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ANTICANCER PROPERTY OF SIMPLE ASCIDIAN *PHALLUSIA NIGRA* SAVIGNY, 1816 AGAINST A-549 CANCER CELL

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ABSTRACT

Background: Lung cancer is one of the most prevalent disease affects the human beings. To assess the anticancer activity of the ethyl acetate, ethanol, methanol, chloroform and aqueous extract of the simple ascidian Phallusia nigra against human lung cancer cell lines (A-549). The extract administration at 100 µl, 50 µl, 25 µl, 12.5µl, 6.25 µl in 100 µl of 5% MEM. 76% of cytotoxicity was observed in cells cultivated at 100 µl concentration. There was a dose dependent increase in number, inhibition of cancer cells and decrease in size of tumor volume. A high reduction in tumor was noted. In vivo studies indicate the presence of bioactive compounds in Phallusia nigra with anticancer property against A-549 cells.

KEY WORDS

Phallusia nigra, anticancer, A-549 cells, cytotoxicity.

INTRODUCTION:

Cancer is a broad group of various disease all involving unregulated cell growth and division. Cancer still remains one of the most serious human health problems and lung cancer is the most common universal death for man. One of the treatments used currently is chemotherapy which kills cancer cells along with the healthy ones. The major drawback associated with chemotherapy is multidrug resistance to anticancer drugs (Borst *et al*., 2002). Therefore, development of a target specific drug without any side effects 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 source of drugs for cancer (Padavala *et al*., 2008). They are an interesting group of marine sedentary organisms commonly called sea squirts found to occur in Vizhinjam coast. Ascidians which are marine sedentary organisms have been largely studied since it has been show that many extracted compounds display potent antitumoral and antiviral activities. ET-743 is a novel chemical compound isolated from the tunicate *Ecteinascidia turbinate* which exhibit potent invitro and invivo antitumor activity in three chemosensitive human tumour Xenografts – MEXF 989, LXFL 529 and 40C22 (Rinechart *et al*., 1990; Jimeno *et al*., 1996; Cragg *et al*., 1997) Ecteinascidin 743 (ET-743) a tetrahydroiso quinolone alkaloid originally isolated from the Caribbean tunicate *Ecteinascidia turbinate*, 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. (Rinehart *et al*., 1990; Wright *et al*., 1990). The invitro growth inhibitory and potential anticancer efficacy of lissoclibadins and lissoclinotoxins isolated from the tropical ascidian. Lissoclinum of badium against nine human cancer cell lines including MDA-MB-23] were evaluated [Oda *et al*., 2007]. The extracts of cystodytes *dellechiajei* showed remarkably high anti proliferative activity in human lung carcinoma A-549 colon cadenocarcinoma H-116, Pancreatic adenocarcinoma PSN-1 and breast carcinoma SKBR3 cell

lines [Garcia *et al*., 2007 Isogranulatimide from *Didemnum granulatum*, Aplidin from *Aplidium albicans*, Clavaminols A-F from *Clavelina phlegraea*, aplidinone A from Aplidium conicum, Cephalostatin from *Cephalodiscus gilchristi*, ritterazine G from Ritterella tokioka, styelin D from *Styela clava*, Lissoctinamides from *Lissoclinum patella*, Tamandarins A and B from the family Didemnidae and 7-oxostaurosporine from *Eudistoma vannamei* showed antitumor activity against various cell lines [Berlinck *et al*., 1998; Fernanotez, 2010, Wojtkielewicz *et al*., 2003; Taylor *et al*., 2000; Schmitz *et al*., 1993; Vervoort *et al*., 2000 and Jimenez *et al*., 2012) 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 availant biofoulant, considered as a nuisance of Vizhinjam coast has been chosen to assess the anticancer activity against A-549 cells.

MATERIALS AND METHODS:

Collection and preparation of ascidian sample

The ascidian *Phallusia nigra* (Chordate: Ascidiacea) were collected during the intertidal Chordata at Vizhinjam south coast of India. The collected samples were rinsed with sterile sea water to remove associated debris and salt. The samples were weighed (10 g) and preserved separately in methanol and ethanol (1:2) and brought to the laboratory.

Preparation of the ascidian extract

The test samples were carefully removed washed with sterile sea water dried under shade and homogenized to get a course powder. The course powder was stored in an airtight container and used for further investigation. 100 g of the powdered animal material was extracted with methanol, ethanol, chloroform ethyl acetate and aqueous using sessile apparatus. The extract was cooled to room temperature evaporated in a rotary evaporator under reduced pressure and a brown sticky residue was obtained (15 g).

Cells for cytotoxic study

A-549 (lung cancer) cells were initially procured from National Centre for cell sciences (NCCS) pune, India and maintained Dulbecos modified Eagles medium (Gibco, Invitrogen). The cell line was cultured in 25 cm^2 tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate and antibiotic solution containing: Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphoteracin B (2.5µg/ml). Cultured cell lines were kept at 37°C in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany).

The viability of cells were evaluated by direct observation of cells by Inverted phase contrast microscope and followed by MTT assay method.

Cells seeding in 96 well plat

Two days old confluent monolayer of cells were trypsinized and the cells were suspended in 10% growth medium, 100µl cell suspension ($5x10^4$ cells/well) was seeded in 96 well tissue culture plate and incubated at 37ºC in a humidified 5% CO_2 incubator.

Cytotoxicity Evaluation

After 24 hours the growth medium was removed, freshly prepared each animal extracts in 5% DMEM were five times serially diluted by two-fold dilution (100µl, 50µl, 25µl, 12.5µl, 6.25µl in 100µl of 5% MEM) and each concentration of 100µl were added in triplicates to the respective wells and incubated at 37ºC in a humidified 5% CO_2 incubator.

Cytotoxicity Assay by Direct Microscopic observation

Entire plate was observed at an interval of each 24 hours; up to 72 hours in an inverted phase contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera) and microscopic observation were recorded as images. Any detectable changes in the morphology of the cells, such as rounding or shrinking of cells, granulation and vacuolization in the cytoplasm of the cells were considered as indicators of cytotoxicity.

Cytotoxicity Assay by MTT Method

Fifteen mg of MTT (Sigma, M-5655) was reconstituted in 3 ml PBS until completely dissolved and sterilized by filter sterilization.

After 24 hours of incubation period, the sample content in wells were removed and 30µl of reconstituted MTT solution was added to all test and cell control wells, the plate was gently shaken well, then incubated at 37ºC in a humidified 5% CO_2 incubator for 4 hours. After the incubation period, the supernatant was removed and 100µl of MTT solubilization solution (DMSO) was added and the wells were mixed gently by pipetting up and down in order to solubilize the formazan crystals. The absorbance values were measured by using microplate reader at a wavelength of 570 nm (Laura B. Talarico *et al.,* 2004).

The percentage of growth inhibition was calculated using the formula:

$$\%\ \text{of viability} = \frac{\text{Mean OD Samples} \times 100}{\text{Mean OD of control group}}$$

RESULTS

1. Cytotoxity Assay by Direct Microscopic observation

The microscopic observation was recorded as images given in Figure 1 to Figure 5.

2. Cytotoxicity Assay by MTT Method

Acute oral toxicity for the period of 24 hours and lethal dose of 6, 12, 25, 50 100 µl and the absorbance values shown in the following Figures 1-5 and Table 1-5.

1. Methanol

Inhibition of tumor cell lines shown in Fig 1. In this graph the O.D values shows that the methanolic extract of Phallusia nigra on the inhibition of tumor reduces with the increase in dosage than control (Table 1).

Table 1 Viability of *Phallusia nigra* observed at different concentrations

Sample volume (µl)	Average OD at 540nm	Percentage Viability
Control	2.7405	
6.25	2.6591	97.02
12.5	2.0001	72.98
25	1.7311	63.16
50	1.5637	57.04
100	1.0096	36.83

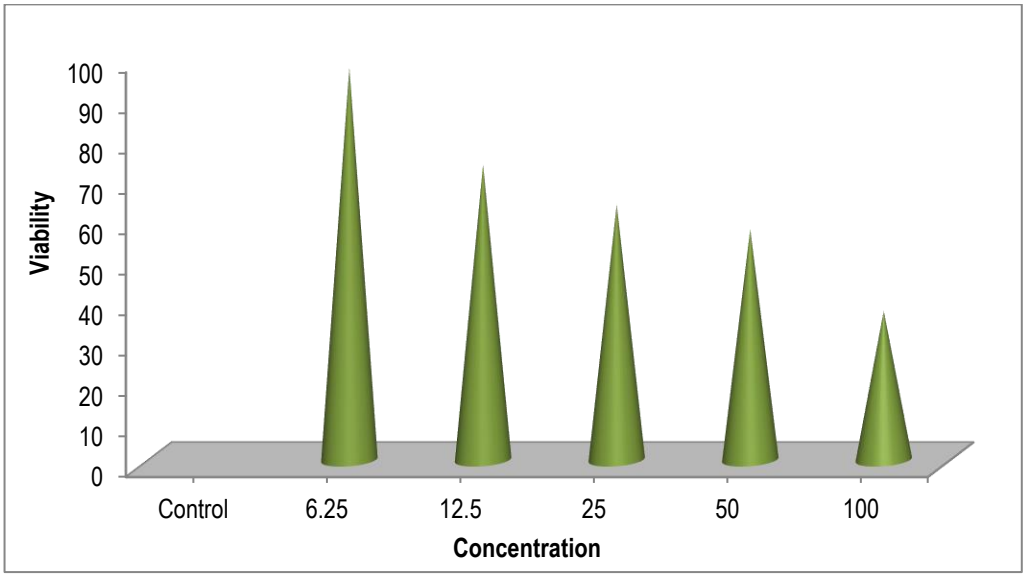


Fig. 2: Viability of *Phallusia nigra* observed at different concentrations

2. Aqueous

Inhibition of tumor cells shown in Fig 2. Here a dose dependent decrease in tumor volume from core 6.25 to 100 µl. At the maximum conc. the growth of tumor is little reduced than other concentration (Table 2).

Table 2 Viability of *Phallusia nigra* observed at different concentrations

Sample volume (µl)	Average OD at 540nm	Percentage Viability
Control	2.2297	
6.25	2.1585	96.80
12.5	2.1399	95.97
25	2.0994	94.15
50	2.0001	89.70
100	1.7297	77.57

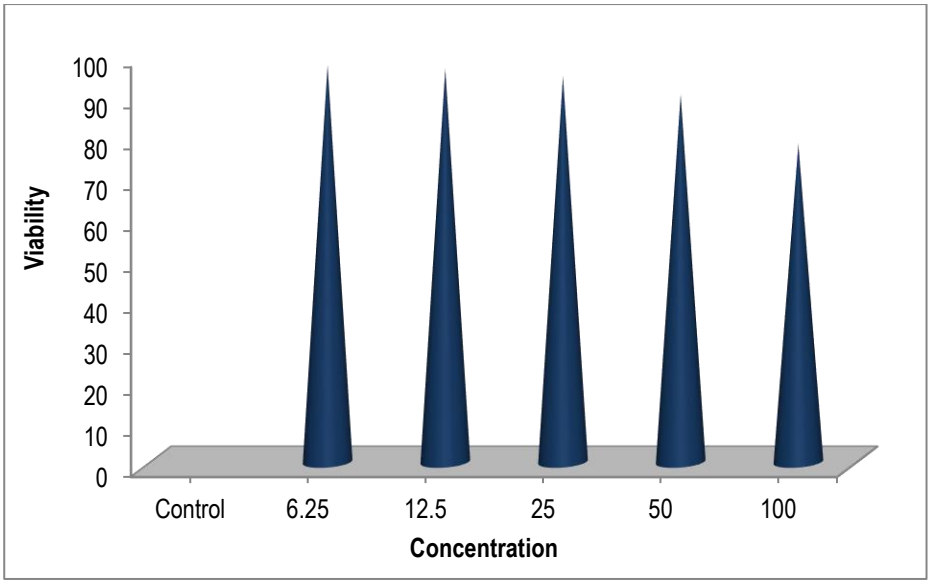


Fig. 2: Viability of *Phallusia nigra* observed at different concentrations

3. Ethanol

Inhibition of tumor cells of ethanol extract shown in Fig 3. Here a gradual increase in concentration of dose upto 100µl concentration the growth of tumor cells is reduced to 30% from lower cone to higher concentration. Viability of cells is less in higher cone of dosage is given in table 3.

Table 3: Viability of *Phallusia nigra* observed at different concentrations

Sample volume (µl)	Average OD at 540nm	Percentage Viability
Control	1.519	
6.25	1.4265	94.52154
12.5	1.3082	86.7395
25	1.1467	75.94433
50	0.8175	54.20146
100	0.5555	36.83897

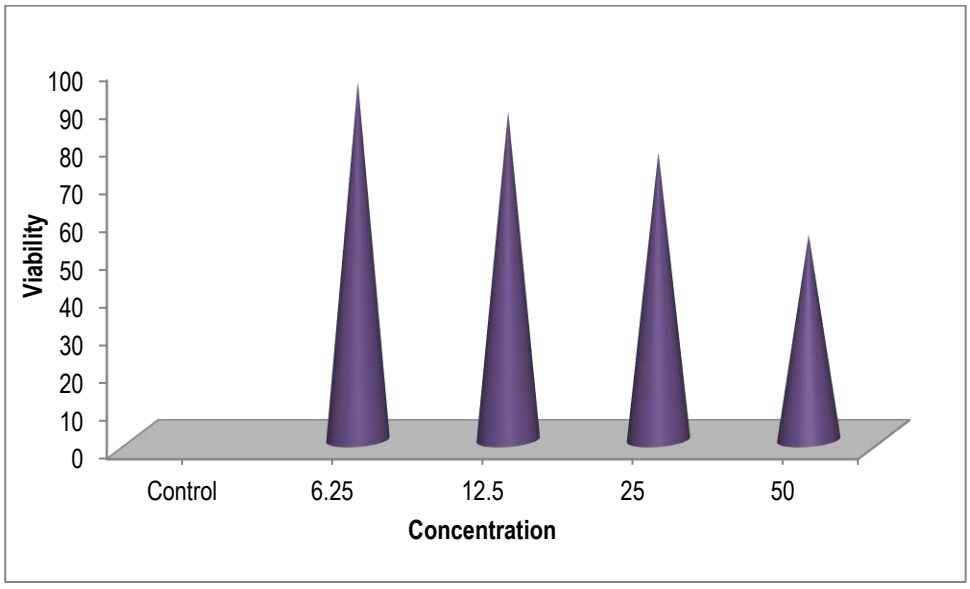


Fig.3: Viability of *Phallusia nigra* observed at different concentrations

4. Ethyl acetate

Inhibition of tumor cells observed in Ethyl acetate extract shown in Fig 4. Here a gradual decrease in concentration of tumor cells when the concentration is increased to 100 µl (ie) it processes that when the concentration of dosage increased the tumor cells number is decreased. In the table 4 and graph 4 shows that only medium amount of cells survives in 100µl concentration.

Table 4 Viability of *Phallusia nigra* observed at different concentrations

Sample volume (µl)	Average OD at 540nm	Percentage Viability
Control	1.8522	
6.25	1.7264	93.20
12.5	1.6286	87.92
25	1.5568	84.05
50	1.4868	80.27
100	0.9005	48.61

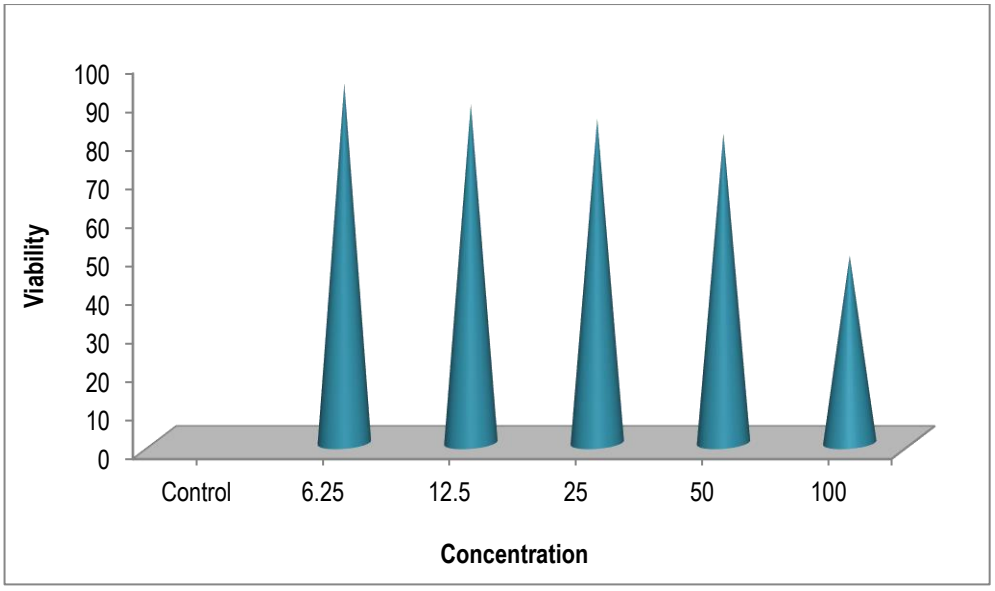


Fig. 4 Viability of *Phallusia nigra* observed at different concentrations

5. Chloroform

Inhibition of tumor cells is shown in Fig 5. In this case the concentration of dosage increased and increase in concentration of tumor cells any less % of cells only death occurs in this chloroform extract (Graph 5) viability of cells is more even in 100µl of concentration

Table 5 Viability of *Phallusia nigra* observed at different concentrations

Sample Concentration (µg/ml)	Average OD at 540nm	Percentage Viability
Control	2.2297	
6.25	2.1972	98.54
12.5	2.0397	91.47
25	1.9999	89.69
50	1.8019	80.81
100	1.6968	76.09

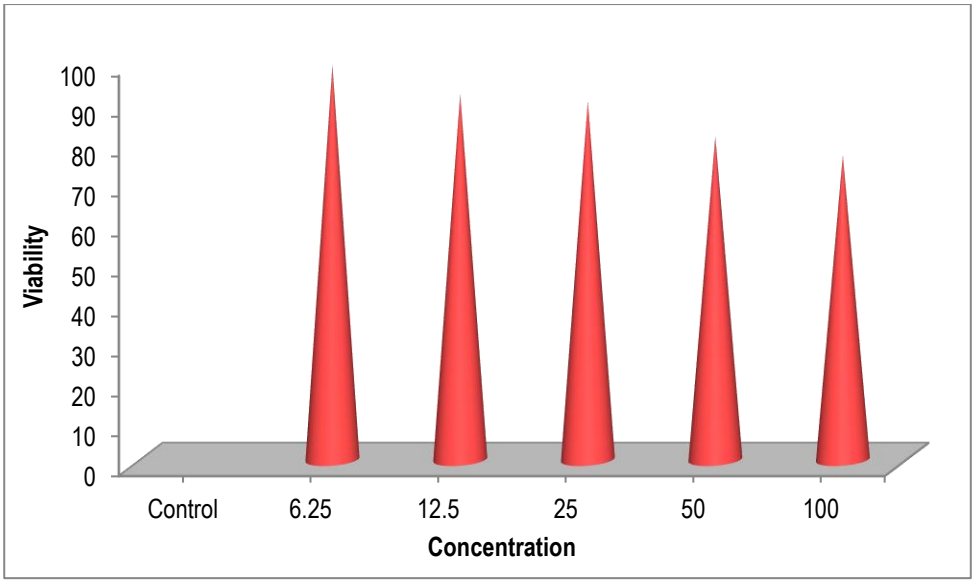
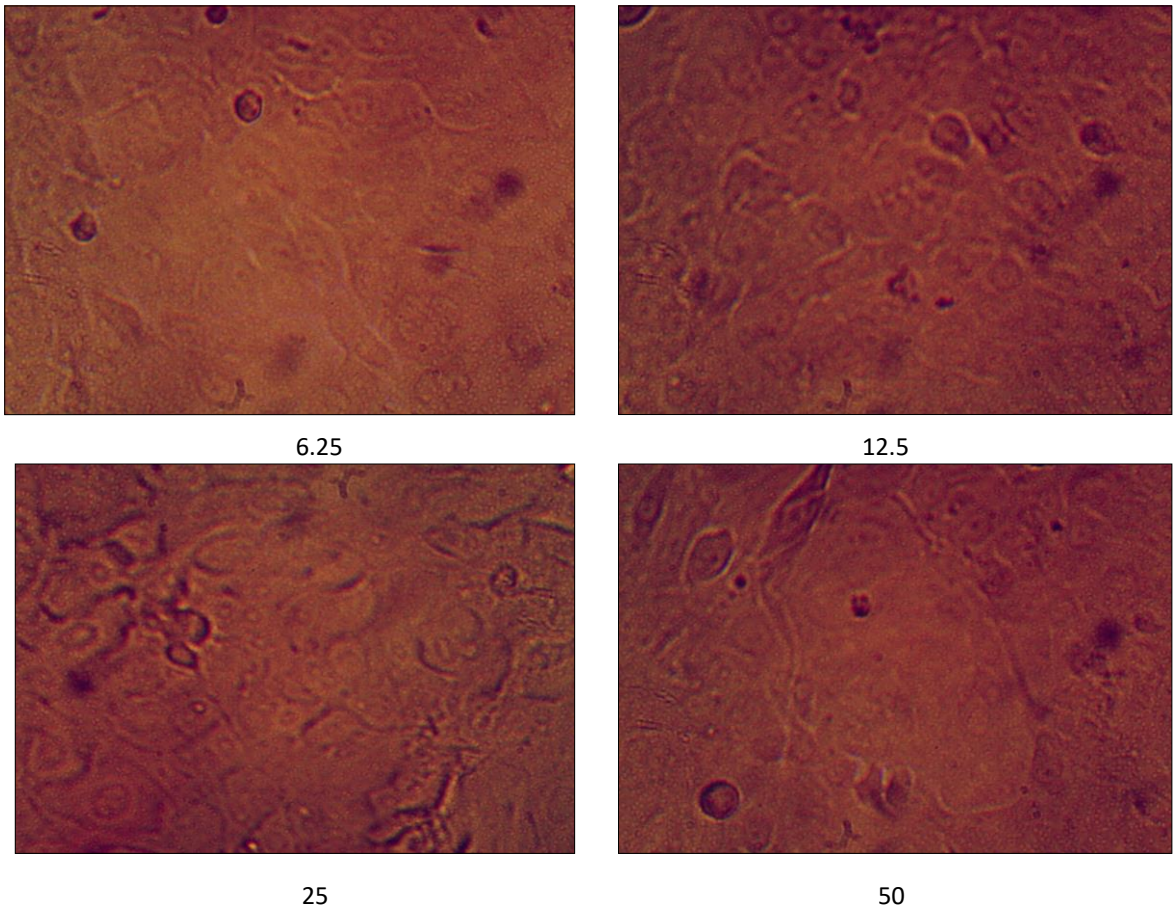


Fig. 5 Viability of *Phallusia nigra* observed at different concentrations

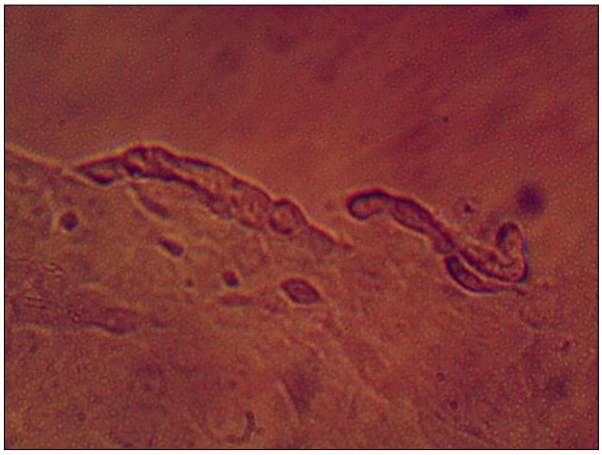
Plate 1

Methanol



6.25 12.5

25 50



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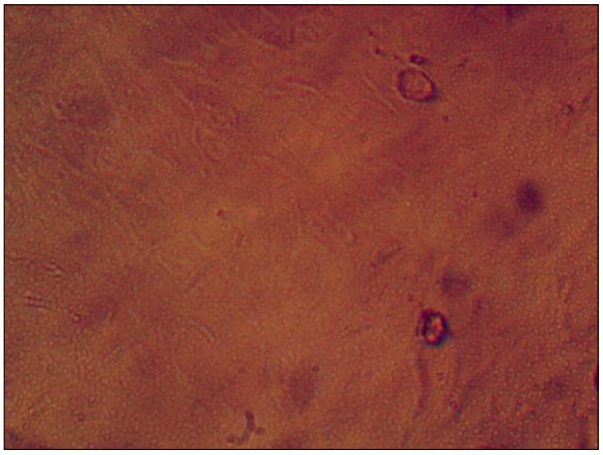
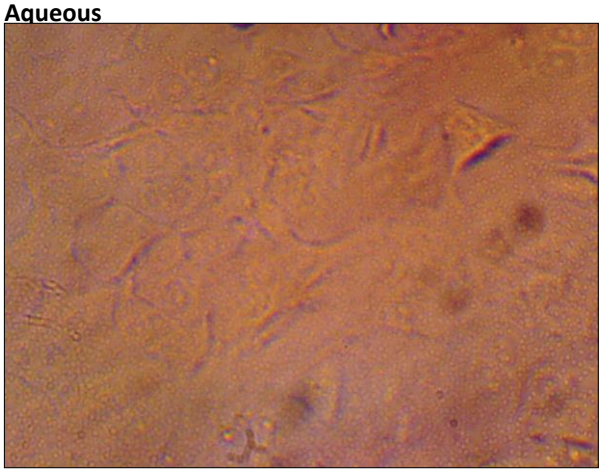


control

Plate 2

Aqueous



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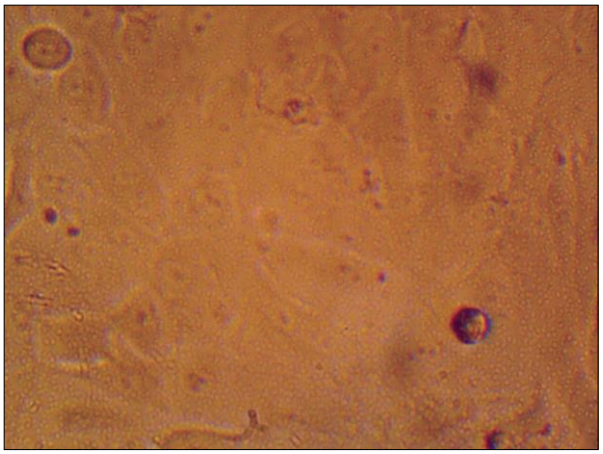

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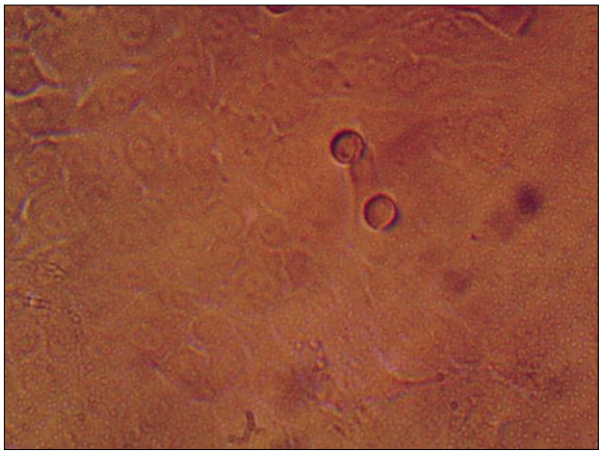

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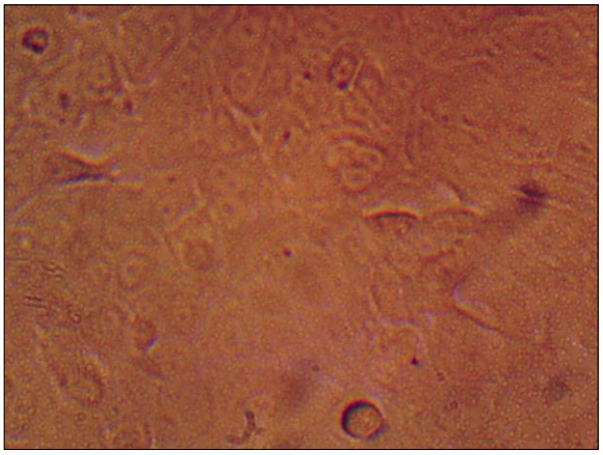

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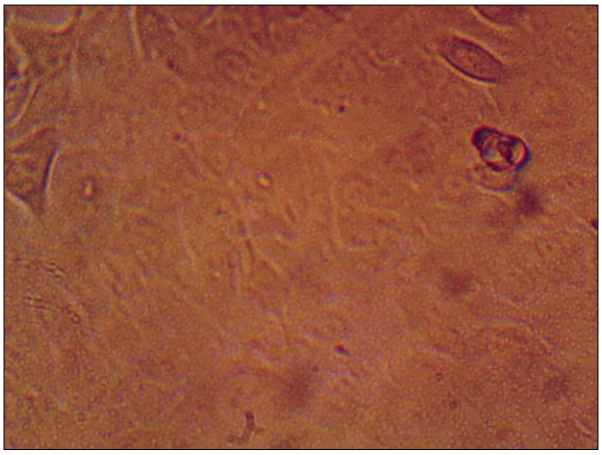

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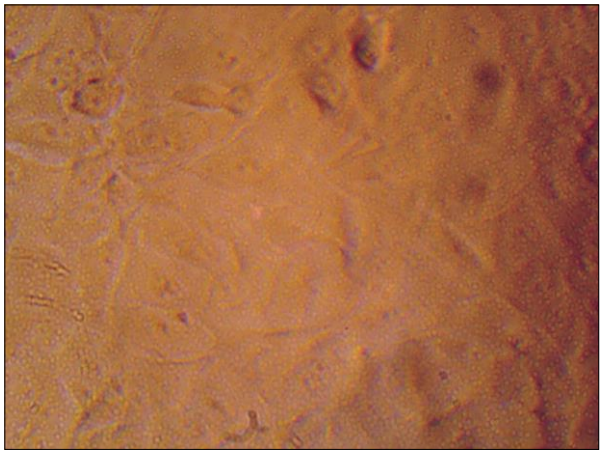

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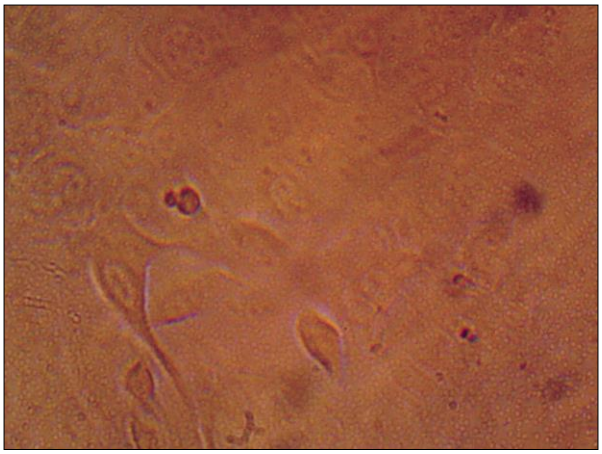
Plate 3

Ethanol



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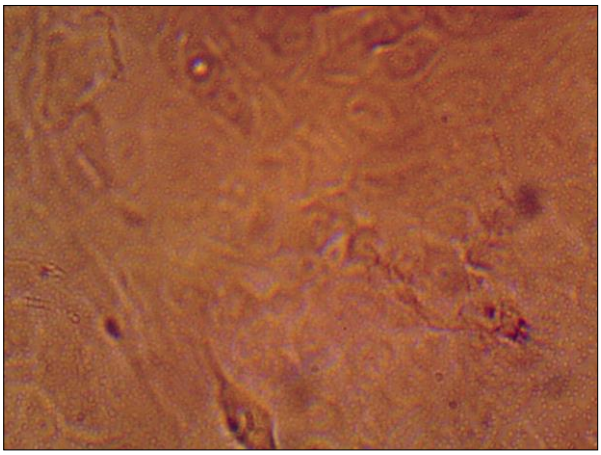

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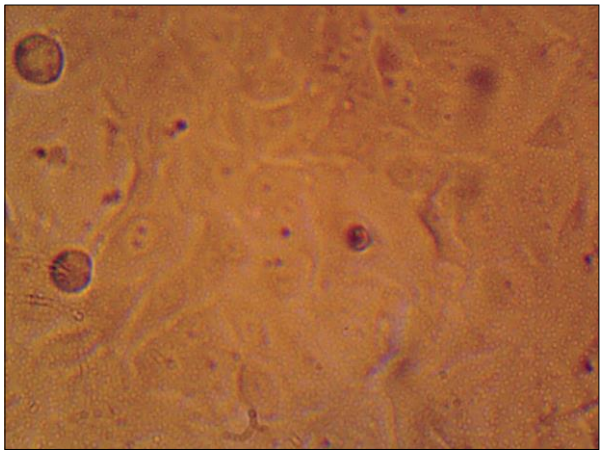

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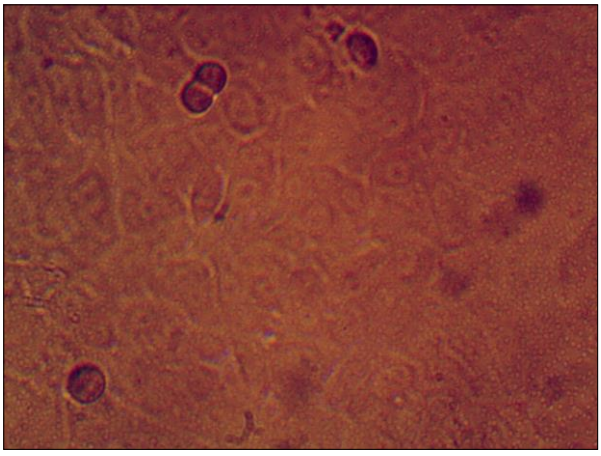

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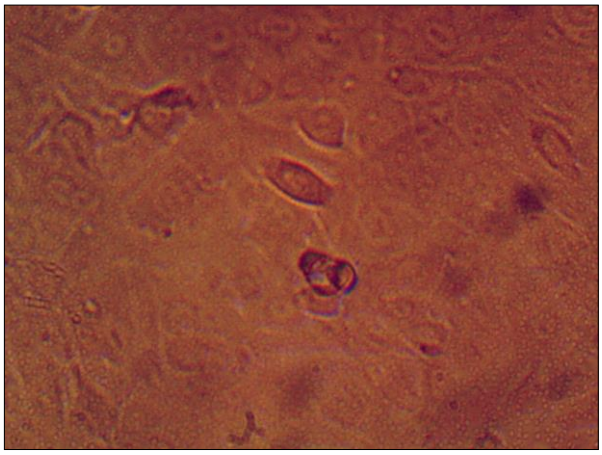

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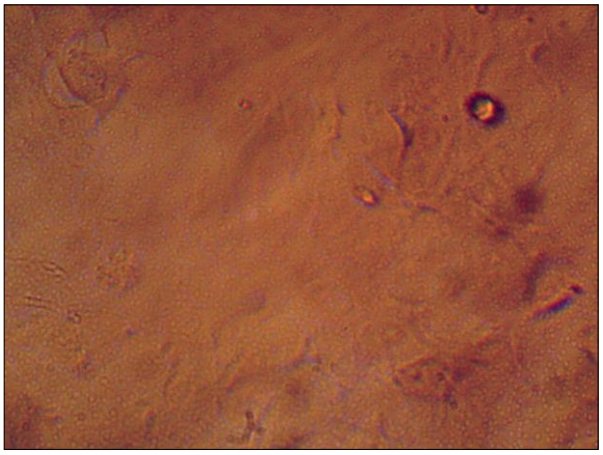

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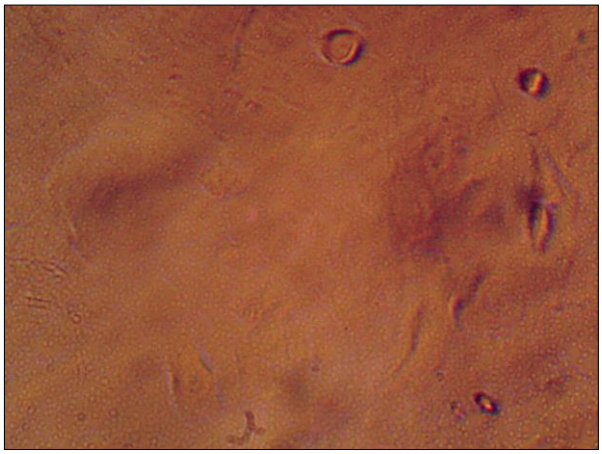
Plate 4

Ethyl acetate



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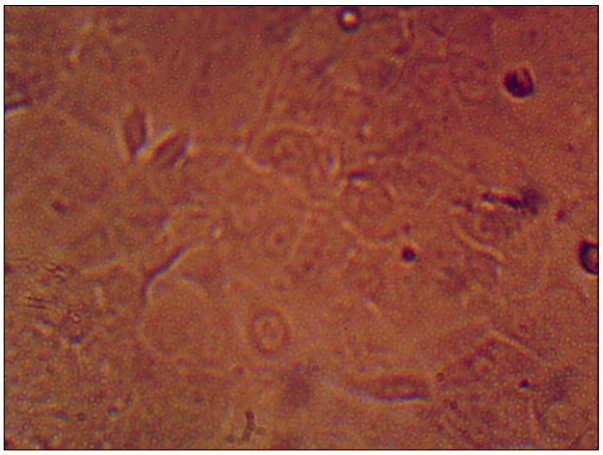

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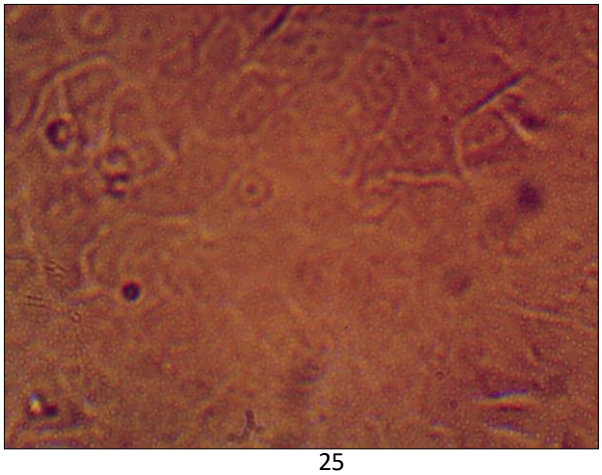

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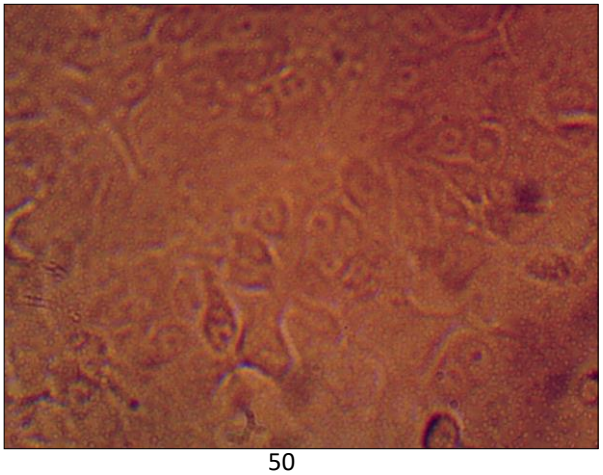

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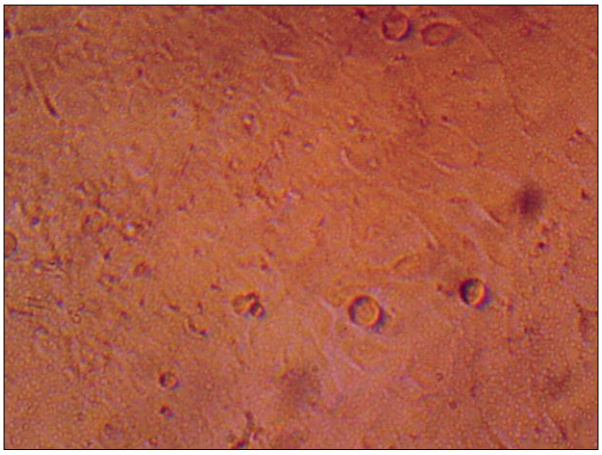

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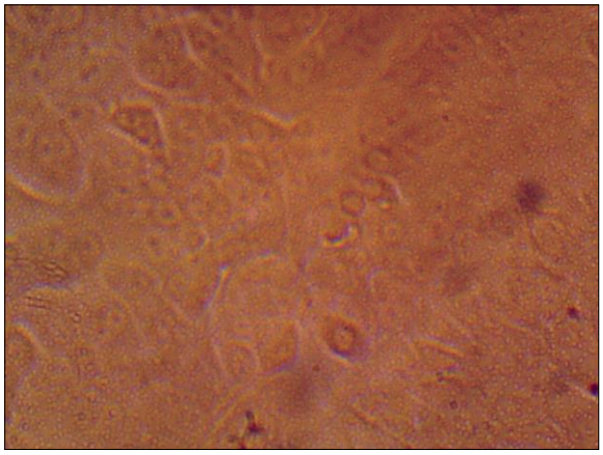

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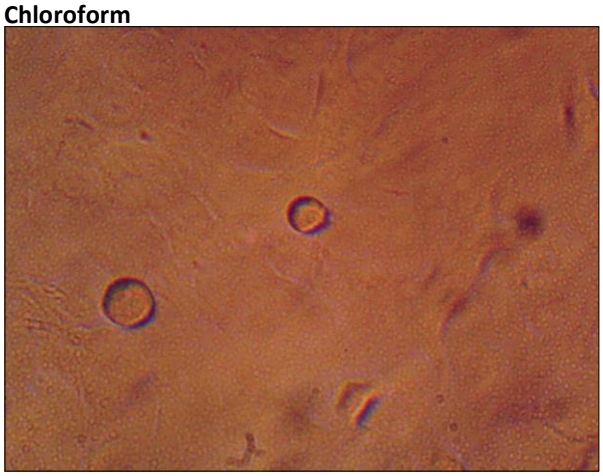
Chloroform



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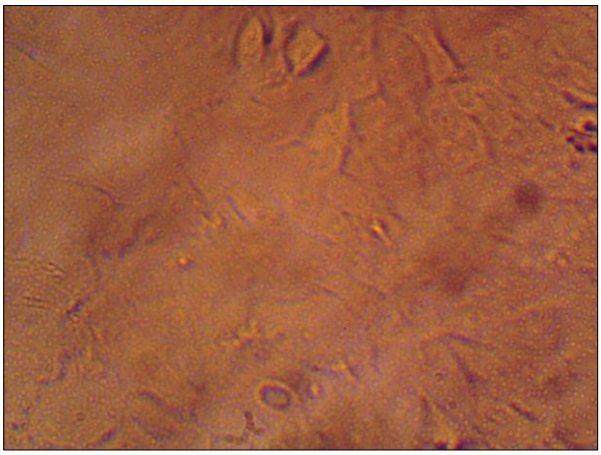

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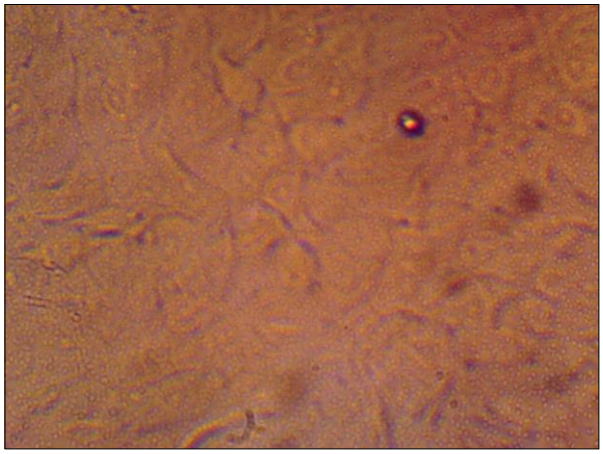

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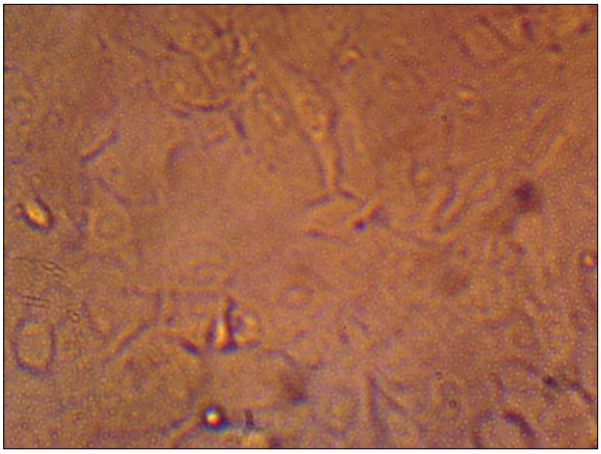

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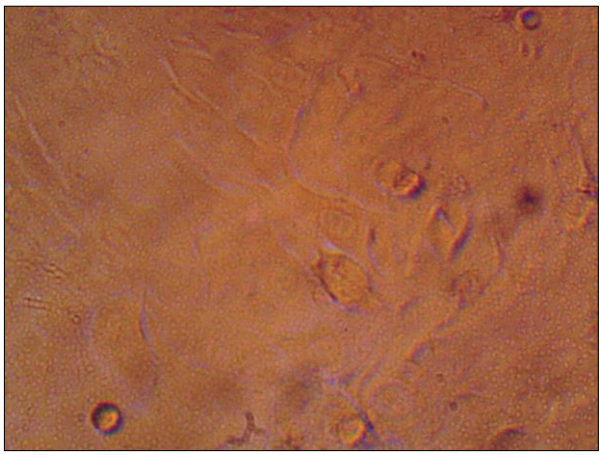

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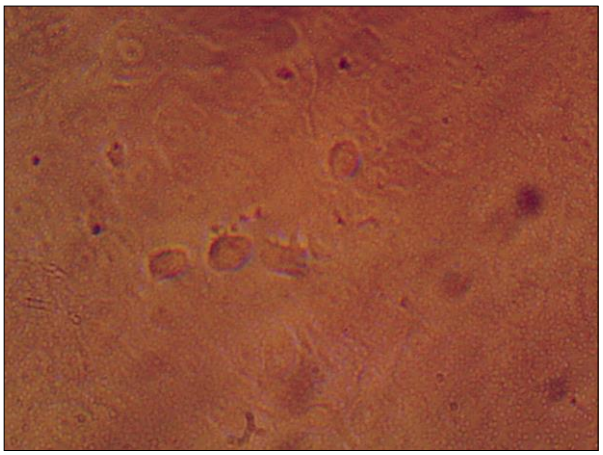

control

DISCUSSION

Studies on the cytotoxicity of methanol, aqueous, ethanol, ethylacetate and chloroform extract of *Phallusia nigra* to A-549 cells showed an increase in the percentage of toxicity with increasing concentration and 100% toxicity was observed with 100 µg/ml concentration. 36.83% viability observed in Methanol extract of *Phallusia nigra* at 100µl concentration, 77% viability observed in Aqueous extract of *Phallusia nigra* at 100µl concentration, 36.83% viability observed in Ethanol extract of *Phallusia nigra* at 100µl concentration, 48.61% viability observed in ethyl acetate extract of *Phallusia nigra* at 100µl concentration, 76-09% viability observed in chloroform extract of *Phallusia nigra* at 100µl concentration. *C. dellechiajei* extracts showed remarkably high anti proliferative activity $IC_{50} \leq$ 5 µg/ml) in human lung carcinoma A-549, colon adenocarcinoma H-116 pancreatic adenocarcinoma PSN-1 and breast carcinoma SlCBR3 cell lines. Moreover we found that the maximum activity was located in the tunic tissue of the colony (Manuel *et al*., 2007).Eusynstyelamide B of ascidian *Eusynstyela latericius* showed cytotoxicity against MDA-MB-231 breast cancer cells by inducing apoptosis (Liberio *et al*., 2013). 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 (Torres *et al*., 2002). Ascididemnin, a pyridoacridine alkaloid derived from a *Didemnum* sp. brought about cytotoxicity by promting oxygen stress related proteins and reactive oxygen species (Matsumoto *et al*., 2003) cytotoxic studies with the colonal ascidian *Cystodytes dellechiajei* indicated high antiproliferative activity to breast cancer S1CBR3 cell lines. (Garcia *et al*., 2007). Ascidians are a rich source of bioactive compounds with cytotoxic properties (Ananthan *et al*., 2011). There was a decrease in 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 highest dose showed in inhibition less than that of standard drug. Hence, though it is effective in inhibiting by the growth of cells, a higher dose might be needed (Oda *et al*., 2007). Gingeral extracted from *Gingiber officinale* has been shown to inhibit cell adhesion invasion and motility of MDA-MB-231 cells (Lee *et al*., 2007). The decrease in number of cancer cells noted was highly significant. Quartets a flavonoid gives protection against chemically induced and spontaneous formation of tumors in animals (Lipkin *et al*., 1999) Quercetin present in the extract of *Phallusia nigra* may be a reason for decrease in tumor volume. The decrease in tumor volume might be due to the cytotoxic action of the extract (Shanmugapriya *et al*., 2012). A chemical screening of *Phallusia nigra* extract showed the presence of saponins. 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 (Man *et al*., 2010). Polyphenolic extract form *Vacinium macrocargus* inhibited tumor growth and proliferation of tumor cells and hence there was an increase in non-viable cells which in turn results in decreased viable cells (Wang, *et al*., 2007).

The decrease in viable cells noted may be the result of inhibition of tumor growth and multiplication by the components present in the extract.

CONCLUSION

Administration of the extract such as Ethylacetate, ethanol, methanol chloroform and aqueous of phallusia nigra decreased the no of tumor cells and increased the percentage of inhibition all due to its anticancer property. In this ethanolic extract of phallusia nigra contains compounds like 2-piperidinone, Benzene acetamide, Tetradecanoic acid, n-Hexadecanoic acid, Phenol 3-pentadecyl, (z,z,z) phenylmethyl ester of 6, 9, 12-octadecatrienoic acid, (z) phenyl metnyl ester of 9-Octadecenoic acid, cholesterol, cholestan-3-ol and 3-hydroxy- (39, 179) spiro (androst -5-ene-17) –(cyclobutary-2-one. One or more of these compounds cancer preventive and anticancer properties observed at present. It is a promising source at present. It is a promising source for isolation of compounds, which can be applied of compounds which can be applied in a prophylaxes and treatment of cancer Meenakhi *el al*. 2012).

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