.24±1.84 and 46.39±1.05). Phagocytosis percentage noted for group IV and V was highly significant.

Effect on Bone Marrow Cellularity and β-Esterase Activity

Table - 2: Effect on bone marrow cellularity and β-Esterase activity

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg bw)</th>
<th>Bone marrow cellularity (10^6 cells/femur)</th>
<th>β-Esterase activity (β-Esterase positive cells /4000 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I - Control</td>
<td>16.87±1.23</td>
<td>784.76±19.34</td>
<td></td>
</tr>
<tr>
<td>II - 50</td>
<td>17.49±2.34</td>
<td>798.33±16.98</td>
<td></td>
</tr>
<tr>
<td>III - 100</td>
<td>20.36±1.84*</td>
<td>814.62±18.12*</td>
<td></td>
</tr>
<tr>
<td>IV - 150</td>
<td>22.90±1.31**</td>
<td>867.38±15.65*</td>
<td></td>
</tr>
<tr>
<td>V - Vincristin (80)</td>
<td>18.84±1.43</td>
<td>1045.11±14.31**</td>
<td></td>
</tr>
</tbody>
</table>

Data represented as mean ±SEM, (N=6). Significance between HLCA-549 control and extract treated groups. *p <0.05; **p <0.01.

Table - 2 shows the effect *Phallusia nigra* on bone marrow cellularity and β-Esterase activity. Bone marrow cellularity in group I (tumor control) was 16.87±1.23 and in group II, III, IV and V it was 17.49±2.34, 20.36±1.84, 22.90±1.31 and 18.84±1.43 x 10^6 cells/femur indicating a significant dose related increase in the experimental groups. The group treated with the standard drug was lesser than that of extract treated.

β- Esterase activity in the treated groups was 798.33±16.98, 814.62±18.12 and 867.38±15.65/4000 cells. In the group which received the standard drug it was 1045.11±14.31. A dose dependent significant increase was noted in the experimental mice.

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 (198.84±1.27) in group IV.
Effect on Plaque Forming Cells

Figure - 2 depict the effect of *Phallusia nigra* on PFC. PFC increased gradually from 3rd day and was maximum on 6th day (197.56±2.84) in group IV. Starting from the 7th day, decrease in the level of antibody was noted. Same trend was noted to standard drug also.

Effect on Serum GGT and NO levels

Effect of the ethanolic extract of *Phallusia nigra* on serum GGT and NO is shown in Table - 3. In group II and III, serum GGT level raised from 5th to 15th day. From 5th to 10th day, an increase followed by a decrease was noted in group IV and V. Groups treated with extract, evidenced a dose related significant elevation in NO. A similar observations were noted on treatment with standard drug.

Table - 3: Effect on the Serum GGT and NO level

<table>
<thead>
<tr>
<th>Group &amp;Dose (mg/kg bw)</th>
<th>GGT (nmol p-nitroaniline/ml)</th>
<th>NO (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5th day</td>
<td>10th day</td>
</tr>
<tr>
<td>I - Control</td>
<td>32.56±0.93</td>
<td>87.88±1.24</td>
</tr>
<tr>
<td>II - 50</td>
<td>24.18±0.84</td>
<td>72.14±1.93</td>
</tr>
<tr>
<td>III - 100</td>
<td>16.93±1.05*</td>
<td>43.81±0.84*</td>
</tr>
<tr>
<td>IV - 150</td>
<td>11.38±0.16**</td>
<td>31.63±1.31**</td>
</tr>
<tr>
<td>V - Vincristin (80)</td>
<td>24.16±0.33</td>
<td>35.88±0.94**</td>
</tr>
</tbody>
</table>

Data represented as mean ±SEM, (N=6). Significance between HLCA-549 control and extract treated groups.*p<0.05; **p <0.01; ***p <0.001.

Effect on Cellular GSH and NO levels

Effect of ethanolic extract of *Phallusia nigra* on cellular GSH and NO is given in Table - 4. From 5th to 10th day, cellular GSH level in group IV and V showed an increase and then a
decrease. In all the experimental groups except group IV, an increase and then a slight decrease in NO level was evident.

Table - 4: Effect on the cellular GSH and NO level

<table>
<thead>
<tr>
<th>Group &amp; Dose (mg/kg bw)</th>
<th>GSH (nmol/mg protein)</th>
<th>NO (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5th day</td>
<td>10th day</td>
</tr>
<tr>
<td>I - Control</td>
<td>9.54±0.85</td>
<td>19.33±0.51</td>
</tr>
<tr>
<td>II - 50</td>
<td>8.68±0.17</td>
<td>14.26±0.63</td>
</tr>
<tr>
<td>III - 100</td>
<td>7.24±0.81</td>
<td>13.16±0.72</td>
</tr>
<tr>
<td>IV - 150</td>
<td>6.40±0.62*</td>
<td>10.94±0.31*</td>
</tr>
<tr>
<td>V – Vincristin (80)</td>
<td>8.54±0.56</td>
<td>10.63±0.74*</td>
</tr>
</tbody>
</table>

Data represented as mean ±SEM, (N=6). Significance between HLCA-549 control and extract treated groups *p <0.05; **p <0.01; ***p <0.001.

Effect on Morphology and Histopathology

Plate – 2. Photomicrograph showing concentration dependent significant morphological changes in HLCA -549 cells.

- a. Typical pathological micrograph; b. Swollen cells with cytoplasmic vacuoles; c. shows apoptotic and karyopknotic cells; d-e. Greater apoptosis, atrophy showing dead cells.
Plate - 2 shows concentration dependent morphological changes in HLCA-549 cells. There was a concentration dependent decrease in cellular growth rate and increase in the number of apoptotic and karyopknotic cells. Cytoplasmic vacuoles and swollen cells were observed in the lowest dose of extract. Moderate and highest dose showed more atrophic and dead cells. The same observation was recorded for Vincristin treated also.

**DISCUSSION**

Quantitative hemolysis using sheep red blood cells indicated a very highly significant increase. Cytotoxic activity of methanolic extract of *Artocarpus heterophyllus* against A-549 cell line by SRB assay has been attributed to flavonoids.\(^{[21]}\) Cytotoxicity of flavonoids is due to phenolic groups.\(^{[22]}\) Report of flavonoids from *Phallusia nigra* indicates that the extract may activate the immune system to produce antibodies as well as lymphocytes.\(^{[23]}\) This in turn might initiate hemolysis by involving the macrophage, NK cells and T cells.

A highly significant dose related increase in lymphocyte proliferation was obvious on extract treatment which can be taken as a clue for the active functioning of the immune system. It is hypothesized that the biologically active compounds present in the extract might have played a significant role in stimulating the proliferation of lymphocytes by the thymus in order to prevent tumor progression.

NK cytotoxic activity showed a highly significant increase in the present investigation. Natural killer cells are antibody independent cells. When they are activated by Interferons and Interleukin-2 secreted by T-helper cells, they kill a range of tumor cells. This is because they recognize altered cell surface and bring about cytolysis and cytotoxicity.

Percentage of phagocytosis was highly significant on treatment. During phagocytosis, hydrolytic enzymes of lysosomes help to digest unwanted cells. Phagocytes can kill neoplastic cells without ingesting them just by membrane contact. An interaction between the macrophage and T helper cells stimulate the macrophage to release Interleukin-1. This activates the T helper cells. The T-helper cells in turn releases lymphokines which include IL-2, macrophage activating factor, macrophage inhibition factor, lymphotoxins and interferons. Macrophage inhibition factor stops the macrophage around the tumor cells and helps in aggregation. Macrophage activating factor and interferons enhance the tumoricidal capacity by stimulating phagocytosis. A combination of all these may result in an increased percentage of phagocytosis observed in the present study.
Bone marrow - a primary lymphoid organ is the site of proliferation of blood and immune reactive cells. An increase in bone marrow cells signifies the ability of Phallusia nigra extract to mobilize immune cells which act against various diseases including cancer. Experimental animals recorded an increase in differentiating stem cells with β-Esterase activity suggesting that extract enhances immunological response.

One of the major problems encountered in those suffering from various types of tumor is a lowering of antibody activity which in turn may lead to progression of tumor. Present investigation revealed that antibody titer increased in a dose dependent manner and was maximum in group which received highest dose on 15th day. Sustained immunological activity can be seen by the increased titer which remained for several days. Stimulation of the humoral immune system to produce antibodies may help to compensate and overcome the decrease in antibody noted in tumor induced mice.

During primary response great number of PFC appeared in the spleen while only a few was seen in the bone marrow.\textsuperscript{24} In secondary response a clear PFC response was noted both in spleen and bone marrow and spleen was found to contain the majority of PFC until about 9 days. The present investigation of plaque assay indicated an increase from 3rd day with a maximum on 6th day in group IV treated with the highest dose. This is an indication of stimulation of primary immune response of treated animals. The reduction in PFC after 9th day was greater in tumor control indicating lesser production of antibodies.

Serum GGT concentration was higher in tumor control compared to treated groups on all days. Cellular GGT is widely distributed in the human body, especially in kidney, liver and is frequently localized in plasma membrane with its active site directed towards the extracellular space playing an important role in antioxidant defence systems. The primary role of GGT is to metabolize extra cellular reduced Glutathione, allowing for precursor amino acids to be assimilated and reutilized for intracellular GSH synthesis.\textsuperscript{25-28} An elevation of GGT in tumor control may be a reflection of oxidative stress due to increased production of reactive oxygen species. Lee \textit{et al.}, also noticed increased serum GGT in a number of tumors.\textsuperscript{29} Administration of extract brought down serum GGT. This may be due to the nature of the extract which is responsible for a reduction in oxidative stress.

Treatment with extract decreased cellular GSH in all the groups compared to control. Increase in GSH can be taken as a sign of oxidative stress which leads to pathological
conditions. Continued proliferation and metabolism of tumor cell need a higher level of GSH. Decrease in cellular GSH noted in treated groups can be used as a marker of tissue recovery. GSH, an important cellular antioxidant, is known to have protective functions against toxic effects of drugs and metals.

Antioxidants are notable for boosting the immune system because immune cells in bloodstream are easily accessed free radicals. They are molecules that can neutralize free radicals by accepting or donating an electron to eliminate the unpaired condition. Glutathione is an important antioxidant. It readily interacts with free radicals, especially hydroxyl and carbon radicals, by donating a hydrogen atom. It also plays an important role in the stabilization of many enzymes. Antioxidant activities are significantly correlated to antiproliferative property on human lung epithelial cancer A-549 cell growth which prevent tumor initiation and act as a protective agent.

Multiple actions of NO in tumor environment is related to heterogenous cell responses with particular attention in regulation of stress response mediated by hypoxia leading to growth arrest, apoptosis or adaptation. NO has been reported to sensitize tumor cells to chemotherapeutic compounds. In the present study an increase of NO in tumor control and a gradual decrease on treatment with the extract of Phallusia nigra may indicate the same role.

Mollamide, a cyclodepsipeptide obtained from the ascidian Didemnum molle against A-549 human lung carcinoma. Didemnin B isolated at first from the Caribbean tunicate Trididemnum solidum has the most potent activity against human prostatic cancer by inhibiting the synthesis of RNA, DNA and proteins. The bioactive compounds present in the extract of Phallusia nigra might be the reason for the inhibition of DNA synthesis and thereby arresting the cell cycle. As a result, many dead cells were observed in the highest dose treated cells.

**CONCLUSION**

In the present study, the ethanolic extract of Phallusia nigra stimulated the Immune system thereby producing significant and dose related increase in hemolysis of SRBC, lymphocyte proliferation, NK cytotoxic activity, phagocytosis rate, bone marrow cellularity and β-esterase activity whereas a decrease was noted in serum GGT-NO and cellular GSH-NO levels against HLCA-549 cells. GC-MS analysis of ethanolic extract of Phallusia nigra by Meenakshi et al., 2012c 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-17,1'-cyclobutan]-2'-one with antioxidant, cancer preventive and anticancer properties. Further studies on isolation, purification and structure determination are needed to conclude on the compound responsible for and the mechanism of action.

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REFERENCES


