

# Isolation and Characterization of *Hermetia Illucens* Larval Protein for the Assessment of Inhibitory Activity against MCF7 and HeLa cell Lines

Nisha Rajasekhar, Ramesh. N, Prashantha C.N



**ABSTRACT:** Cancer has a major impact on society having the highest morbidity and mortality rate. Approximately 70% of deaths are predominantly caused due to cancer and it prevails in low and middle-income countries, as they are unaware of the signs and symptoms, treatment methods, non-availability of cancer drugs and the incurrance of huge economic burden on the family. As per the literature, the chemotherapeutic drug has numerous side effects and occasionally it may lead to death. Based on these inferences, there is a requirement to identify therapeutic peptides that are more effective for cancer treatment with less toxic effects on the body. Peptides have a great advantage to fight against the disease efficiently. Several flies have anticancer, antibacterial, antifungal and antioxidant properties that help to control disease and inhibit cancer progression. In the present research, we have utilized *Hermetia illucens* has great anticancer peptides that can control cancer growth.

In the current research, we utilized crude protein extraction from *Hermetia illucens* larvae, to discover the inhibitory activity against MCF7 and HeLa cell lines. by adopting the MTT assay method, along with reference drug Camptothecin. Bradford assay results helped to identify that 23.5µg/ml concentration of protein is present in the crude sample. Flow cytometry was used to calculate the % of cell violations and IC50 values were observed in HeLa (19.88±50.3µg/mL) and MCF7 (224.68±50.3µg/mL) cells. Cell cycle inhibition was observed in HeLa cells of G1 (57.3±1.2) and, S (19.8±1.3) phase of dose-dependent with the significance of (P<0.05). *Hermetia illucens* crude protein has significant inhibition with HeLa cells that have the best anti-cancer proteins in human cervical carcinoma cells.

**KEYWORDS:** *Hermetia Illucens*. Black soldier fly. MTT Assay. HeLa cells. MCF7 cells.

## I. INTRODUCTION

Based on the literature, it is perceived that cancer is characterized by the mutation of somatic genes which in turn alter the functioning of the protein. This will lead to uncontrolled cell growth with the potential to invade or spread to other parts of the body, which results in the death of an individual if not treated. Over 1.7 million new cancer cases are expected to be diagnosed in 2019 [1]. It is estimated that 63% of cancer deaths were observed in low and middle-income countries [2].

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There is an exponential increase in the human population and several risk factors exist that may act simultaneously to initiate and promote cancer growth, which leads to death. In developed countries like America, it has been reported that 606,880 people are expected to die of cancer in the year 2019[1]. As a consequence of such a phenomenon, it is imperative to produce a subsequent intensification of adding to the liability on pharmaceuticals, particularly in poorly developed countries [3].

In the current scenario, multiple treatment methods have been attempted based on chemo, radiation, immune and targeted therapies. However, chemotherapy is the most relevant and familiar method adopted for cancer treatment, prevention, and disease progression. The irrefutable fact observed in chemotherapy treatment challenge is the difficulty in controlling disease progression, without completely destructing the cancer cells [4]. In concrete, the existing treatment methods are not effective enough to provide full protection from the disease, so it is necessary to expand the therapeutic research and exploration to keep on imparting anticancer activity [5]

In human's genetic mutation or amplification, alter the function of the protein in cells that cause damage and thereby cause cancer[6]. Being able to degrade the protein inside the cell using another protein provides a unique opportunity for biomedical invention for the treatment of cancer [7]. Protein therapy techniques would be a new and effective treatment of cancer without causing any side effects unlike chemotherapy [8]. Protein has the capability to effectively destroy cells which are linked to the progression and development of cancer [9]. Present days peptides are gaining more importance and are viewed as future chemotherapeutic drugs, with the advantage of low propensity to resistance, which started this paradigm in the pharmaceutical market [10]. Peptides are the naturally occurring biological molecules that are used in drugs to improve the patient's medical condition. They are highly safe and effective due to which they are widely accepted by physicians and patients for treating several ailments. Peptides are small molecules that have <50 amino acids which have a significant effect on the treatment of cancer.

Previous studies state that insects have a great advantage for the treatment of various cancers and other diseases [11]. The current research is focused on *Hermetia illucens*, also known as the black soldier fly. *Hermetia illucens* is an invertebrate belonging to the Kingdom: Animalia, Phylum: Arthropoda and Class: *Insecta*, Subfamily: *Hermetiinae*, Genus: *Hermetia*, Species: *Hermetia illucens*. This work represents an enormous opportunity to drive value creation through a hierarchical strategy of organic waste management.



*Hermetia illucens* is of great ecological importance as its larvae act as a decomposer which helps in the degradation of organic waste. The larvae of insect *Hermetia illucens* are utilized for animal consumption and the by-products formed during the degradation are used as micronutrients to plants[12]. The larvae possess a rich source of protein and chitin which increases the nutrient value of animals when provided. However, the administration of 40-44% of protein supplements can balance the nutrient value as well as will increase the efficacy of the immune system. There are numerous health benefits for protein-rich content of the larvae which facilitate in suppressing the expression of colon cancer and also help to diminish cervical cancer cells[13].

## II. MATERIAL AND METHODS

### A. Rearing, Harvesting, and isolation of larvae

*Hermetia illucens* was cultured using vegetable-based media like fresh vegetable waste, peels of carrot, potato, green peas, green leafy vegetables, the remnant of fruit juice extract and a sufficient quantity of water are added to a large closed container for culturing of larvae. The Adult black soldier fly does not possess mouthpart or digestive organs. Hence the larvae and adults are not considered as vectors and pests. The adult soldier fly lacks functional mouthparts; it spends its time for mating and reproducing. They are not attracted to human habitation or food.

The trough was conserved for fermentation and maintained sufficient moisture content for the growth of larvae and observed that the trough contains a huge aggregate of larval growth after two weeks. The genus was confirmed by identifying the features mentioned. The huge aggregate of larvae was collected in a plastic bucket by connecting a single ventilation hole to the bucket which was closed with a plastic file and kept in a chamber at 27°C. The collected samples containing the pre-pupae and larvae were harvested manually using forceps. The larvae were collected to a bottle and killed by applying chloroform. The dead larvae were immediately washed in sterile distilled water and dried in an oven at 37°C. After thorough drying the larval matter was measured and powdered using pestle and mortar, the powder was sieved and stored in the bottle until further use[14].

### B. 2.2. Protein extraction and estimation

The powdered sample was measured (5.0g) and mixed with 50mL of Ultra-pure Milli-Q water and allowed to dissolve for 16-18 hours using magnetic stirrer at room temperature followed by incubation of 18 hours at 37°C. The dissolved solution was centrifuged at 12000 rpm for 10 minutes in a cooling centrifuge. The supernatant was collected and filtered using a 0.2-micron PVDF membrane. This sample was further participated by adding ammonium sulfate (NH<sub>4</sub>SO<sub>4</sub>) to 30%, 50%, 70%, and 80% saturation. The mixture was centrifuged at 12000 rpm for 30 min at 4°C and the pellet was resuspended in PBS (7.4pH). The pellet was then buffer exchanged using a 4 mL Zeba desalting column (Thermo Scientific) and sample lysate was stored [15]. The protein can estimate using the Bradford method [16]. The standard bovine serum albumin of 5-25µg/ml was dissolved using 25-100ml of distilled water, 0.5 µg/ml of samples were prepared by serial dilution. The assay was performed according to Table 1(below table) using Bradford

reagent. Optical density was measured at 595nm using a UV visible spectrophotometer. The graph was plotted on average against the concentration of standard protein. Further facilitated the calculation of the percentage of protein present in the larval protein.

### C. Protein estimation using polyacrylamide gel electrophoresis

For determining the molecular weight of the protein using Sodium dodecyl sulphate–polyacrylamide gel electrophoresis was conducted (SDS-PAGE) [17]. Using standard BSA protein as reference and larval protein sample of n=5 were in a ratio of 1:10 with sample buffer (0.0125 M Tris buffer at pH 6.8 containing 0.005 M EDTA at pH six.8–7.0, 1% of sodium dodecyl sulphate, 10% of glycerol, 1% of 2-mercaptoethanol and 0.005% of Bromophenol Blue). For denaturation of protein, the sample was heated at 95°C for 3min before the examination. Vertical electrophoresis equipment (Biobee) was used to run the gels. As commonplace the Page Ruler™ Unstained Broad varies supermolecule Ladder (Thermo Scientific, Vilnius, Lithuania) was used. The band intensity of five µl/10 µl of the sample was separated in twelve-tone music T gels was calculable following staining the gels with Coomassie good blue and quantification was conducted using UV visible spectrometer.

### D. Cytotoxicity studies

Identification and functional examination of the crude protein sample were used for cytotoxicity studies to understand the inhibition against cancer cells. Using MTT assay, the colorimetric method for determination of the cell proliferation and cytotoxicity, based on the reduction of the yellow-coloured water-soluble tetrazolium dye MTT to formazan crystals[18]. Dehydrogenase produced by Mitochondria in the live cells reduces MTT to insoluble formazan crystals, which upon dissolution into an appropriate solvent exhibits purple colour, the intensity of which is proportional to the number of viable cells and may be measured spectrophotometrically at 570nm. The tumour cell lines of MCF-7 (Breast cancer) and HeLa (Cervical cancer) were taken from the *National center for cell science (NCCS)*, Pune and were cultured with DMEM- high glucose medium (#AL111, Himedia) (#AL111, Himedia). The cells were seeded (20,000 per well) in 96-well and allowed to grow for 24 hours[19]. The crude protein was dissolved in DMEM at the required concentrations (6.25, 12.5, 25, 50 and 100 µg/mL) and incubated for 24 h at 37 °C and 5% CO<sub>2</sub> [20]. Camptothecin at a concentration of 25 µM was used as a positive control for interpretation. After the incubation period, the spent media was removed and 100 µL MTT reagent (Cat No: 4060, HiMedia Mumbai, India) was added to the cells and incubated for 3 h at 37 °C. The resulting formazan R crystals were dissolved in 100 µL of dimethyl sulfoxide (DMSO; Cat No.1309, Sigma, USA) and its absorbance was measured using a microplate plate reader (ELX 800, Biotek) at 570 nm [21]. The IC<sub>50</sub> value was calculated using the linear regression equation ( $y = mx+c$ ) obtained from the cell viability graph. The viability of the cells was determined by the following formula:

$$\% \text{ of viability} = \frac{\text{Absorbance reading of Sample}}{\text{Absorbance reading of control}} \times 100$$

### E. Effect of Crude Protein treated with HeLa Cell Line by Flow Cytometry

Cell Cycle interpretation of the crude protein extract was determined in HeLa cells. HeLa cells were cultured in a 6 well plate at a density of  $2 \times 10^5$  cells/2 ml and incubated at  $37^\circ\text{C}$  for 24 h. After incubation, the cells were treated with  $\text{IC}_{50}$  concentration of 12.5, 25, 50, and 100  $\mu\text{g/mL}$ , Cell Control and standard Control with the concentration of 12.5, 25, 50, and 100  $\mu\text{g/mL}$  in 2ml of DMEM Medium with high glucose and incubated the cells for 24 hours. After the incubation period, removed spent medium and washed cells with 2mL of 1X DPBS. Cells in each well were resuspended in 500  $\mu\text{l}$  trypsin and incubated at  $37^\circ\text{C}$  for 3-4 minutes. Followed by the addition of 2 ml of culture medium and the cells were transferred directly into 12x75 mm tubes and centrifuged for 5 min at  $300 \times g$  at  $25^\circ\text{C}$  (REMI R-8C, REMI, India). Pelleted cells were washed with 1 ml of phosphate-buffered saline [22]. Cells were fixed and permeabilized with 70% of Prechilled Ethanol for 30mins in  $-20^\circ\text{C}$  deep Freezer and again the cells were centrifuged to remove ethanol and washed with 1X DPBS and stained the Cells with 400 $\mu\text{L}$  of Propidium-RNase Solution (BD Biosciences, USA) and incubated the cells for 20-30mins under dark environment at room temperature (28 to  $30^\circ\text{C}$ ). Analyzed the cells immediately with a Flow cytometer (BD FACS Calibur, BD Biosciences)[23].

## III. RESULTS

The *Hermetia illucens* was cultured aerobically using bio-components with constant moisture. The larvae are grown, collected, killed, washed, dried and powdered using standard protocols. The concentration of protein present in the crude extract is predicted using the Bradford method, the unknown sample\_1 has 23.5 $\mu\text{g/ml}$  and unknown sample\_2 has 24.5 $\mu\text{g/ml}$  of protein were identified (Figure: 1). SDS-PAGE interpretation was performed with crude protein and the results show 90kda of protein fragments. Further, we have studied the cytotoxicity assessment of crude extract with cancer cell lines MCF7 and HeLa cells with different concentrations along with reference drug Camptothecin using the MTT assay method. Based on the observation, MCF7 cells have a constant decrease in the number of cells when compared with positive control of  $\text{IC}_{50}$  224.68 $\pm$ 50.3 $\mu\text{g/mL}$  (Figure: 2a, 2b) and HeLa cells have the highest viability of cells by comparing with positive control of  $\text{IC}_{50}$  19.88 $\pm$ 50.3 $\mu\text{g/mL}$  (figure: 3a, 3b).

### Cell Cycle interpretation by Flow Cytometry

Based on the cytotoxicity of HeLa and MCF7 cell lines with MTT assay results shows HeLa cells have greater cell viability. To further prove the Results of Apoptosis of crude protein against the MCF7 and HeLa Cancer Cell lines, we assessed the cell cycle arrest by Flow Cytometry. The results showing more % of Cell Cycle arrest at S Phase compared to STD Control used for the present research(figure:4).

## IV. DISCUSSION

Research on Protein therapeutic exploration paved a way to understand the mechanism of molecular interactions with therapeutic applications. In the current research, we utilized the proteins extracted from *Hermetia illucens* larvae, which has great precedence in multiple biological functions such as anti-oxidant and anticancer properties. The rearing of back soldier fly using vegetable-based media with a proper aerobic condition for 44 days of the life cycle was the first step of the research. The larvae were produced in large quantities and are collected in a container from 22 to 24 days and a quantity of 500gm was measured and dried thoroughly to make powder that measured 150 grams Further the quantitative examination of protein using Bradford method was carried out by preparing 5.0 grams with 50 ml of double distilled water and the resultant sample is used to the experiment. The BSA protein is used as a reference with different concentrations 5, 10, 15, 20 and 25 $\mu\text{g/ml}$  along with crude protein using standard protocol and the absorbance of 596nm is measured and drawn the graph to measure the unknown concentration. The results show 23.5  $\mu\text{g/ml}$  was observed (Figure: 1). Further SDS-PAGE was conducted by treating with trypsin digestion method and loaded using SDS-PAGE gel and 80v of electricity is supplied and the result manifests that the molecular weight of protein constituted 90 kDa.

The cytotoxicity examination was conducted with MCF7 (Breast cancer) and HeLa (cervical cancer) cell lines treating with crude protein and with reference drug Camptothecin of 25 $\mu\text{g/ml}$ . The treatment of cancer cell lines with protein at different concentrations of 6.25, 12.5, 25, 50 and 100 $\mu\text{g/mL}$ , apparently indicated cell growth inhibition. The result manifested shows the fact that a constant decrease in the number of cells takes place and it was measured using flow cytometry.

$\text{IC}_{50}$  values were calculated by drawing the straight-line curve of  $y=mx+c$  to measure the linear decreasing of cell counts. Based on the observation of MCF7 cells the constant decreasing of cells showcased from (6.25  $\mu\text{g/mL}$ ) 97.219 $\pm$ 49.179, (12.5 $\mu\text{g/mL}$ ) 94.348 $\pm$ 49.179, (25 $\mu\text{g/mL}$ ) 88.514 $\pm$ 49.179, (50 $\mu\text{g/mL}$ ) 83.363 $\pm$ 49.179, (100 $\mu\text{g/mL}$ ) 77.073 $\pm$ 49.179 of violations (Figure: 2a). The results compared with Camptothecin standard drug that shows less % of cell violations. The  $\text{IC}_{50}$  values were calculated compared with the standard drug and indicated the value of 224.68 $\pm$ 50.3 $\mu\text{g/mL}$  (Figure: 2b). The HeLa cells also treated with protein of different concentrations and indicated the outcome as (6.25 $\mu\text{g/mL}$ ) 72.350 $\pm$ 49.141, (12.5 $\mu\text{g/mL}$ ) 60.159 $\pm$ 49.141, (25 $\mu\text{g/mL}$ ) 46.543 $\pm$ 49.141, (50 $\mu\text{g/mL}$ ) 29.283 $\pm$ 49.141, (100 $\mu\text{g/mL}$ ) 17.888 $\pm$ 49.141 (Figure: 3a). The results compared with Camptothecin standard drug of  $\text{IC}_{50}$  values shows 19.88 $\pm$ 50.3 $\mu\text{g/mL}$  (Table: 3b). By comparing the MCF7 and HeLa cell lines the % of violations is less in MCF7 and  $\text{IC}_{50}$  values were high compared with the standard drug (Table: 4). In HeLa cells, the cell violations are more that shows the more inhibition of cells of % of cells is more compared with the standard drug. The result manifested that the crude protein has the highest inhibition with HeLa cells.

Further experimented the cell cycle inhibitory activity of HeLa cells treated with standard drug and protein sample at different concentrations and portrayed the results as Sub-G1 =  $0.87 \pm 0.2$ , G1 =  $51.3 \pm 1.2$ , S =  $29.8 \pm 2.3$ , G2/M =  $18.2 \pm 1.4$  of control data that compared with different concentrations of protein treated shows (12.5µg/mL) Sub-G1 =  $1.83 \pm 0.4$ , G1 =  $56.2 \pm 1.5$ , S =  $27.3 \pm 1.6$ , G2/M =  $14.3 \pm 0.8$ , (25µg/mL) Sub-G1 =  $4.98 \pm 0.7$ , G1 =  $62.5 \pm 1.8$ , S =  $23.8 \pm 1.7$ , G2/M =  $08.6 \pm 1.6$ , (50µg/mL) Sub-G1 =  $16.8 \pm 1.6$ , G1 =  $57.3 \pm 1.2$ , S =  $19.8 \pm 1.3$ , G2/M =  $06.7 \pm 0.6$  (100µg/mL) Sub-G1 =  $25.87 \pm 0.2$ , G1 =  $54.3 \pm 1.2$ , S =  $19.8 \pm 2.3$ , G2/M =  $1.2 \pm 0.5$  cell inhibitions (Figure: 5). The results shows 50µg/mL is the best concentrations to inhibit the cell growth at G1 and S phase compared to the 100µg/mL of G1 and S phase. Based on the observation the dosage concentration 50µg/mL is the best interaction for the cervical cancer inhibition.

### V. CONCLUSION

Peptides are considered to possess high clinical therapeutic value. Peptides are significant in the therapeutic field as it can be customized to achieve desired biocompatibility that brands it to a potent therapeutic grade. Currently, peptide therapeutics is a major research category in the field of identification of peptide sequences for the treatment of cancer and other diseases. In cancer therapeutics, peptides demonstrate a significant role because of its high target specificity and selectivity, high biological and chemical diversity, high solubility, diminutive toxicity, and inconsequential side effects. In this research, we resorted to *Hermetia illucens* insect larva protein and analyzed that the peptides offer enormous potential for anticancer activities. The isolation, identification, and characterization conducted by employing various biochemical and cytotoxicity methodology with MCF7 and HeLa cancer cell lines predicted and established the fact that crude *Hermetia illucens* insect protein has substantial inhibitory activity with HeLa cell lines (IC50 =  $19.88 \mu\text{g/mL}$ ). Further, the cell cycle inhibitory activity of HeLa cells with different concentrations were conducted and observed that the concentration of 50µg/mL shows immense cell cycle inhibitory effect at G1/S phase G1 =  $57.3 \pm 1.2$ , S =  $19.8 \pm 1.3$ . The current research consummates that the cytotoxic activity of *Hermetia.Illucens* crude protein has a great inhibitory effect on cervical cancer. The conclusion provides a great scope for the future researchers to isolate individual protein/ peptide and consider the in-vitro and in-vivo exploration to predict the strong inhibitory effect with cervical cancer and help validate the innovative therapeutic function.

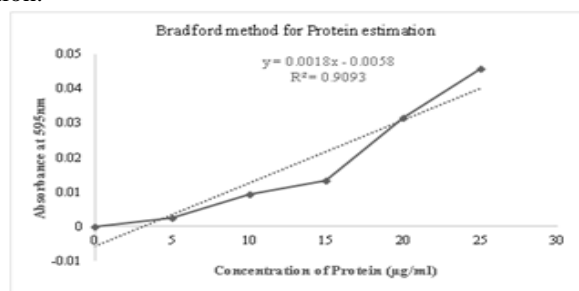


Figure: 1. Estimation of protein concentration present in the crude extract of *Hermetia illucens* using the Bradford method.

The concentration of protein present in the crude extract of *Hermetia illucens* using Bradford method

Sample 1 (After Ammonium sulphate treatment 1) - 23.5 µg/ml Protein

Sample 2 (After Ammonium sulphate treatment 2) - 24.5 µg/ml Protein

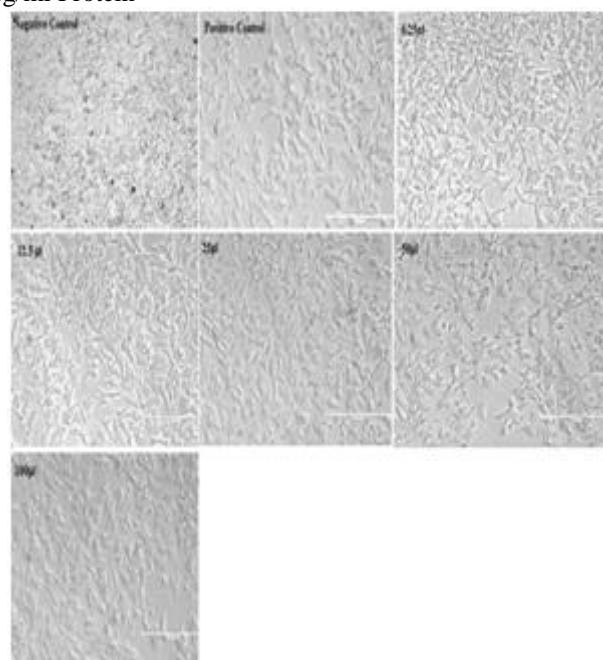


Figure: 2a. Cytotoxicity assessment of *Hermetia illucens* crude protein induced inhibition in MCF7 cell lines using MTT Assay.

Negative Control: MCF7 cells without reference drug and crude protein

Positive Control: MCF7 cells treated with reference drug (Camptothecin  $25 \mu\text{m} \pm 0.511 \mu\text{g/ml}$ )

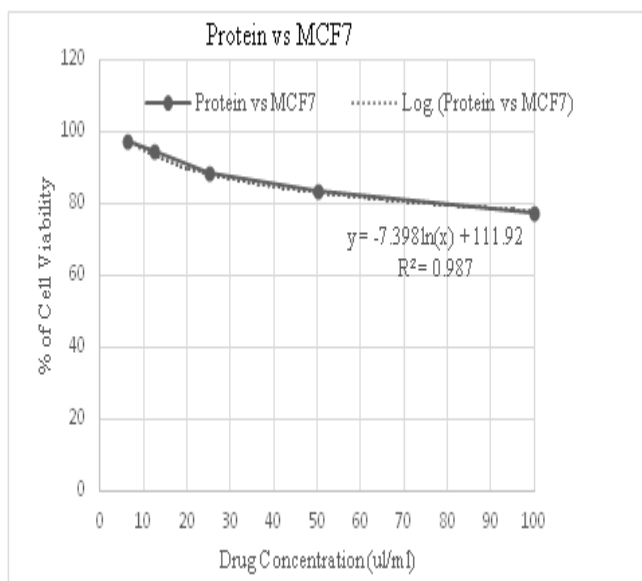
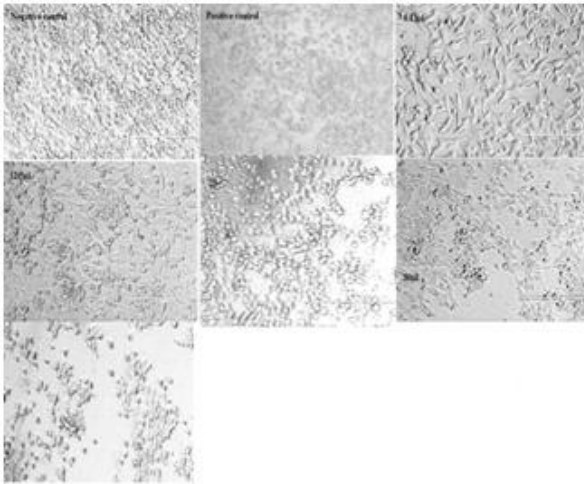


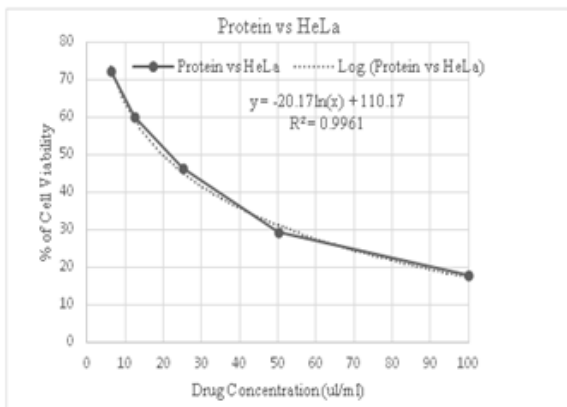
Figure: 2b Cytotoxic effect of crude protein against MCF7 cancer cell line.



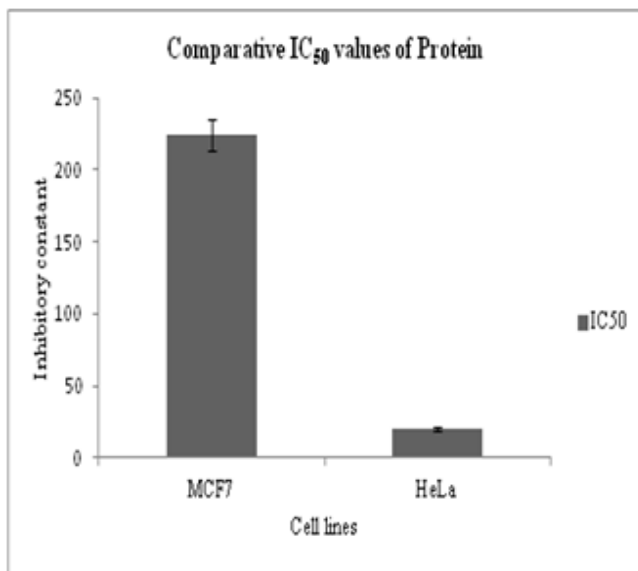
**Figure: 3a** Cytotoxicity activity of *Hermetia illucens* crude protein induced inhibition in HeLa cell lines using MTT Assay.

Negative Control: HeLa cells without reference drug and crude protein

Positive Control: HeLa cells treated with reference drug (Camptothecin 25µm ± 0.511µg/ml)

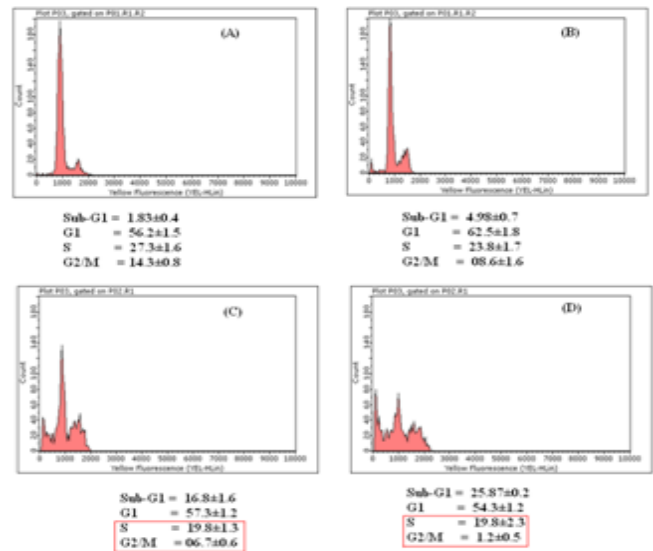


**Figure: 3b.** Cytotoxic effect of crude protein against HeLa cancer cell line



**Figure: 4.** Correlative graphs of MCF7 and HeLa cell lines treated with crude protein

**Figure Legend:** The values were expressed as mean ± SEM of MCF7 (Breast), and HeLa (Cervical) cancer cell lines treated with reference drug and crude protein and the statistical significance of the data at the level of P < 0.05. (a) Denote collation of control vs MCF7 cell lines; (b) denote collation of HeLa (Cervical) vs control cell lines.



**Figure: 5.** Cell Cycle inhibitory activity of HeLa cells treated with crude protein

A). 12.50µg/mL of crude protein with HeLa cell lines, B). 25µg/mL of crude protein with HeLa cell lines, C). 50.00µg/mL of crude protein with HeLa cell lines, D). 100µg/mL of crude protein with HeLa cell lines.

**Figure: 1.** Estimation of protein concentration present in the crude extract of *Hermetia illucens* using Bradford method.

**Figure: 2a** Cytotoxicity activity of *Hermetia illucens* crude protein induced inhibition in MCF7 cell lines using MTT Assay.

**Figure: 2b** Cytotoxicity effect of *Hermetia illucens* crude protein induced inhibition in MCF7 cell lines.

**Figure: 3a** Cytotoxicity activity of *Hermetia illucens* crude protein induced inhibition in HeLa cell lines using MTT Assay.

**Figure: 3b** Cytotoxicity effect of *Hermetia illucens* crude protein induced inhibition in HeLa cell lines.

**Figure: 4** Correlative graphs of MCF7 and HeLa cell lines treated with crude protein.

**Figure: 5** Cell Cycle inhibitory Studies of HeLa cells treated with crude protein.

**Abbreviations**

MCF7- Michigan Cancer Foundation-7; HeLa- Henrietta Lacks; MTT- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; PVDF- polyvinylidene difluoride; PBS-Phosphate-buffered saline; PBS- Phosphate-buffered saline; SDS- PAGE- sodium dodecyl sulfate polyacrylamide-gel electrophoresis; DMEM- Dulbecco's Modified Eagle Medium.



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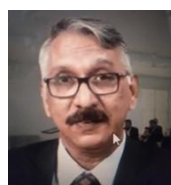
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