



**INHIBITION OF HLCA-549 CELL PROLIFERATION AND SURVIVAL  
BY ETHANOLIC EXTRACT OF *PHALLUSIA NIGRA* SAVIGNY, 1816**

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Article Received on 29/01/2015

Article Revised on 18/02/2015

Article Accepted on 09/03/2015

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**ABSTRACT**

Lung cancer is one of the leading cancers affecting and reducing the life span of people in India, hence there is an urgent need for drug development. The present research article was aimed to study the antiproliferative property of the ethanolic extract of *Phallusia nigra* against HLCA-549 bearing adult Swiss albino mice. Cytotoxicity, relative organ weight, solid tumor volume, median survival time, increase of lifespan, packed cell volume, viable and non-viable cell

count and hematological parameters were studied using the extract. The extract was 100% toxic at 0.80 mg/ml concentration. The results showed a dose dependent decrease in tumor volume, packed cell volume, viable cell count and an increase in non viable cell count and mean survival time there by increasing the life span by 67.77% in group IV treated with 150 mg/kg body weight of the extract. Administration of the extract resulted in an increase in Hb, WBC and lymphocytes whereas a decrease was noted in neutrophil and eosinophil count. The present study indicates that *Phallusia nigra* is a significant source of bioactive compounds with antiproliferative property against HLCA-549.

**KEYWORDS:** cytotoxicity, antiproliferative, *Phallusia nigra*, HLCA-549.

**INTRODUCTION**

Lung cancer is a disease characterized by uncontrolled cell growth in tissues of one or both lungs. The abnormal cells divide rapidly and form tumors. As tumors become larger and more numerous, they undermine the lung's ability to provide the blood stream with oxygen. It is the most common cancer in the world representing a major public health problem and

accounts for 1.18 million deaths including both men and women.<sup>[1,2]</sup> Most lung cancer patients are diagnosed at late disease stages when surgery is not a viable option.<sup>[3]</sup> The common regimen for treating patients with advanced lung cancer is either cisplatin or platinum-based chemotherapy. However, these drugs are highly toxic with a low survival profile against non-small cell lung cancer.<sup>[4,5]</sup> In the field of cancer drug discovery, development of a target specific drug without any side effect to normal cells is an ongoing effort. Bioactive compounds with cytotoxic activities have been isolated from plants. Many marine sedentary organisms produce components with unique structural pattern, for their chemical defence. As per literature survey anticancer work on ascidians in India is scanty. A significant antiproliferative activity to DLA, EAC and S-180 cells was obtained with the ethanolic extract of *Phallusia nigra*.<sup>[6-8]</sup> Earlier studies have shown that *Phallusia nigra* is abundant throughout the year from Tuticorin coast. As they are an important component of biofouling community, they are considered as a nuisance and are usually thrown away. Still it was decided to study the antiproliferative property of the ethanolic extract of *Phallusia nigra* against HLCA-549 cells.

## 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 the species level was carried out based on the key to identification of Indian ascidians.<sup>[9]</sup> A voucher specimen AS 2083 has been submitted to the museum, Department of Zoology, A.P.C. Mahalaxmi College for Women, Tuticorin - 628002, Tamilnadu, India.

### Systematic position

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

### Animal material

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



**Plate -1 *Phallusia nigra* Savigny, 1816**

### **Preparation of powder and extract**

The animal was dried at 45° C and powdered. Ten grams of the powder was soaked overnight in 100 ml of 70 % ethanol and filtered. The filtrate was 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* studies. For *in vivo* studies, it was resuspended 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±3° C, 12 hour photoperiod, humidity 60-70%, fed with normal mice chow, water '*ad libitum*' and were kept under fasting for 16 hrs before the experiment. Protocol used for *in vivo* anticancer study was in accordance with the standards of the 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.<sup>[10]</sup> Adult Swiss albino mice of either sex weighing 20-25 g were used. Three animals were selected and an oral dose of 2000 mg/kg bw of the 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

3 hours. The experimental animals were administered the extract daily upto 7 days and were monitored at regular intervals upto 14 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. HLCA-549 cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated calf serum, 100 U/ml penicillin G and 100 U/ml streptomycin, pH 7.4 in a Water Jacketed CO<sub>2</sub> incubator with a humidified atmosphere of 5% CO<sub>2</sub> at 37° C.

### In vitro cytotoxicity to HLCA-549 cells

HLCA-549 cells (1x10<sup>6</sup> cells) were incubated with various concentrations (0.05, 0.10, 0.20, 0.40 and 0.60 mg/ml) of the 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.<sup>[11]</sup> The percentage cytotoxicity was calculated using the formula.

$$\% \text{ cytotoxicity} = \frac{\text{No. of dead cells}}{\text{No. of viable cells} + \text{No. of dead cells}} \times 100$$

**Experimental protocol:** Adult Swiss albino mice were divided into 5 groups of six animals (n=6) each. Viable HLCA-549 cells were adjusted to 1x10<sup>6</sup> cells/mL. The animals were injected intraperitoneally on day zero with 0.1 mL of HLCA-549 cells per 10 g body weight. A day of incubation was allowed for multiplication of the cells. Group I acted as control and was given normal saline. Group II, III and IV were treated daily with ethanolic extract of *Phallusia nigra* at 50, 100 and 150 mg/kg body weight respectively for 9 days and group V with Vincristin at 80 mg. The extract was blended with 1% gum acacia and vanillin solution and administered intra gastrically. The animals were kept on fasting 18 hours before sacrificing. The antitumor effect of the ethanolic extract of *Phallusia nigra* was measured in HLCA-549 induced animals with respect to the following parameters:

**Induction and measurement of Solid Tumor Volume:** Tumor was induced by injecting HLCA-549 cells (1x10<sup>6</sup> cells/mouse) intraperitoneally for five groups of animals. The radii of the tumors were measured using Vernier Calipers at 5 days intervals for one month after 15th day of the start of experiment and the volume of the tumor was calculated using the formula  $V=4/3 \Pi r_1 r^2$ , where 'r<sub>1</sub>' and 'r' represent the major and minor diameter respectively.<sup>[12]</sup>

### 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 6 weeks and percentage increase in life span was calculated by the following equation.<sup>[13]</sup>

$$\text{MST \%} = \frac{\text{MST of treated group} - \text{MST of control group}}{\text{MST of control group}} \times 100$$

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

$$\text{Increase in lifespan} = \frac{T-C}{C} \times 100$$

**Packed cell volume:** Blood for hematology was collected using EDTA as an anticoagulant and immediately mixed well to avoid clotting. Two microhematocrit tubes were filled 2/3 full for each EDTA sample and/or control. Each test was done in duplicate. The dry end of each tube was clayed and was put in hematocrit sheet holes. The sheet was labeled and carefully transported to centrifuge area. The tubes in centrifuge were balanced, the lid put on and centrifuged for 5 min. The groove used for each tube on sheet was recorded. After centrifugation, the hematocrit was used to set 0 and 100, and then the hematocrit % was read at the top of the packed red cells to the nearest 0.5%. The reading obtained for each tube was recorded on the sheet.

### Viable and Non viable cell count

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 (haemocytometer).

### Effect on Hematological Parameters

With the remaining set of mice the experiment was continued. 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.<sup>[14]</sup> 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.

## RESULTS

**Cytotoxic activity to HLCA-549 cells:** Figure - 1 shows the percentage cytotoxicity of the ethanolic extract of *Phallusia nigra* to HLCA-549 cells. Ethanolic extract of *Phallusia nigra* was found to be 100% toxic at the concentration of 0.80 mg/ml to HLCA-549 cells.

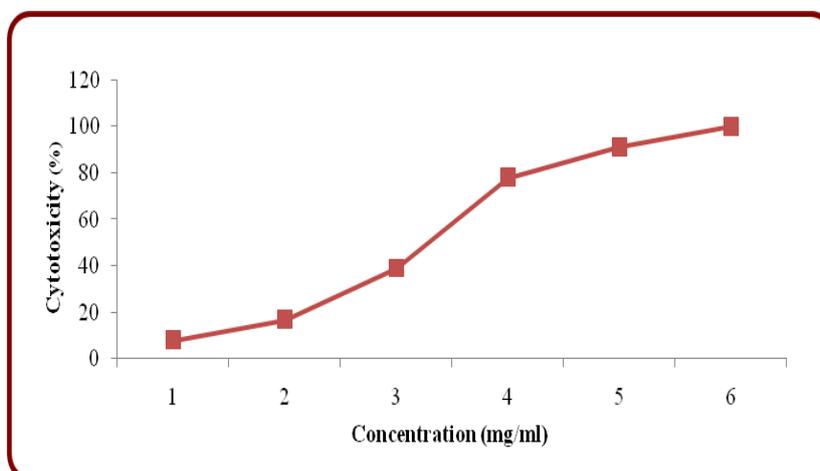


Figure - 1. Cytotoxicity of ethanolic extract of *Phallusia nigra* to HLCA-549

### Effect on Relative Organ Weight

The effect of the ethanolic extract of *Phallusia nigra* on the weight of body, tumor weight (Figure - 2), percentage inhibition of tumor growth and relative organs in HLCA-549 tumor bearing mice is shown in Table - 1. The reduction in tumor weight in the mice treated with 150 mg/kg body weight and that with 80 mg/kg body weight of Vincristin was found to be same. The percentage inhibition of tumor growth in group IV was 46.51 whereas in group V, it was 48.06.

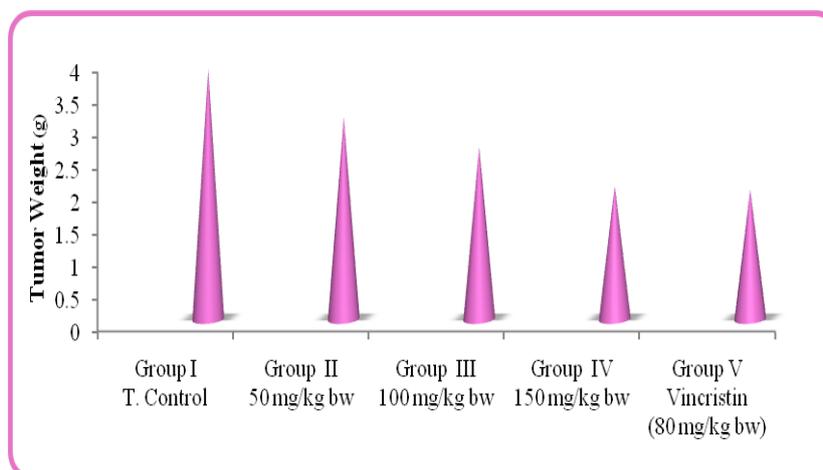


Figure - 2. Effect on Tumor Weight

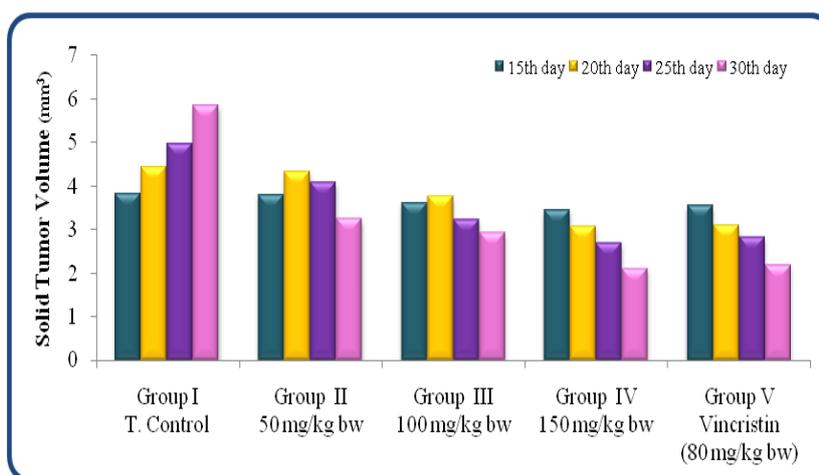
**Table - 1: Effect on Relative Organ Weight**

Group & Dose (mg/kg bw)	Relative Organ Weight (g/100 g body weight)							Inhibition (%)
	Body weight		Spleen	Thymus	Liver	Kidney	Tumor weight (g)	
	Before	After						
I - Control	24.84±1.13	27.20±1.31	0.57±0.021	0.37±0.029	4.94±0.240	4.34±0.17	3.87±0.74	-
II - 50	22.16±0.98	24.93±1.14	0.44±0.014	0.36±0.016	4.66±0.160	3.86±0.12	3.13±0.72	19.12
III - 100	23.93±1.27	26.14±0.98	0.45±0.016	0.24±0.011	4.54±0.140	4.26±0.24	2.67±0.12*	31.00
IV - 150	20.64±0.89	24.33±1.08	0.42±0.012	0.20±0.051	4.21±0.022	4.03±0.11	2.07±0.29**	46.51
V - Vincristin (80)	25.18±0.14	27.67±1.36	0.41±0.017	0.21±0.026	3.94±0.014	4.12±0.27	2.01±0.32**	48.06

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

**Effect on Solid Tumor Volume**

Figure - 3 shows the results of the antitumor activity of the extract of *Phallusia nigra* on solid tumor volume of HLCA-549 bearing mice. A highly significant dose related marked reduction in tumor volume was evident compared to the control. The values observed for group IV and V were very highly significant.



**Figure - 3. Effect on Solid Tumor Volume**

**Effect on 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, viable and non viable cell count 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 (15.24±0.21, 19.84±0.13 and 24.16±0.28). The percentage increase in the life span was 5.83, 37.77 and 67.77 in the treated groups whereas in the group which received Vincristin, it was 68.54. In the extract administered groups the packed cell volume was 3.14±0.24, 2.86±0.17

and 2.40±0.26 ml. The viable cell count decreased whereas nonviable cell count increased in all the treated groups.

**Table - 2: Effect on Median Survival Time, Percentage increase of Life span, Packed cell volume, Viable and Non viable cell count**

Group & Dose (mg/kg bw)	Median Survival time (Days)	Increase of life span (%)	Packed cell volume (ml)	Viable cells 1x10 <sup>6</sup> cells/ml	Non viable cells 1x10 <sup>6</sup> cells/ml
I - Control	14.40±0.34	-	3.67±0.62	12.36±1.18	0.88±0.024
II - 50	15.24±0.21	5.83	3.14±0.24	10.17±1.05	0.93±0.016
III - 100	19.84±0.13*	37.77	2.86±0.17*	9.38±0.94*	1.13±0.027
IV - 150	24.16±0.28**	67.77	2.40±0.26*	7.16±0.14**	1.43±0.041*
V - Vincristin (80)	24.27±0.14**	68.54	1.03±0.34**	3.18±0.27***	2.36±0.033**

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 Hematological Parameters

The changes in the hematological parameters in HLCA-549 bearing mice treated with the extract of *Phallusia nigra* is shown in Table - 3.

**Table - 3: Effect on Hematological Parameters**

Group & Dose (mg/kg bw)	Hb (gm%)	RBC (million/mm <sup>3</sup> )	WBC (10 <sup>3</sup> cells/ mm <sup>3</sup> )	Differential Count (%)		
				Lymphocytes	Neutrophils	Eosinophils
I - Control	8.28±0.13	3.14±0.17	14.22±0.29	42.39±0.73	53.29±0.14	4.32±0.25
II - 50	8.94±0.22	3.65±0.13	12.94±0.18	49.68±0.26	58.18±0.21	7.86±0.14
III - 100	9.56±0.19*	3.95±0.22*	11.86±0.34*	52.18±0.32*	43.14±0.19*	4.68±0.51
IV - 150	10.93±0.34*	4.08±0.42*	11.24±0.13*	56.24±0.18**	40.95±0.22**	2.81±0.22*
V - Vincristin (80)	12.16±0.27**	3.97±0.18*	8.60±0.33**	54.90±0.34**	43.84±0.19*	1.26±0.13**

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

In the groups which received the extract, a significant dose dependent increase was noted in the percentage of Hb when compared to the tumor control. The content of RBC in group I was 3.14±0.17 million/mm<sup>3</sup> while in group II, III and IV it was 3.65±0.13, 3.95±0.22 and 4.08±0.42 respectively. In group V, it was 3.97±0.18. The reduction in the number of WBC noticed in group V treated with 80 mg/kg bw of Vincristin was highly significant than that of group IV administered with 150 mg/kg bw of the extract. Lymphocyte content in the extract treated groups was 49.68±0.26, 52.18±0.32 and 56.24±0.18 whereas it was 54.90±0.34 in the group which was given the standard drug. In group II, there was an increase in neutrophil count compared to control after which a decrease was observed in a dose dependent manner.

A sudden increase in eosinophil count was observed in group II whereas in the other groups administered with the extract there was a fall in the eosinophil count which was gradual.

## DISCUSSION

An attempt was made to assess the antiproliferative activity using the extract of *Phallusia nigra* on HLCA-549 tumor bearing mice and the results obtained are discussed as follows. In the present study on cytotoxicity of the ethanolic extract of *Phallusia nigra* to HLCA-549, it was found that the percentage of cytotoxicity increased with increasing concentration of the extract and 100 percentage toxicity was observed with 0.80 mg/ml. Cytotoxic studies with the colonial ascidian *Cystodytes dellechiajei* have shown high antiproliferative activity to HLCA-549 cell lines.<sup>[15]</sup> A similar cytotoxic activity mediated through apoptosis has been reported using the extract of *Polyclinum indicum* to HeLa cells.<sup>[16]</sup> *Polyclinum madrasensis* Sebastian, a colonial ascidian often exhibit potent cytotoxic activities against cervical cancer cell line because of the presence of unusual cytotoxic metabolites.<sup>[17]</sup> The same role may be suggested for the cytotoxicity of *Phallusia nigra* on HLCA-549.

*Phallusia nigra* extract stimulated the weight of the body which can be taken as an indication of regular intake of food resulting in normal growth. The relative weight of vital organs like spleen, thymus, liver and kidney recorded a dose dependent decrease compared to tumor control. The increase observed in control may be due to pathological condition in the tissues and the decrease observed in the experimental group on treatment reveals bringing the organs to normal status and initiating the production of immune related cells to fight against rapidly proliferating cells.

A dose dependent decrease in the tumor weight with increasing concentration of extract was evident on administration of the extract. The percentage inhibition of tumor progression was 46.51 in group IV which was near to that observed for group V treated with standard drug Vincristin indicating the effective role of the extract of *Phallusia nigra* in controlling tumor growth. The effect of *Cassia alata* on A-549 lung cancer cell by the inhibition of tumor progression is via a mechanism that is Caspase 8 dependent.<sup>[18]</sup> The same role may be played for the decrease in the tumor weight.

Studies on solid tumor volume in HLCA-549 bearing mice indicated a gradual decrease from 15<sup>th</sup> to the 30<sup>th</sup> day in the treated groups. Flavonoids are noted for their cytotoxic activity due to presence of phenolic groups.<sup>[19]</sup> Increasing scientific evidence shows that polyphenols are

good anti-oxidants preventing initiation of the carcinogenic process and act as cancer-suppressing agents, inhibiting cancer promotion and progression.<sup>[20]</sup> Cytotoxic activity of methanolic extract of *Artocarpus heterophyllus* by various *in vitro* assays like MTT and SRB against A-549 cell line has been attributed to the presence of flavonoids having mono to poly phenolic groups.<sup>[21]</sup> Triterpenes isolated from the stem bark of *Physocarpus intermedius* is responsible for strong cytotoxic activity against A-549 cells.<sup>[22]</sup> The presence of flavonoids - isoquercitrin, quercitin and phenolic compounds - gallic, ferulic and caffeic acids reported in *Phallusia nigra* might be the reason for the significant suppression of tumor growth.<sup>[23]</sup> Flavonoids have been reported to inhibit A-549 cell proliferation in a dose dependent manner.<sup>[24]</sup> The presence of flavonoids in *Phallusia nigra* may be a reason for the reduction in tumor volume.

The percentage increase of life span (ILS) and mean survival time showed an increase in all the treated groups compared to control. A maximum of 67.77% ILS was noted in group IV. The reliable criterion for judging the value of any anticancer drug is the prolongation of the life span of animals.<sup>[25]</sup> Oral administration of root extract of *Lindera strychnifolia* prolonged survival time and inhibited tumor growth in a dose dependent manner by apoptosis in mice model.<sup>[26]</sup> The rate of tumor growth depends on an equilibrium between the production and death rate of tumor cells. In the present study also it may be that the extract induced greater level of apoptotic activity which resulted in slow tumor growth and better survival rate. The increase in life span recorded in the present study can be taken as an evidence for the effectiveness of the extract. Similar results were noted on studies involving DLA, EAC and S-180 tumor bearing mice.<sup>[6-8]</sup>

The decrease in the packed cell volume is an indication of the antiproliferative activity of the extract. The compounds present in the extract may act on the tumor cells inhibiting their growth and multiplication resulting in a reduction in the total number of cells.

In the extract administered groups the viable cells decreased and non viable cells increased. Matrine, an alkaloid compound isolated from *Sophora* roots significantly induced cell cycle arrest at G2/M phase in A-549 cell growth.<sup>[27]</sup> Chamaejasmine, a natural flavonone extracted from the root of *Stellera chamaejasme* inhibited the growth of A-549 cells in a dose dependent manner and the reduction in cell viability resulted from cell cycle arrest.<sup>[28]</sup> The decrease in viable cells noted in the present investigation may be due to the inhibition of multiplication, arrest of growth or other multiple factors preventing the maturation of cells.

Administration of *Phallusia nigra* extract increased the level of hemoglobin and RBC significantly indicating stimulation of the hemopoietic system. Hemoglobin is the important part of RBC. The major function of hemoglobin is carrying oxygen. When the RBC count is reduced, the oxygen level is also decreased. Direct or indirect involvement in detoxification of some toxic products of tumor cells after treatment in DLA bearing mice with the extract of *Dillenia pentagyna*, may increase in the level of red blood cells and hemoglobin.<sup>[29]</sup> The same role can be suggested in the present observation also.

During tumor growth progression, usually a significant increase in WBC count is noticed which indicates the stimulation of immune response and their ability to kill tumor cells. In the present study also the tumor control showed an increased level of WBC which on treatment with the extract registered a significant decrease. The decrease in the level of WBC and bringing it back to normal values can be attributed to the decrease in the tumor volume induced by the effect of the extract which in turn shows the protective action on the hemopoietic system.

Studies on the differential count showed an increase in the lymphocytes and decrease in the neutrophil and eosinophil count compared to tumor control on extract administration. Lymphocytes play a key role in maintaining antitumor immunity and they provide an important opportunity for immunotherapy of cancer.<sup>[30,31]</sup> An increase in lymphocyte observed here is supportive of the role of the extract in immune activation. A decrease in neutrophil and eosinophil level is an indication that they started fighting off tumor progression on studies with EAC bearing mice.<sup>[7]</sup>

Phenolic compounds acting as antioxidants may function as terminators of free radical chains and as chelators of redox-active metal ions that are capable of catalysing lipid peroxidation.<sup>[32]</sup> The phenoxy radical intermediates are relatively stable so they do not initiate (propagate) further radical reactions. They even act as terminators of the reaction chain by interacting with other free radicals.

Flavonoids are ideal scavengers of peroxy radicals due to their favorable reduction potentials relative to alkyl peroxy radicals and thus, in principle, they are effective inhibitors of lipid peroxidation.<sup>[33]</sup> Lipid peroxidation can trigger the process of apoptosis, activating the intrinsic suicide pathway present within the cells.<sup>[34]</sup>

## CONCLUSION

The present study showed that ethanolic extract of *Phallusia nigra* inhibited the growth of HLCA-549 cells in a dose-dependent manner. Administration of the extract decreased the volume of the tumor. An increase on median survival time, percentage increase of life span, non viable cells, haemoglobin, RBC, lymphocyte and decrease in packed cell volume, viable cells WBC, neutrophils, eosinophils in HLCA-549 tumor bearing mice which indicates the antitumor property. The 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.<sup>[35]</sup> Further studies on the 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 University Grants Commission – F.No. 39-588/2010 (SR) New Delhi for financial assistance and Dr. R. Sampath Raj Ph.D., Dr Samsun Immuno Clinical laboratory for providing facilities to conduct the experiments.

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