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Potential Of Syringodium Isoetifolium On Hela Cell Lines

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Abstract

Seagrass is a plant that lives in tidal areas, possesses unique secondary metabolites, which have roles as anticancer bioactive compounds. The aim of the present investigation was to evaluate the effect of in vitro anticancer and cytotoxic activity of the methanol extracts of Syringodium isoetifolium leaves, against the human HeLa cervical cancer cell line and to compare to the normal African green monkey kidney epithelial cell line (Vero) using the MTT colorimetric assay. Results showed that the methanol extract exhibits their potential as an anticancer agent. HeLa cell lethality by semi polar extract, polar extract and doxorubicin are 48.11%, 15.32 % and 48.75%, respectively. Ethyl acetic crude extract of fresh seagrass contain phytochemical compounds of alkaloid, terpenoid, polyphenol and flavonoid. The results suggest that the methanol extracts of S.isoetifolium exhibit greater activity on the HeLa cell line, meaning that these plant can be evaluated for potential promising anticancer activity.

Keywords: Anticancer activity; crude extracts; HeLa cell line.

1.0 introduction

Natural products, especially medicinal plants, have played a significant role in drug discovery and development of therapeutic agents. Plants contain many biologically active compounds that have potential for development of therapeutic agents. More

than 35,000 plant species have been used in various regions around the world for medical purposes (Al-Faifi et al.,2017). Natural ingredients contain several active compounds which give pharmacological effects. In general, those active compounds are secondary metabolites (Stepp and Moerman, 2001). Secondary metabolites have been known as sources of medical therapy, for example as antibacterial and anticancer medicines, etc (Harvey, 2000).

The idea of screening therapeutic plants is to search for bright lethal anticancer agents to human cancers. Despite the amazing developments in modern treatment, cancer remains a global health problem, thus attempting the exploration for new substitute approach. The nature as a vast treasured supplier of possible origin for chemotherapeutic agents has been reviewed (Newman and Cragg, 2007).

The current global burden of cancer is quite alarming, and this worrying prospect is even bound to increase significantly in the near future. Cancer is a dreadful disease and any practical solution in combating this disease is of paramount importance to public health. Plants have been used as folk remedies and ethno botanical literature has described usage the sage of plant extracts. There is an increasing need for search of new compounds with cytotoxic activity as the treatment of cancer with the available anticancer drugs is often unsatisfactory due to the problem cytotoxicity to the normal cells. For the last few decades, phytochemical examination has been making rapid progress and herbal products are becoming popular as sources of possible anticancer compounds (Patel et al., 2010). Antitumor activity against sarcoma and ascites, leukemia in mice and cytotoxic activity against certain cancer cell lines were also observed (Prakash and Gupta, 2013).

Seagrass is a hydrophyte that lives in tropical or subtropical tidal coastal areas. Around the world. Several researches showed that seagrass is potential as antioxidant, anticancer and antibacterial agents. Compounds in seagrass that are suspected to be able to inhibit cancer cell proliferation and inactivate pathogenic bacteria are flavonoid, saponin, steroid, terpenes, tannin and alkaloid. Azmi et al., (2013) stated that seagrass Enhalus acoroides contains stigmasterol, sitosterol and alkaloid. Seagrass Thalassia testudinium contains glycosides and phenol compounds which are potential to act as antifungi and anticancer, while seagrass Halodule uninervis contains steroid compounds

which acts as antibacterial and anticancer. The was conducted to evaluate the in vitro anticancer and cytotoxic activity of the methanol extract of Syringodium isoetifolium leaves, against human HeLa cervical cancer cell line.

2.0 Material and methods

2.1 Collection of Seagrass

In the present study, fresh leaves of seagrass Syringodium isoetifolium, (Ascherson) Dandy was collected at a depth of 1m from Tuticorin coast, Tamilnadu, India. The taxonomic position of the candidate seagrass S. isoetifolium were as follows: Syringodium isoetifolium leaves are tubular, narrowed at base and pointed at the apex. They are herbaceous plants; rhizomes creeping, shoots erect, branched, bearing 2-3 leaves; rhizomes and shoots have scars; rhizomes produce branched roots at each node. It generally grows well on coral flats, but also grows on sandy to muddy bottoms up to 15 m depth. It is not seen in backwaters and estuaries. They are mainly reported to be distributed in India at Lakshadweep Islands, Gulf of Mannar, Palk Bay, Andaman and Nicobar Islands.

2.3 Preparation of extracts

Collected seagrass S.isoetifolium was brought to laboratory in a polythene bag. Then the seagrass sample was washed thoroughly with running tap water and surface sterilized with 3% ethanol to remove the dirt and epiphytes. Then seagrass was dried at room temperature and then pulverized into fine powder with electrical grinder. In the present study, 100 g of seagrass powder sample was taken and subjected to percolation individually in 300 ml of solvents (methanol, chloroform and acetone) of varying polarity at room temperature. The process was repeated twice; extracts were pooled together and filtered through Whatman No:1 filter paper. Each filtrate was concentrated to dryness under reduced pressure using a rotary vacuum evaporator. Dried extracts were weighted and stored in screw cap bottles under refrigerated condition for further study.

2.4 Anticancer activity

Anticancer activity of crude methanol extract of S. isoetifolium as well as standard was determined through MTT cytotoxicity assay.

2.4.1 Cell lines used for the present study

2.4.1.1 HeLacell Line

HeLa (Human epithelial carcinoma) is a cell type in an immortal cell line used in scientific research. It is the oldest and most commonly used human cell line. The cell line was derived from cervical cancer cells taken on February 8, 1951 from Henrietta Lacks, a patient who eventually died of her cancer on October 4, 1951. The cell line was found to be remarkably durable and prolific. This was the first human cell line to prove successful in vitro which was a scientific achievement with profound future benefit to medical research.

2.5 MTT Cytotoxicity assay

2.5.1 Media preparation for animal tissue culture

The reagents required for media preparation are MEM (Minimum Essential Media), Sodium Bicarbonate, EDTA, FCS (Foetal Calf Serum), L–Glutamine, Antibiotics, and TPVG. The reagents required for media preparation was illustrated in Table 1.

The MEM was dissolved in the pre-sterilized millipore double distilled water, mixed well, closed and sterilized at 121°C for 15 minutes. It was then cooled to room temperature. The ingredients were added in quantities as indicated in Table 1. Depending on the concentration of Foetal Calf Serum (2%, 5%, 10%). The ingredients were mixed well by shaking. The bottle was shaken and the pH was adjusted to the range of 7.2 to 7.4. It was then stored at 4°C. One aliquot of the prepared MEM was kept for 2 days at 37°C and checked for sterility, pH drop and floating particles. The mixture was then transferred to the refrigerator.

2.5.2 Penicillin and Streptomycin

Both antibiotics were dissolved in sterile millipore distilled water to give a final concentration 100 IU/ml of penicillin and 100 mg/l of streptomycin. It is then mixed well, distributed in 1 ml aliquots and stored at -20°C.

2.5.3 Kanamycin acid sulphate

Kanamycin acid sulphate was dissolved in millipore distilled water to give a final concentration of 20 µg/ml. It is then mixed well, distributed in 1 ml aliquots and stored at -20°C.

2.5.4 Fungizone

Fungizone (Amphotericin B) was dissolved in sterile millipore distilled water so as to give a final concentration of 20µg/ml and distributed in 1ml aliquots in vials. It was stored at -20°C.

2.5.5 L-Glutamine: (3%)

3 g of L-Glutamine was weighed accurately and dissolved in sterile distilled water and mixed well. It was filtered through millipore membrane filter 0.22 µm and distributed in 5 ml aliquots in vials and stored at -20°C.

2.5.6 Sodium-bicarbonate solution (7.5%)

Required quantity of sodium bicarbonate (to give 7.5% solution) was weighed accurately and dissolved in 200 ml of sterile millipore distilled water. It was filtered through Whatmann filter paper No.4, distributed into 6 X 33 ml bottles and autoclaved at 121ºC for 10min.It was then cooled and stored at -4°C.

2.5.7. Foetal Calf Serum

The FCS was brought to room temperature, inactivated at 56°C in water bath for 30 min and cooled to room temperature. If floating particles are seen, filter through Seitz filter. It was distributed in 100, 50l and 20 ml quantities in sterile bottles and stored at –20°C.

2.5.8 Trypsin, PBS, Versene, Glucose solution (TPVG)

Trypsin (2%)

2 g of Trypsin was weighed accurately, dissolved in 100 ml sterile millipore distilled water with magnetic stirrer for 30 min and then filtered through 0.22µm membrane filter and stored at -20°C.

EDTA (0.2%)

200 mg of EDTA was weighed accurately, dissolved in 100 ml of sterile millipore distilled water and autoclaved at 12ºC for 15 min.

Glucose (10%)

1 g of glucose was weighed accurately, dissolved in 10 ml of sterile millipore distilled water, filtered through Whatman filter paper and autoclaved at 121ºC for 15 min.

TPVG

All the Ingredients were mixed and adjusted to pH 7.4 by 0.1N HCl or 0.1N NaOH, distributed in 100 ml aliquots and stored at -20°C.

Procedure

Maintenance of Cell Lines

1) The cell lines used were (VERO and MCF-7) obtained from King Institute of Preventive Medicine, Guindy, Chennai, TamilNadu.

2) The tissue culture bottles that showed confluent monolayer were selected by observing them under an inverted microscope.

3) Growth medium was removed from the bottle, washed with PBS/MEM without FCS and 5 mL of TPVG (for 25 cm^2) was added dispersing evenly on the monolayer and left in contact with the cells for 2-3 minutes until there is a cloudy appearance on the monolayer.

4) TPVG was removed and the cells were resuspended in 5 ml of growth medium (MEM containing 10% FCS).

5) The suspension was aspirated few times to break the cell clumps.

6) The cell suspension was then transferred to a 24 well plate. 1ml (1 lakh cells/ml) of the cell suspension was added to each well.

7) The plate was then incubated in a CO_2 incubator maintained with 5% CO_2.

Table 1. Ingredients required for Media Preparation

Ingredients	10% Growth Media	5% Maintenance Media	2% Wash Media
MEM	862 ml	912 ml	942 ml
Penicillin (a) and Streptomycin (b)	1 ml	1ml	1 ml
Kanamycin	1 ml	1 ml	1 ml
Fungizone	1 ml	1 ml	1 ml

L-Glutamine	10 ml	10 ml	10 ml
Foetal Calf Serum (FCS)	100 ml	50 ml	20 ml
$NaHCO_3$	20 ml	20 ml	20 ml
Hepes buffer	5 ml	5 ml	5 ml
Total volume	1000 ml	1000 ml	1000 ml

2.6 MTT assay (Mossman, 1983)

Cells (1×10^5/well) were plated in 24-well plates and incubated in 37ºC with 5% CO_2 condition. After the cell reaches the confluence, the various concentrations of the crude extracts were added and incubated for 24 h. After incubation, the sample was removed from the well and washed with phosphate-buffered saline (pH 7.4) or MEM without serum. Then 100µl of 0.5% 3- (4, 5-dimethyl-2-thiazolyl) -2, 5-diphenyl - tetrazolium bromide (MTT) was added individually to each well and incubated for 4 hrs. After incubation, 1 ml of DMSO was added in all the wells. The absorbance at 570 nm was measured with UV-Spectrophotometer using DMSO as the blank. Measurements were performed and the concentration required for a 50% inhibition (IC_{50}) was determined graphically. The % cell viability was calculated using the following formula:

Cell viability (%) = A570 of treated cells / A570 of control cells X 100

Graphs are plotted using the % of cell viability at Y-axis and concentration of the sample in X-axis. Cell control and sample control is included in each assay to compare the full cell viability in cytotoxicity assay.

2.7 Statistical analysis

The data obtained in the present study were subjected to the following statistical analysis using SPSS 16.0.

3.0 Results

3.1 Anticancer activity of methanol extract of S. isoetifolium and 5-Fluorouracil against HeLa cells

In the present study, an attempt was undertaken to evaluate the anticancer activity of crude methanol extract of S. isoetifolium and 5-Flurouracil against HeLa cell line through MTT cytotoxicity assay. Results showed dose dependant growth inhibition of cell line. As it can be seen from the Fig.1 that, increase in concentrations of crude extract (7.8 to 1000 µg ml^{-1}) had significantly inhibited the proliferation of HeLa cells in vitro. For instance, at 7.8 µg ml^{-1} concentration of extract, 18.56% growth inhibition of HeLa cells was noticed. However, further increase in concentration of extract showed a marked reduction in HeLa cells and recorded IC_{50} value of 112.77 µg ml^{-1} (Tables 2 & 3 and Fig.1). On the other hand, anticancer activity of 5-Flourouracil (positive control) on HeLa cell line revealed that, it had effectively inhibited HeLa cells with higher cytotoxic activity than the extract at minimal concentrations. Indeed, at 7.8 µg ml^{-1}concentration, it showed 42.34% growth inhibition of HeLa cells and recorded IC_{50} value of 18.49 µg ml^{-1} (Fig.2). The negative control DMSO had no inhibitory effect on the HeLa cells.

4.0 Discussion

In spite of the outshine by current synthetic chemistry as a technique of drug detection and invention, the influence of new products from probable bioactive plants or their extracts for disease handling and inhibition is still enormous (Kviecinski et al.,2008). Besides, plant-derived medications had led to utmost spread within the vicinity of antitumor upon where drugs were described to improvise the chemotherapy of some cancers (Yousefzadi et al.,2010). Plants comprise nearly limitless capacity to create phytoconstituents that attract scientists in the search for innovative and original chemotherapeutics (Reed and Pellecchia, 2005). The exploration for new anticancer compounds in plant remedies and traditional foods is a truthful and auspicious strategy for its inhibition (Hu et al., 2009).

The spreading of cancer is increasing worldwide, and the percentage of deaths caused by this fatal disease is rising, especially in the developing countries. Scientists and researchers are now giving more of their attention to the marine organisms medicine to provide treatment for more difficult diseases like cancer due to the fact that, the treatments of cancer patients with chemical therapy have serious side effects. Recently, marine medicines are coming to play a more vital role in the reduction and prevention of cancer.

Natural derivatives play an important role to prevent the cancer incidences as synthetic drug formulations cause various harmful side effects to human beings. Of the anticancer compounds extracted so far, the marine algal contribution is 65.63%. Owing to a diverse chemical ecology, the marine organisms especially marine flora have a great promise for providing potent, cheaper, and safer anticancer drugs, which deserve an extensive investigation (Chahar et al., 2011).

In the current study, the most promising methanol extract of seagrass S. isoetifolium and 5-Fluoruracil (positive control) was used to check whether it had the capability of inducing cytotoxic effects on the HeLa cell line. In the MTT assay method both extract and 5-Fluorouracil were tested on cell lines by using concentration in the range of 7.8-1000 µg ml^{-1}. MTT assay was conducted to evaluate the growth inhibitory effects of extract from S. isoetifolium and 5-Fluoruracil on the cell viability of HeLa cell line, which is based on the reduction of MTT at different concentrations (7.8 to 1000 µg ml^{-1}). After 48 h of treatment, methanol extract of S. isoetifolium exhibited higher inhibitory effect against all tumor cells, with varying efficiencies and selectivities while others caused marginal cell inhibition. The evaluation of the anticancer activity of plant extracts is essential for safe treatment. It enables identification of the intrinsic toxicity of the plant and the effects of acute overdose (Padmaja et al., 2002; Rahman et al., 2016). The MTT assay is used in screening the crude extracts to assess the toxicity. It could also provide an indication of possible cytotoxic properties of the tested plant extracts. MTT assay is based on the reduction of MTT by mitochondrial dehydrogenase by purple formazan product. It is frequently used as an in vitro model system to measure cytotoxic effects of variety of toxic substances and plant extracts against cancer cell lines (Morshed et al.,2011). The concentration of the extract causing 50% cell death (IC50) values of test extract are given in Table 1. The IC50 values of methanol extract of S. isoetifolium was found to be 112.77 µg ml^{-1}against HeLa, respectively.

The anticancer activity of methanol extract of S. isoetifolium also have been demonstrated with various cell lines including HeLa (Cervical cancer). Inducing the production of reactive oxygen compounds in cancer cells, arresting the cell cycle at G1 phase by apoptosis activation were the possible proposed mechanisms of anticancer action (Rajamaheswari et al., 2017).

The promising cytotoxicity rendered by crude ethyl acetate extract in the present study may be due to synergistic effect of active components within the extract. This result was corroborated with the findings of Hardoko et al.,(2016) investigated anticancer potential of non-polar (n-hexane), semipolar (ethyl acetate) and polar (ethanol) solvent extract against HeLa cells and inferred that ethanol solvent had good anticancer activity as compared to ethyl acetate and hexane extract. They further suggested that the promising anticancer activity exhibited by ethanol solvent might be due to presence of numerous phytochemicals having antitumor ability. Thus overall results of present investigation, suggested that methanol extract of seagrass S. isoetifolium possess promising bioactive molecules responsible for potent antimicrobial, antioxidant and anticancer activities. However, further studies on purification may definitely help to isolate pure compounds to overall health related issues.

5.0 Conclusion

Anticancer activity of crude extract methanol extract of S. isoetifolium against HeLa cells revealed that it had significantly inhibited the growth of cells in dose dependent manner and displayed better cytotoxicity with the IC_{50} value of 112.77 µg ml^{-1}. Nevertheless, 5-Fluorouracil which was used as positive control has showed potent cytotoxicity at least concentration with the IC_{50} value of 18.49 µg ml^{-1}. Further studies are warranted to elucidate the molecular mechanisms of isolated compounds of S. isoetifolium.

Table 2. Anticancer activity of methanolic extract S. isoetifolium on HeLa cell line

Concentration (µg ml^{-1})	**Cell viability (%)**	**Cell inhibition (%)**	**IC_{50} (µg ml^{-1})**
7.8	81.44	18.56	IC_{50} = 112.77
15.6	72.21	27.79	
31.2	62.98	37.02	
62.5	59.23	40.77	
125	50.40	49.60	
250	42.57	57.43	

500	31.44	68.56	
1000	22.20	77.80	
Vehicle control (DMSO)	100.00	0.00	

Table 3. Anticancer activity of 5-Fluorouracil on HeLa cell line

Concentration (µg ml^{-1})	**Cell viability (%)**	**Cell inhibition (%)**	**IC_{50} (µg ml^{-1})**
7.8	57.66	42.34	IC_{50} = 18.49
15.6	54.25	45.75	
31.2	45.7	54.3	
62.5	33.82	66.18	
125	28.87	71.13	
250	22.05	77.95	
500	16.98	83.02	
1000	11.86	88.14	
Vehicle control (DMSO)	100.00	0.00	

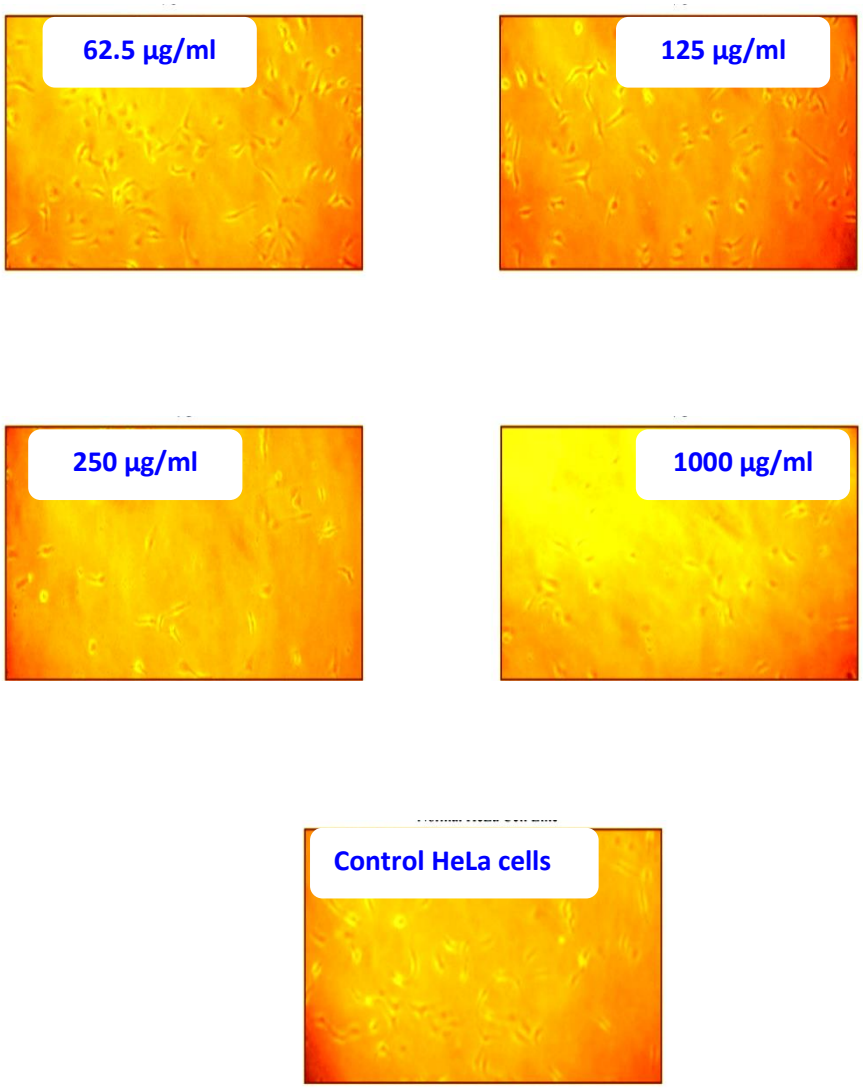

Fig.1. Anticancer activity of methanolic extract of S. isoetifolium on HeLa cells.

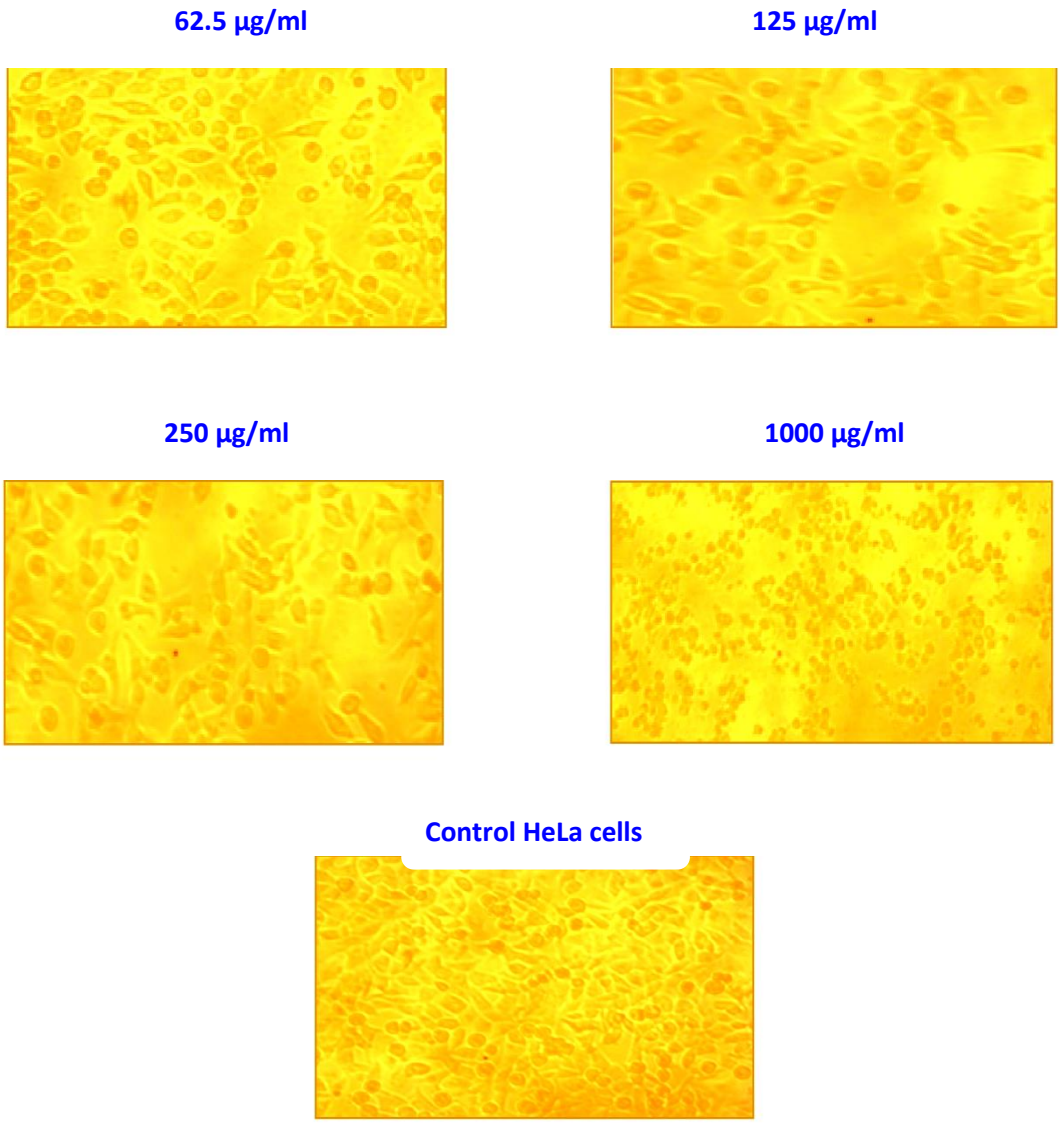

Fig.2. Anticancer activity of 5-Fluorouracil on HeLa cells.

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