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IN VITRO CYTOTOXICITY EFFECT OF MANGROVES AGAINST NON-SMALL CELL LUNG CARCINOMA A549 AND NCI-H522

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ABSTRACT

This is the study mainly focused on to find the anti-prolifertative effect of field grown and tissue cultured callus materials. The mangrove species *Acanthus ilicifolius, Callophyllum inophyllum and Excoecaria agallocha* and two different non-small cell lung cancer cell lines A549 and NCI-H522 were used for this present investigation. Cytotoxicity studies were conducted with root extracts of *Acanthus ilicifolius, Callophyllum inophyllum and Excoecaria agallocha* and its respective calli. The results were recorded from different concentration at different time interval. It is likely to point out that all the roots and root calli extracts these mangroves showed the toxicity activity. The root callus of all the studied mangrove extracts exhibited the better activity against both A549 and NCI-H522 than the field grown root extracts. Among the different concentrations tried, 6.5 mg/ml was found to be the optimum concentration for both the A549 and NCI-H522 lung carcinoma. This present study revealed that the mangrove species *Acanthus ilicifolius, Callophyllum inophyllum* and *Excoecaria agallocha* have the anti-proliferative effect and it may lead to new drug development for this leading cause of global disease.

KEY WORDS: mangroves, A549 and NCI-H522 lung carcinoma, anti-prolifertative effect, root callus.

INTRODUCTION

The coastal communities utilized wide variety of traditional products of mangroves [1-5]. Traditional uses of mangroves recently attracted the scientific communities to find out the pharmaceutical products to combat a number of serious diseases and the mangrove species

like *Avicennia Africana, A. nitida, Bruguiera exaristate, B.sexangula and Buddlya parviflora* and some other mangrove associate are also used to cure the cancer disease by the fishermen communities [6]**.** Based on traditional knowledge and preliminary scientific work about sixteen mangroves are the possible source of anticancer drugs[7]. Similar scientific report on anticancer activity of tissue cultured mangrove *Acanthus ilicifolius* is documented [8].

Lung cancer is a most common cancer in the world and it cause major cancer-related mortality worldwide [9]. Lung cancer accounts for more death than any other cancer in both male and female. An estimated death due to lung cancer is 159.480, its accounts for 27 % of all cancer death in 2013. Chemotherapy is still standard treatment for the majo- rity of patients with advanced NSCLC, who do not have specific molecular markers [10]. For localized non-small cell lung cancers surgery is usually the treatment of choice for most of the patients. The important thing is survival is improved when chemotherapy is given after surgery. In adavanced stage non-small cell lung cancer patients are usually treated with chemotherapy [11].

Based on this idea, to prove the traditional medicinal application of mangroves, the present study was designed to evaluate the anti-proliferative effect of mangroves *Acanthus ilicifolius, Callophyllum inophyllum and Excoecaria agallocha* and its tissue cultured callus materials against human non-small cell lung cancer cell lines A549 and NCI-H522.

MATERIALS AND METHOD

Surface sterilization and Callus induction

The young root explants of *Acanthus ilicifolius, Callophylum inophyllum, Excoecaria agallocha* were collected from Pichavaram mangrove forest and surface sterilized with detergent solution (2% Teepol, Reckill and Colman, India) for 5 min. Then washed they were in 0.1% mercuric chloride for 1.5 min followed by 70% ethanol for 45 seconds. After sterilization, all the explants were cut into small pieces (1.0 to 1.5 cm long) and were individually placed on MS medium supplemented with various concentration and combinations of cytokinins and auxins, 3.0% sucrose and 0.8% agar.

Extraction

The shade-dried root and *in-vitro* derived root callus samples of *Acanthus ilicifolius, Callophyllum inophyllum, Excoecaria agallocha* were extracted with methanol:chloroform in the ratio of 8:2 at room temperature for 24 hours in a Soxhlet apparatus and this extracts

were subsequently concentrated on a watch glass in an air draught for removing the chloroform and other impurities [12].

Culture of A549 and NCI- H522 lung carcinoma

Cytotoxicity studies were performed using 3-[4,5-dimethylthiazol-2-yl]- 2,5-diphenyl tetrazolium bromide (MTT,Sigma) assays. It is a colorimetric assay in which the MTT reduce the yellow tetrazolium with help of active mitochondrial enzyme of a live cell into the purple formazon product measured at 570 nm. A549 and NCI- H522 lung carcinoma cells were purchased from national centre for cell science (NCCS, Pune, India) and grown in DMEM (Sigma). All media were supplemented with 10% FBS (Biowest) and 1% antibiotic and antimycotic solution (sigma) and cultured in 5% CO_2 atmosphere at 37°C. In this study 1x104 cells were seeded in each well of 96 well plates (nunc). Four different chemicals dispersed in PBS were added in various concentrations (20, 40, 60, 80,100µl) along with control. After 24 h incubation, the supernatant of each well was replaced with 100µl MTT diluted in serum-free medium (0.5mg/ml) and the plates incubated at 37°C for 4 h. After aspirating the MTT solution, Dimethyl sulfoxide (DMSO, Sigma) was added to each well and pipette up and down to dissolve all of the dark blue crystals (formazon) and then left at room temperature for few minutes to ensure all crystals are dissolved. Finally, absorbance was measured at 570nm using synergy HT-1 micro reader. The same has done in 48h, 72h duration in triplicates viability of non-treated control cells was arbitrarily defined as100%. The results are expressed as mean± (SEM) of the absorbance.

Cell viability test

Cell viability of A549 and NCI-H522cells were assessed by MTT assay (Mosmann, 1983). MTT (3-[4,5-dimethythiazol-2-yl] 2,5-diphenyl tetrazolium bromide) solution was prepared by adding 0.5 mg MTT/ml of serum-free RPMI-1640 medium, solubilization solution was prepared by adding DMSO –dimethyl sulfoxide and Phosphate Buffered Saline (PBS; pH 7.4)

The cells A549 and NCI- H522were plated separately in 96 well plates at a concentration of 1 × 104 cells/well. After 24 h of plating, cells were washed twice with 100 µl of serum-free medium and starved by incubating the cells in serum-free medium for an hour at 37oC. After starvation, cells were treated with different concentrations of drug (range of concentrations 50mg/ml, 12.5 mg/ml, 6.25 mg/ml and 3.13 mg/ml) for 24, 48 and 72 h. At the end of treatment, the medium from control and drug treated cells were discarded and 100 µl of MTT

containing DMEM medium (0.5 mg/ml) was added to each well. The cells were then incubated for 21/2 h - 4 h at 37ºC in the CO2 incubator.

The MTT containing medium was then discarded and the cells were washed with 1× PBS (200 µl). The crystals were then dissolved by adding 100 µl of DMSO and this was mixed properly by pipetting up and down. Spectrophotometrical absorbance of the purple blue formazon dye was measured in microplate reader at 570 nm. The OD of each sample was then compared with the control OD and the graph was plotted. The cell viability was calculated using the following formula

$$\text{Cell viability (\%)} = \frac{\text{Mean OD}}{\text{Control OD}} \text{ X } 100$$

RESULT AND DISCUSSION

Cytotoxic effect of *Acanthus ilicifolius, Callophyllum inophyllum, Excoecaria agallocha* on lung cancer cell lines

Mangroves extracts have been used in indigenous medicine for long time. They are diverse, productive, biologically active and chemically unique, offering a great scope for discovery of new drugs [14]. Extracts from mangroves seems to have a potential for human, animal and plant pathogens and for the treatment of incurable diseases such as AIDS and cancers [13].Mangroves have many bioactivities such as antioxidant, antibacterial, anticancer due to the presence of numerous phytochemicals [15]. The remarkable amounts of free radical inhibition activity was already stated in the species of *Acanthus ilicifolius, Callophylum inophyllum* and *Excoecaria agallocha* plant parts using DPPH assay methods [16].

In this present research, initially callus biomass was produced using different combination and concentration of growth regulators. The best callus biomass (Fig.1) was obtained from the 2,4-D and BAP combination, results shown in the table.1. The partially purified root and root callus extract of the *Acanthus ilicifolius, Callophylum inophyllum* and *Excoecariaag allocha* was characterized and furthermore the cytotoxic effect of the those extracts were tested against A549 and NCI- H522 non-small cell lung carcinoma. A549, NCI- H522 and normal fibroblasts cells were treated with root and root callus of *Acanthus ilicifolius, Callophylum inophyllum* and *Excoecaria agallocha* extract at various concentrations (3.05 - 50mg/ml) for 24,48 and 72 h. The extract significantly inhibited viability of both lung carcinoma cell lines tested in a dose-dependent manner. Among the tow cancer cell lines used Acanthus ilicifolius, Callophylu minophyllum and Excoecaria agallocha exhibited the

strongest potency of cytotoxicity in A549 at a dose of 3.05 mg/ml when compare to the NCI-H522. IC50 and CC 50 along with the therapeutic index were calculated based on the experimental data and shown in (Table 2;Fig 2,2a & 3). The IC50 and CC 50 and therapeutic index values are varied significantly between samples and cell line. The maximum (14.34) therapeutic index was observed in C2 against the NCI-H522 cell line, it revealed that the sample C2 was high potent to cure the NCI H522 type of lung cancer. This observation is in accordance with previous antioxident studies in fungus, plants and bacterial extracts [17-18]. phenolic derivatives are responsible for such antioxidant activity in removal of toxic free radicals [19-20]. A drug is considered to be worthy of further testing if it has a therapeutic index value of 16 or greater [21] .In the present study, therapeutic index value was found to be near of 16 and that was 14 against NCI H522 cell line (Table.2). Further purification of active compounds present in the crude will provide potent activity for its further development as anticancer drugs. These results indicated that the p53+/+ cancer cells (A549) were more sensitive to the treatment than p53-/- cancer cells (NCI- H522) Table-2&3.To ascertain whether the extract has any selectivity for normal versus cancer cells, human normal lung fibroblasts WI-38 were treated with the extract. The extract did not show significant growth inhibition of normal lung fibroblast WI-38 upto50mg/ml concentration. Hence, the IC50 value for normal fibroblast cell line was significantly higher than those obtained for human lung cancer cell lines. In this study, root callus extracts of all the plant extracts tested had obvious cytotoxicity on A549 and NCI- H522 cells when compare to the root extracts.

Microscopic observation of A549 and NCI- H522 carcinoma apoptosis

In this study A549 and NCI- H522 lung cancer cell lines were seeded (0.5 × 106) in T-25 flasks with various concentrations 3.05- 50mg/ml of chloroform and methanol extract of of root and root callus of the A*canthus ilicifolius, Callophylum inophyllum and Excoecaria agallocha* were photographed under a phase-contrast microscope after 24 h treatment. Significant decrease of the number of cells treated with all the five concentrations of the extract for 24 h was observed compared with the control group. The control cells showed a typical monolayer appearance there was no changes appeared. The treated cells initiated to have morphological changes Fig-2, which showed theround-shaped cells less adhered at the bottom of the culture flasks.Also the nucleus and the cell membrane appeared asshrunken and the chromatin appeared brighter due to condensation. Nuclear blebbing was also apparent in the treated cells of all the concentrations.

Table 1. Effect of different concentrations of auxins and cytokinins on root callus biomass production in *Acanthus ilicifolius, Callophylum inophyllum* and *Excoecaria agallocha*

Growth hormones(mg/l)	% of callus response in *Acanthus ilicifolius*	% of callus response in *Callophylum inophyllum*	% of callus response in *Excoecaria agallocha*
NAA			
0.1	11±0.1	22±0.2	12±0.0
0.3	20±0.3	28±0.1	15±0.3
0.5	27±0.2	21±0.3	16±0.1
IAA			
0.1	10±0.1	14±0.1	21±0.2
0.3	14±0.5	11±0.2	22±0.0
0.5	17±0.2	30±0.5	22±0.5
2-4-D			
0.1	25±0.2	29±0.4	31±0.2
0.3	48±0.1	31±0.0	33±0.4
0.5	69±0.7	47±0.6	35±0.3
NAA+KIN			
0.5+0.1	11±0.2	12±0.3	35±0.1
0.5+0.3	29±0.6	26±0.2	32±0.3
0.5+0.5	51±0.2	28±0.4	25±0.3
NAA+BAP			
0.5+0.1	21±0.8	19±0.1	29±0.5
0.5+0.3	37±0.7	30±0.2	23±0.0
0.5+0.5	52±0.5	31±0.5	25±0.7
IAA+KIN			
0.5+0.1	07±0.3	15±0.1	10±0.1
0.5+0.3	12±0.2	19±0.1	10±.0.6
0.5+0.5	21±0.2	17±0.4	11±0.1
IAA+BAP			
0.5+0.1	25±0.7	16±0.4	22±0.4
0.5+0.3	36±0.6	51±0.1	24±0.3
0.5+0.5	39±0.5	42±0.0	26±0.5
2,4-D+KIN			
0.3+0.1	20±0.2	17±0.6	34±0.4
0.3+0.3	49±0.0	43±0.9	33±0.2
0.3+0.5	70±0.8	51±0.1	36±0.3
2,4-D+BAP			
0.3+0.1	39±0.3	31±0.3	44±0.3
0.3+0.3	57±0.1	59±0.2	47±0.0
0.3+0.5	89±0.6	81±0.0	58±0.6

Note: Data are expressed as fresh weight of callus, 50 explants were taken for each experiment. Each experiment was repeated five times.

Table 2 .IC_{50}, CC_{50} and therapeutic index values of the *Acanthus ilicifolius, Callophylum inophyllum and Excoecaria agallocha* and its respective calli (CC_{50} is the concentration at which 50% normal cells survived and IC_{50} is the concentration at which 50% cancer cell death occurred)

Test samples	Normal cell line-FIB (CC_{50}) mg.l^{-1}	Lung cancer cell line-NCI-H522 (IC_{50}) mg.l^{-1}	Lung cancer cell line-A549 (IC_{50}) mg.l^{-1}	Therapeutic index (CC_{50}/IC_{50})	
				NCI-H522	A549
A1	04.65	15.6	37.5	0.29	0.12
A2	06.21	12.1	15.6	0.52	0.39
C1	03.85	12.5	3.05	0.31	1.26
C2	43.75	3.05	9.37	14.34	4.67
E1	03.58	25.0	43.75	0.14	0.081
E2	37.50	6.25	50.00	6.00	0.75

Note: A1- Root extract of *Acanthus ilicifolius*, A2- Root callus extract of *Acanthus ilicifolius*, C1- Root extract of *Callophylum inophyllum*, C2- Root callus extract of *Callophylum inophyllum*,E1- Root extract of *Excoecaria agallocha*, E2- Root callus extract of *Excoecaria agallocha.*

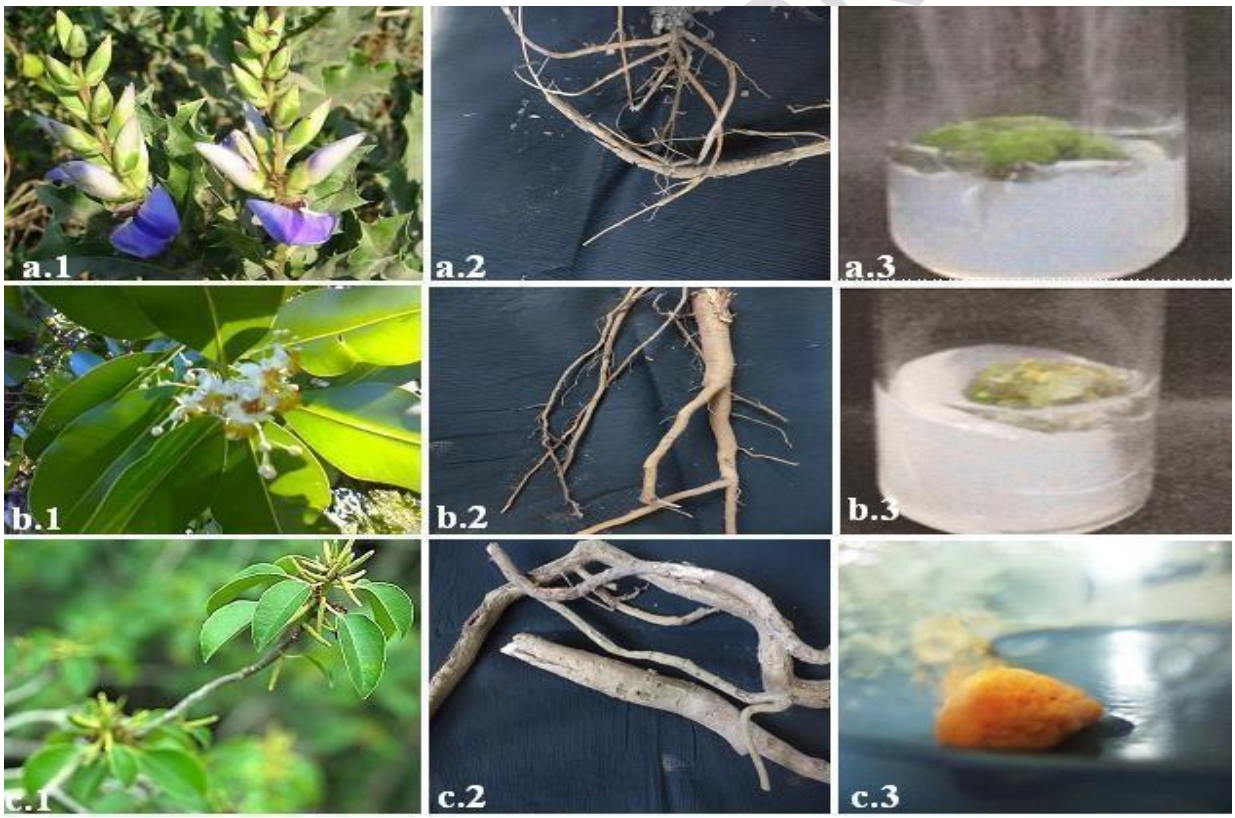

Fig.1. **Callus biomass production in root explants of *Acanthus ilicifolius, Callophylum inophylum* and *Excoecaria agallocha.* a.1. Natural habitat of *Acanthus ilicifolius*, a.2. Root of *Acanthus ilicifolius*, a.3. Callus biomass derived from the root explant of *Acanthus ilicifolius*. b.1. Natural habitat of *Callophylum inophylum*, b,2, Root of *Callophylum inophylum*, b.3. Callus biomass derived from the root explant of *Callophylum inophylum*. C.1. Natural habit of *Excoecaria agallocha*, c.2. Root of *Excoecaria agallocha*, c.3. Callus biomass derived from the root explants of *Excoecaria agallocha*.**

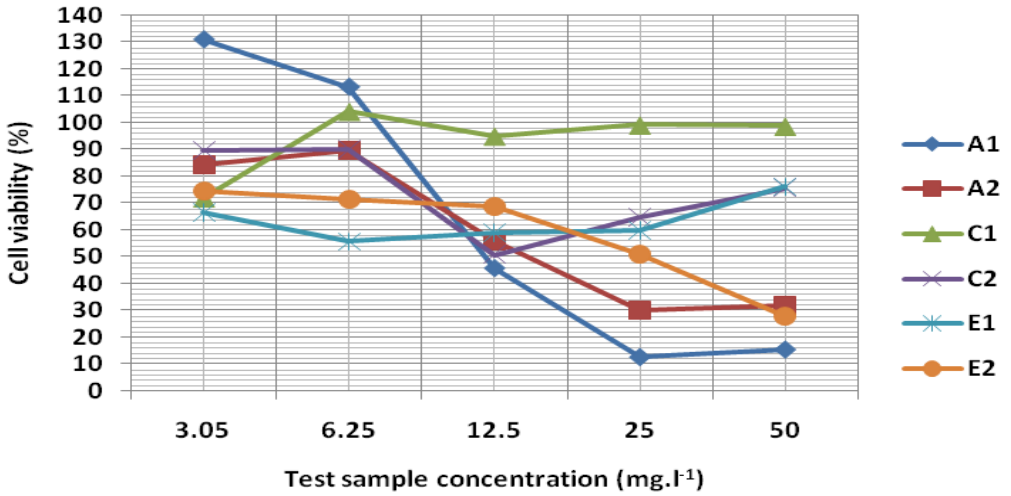

Fig-2. IC50 value of root and root callus extract of *Acanthus ilicifolius, Callophylum inophyllum* and *Excoecaria agallocha* on lung cancer Lung cancer cell line- NCI-H522

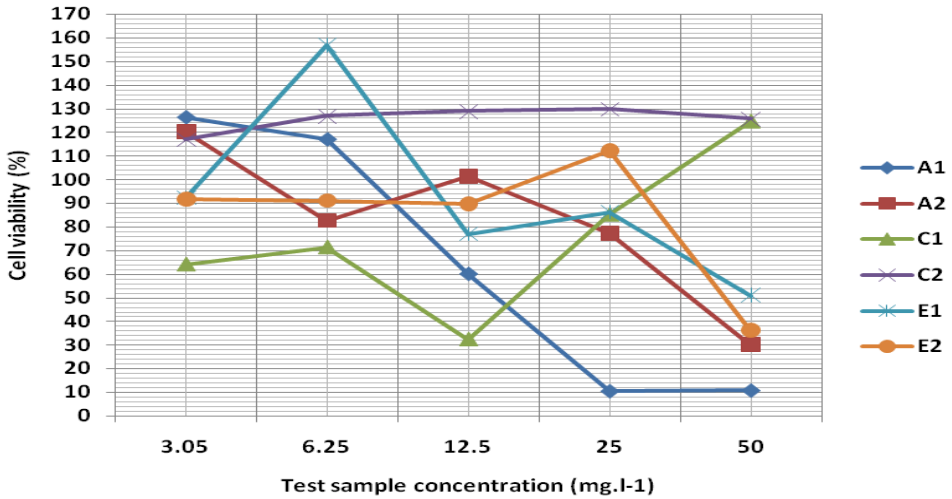

Fig-2a. IC50 value of root and root callus extract of *Acanthus ilicifolius, Callophylum inophyllum* and *Excoecaria agallocha* on lung cancer cell line-A549

Note: A1- Root extract of *Acanthus ilicifolius*, A2- Root callus extract of *Acanthus ilicifolius*, C1- Root extract of *Callophylum inophyllum*, C2- Root callus extract of *Callophylum inophyllum*,E1- Root extract of *Excoecaria agallocha*, E2- Root callus extract of *Excoecaria agallocha.*

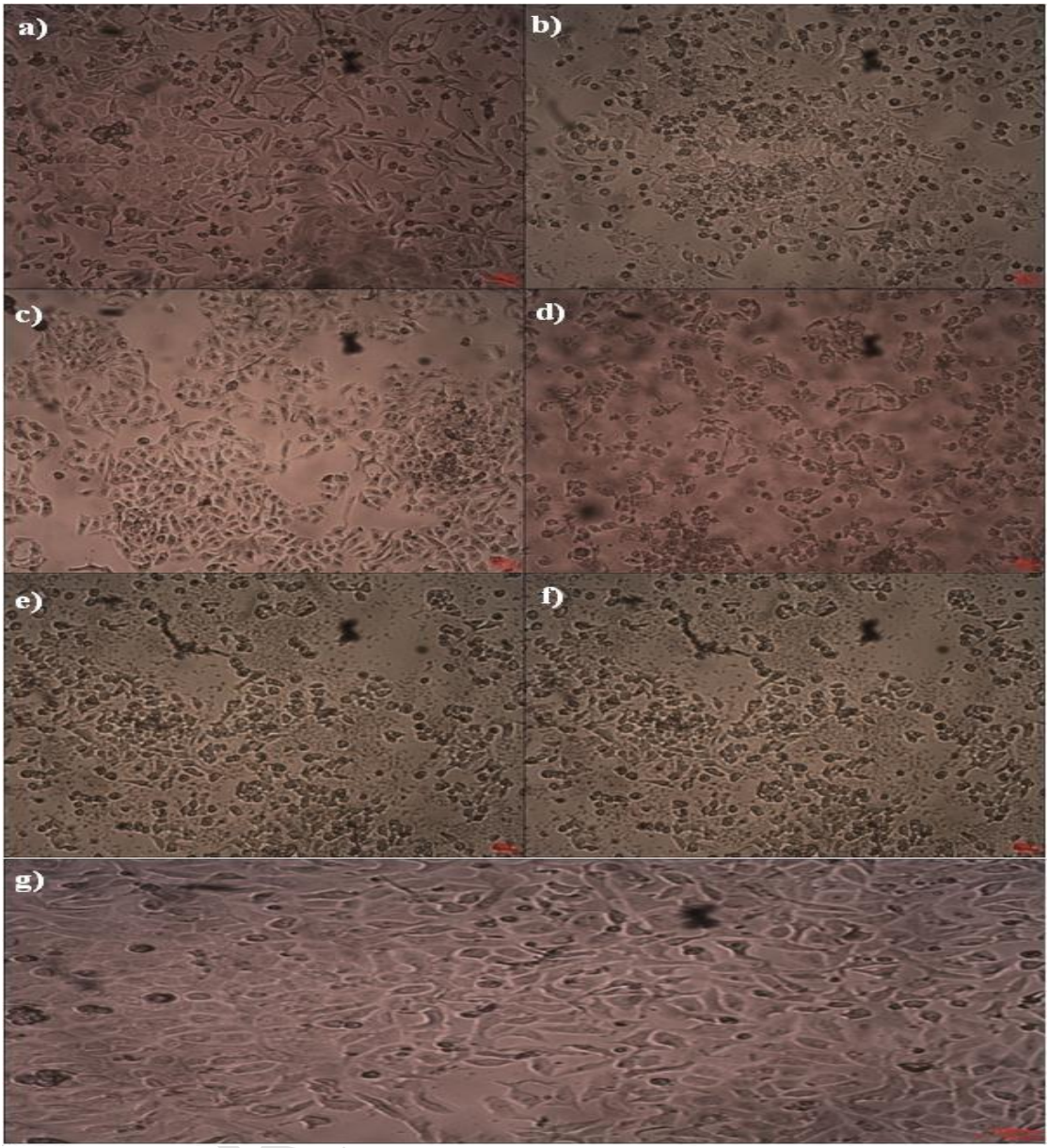

Fig. 3. Phase contrast micrographic observation of A549 cellular morphological changes induced by *Acanthus ilicifolius, Callophylum inophyllum and Excoecaria agallocha*.

Note: a. Root extract of *Acanthus ilicifolius*, b. Root callus extract of *Acanthus ilicifolius*, c. Root extract of *Callophylum inophyllum*, d. Root callus extract of *Callophylum inophyllum*, e. Root extract of *Excoecaria agallocha*, f. Root callus extract of *Excoecaria agallocha*, g. Normal cell fibroblast of A549.

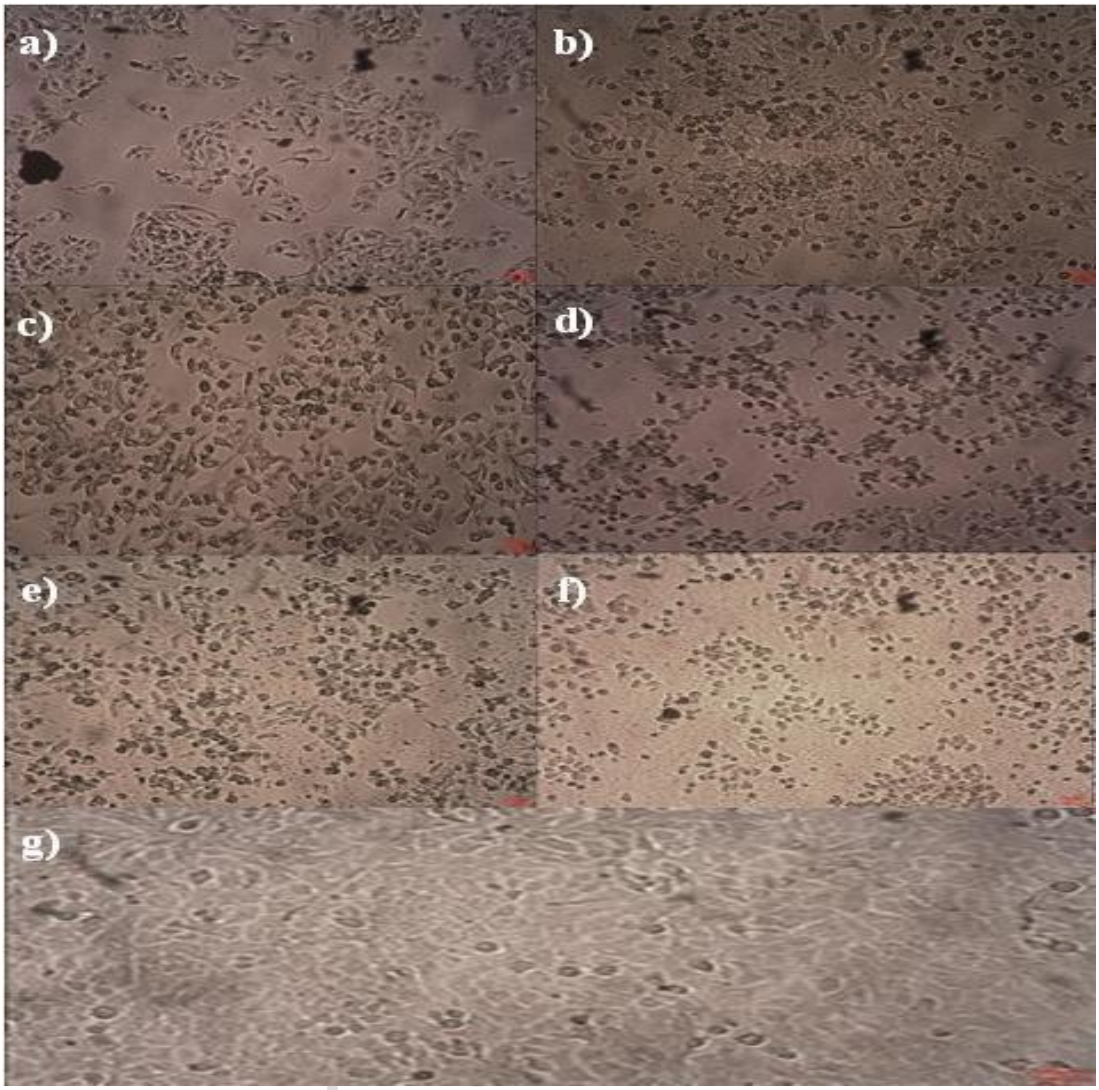

Fig.3. Phase contrast micrographic observation of NCI-H522 cellular morphological changes induced by *Acanthus ilicifolius, Callophylum inophyllum and Excoecaria agallocha*.

Note: a. Root extract of *Acanthus ilicifolius*, b. Root callus extract of *Acanthus ilicifolius*, c. Root extract of *Callophylum inophyllum*, d. Root callus extract of *Callophylum inophyllum*, e. Root extract of *Excoecaria agallocha*, f. Root callus extract of *Excoecaria agalloch*, g. Normal fibroblast of NCI-H522.

CONCLUSION

The study has revealed the potential bioactivities of *Acanthus ilicifolius, Callophylum inophyllum, Excoecaria agallocha* which showed significant cytotoxic effect, which could be successfully elevated by *in vitro* callus culture method. The mangroves are promising source of lead compounds to develop drugs in future to combat dreadful human diseases. This deserves further research on *in vitro* callus culture method on mangroves.

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