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TO INVESTIGATE CELL VIABILITY, CYTOTOXICITY AND ANTI-CANCEROUS PROPERTIES OF PHYLLANTHUS EMBLICA EXTRACT ON HUH-7 AND L929 CELL LINES

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ABSTRACT

Phyllanthus emblica commonly known as Indian gooseberry or amla, has been widely recognized for its medicinal properties, including its potential anticancer effects. This study investigates its impact on Huh-7 liver cancer cells and L929 normal fibroblast cells by evaluating cell viability, cytotoxicity and oxidative stress. The MTT assay was used to assess cell viability, revealing a dose-dependent decrease in cancer cell survival while sparing normal cells. Reactive oxygen species (ROS) analysis indicated increased oxidative stress in Huh-7 cells, suggesting a role in apoptosis induction. These findings suggest that P. emblica selectively targets cancer cells while causing minimal harm to normal cells, making it a promising candidate for natural anticancer therapies. However, further studies are needed to explore its molecular mechanisms and in vivo efficacy.

Keywords: Extracellular Matrix, Huh 7 Cell Line, L929 Cell Line, Hepatocellular Carcinoma, Phyllanthus Emblica, Cytotoxicity

1. INTRODUCTION

Humanity has faced several health issues since its beginning. Several diseases pose significant threats to human life. Although medical science has witnessed significant progress in the twenty-first century, there are still several diseases that are incurable. Therefore, cures for these diseases must be investigated. Cancer is a fatal health issue that affects people all over the world. It can arise in almost any tissue/organ of the body when anomalous cells grow uncontrollably, migrate to other organs through metastasizing process and cause death.

Hepatocellular carcinoma (HCC), one of the most prevalent and deadliest cancers, has limited treatment options and poor patient prognosis (Vogel, A. et al., 2022). The five-year survival rate of localized HCC is 31% and falls to 2% for metastatic HCC (Singal, A. G., 2020). To prolong the length of life and improve its quality, the work done here aims to contribute to the cancer research field and design of novel therapy approach. Thousands of investigations of hepatocellular carcinoma have been performed employing the human hepatoma Huh-7 cell line. The extensive body of

knowledge produced the importance and value of this in vitro cell system to study the characteristics of hepatomas and the potential of natural and synthetic compounds to prevent and eliminate liver cancer (Krelle et al., 2013; Triyatni M., et al., 2011).

The fruits of Phyllanthus emblica Linn or Emblica officinalis (Phyllanthaceae), (FPE) commonly known as Indian gooseberry or Amla, gained immense importance in indigenous traditional medicinal systems, including Ayurveda, for their medicinal and nutritional benefits. It is used to cure several diseases such as common cold, fever, cough, asthma, bronchitis, diabetes, hyperacidity, peptic ulcer, erysipelas, skin diseases, etc. (Saini, R. et al., 2022).

The fruit extract of the Phyllanthus emblica tree, possesses strong anticancer capabilities, according to a plethora of data from both in vitro and in vivo investigations. Polyphenols, particularly tannins and flavonoids, are regarded to be the main mediators of the bioactivity in this extract. Phyllanthus emblica possesses anticancer and cancer-preventive qualities. Some of the anticancer activity of Phyllanthus emblica may be attributed to its antioxidant function, but other pathways are undoubtedly just as significant (Zhao, T. et al., 2015). Amla suppressed cell proliferation by targeting Wnt/β-catenin signaling as seen by decreased nuclear translocation of β-catenin. Additionally, this led to suppressed of expression of c-Myc and cyclin D1, key proteins involved in cell proliferation (Vadde, R., et al., 2016).

Cell lines that were used were Huh-7 and L929. Huh-7 is a well-established and differentiated hepatocyte derived cellular carcinoma cell line that was originally taken from a liver tumor. Basically, it is an immortal cell line composed of epithelial-like, tumorigenic cells. Another cell line is L929, these cells are fibroblast-like cell line derived from the subcutaneous connective tissue of a 100-day-old male mouse. L929 cells are characterized by their spindle-shaped, fibroblastic morphology, and adherent growth.

As the effects of different concentrations of amla on Huh-7 and L929 cell lines on cell viability, cell cytotoxicity, and oxidative stress have not been studied previously hence in this study, the effects of different concentrations of amla on Huh-7 and L929 cell line on the various parameters like cell viability, cell cytotoxicity, oxidative stress were determined.

2. MATERIAL AND METHOD

2.1. PREPARATION OF AMLA'S STOCK SOLUTION

The fruits of Amla (Phyllanthus emblica) were collected from the Botanical Garden of Panjab University Chandigarh, India. The pulp of fruits of Phyllanthus emblica was taken out and blended, followed by filtration through Whatman paper no. 1 to collect the filtered aqueous extract. Then this filtered aqueous extract was evaporated at reduced pressure using a Rotary vacuum evaporator at 50°C and lastly freeze-dried with the help of freeze drier. A stock solution (5mg/mL) of Phyllanthus emblica extract was prepared in Dulbecco's Modified Eagle Medium (DMEM) and sterilized by filtration through a 0.22µm pore size hydrophilic polyethersulfone membrane. The stock solution was then stored at -20°C and thawed immediately before use.

The stock solution was used to prepare the following doses: -

Table 1

Dosage 1	50 µg/ml
Dosage 2	100 µg/ml
Dosage 3	150 µg/ml
Dosage 4	200 µg/ml
Dosage 5	250 µg/ml

2.2. PASSAGING AND SUB-CULTURING OF CELLS

All the chemicals used in the study were of tissue culture and molecular grade. Dulbecco's Phosphate Buffer Saline (DPBS) (TS1006), penicillin-streptomycin (Pen-strep) (A001-5X), phosphate-buffered saline (PBS), fetal bovine serum (FBS) (RM10832), Trypsin-EDTA (TCL007), MTT Reagent (RM1131), and Dulbecco's Modified Eagle's Medium (DMEM) (AL007A), Trypan blue dye (TCL005), were purchased from HI Media laboratories, Mumbai. Both L-929 fibroblast cell line and HuH-7 hepatic cancer cell line were procured from the National Centre for Cell Science, Pune, India, and

maintained as per the supplier's protocol. Both cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 2 mM L-glutamine i.e. full growth media (FGM). Cells were incubated at 37°C in a humidified atmosphere with 5% CO2. After approx. 80% confluence the cells were subjected to passage. For this purpose, the media was drained from the T25 tissue culture flask, and the cells were washed twice with Dulbecco's Phosphate-Buffered Saline (DPBS) (2 mL each). After washing, 1 ml of 0.25% Trypsin-EDTA was added, and the culture flask was incubated for 3 min at 37°C and 5% CO2. Then, 4 ml of FGM was added to inhibit trypsinization. The cell suspension was then transferred to a 15 ml centrifuge tube for centrifugation (4 min., 4000 R.P.M., 4oC). After centrifugation, the supernatant was discarded, and 1 ml full growth media (FGM) was added, and cells were re-suspended by gentle tapping. Cells were then counted using a hemocytometer. Then appropriate number of cells were transferred to T25 tissue culture flasks and FGM was added to make the final volume to 5ml. Cells were then incubated for 24-48 hr at 37°C and 5% CO2 until 80% confluency.

2.3. CELL CYTOTOXICITY ASSAY (MTT) (MOSMANN, T., 1983)

Measurement of cell viability and proliferation forms the basis for numerous in vitro assays of a cell population's response to external factors. The reduction of tetrazolium salts is a reliable way to examine cell proliferation.

Re-suspended 8000 cells/100µL (Huh-7 cells) and 8000 cells/100µL (L929 cells) in 100µL FGM each were transferred to each well of a 96-well culture plate and incubated for 24 hours at 37˚C and 5% CO2. After 24hrs Amla extract was added at different concentrations (50 µg/ml, 100 µg/ml, 150 µg/ml, 200 µg/ml, 250 µg/ml) in 100µL FGM to each cell line in respective well and then again incubated for 72hrs at 37˚C and 5% CO2. After 72 hours, 10µL MTT Reagent (5mg/mL) was added to each well and incubated for 3 hours at room temperature. After incubation purple precipitate was visible after the addition of 100µl of dimethyl sulphoxide (DMSO). The absorbance was observed at 570 nm using an ELISA plate reader.

2.4. ROS (REACTIVE OXYGEN SPECIES) ASSAY (KIM & XUE, 2020) FOR MEASURING OXIDATIVE STRESS

- Preparation of the DCFH-DA solution
- Dissolve 4.85 mg of DCFH-DA in 5 mL of dimethyl sulfoxide (DMSO) to make a 10mM stock solution.
- Dilute the stock solution with pre-warmed DMEM to 10µM working solution right before addition to the wells.
- Vortex the working solution for 10s.
- The intracellular ROS levels were measured using the dichlorofluorescein diacetate (DCFH-DA) assay. HuH-7 cells were seeded in black-bottom 96-well plates at a density of 3×10^4 cells per well in 100µL FGM for 24hrs and then cells were washed with DPBS and then treated with in 100µL FGM containing Amla extract at different concentrations (50 µg/ml, 100 µg/ml, 150 µg/ml, 200 µg/ml, 250 µg/ml) for next 24 hours. After treatment, cells were washed twice with DPBS and incubated with 10 µM DCFH-DA for 30 minutes at 37°C in the dark. Fluorescence intensity was measured using a fluorometer with excitation and emission wavelengths of 485 nm and 530 nm, respectively.

3. RESULTS AND DISCUSSION

The purpose of this study was to investigate the primary effect of Phyllanthus emblica on Huh-7 and L929 cells by observing its effects on its viability, cytotoxicity, oxidative stress and checking anti-cancerous property on cancerous and non-cancerous cells.

To attain these conditions, Amla pulp extract was prepared and added on to both Huh-7 and L929 cells in varying concentrations i.e. 50µg/ml, 100µg/ml, 150µg/ml, 200µg/ml, and 250µg/ml, for 24 hr. MTT was performed on Huh-7 and L929 cells. Throughout this study, 3rd passage cells were employed for all the experimental studies. These cells have epithelial like shape and are adherent and were growing well. After this the reactive oxygen species assay was laid down on Huh-7.

3.1. REVIVAL AND SUBCULTURE

Huh-7 and L929 cells were revived and plated in T-25 flask. Fig 1 and Fig 2 show the photomicrographs of Huh-7 cells and L929 cells respectively.

Figure 1

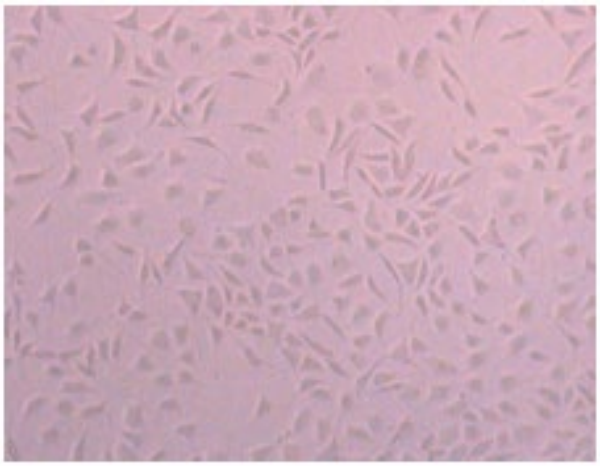

Figure 1 Photomicrograph at 40x: Huh-7 cells after 48 hrs. of revival.

Figure 2

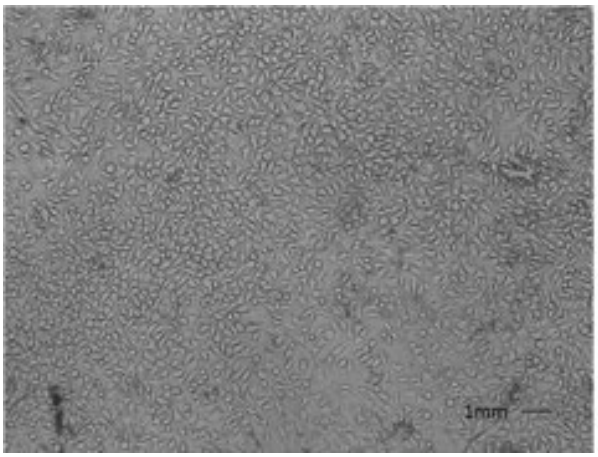

Figure 2 Photomicrograph at 40x: L929 cells after 48 hrs. of revival.

3.2. MTT ASSAY (3-(4, 5-DIMETHYLTHIAZOL-2-YL)-2,5-DIPHENYL TETRAZOLIUM

bromide).

The cytotoxicity of Phyllanthus emblica on Huh-7 and L929 cells was assessed by performing an MTT assay. In this assay, the amount of formazan crystals formed by the reduction of MTT dye by oxidoreductase enzymes in mitochondria, states how metabolically active the cell is, hence indirectly measuring viability of cells. The results obtained are shown in Figure 3. Viability was measured by the colorimetric change using a plate reader at 570 nm. It was observed that cell viability was affected on addition of amla extract and at different dosage, cells showed different cytotoxic behavior. For Huh-7 cells at 50 µg/ml of concentration, cell viability was highest i.e. 65.7% whereas at 250µg/ml of concentration, cell viability was recorded lowest i.e. 9.5%. It was observed that the cell viability of Huh-7 cells decreased with increase in concentration of amla extract.

Figure 3

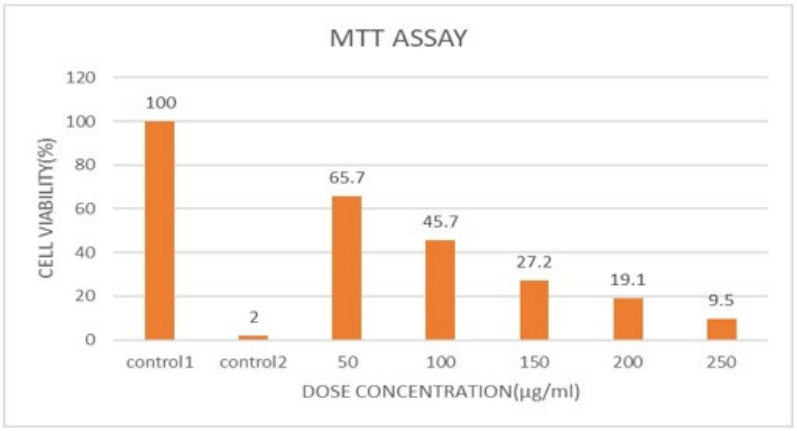

Figure 3 Graphical representation of percentage cell viability of Huh-7 cells on treatment with Phyllanthus emblica.

As shown in Figure 4, it is seen that for L929 cells, at 50 µg/ml dose treatment, the cell viability of was highest i.e. 77%. At 250 µg/ml, the cell viability was decreased to 16.7% which is still higher than Huh-7 MTT case because Huh-7 is a cancerous cell line whereas L929 is a non-cancerous one. The control 1 was basically cells with DMEM media and control 2 was cells without DMEM media. No extract was added to both the controls. Hence the cell viability of L929 cells decreased with increase in concentration of amla extract.

Figure 4

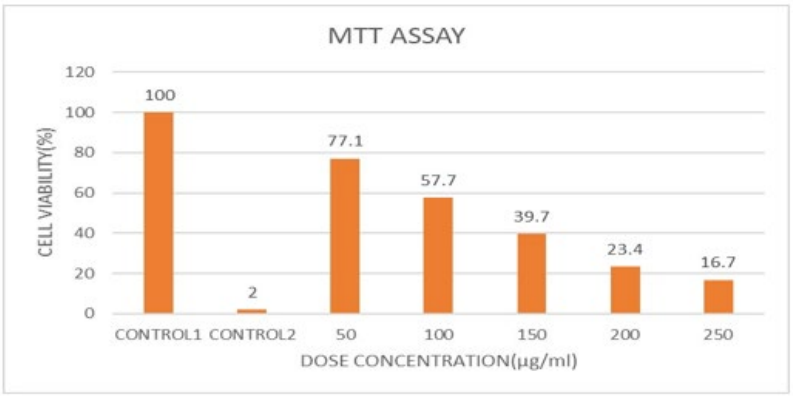

Figure 4 Graphical representation of percentage cell viability of L929 cells on treatment with Phyllanthus emblica.

3.3. REACTIVE OXYGEN SPECIES (ROS)

Superoxide anion, hydroxyl radical, and hydrogen peroxide are the three main physiologically significant reactive oxygen species (ROS) produced by cellular metabolism. They take part in normal cell functions when present in low quantities, but when present in excessive concentrations, they negatively impact cell signaling pathways (Birben et al., 2012). The results obtained are shown in Figure 5. Viability was measured by the colorimetric change using an Elisa plate reader at 485 nm. It was observed that cellular reactive oxygen species was high in Huh-7 cells treated with higher concentration of amla extract (250 µg/ml) showing 18709 reactive oxygen species value whereas Huh-7 cells treated with lower concentrations of amla extract showed lesser amount of ROS. i.e. 50µg/ml showed 4977 ROS value. Further Huh-7 cells treated with 100 µg/ml, 150 µg/ml and 200 µg/ml showed 7448, 13112 and 16994 ROS values respectively. Control showed even lesser amount of ROS i.e. 1123 as compared to treatment groups. Hence it was observed that the ROS levels increased in Huh-7 cells with increasing dose of the amla extract. The overproduction of ROS i.e. low level or

deactivation of antioxidant machinery at higher doses of amla extract caused increased oxidative stress in Huh-7 cancer cells and might have resulted in cytotoxicity at higher doses.

Figure 5

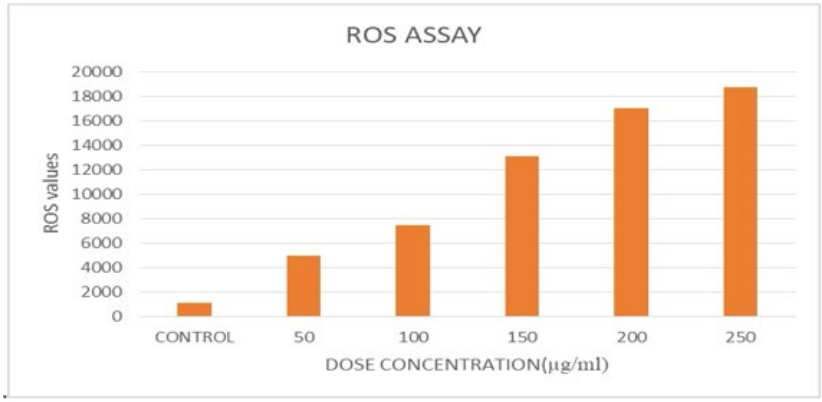

Figure 5 Graphical representation of R.O.S. on Huh-7 cells after 72 hrs. of treatment with increasing dose of amla extract.

4. CONCLUSION

It is clear from the result evidences that in the present study, amla extract prepared in different concentration showed cell viability, cytotoxicity and anti-cancerous nature when experimented with Huh-7 and L929 cells. MTT assay on Huh-7 and L929 showed dose dependent effect of amla extract on cell viability of both cell lines. It was found that cell viability of both cell lines decreased with increasing dose of amla extract. Hence the higher dose (250 µg/ml) showed more cytotoxic effect as compared to lower doses. Cancerous cell line i.e. Huh-7 cells showed less growth rise than non-cancerous cells i.e. L929 cell line which showed that Huh-7 cells were more sensitive to amla. Similarly, number of reactive oxygen species generated by Huh-7 cells after administration of amla extract at high amla dose (250µg/ml) produced more reactive oxygen species as compared to low amla doses like 50µg/ml, 100µg/ml, 150µg/ml and 200µg/ml. Hence the overproduction of ROS i.e. low level or deactivation of antioxidant machinery by amla caused increased oxidative stress in Huh-7 cancer cells.

Researchers around the world are looking at the use of plant-derived medicines as a healthcare supplement. Identification of biologically targetable compounds that take advantage of divergence between normal and cancerous cells could permit superior specificity for cancer treatment with minimal disadvantage to healthy cells is still the eventual objective in antitumor drug development. Hence, with more and better experimentations we can surely say that amla possesses anti-cancerous properties.

DECLARATION OF COMPETING INTEREST

None

CONFLICT OF INTERESTS

None.

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