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IN-VITRO STUDIES OF EXTRACTS OF *PLUMBAGO ZEYLANICA* IN CERVIX CARCINOMA CELLS

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Keywords:

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ABSTRACT: The present study evaluated the antimicrobial and cytotoxic activities of chloroform, hexane methanol, and petroleum ether extracts of *Plumbago zeylanica* (PEC, PEH, PEM, and PEP). Antimicrobial activities of the extracts were evaluated against gram-positive bacteria (*Staphylococcus aureus*-ATCC 6538 and *Bacillus cereus*-NCIM 2155) and gram-negative bacteria (*E. coli*-ATCC 8739) along with cytotoxicity activity against cervix carcinoma cell lines (HeLa and SiHa cell lines). Out of the four extracts tested, PEM and PEC extracts showed significantly high value of zone of inhibition against tested bacteria. PEM showed zone of inhibition of 10 mm, 13mm and 8 mm in ATCC 6538, NCIM 2155 and ATCC 8739, respectively. On the other hand, chloroform extract showed a zone of inhibition of 8mm, 10mm and 7 mm in ATCC 6538, NCIM 2155 and ATCC 8739, respectively. The four extracts PEC, PEH, PEM, and PEP showed a significant cytotoxicity activity with IC_{50} concentration at 238.93ug/ml, 694.44ug/ml, 580.40ug/ml and 593.04ug/ml against the HeLa Cell line and 142.71ug/ml, 547.16ug/ml, 448.04ug/ml and 424.27ug/ml respectively against the SiHa Cell line after the treatment of 24 h. Based on the results of the anti-microbial and anticancer analysis, it was identified that extracts of *P. zeylanica* may show anticancer activity and also suggest that the PEC has satisfactory cytotoxic potential properties against both cervix carcinoma cell lines based on the dosage of the drug after the incubation period of 24 h. Therefore our results strongly support the antimicrobial and cytotoxicity activities of *P. zeylanica* provide a scientific basis to develop novel drug formulations from this traditional medicinal plant.

INTRODUCTION: One of the richest sources of phytochemicals is the roots of *Plumbago zeylanica* [1]. *P. zeylanica*, a wide-ranging curative herb, is available all over Africa and Asia. It is a long-winded, lasting herb belonging to the Plumbaginaceae family (Plumbago L. genus).

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Enormous literature is available that explains the medicinal uses of *P. zeylanica* [2]. It is used as a remedy for skin infections and intestinal worm's *viz*. scabies, hookworms, leprosy, dermatitis, acne, sores, ringworm, and ulcers [3].

The ancient systems of medicine in different places have been using *P. zeylanica* for a variety of treatments. The powdered root and the bark are used to treat syphilis, gonorrhea, tuberculosis, swellings, rheumatic pain, and wound healing [4]. The decoction obtained from roots with boiled milk is swallowed to treat inflammation in the mouth, chest and throat.

A paste of the root in vinegar, water, and milk shows a significant effect against influenza and black water fever, although root extracts are taken orally to treat shortness of breath [5]. In Ayurveda, it was mentioned that the extracts of *P. zeylanica* plant have anticancer [6, 7], antitumor, anti-inflammatory, antioxidant, anti-mycobacterial, and antimicrobial activities [8-15].

The constituents in the roots of the plant are credited with potential properties towards therapeutic uses, including hepatoprotective, anti-atherogenic, neuroprotective, cardiotonic, and stimulating the central nervous system [16]. Different extracts from *P. zeylanica* have various vital activities like acetone extract of *P. zeylanica*, which shows effects on chromosomal aberrations produced by ethinyl estradiol in human lympho-cytes culture [17].

In view of the vast applications of *P. zeylanica* to treat various diseases as a medicine, the present investigation aimed to analyze anti-microbial activity against gram-positive (*Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (NCIM 2155)) and gram-negative (*E. coli* (ATCC 8739)), and anticancer activities in cervix carcinoma cell lines (HeLa and SiHa cell lines) of chloroform, hexane methanol, and petroleum ether extracts (PEC, PEH, PEM, and PEP) of *P. zeylanica*.

MATERIALS AND METHODS:

Chemicals and Cell Lines: Gram-positive bacteria (*Staphylococcus aureus* (ATCC 6538), bacillus cereus (NCIM 2155)), gram-negative bacteria (E.coli (ATCC 8739)), Cell lines - HeLa-Human Cervix Adenocarcinoma Cell line (NCCS, Pune), SiHa-Human Cervix Squamous Carcinoma Cell line (NCCS, Pune), Cell culture medium: DMEM-High Glucose - (#AL111, Himedia), Adjustable multichannel pipettes and a pipettor (Benchtop, USA) , Fetal Bovine Serum (#RM10432, Himedia), MTT Reagent (5 mg/ml) (# 4060 Himedia) , DMSO (#PHR1309, Sigma), Camptothecin (#C9911, Sigma), D-PBS (#TL1006, Himedia), 96-well plate for culturing the cells (From Corning,USA), T25 flask (# 12556009, Biolite - Thermo), 50 ml centrifuge tubes (# 546043 TORSON), 10 ml serological pipettes (TORSON), 10 to 1000 ul tips (TORSON). Chloroform, Methanol, Pet Ether, and Hexane solvents were purchased from Sigma-Aldrich Co., USA.

Equipment: Centrifuge (Remi: R-8 °C), Pipettes: 2-10µl, 10-100µl, and 100-1000µl, Inverted micro-scope (Biolink), 37°C incubators with a humidified atmosphere of 5% CO_2 (Healforce, China).

Anti-microbial Analysis: Paper discs impregnated with specific antibiotics or the test substances are placed on the surface of the Muller Hinton agar medium inoculated with the target organisms, which is recommended for the diffusion of antimicrobial agents as described in NCCLS approved standard. The plates are incubated, and the zones of inhibition around each disc are measured. Muller Hinton Agar plates were prepared, and the test microorganisms were inoculated by the spread plate method. Filter paper discs approximately 6mm in diameter were soaked with 15µl of the plant extract and placed in the previously prepared agar plates. Each disc was pressed down to ensure complete contact with the agar surface and distributed evenly so that they are no closer than 24 mm from each other, center to center. The agar plates were then incubated at 37ºC. After 16 to 18 h of incubation, each plate was examined. The resulting zones of inhibition were uniformly circular with a confluent lawn of growth. The diameters of the zones of complete inhibition were measured, including the diameter of the disc where the chloramphenicol was used as control.

Cytotoxicity Studies:

MTT Assay: Briefly, cells were seeded at 1 × 105 cells/mL in 96 well microtiter plates in Minimum Essential Medium with fetal bovine serum. The cells were incubated overnight for attachment. Drug concentrations in serial three-fold dilutions were added in triplicates and incubated for 48h at 5% CO_2 at 37°C (see list of drugs and corresponding cell line used in **Table 1**). Thereafter, the cells were treated with 3-[4,5-dimethylthiazol- 2- yl]- 2, 5- diphenyltratrazolium bromide (MTT) (Sigma Chemical Co., St. Louis, MO). Four hours later, all of the medium, including MTT solution (5 mg/mL) was aspirated from the wells. The remaining formazan crystals were dissolved in DMSO, and the absorbance was measured at 570 nm using a 96 well microplate reader (SynergyTM HT, Bio-Tek Instruments, Inc.). The cytotoxicity index was determined using the untreated cells as a negative control. The percent age of cytotoxicity was calculated using

the background-corrected absorbance follows (3, 4): IC_{50} determination The IC_{50} was extrapolated from the dose-response graph. The drug concentration that reduced the viability of cells by 50% (IC_{50}) was determined by plotting triplicate data points over a concentration range and calculating values using regression analysis of PRISM program. MTT assay is a colorimetric assay used for the determination of cell proliferation and cytotoxicity, based on the reduction of the yellow-colored water-soluble tetrazolium dye MTT to formazan crystals. Mitochondrial lactate dehydrogenase produced by live cells reduces MTT to insoluble formazan crystals, which upon dissolution into an appropriate solvent exhibits purple color, the intensity of which is proportional to the number of viable cells and can be measured spectrophotometrically at 570 nm [20-22].

TABLE 1: DETAILS OF PLANT EXTRACTS

S. no.	Sample Name/Code	Concentrations	Cell line
1	PEC (Chloroform Extract)	5(25,50,100,200,400ug/ml)	HeLa and SiHa
2	PEH (Hexane Extract)	5(25,50,100,200,400ug/ml)	HeLa and SiHa
3	PEM (Methanol Extract)	5(25,50,100,200,400ug/ml)	HeLa and SiHa
4	PEP (Pet. Ether Extract)	5(25,50,100,200,400ug/ml)	HeLa and SiHa

The IC_{50} value was determined by using a linear regression equation *i.e.*

$$Y = Mx + C.$$

Here, Y = 50. M and C values were derived from the viability graph [23-25].

Concentrations Used for the Study: In this study, four test compounds were used to check the cytotoxicity in two different carcinoma cell lines, namely, HeLa and SiHa **Table 1**. The used concentrations of the compound to treat the cells are tabulated in **Table 2**.

TABLE 2: DETAILS OF DRUG TREATMENT TO RESPECTIVE CELL LINES USED FOR THE STUDY

S. no.	Test Compounds	Cell Line	Concentration treated to cells
1	Untreated	HeLa and SiHa	No treatment
2	Standard (Camptothecin)	HeLa and SiHa	25uM
3	Blank	-	Only Media without cells
4	PEC	HeLa and SiHa	25,50,100,200,400uG/mL
5	PEH	HeLa and SiHa	25,50,100,200,400uG/mL
6	PEM	HeLa and SiHa	25,50,100,200,400uG/mL
7	PEP	HeLa and SiHa	25,50,100,200,400uG/mL

RESULTS AND DISCUSSION:

Anti-microbial Analysis: Antimicrobial activity of four different plant extracts (PEC, PEH, PEM, and PEP) against *Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (NCIM 2155), and *E. coli* (ATCC 8739) were analyzed by means of the zone of inhibition and compared with the standard antibiotic (streptomycin). The plant extracts showed the inhibition of the growth of both gram-positive and negative bacteria **Fig. 1**.

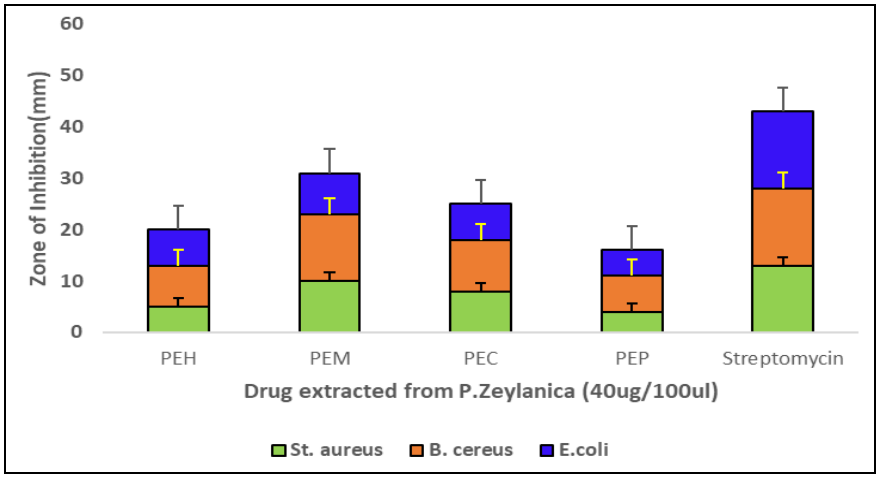

FIG. 1: ANTIMICROBIAL ANALYSIS OF FOUR DIFFERENT EXTRACTS OF *P. ZEYLANICA* (PEH, PEM, PEC AND PEP AT A CONCENTRATION OF (40ug/100ul) AGAINST THE GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA

Out of the four extracts tested, methanol and chloroform solvent extracts showed a good zone of inhibition against the gram-positive and gram-negative bacteria **Fig. 1** and also revealed that secondary metabolites resolved in methanol and chloroform extracts of *P. zeylanica* are showing significant antibiotic efficiency on par with standard antibiotic streptomycin.

Cytotoxicity Studies: The direct microscopic observations of drug-treated images of HeLa and SiHa Cell lines by Inverted biological microscope after incubation of 24 h were present in plate 1 and 2. Percentage of cell viability of HeLa and SiHa Cell lines at different concentrations of PEC, PEH, PEM, and PEP showed variation in their cytotoxicity efficacy **Fig 3**. Statistical analysis of cell cytotoxicity study by ELISA reader suggest that against HeLa Cells, the test Compounds *viz*., PEC, PEH, PEM, and PEP, showing significant cytotoxicity with the IC_{50} concentrations at 238.93 µg/ml, 694.44µg/ml, 580.40µg/ml and 593.04 µg/ml respectively. And 142.71µg/ml, 547.16 µg/ml, 448.04µg/ml and 424.27µg/ml respectively in SiHa cell line after the treatment of 24 h of incubation at 37 °C temperature **Fig. 2**.

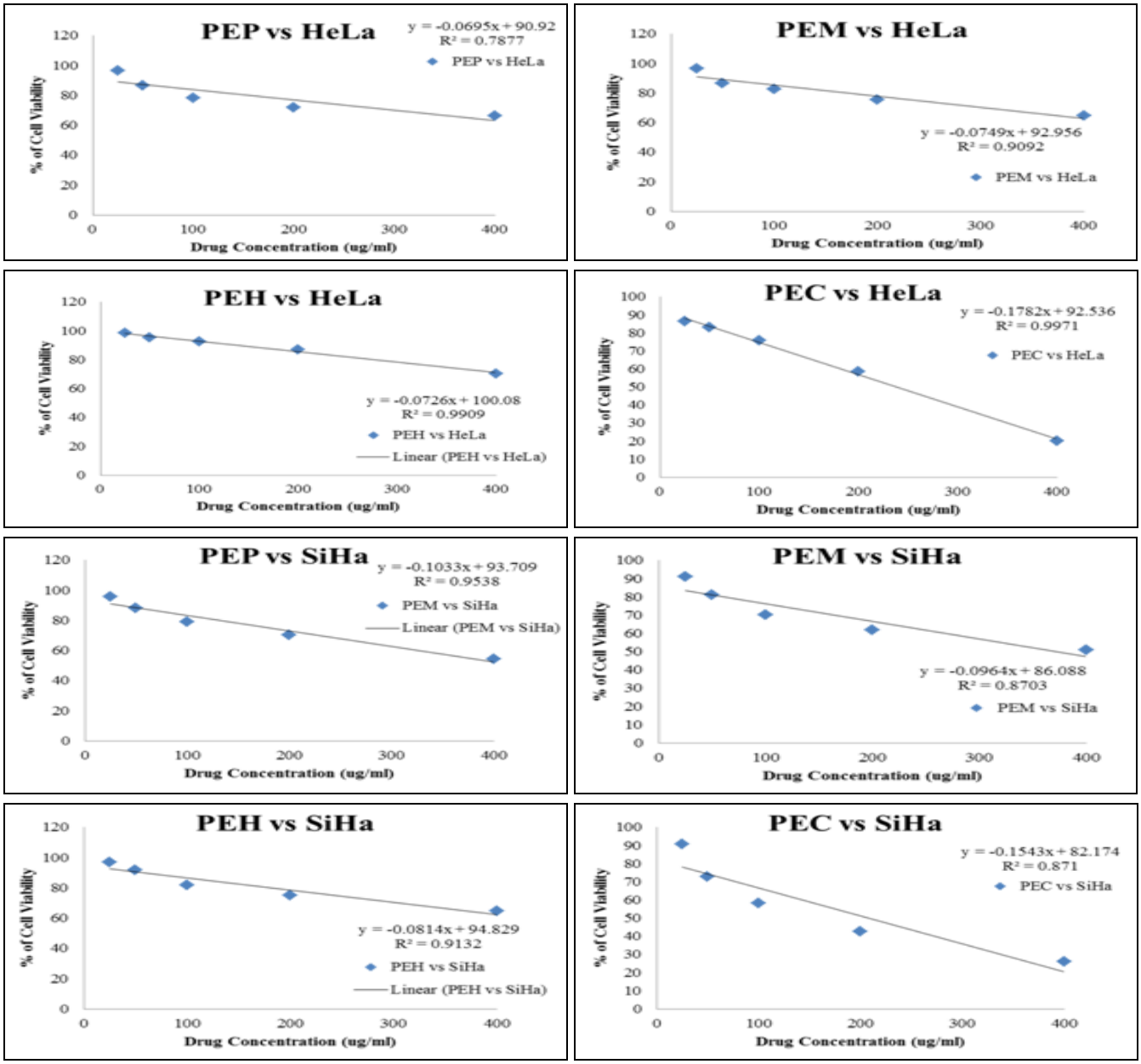

FIG. 3: % OF CELL VIABILITY OF HeLa AND SiHa CELL LINES TREATED WITH DIFFERENT CONCENTRATIONS OF PEP, PEM, PEH AND PEC AFTER 24 h Of THE INCUBATION PERIOD

Earlier studies reported the antibacterial activities of *P. zeylanica* against many human and agricultural pathogens [18] and cytotoxic activities in HGE-17 cell lines [19]. It was found that the cytotoxic activity of methanol extracts from different parts of five Arthemisia species was evaluated by Gordanian [26]. *A. absinthium* was found to have a greater cytotoxic effect on MCF-7 cells with an IC_{50} value of 221.5 µg/mL [27]. The highest cytotoxicity of Consolida orienta-lis L. extract against HeLa cell was found in 5 and 2.5 mg/ml (500 µg/ml and 250 ug/ml), and it was found that the percentage of growth inhibition is increasing with increasing the concentration of test compounds, and IC_{50} value was 1.6 mg/ml (1600 µg/ml) [28]. Our results also revealed that chemical constituents are solvent specific and showed variation in their activity against two different cancer cell lines (Hela and SiHa) causing cervical cancer and also strongly suggest that the chloroform extracts (PEC) have satisfactory cytotoxic potential properties against the both cervix carcinoma cell lines based on the dosage of the extracted sample after the incubation period of 24 h. Therefore, the *chloroform extracts of Plumbago zeylanica* are useful to treat the cervix cancers caused by HeLa and SiHa cell lines effectively.

CONCLUSION: Although the secondary metabolites of *P. zeylanica* are known for their drug properties, their bioactive potential solvent-specific activity. Chloroform extract (PEC) has satisfactory cytotoxic potential properties against both cervix carcinoma cell lines and antimicrobial activities against both gram-positive (*Staphylococcus aureus (ATCC 6538*) and *Bacillus cereus* (NCIM 2155)) and gram-negative (*E. coli (ATCC 8739*)) clinical pathogens and provide a scientific basis to develop novel drug formulations from this traditional medicinal plant.

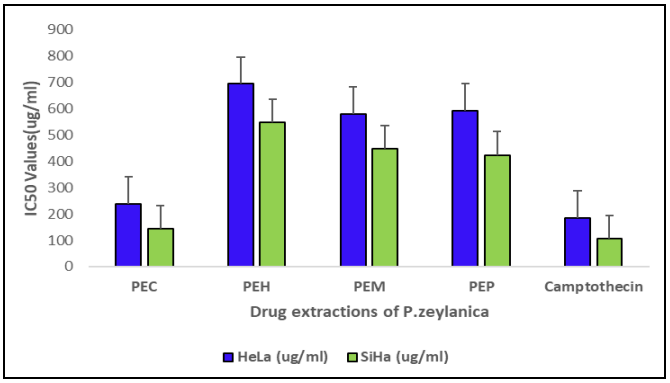

FIG. 2: IC_{50} CONCENTRATIONS OF THE TEST COMPOUNDS AGAINST THE DRUG TREATED HeLa AND SiHa CELL LINES AFTER THE INCUBATION PERIOD OF 24 h

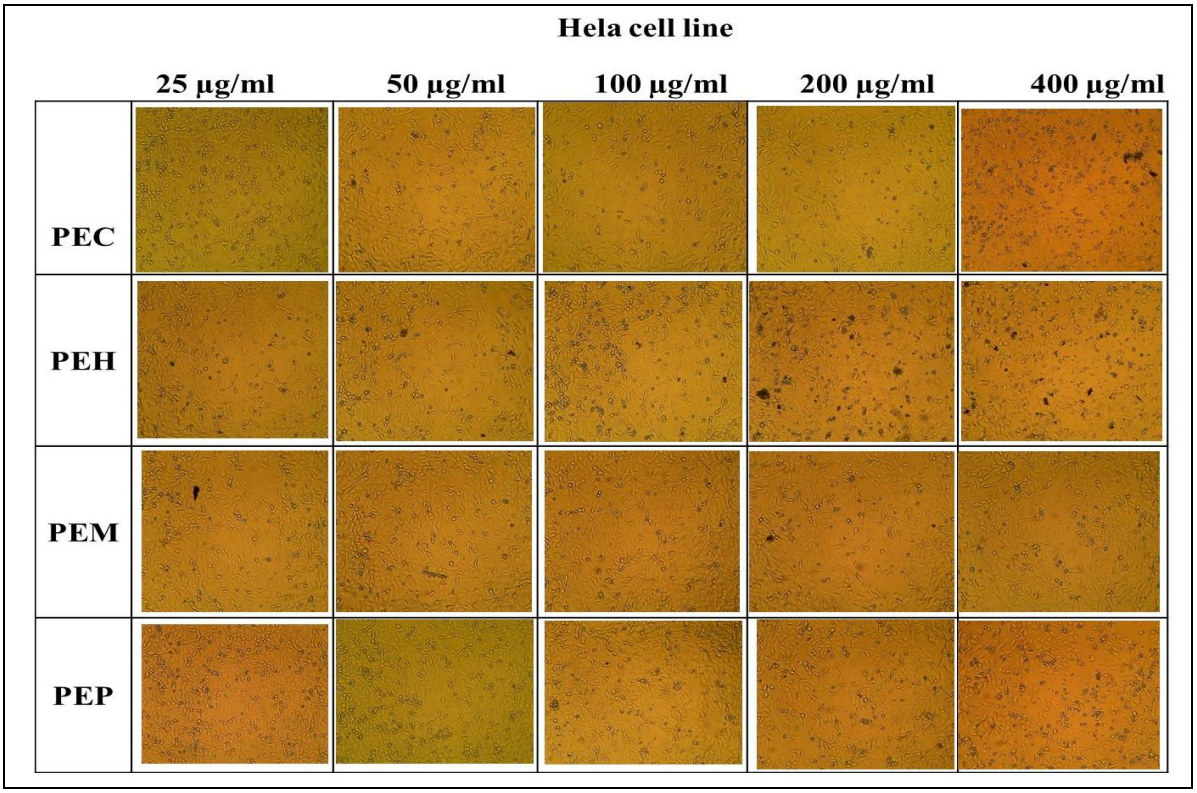

PLATE 1: CYTOTOXICITY STUDIES OF THE PLANT EXTRACTS (PEC, PEH, PEM, AND PEP) IN THE HELA CELL LINE AT DIFFERENT CONCENTRATIONS AFTER INCUBATION FOR 24 h

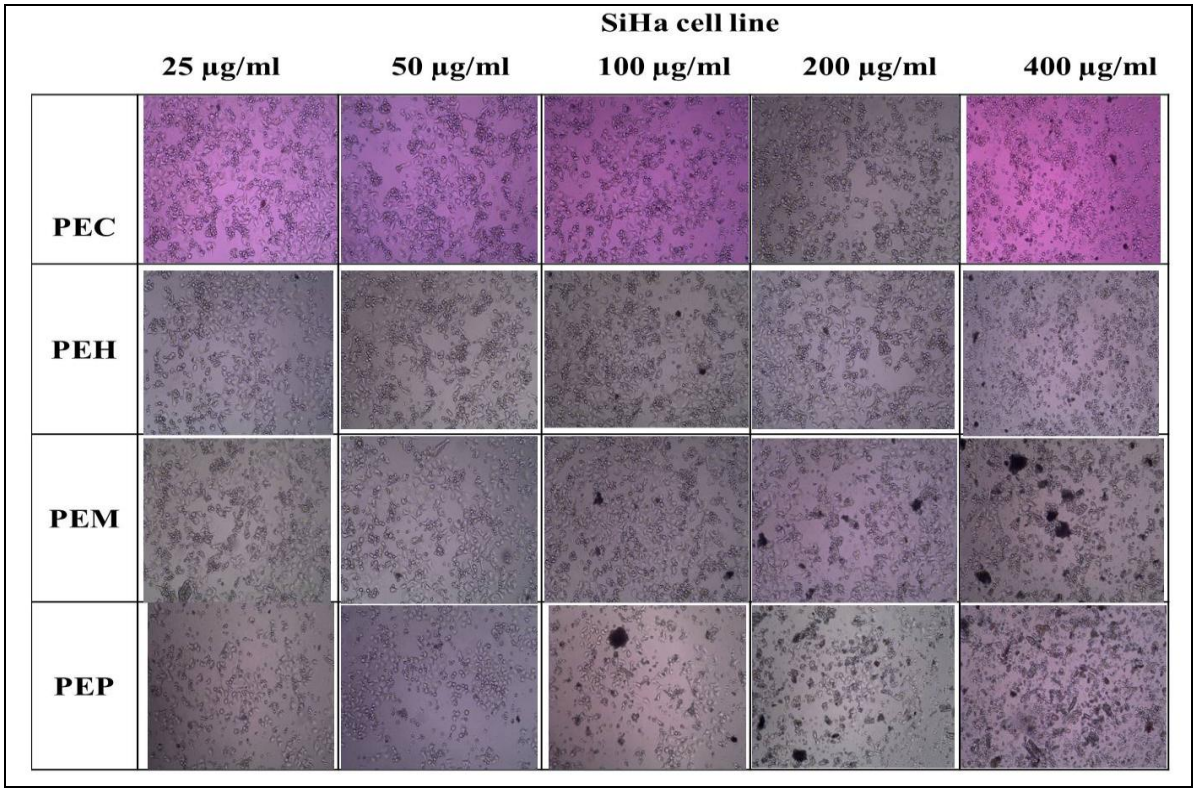

PLATE 2: CYTOTOXICITY STUDIES OF THE PLANT EXTRACTS (PEC, PEH, PEM, AND PEP) IN THE SiHa CELL LINE AT DIFFERENT CONCENTRATIONS AFTER INCUBATION FOR 24 h

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CONFLICTS OF INTEREST: No conflict of interest.

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