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INFLUENCE OF FUNGAL DERIVED CAMPHTOTHECIN ON THE CELL CYCLE ANALYSIS OF CERVICAL CANCER CELL LINE (HELA)

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ABSTRACT:

Cancer is one of the major health problems worldwide and its current treatments have a number of undesired adverse side effects. In the past two decades, many valuable bioactive compounds with antimicrobial, insecticidal, cytotoxic, and anticancer activities have been successfully discovered from Natural compounds. Currently, a few of them are being used to treat cancer. Some endophytes fungi are have the ability to produce the same or similar bioactive compounds as those originated from their host plants. Camptothecin (CPT), the third largest anticancer drug, is produced mainly from Camptotheca acuminata and Nothapodytes foetida. In the present study is focussed to develop Camptothecin from endophyte Fungi and to elevate their potential anti-cancer properties against Hela cervical cancer cells. Cervical cancer cells were treated with the isolated camptothecin and its effect was analysed using the cell cycle analysis. From the investigation the cytotoxic properties against cervical cancer and anticancer activities have been successfully discovered from endophytic fungi. Thus appears to display potent antitumor activity against human cancer via the induction of apoptosis, and may be a useful to develop alternative drug for different cancer therap.

KEY WORDS: Bioactive compounds, Fungi derived Camptothecin, Cervical cancer, HeLa Cell Lines, Anti-Cancer.

INTRODUCTION:

Cancer is a widespread disease. Cancer is uncontrolled growth of cells. It can affect almost any part of the body. The growth often invades surrounding tissue and can metastasize to distant sites (WHO, 2011). Cancer is caused by mutations in the DNA. Normal cells repair the mutation or simply die when a mutation occurs whereas cancerous cells continue to survive with the mutations and they grow in an uncontrolled manner until a mass of cells known as tumor is created. Often the tumor interferes with the normal functioning of healthy

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tissues and can spread to other parts of the body (Tompa, 2007). Many efforts to combat cancer have been undertaken through many decades. The occurrence of cancer in varied parts of the body adds to the difficulty in its cure. Many types of cancers with different mechanisms have been studied in the past years. Due to different mechanisms in action, the effectiveness of drugs varies from type to type. Hence extensive research has been carried out for various drugs and their effect on different types of cancer.

Chemotherapeutic agents are synthetic compounds which are now widely used to treat cancers across the world. They are also known to have many side effects. With increasing awareness about the side effects alternative sources are being examined. Many natural sources are now being examined for compounds with potential anti-cancer activity. Many such compounds exist in nature, which have anti-cancer activity. However due to the lack of substantial evidence, they are not being put to effective use. Studies identifying such compounds are the need of the hour. The identification and validation of such natural compounds will be immensely beneficial. The compounds with appropriate properties can be then be used as lead compounds in the pharmacological industry. Hence many natural compounds or their modifications can be used as drugs. The right use of these natural compounds will lower the risk of side effects and act as efficient drugs.

Endophytes resided in the internal tissues of living plants occur in almost every plant on earth from the arctic to the tropics, and they are rich sources for bioactive natural products (Qin *et al*. 2011). It is generally recognized that endophytes represent an important and largely untapped reservoir of unique chemical structures that have been modified through evolution and exhibit the capability to produce the same functional compounds as their hosts, some examples include taxol, podophyllotoxin (Puri *et al*. 2006), hypericin (Kusari *et al*. 2008), and azadirachtin (Findlay *et al*. 1997). In 2005, Puri *et al*. reported the first discovery of CPT-producing endophytic fungus Entrophospora infrequens from the inner bark of N. foetida, which set the stage for a more comprehensive examination of other plants for the presence of CPT-producing endophytes; (Shweta *et al*., 2013). These intriguing results have undoubtedly renewed the interests in exploring other endophytic sources of CPT and its analogues from various plant sources. Endophytic Fungi are microorganisms that live asymptomatically inside plant tissues for all or part of their life cycle. Endophytes are ubiquitous within the plant kingdom. Fungal endophytes are hyperdiverse and abundant groups. More importantly, endophytic fungi can produce a great number of plant derived and novel bioactive compounds any of which could be developed into novel antimicrobial and anticancer agent. Endophytes resided in the internal tissues of living plants occur in almost every plant on earth from arctic to the tropics, and they are rich sources for bioactive natural products. Endophytic fungal communities are influenced by many factors such as geographic locations, climatic pattern, physiology and specificity of the colonized tissue. As a result of adaptation to these different environmental conditions, different fungi forming distinctive endophytic communities are specific to each environmental condition and tissue type. Endophytic Fungi represent an important and largely untapped reservoir of unique chemical structures that have been modified through evolution and exhibit the capability to produce the

same functional compounds as their hosts, some examples include taxol, podophyllotoxin, hypericin and azadirachtin. Despite the fact that endophytic fungi create enormous biological and chemical diversity, only a few of these plant associated microorganism has been studied and it may provide new opportunities for discovering diverse species and natural products for exploitation in medicine, agriculture and industry. (Shankar Naik *et al.*2012).

Bioactive compounds are found in both plant and animal products or can be synthetically produced. Examples of plant bioactive compounds are carotenoids and polyphenols (from fruits and vegetables), or phytosterols (from oils). Examples in animal products are fatty acids found in milk and fish. Some examples of bioactive components are flavonoids, caffeine, carotenoids, choline, carnitine, coenzyme Q, creatine, polysterols, polyphenol.

In the past two decades, many valuable bioactive compounds with antimicrobial, insecticidal, cytotoxic, and anticancer activities have been successfully discovered from endophytic fungi. Some endophytes have the ability to produce the same or similar bioactive compounds as those originated from their host plants. It mainly deals with the research progress on endophytic fungi for producing plant-derived bioactive compounds such as paclitaxel, podophyllotoxin, camptothecine, vinblastine, hypericin, and diosgenin.

Camptothecins are among the most promising antitumor agents (Zunino F and Pratesi G 2004). On the basis of their therapeutic interest, intense research efforts have provided insights to understand their mechanism of action and to exploit their anti-tumor potential (Zunino *et al.*, 2003). *Camptothecins* are DNA-damaging agents characterized by a unique mechanism of action because they are target-specific inhibitors of DNA topoisomerase I by stabilizing the covalent enzyme–DNA complex (cleavable complex) (Pommier *et al.*, 1998). Due to the specific mechanism of topoisomerase I–mediated cytotoxicity, a characteristic feature of camptothecin action is their preferential or selective toxicity to proliferating cells.

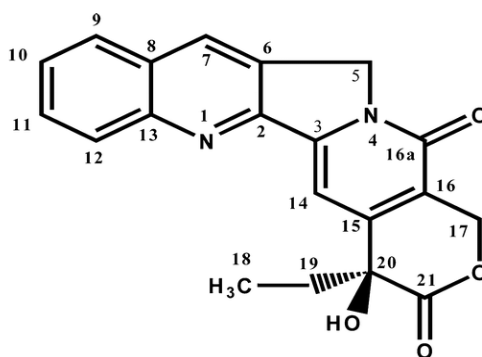

Figure 1: Structure of Camptothecin

Camptothecins exhibit a wide range of anti-neoplastic activity. They exert their cytotoxic effect through a single intracellular target, the nuclear enzyme topoisomerase I

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(topo I) (Hsiang and Liu, 1988), which relieves torsional strain introduced in the DNA duplex by active replication and transcription. The enzyme cleaves one of the strands of the duplex DNA, allowing the 5′-end of the cleaved strand to rotate around the inter nucleotide bond of the intact strand. Resealing of the cleaved strand after one or several strand passages completes enzyme action (Liu, 1989). CPT and its analogues slow the relegation step of the topo I catalytic cycle without affecting the DNA cleavage reaction. As a result, topo I-DNA adducts (cleavable complexes) are stabilized in the presence of CPT, resulting in single-strand DNA breaks.

Camptothecin (CPT; Fig. 1(1)), a pentacyclic pyrroloquinoline alkaloid, was isolated firstly from a China native tree Camptotheca acuminata (Wall *et al*. 1966). CPT induces protein-linked DNA breakage via mammalian DNA topoisomerase I, and thus, it was used as an efficient anticancer drug against a broad band of tumor types such as small lung and refractory ovarian cancers. CPTs represent an important class of anticancer drugs with a wide spectrum of activities in many solid tumors, including lymphoma, gastric cancer and colorectal cancer. CPT-type drugs (Sirikantaramas *et al*. 2007) used clinically, including 10-hydroxycamptothecin, topotecan, irinotecan, and SN-38 were chemically derived from CPT, although a small amount of 10- hydroxycamptothecin could be isolated from plant sources (Thomas *et al.* 2004). CPT, the multi-billion dollar anticancer natural compound, is the third largest anticancer drug from plant sources in the world market. CPT itself was produced mainly by C. acuminata in China and Nothapodytes foetida in India. (Lorence and Nessler 2004), though several plant species of the Asterid clade, including Icacinaceae (Pyrenacantha klaineana and Merrilliodendron megacrapum), Rubiaceae (Ophiorrhiza pumila and Ophiorrhiza mungos), Apocynaceae (Ervatamia heyneana), and Gelsemiaceae (Mostuea brunonis) have been reported to produce CPT (Shaanker *et al*. 2008). In recent years, the heavy demand for CPT has resulted in destructive harvesting of these trees in China and India. As an alternative approach, efforts are underway to identify new sources of CPT from both new plant sources and endophytic microorganism associates of CPT-producing plants (Shweta *et al*. 2013). The endophytic fungi *Aspergillus niger* has been found to be an effective source of camptothecin.

Cervical cancer is a malignant neoplasm of the cervical area. It is an important women's health problem in developing countries, killing 270,000 women each year. It is the third most common cancer overall and the leading cause of death from cancer among women in developing countries. At least 370,000 new cases are identified each year (WHO, 2010). Current cancer chemotherapy can damage or kill the rapid dividing and healthy cell but causes serious side effects such as nausea, anemia, and hair loss. In addition, the cost of chemotherapy drug is high as compared to the natural compound from medicinal plants.

Components of the cell cycle machinery are frequently altered in human cancer. Central players are the cyclin-dependent kinases (cdks), which govern the initiation, progression, and completion of cell cycle events. The scheduled activity of the cdks, which allows orderly transition between cell cycle phases, is controlled by their association with

cyclins and cdk inhibitors, by their state of phosphorylation, and by ubiquitin-mediated proteolysis. As malignant cells evolve, both genetic and epigenetic mechanisms commonly affect the expression of cell cycle regulatory proteins, causing over expression of cyclins and loss of expression of cdk inhibitors. A major consequence is deregulated cdk activity, providing cells with a selective growth advantage. The crucial role of the cdks has prompted great interest in the development of specific kinase inhibitors that would be expected to block cell cycle progression and induce growth arrest. Thus the effect of the drug on the cancer cells can be studied through its cell cycle analysis.

In this study, camptothecin was isolated from the endophytic fungi *Aspergillus niger*. Cervical cancer cells were treated with the isolated camptothecin and its effect was analysed using the cell cycle analysis.

MATERIALS AND METHODS

EXTRACTION OF CAMPTOTHECIN

Fungal Endophyte Aspergillus spp. was obtained from Life Teck Research Centre for the present study. For the production of Camptothecin, spore suspensions of the culture were inoculated in Erlenmeyer flask (500 ml) with SDA broth enriched by 1% peptone and yeast extract (200 ml). The cultures were incubated in a rotary shaker (220 rpm) at 28 o C for, 96 hours.

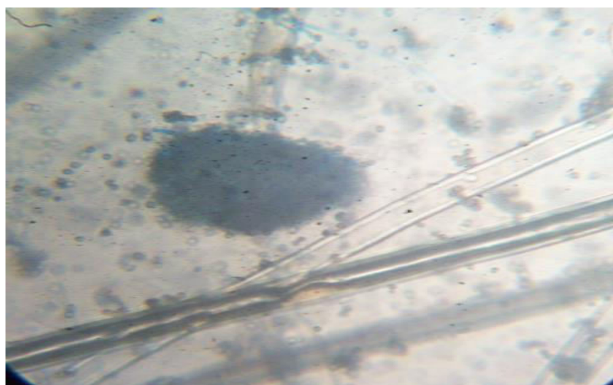


Figure 2: *Aspergillus niger*

EXTRACTION OF MYCELIA:

The fully grown mycelia (after four days of incubation) were harvested. The mycelia and broth were separated by filtration and the mycelia were thoroughly washed with sterile distilled water and then homogenized. The resulting homogenate were extracted with equal volume of chloroform: methanol (4:1 v/v) solvent mixture. The extraction was carried out with cellulase enzyme and without cellulase enzyme separately. The residue was obtained after stripping off the solvent. The crude camptothecin obtained was used for further analysis (Puri *et al*., 2005).

ANTICANCER ACTIVITY

HeLa cell line was obtained from National Centre for Cell Sciences, Pune (NCCS). The cells were maintained in DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 50 µg/ml CO_2 at 37 °C.

In Vitro assay for anti cancer activity: (MTT assay) (Mosmann, 1983)

Cells (1×10^5/well) were plated in 24-well plates and incubated in 37^0C with 5% CO_2 condition. After the cell reaches the confluence, the various concentrations of the samples were added and incubated for 24hrs. After incubation, the sample was removed from the well and washed with phosphate-buffered saline (pH 7.4) or DMEM without serum. 100µl/well (5mg/ml) of 0.5% 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl--tetrazolium bromide (MTT) was added and incubated for 4 hours. After incubation, 1ml of DMSO was added in all the wells .The absorbance at 570nm was measured with UV- Spectrophotometer using DMSO as the blank. Measurements were performed and the concentration required for a 50% inhibition (IC50) was determined graphically. The % cell viability was calculated using the following formula:

% Cell viability = A570 of treated cells / A570 of control cells × 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 assessments.

Cell cycle analysis:

Cell Cycle Analysis provides a rapid and convenient assay for cell cycle and cell proliferation. For normal cells, the content of DNA is changed with the process of cell cycle. Observed DNA stained by dyes using flow cytometry to calculate percentage of G0/G1, S, and G2/M. It will be clear known that how about the distribution of cell cycle and the activity of proliferation. For apoptotic cells, DNAs in cells is degraded by endogenous nuclease activated and diffuse out of cells with the process of apoptosis. A highly definable sub-G1 peak occurs and is easily quantified by dyes. The change of DNA in apoptotic cells is also assayed for sorting and further analyzing apoptotic cells. After RNA is degraded by RNase, the nucleic acid dye in this kit bind with DNA composed of chromatin in the nucleus. And the results can be analyzed by flow cytometry.

Protocol:

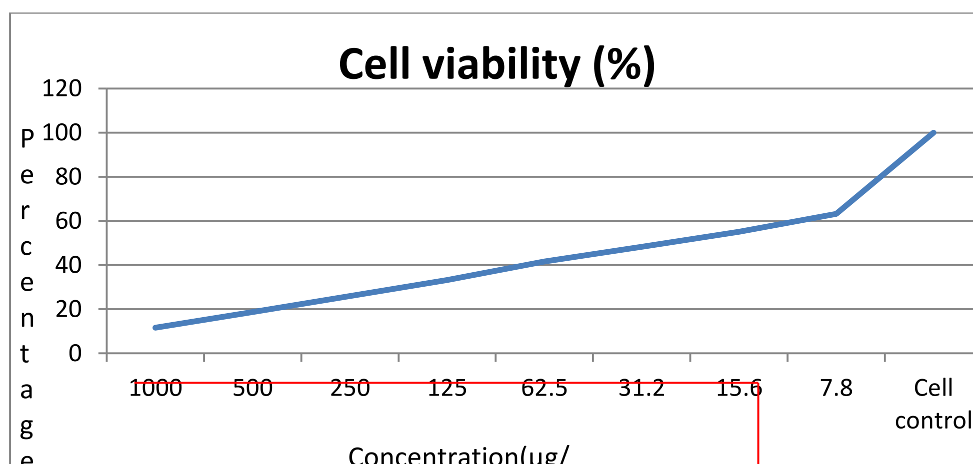
- Induce cell apoptosis using proper method and set a negative control. Harvest cells.
- Add PBS to wash cells once. Then, centifugate cells at 2000 rpm for five minutes.
- Add PBS to resuspend cell and adjust cell concentration to 1×106/ml.
- Centrifuge cells at 2000 rpm for five minutes and discard the supernatant.
- Fix cells using 70% ethanol at 4°C for two hours or overnight.

- Use PBS to wash cells for removing fixing solution. If necessary, filter cell suspension once using sieve with 200 meshes.
- Add 100 µl of RNase A to cells suspension and incubate cells at 37 °C for 30 minutes.
- Add 400 µl of PI (Propidium Iodide) to stain. Incubate cells at 4 °C for 30minutes and protect from light.
- Observe at 488 nm of excitation wavelength by flow cytometry.

RESULT AND DISCUSSION:

Table 1: Anticancer effect of Camptothecin standard *on HeLa* cell line

S.No	Concentration (µg/ml)	Dilutions	Absorbance (O.D)	Cell viability (%)
1	1000	Neat	0.194	11.65
2	500	1:1	0.311	18.68
3	250	1:2	0.433	26.02
4	125	1:4	0.552	33.17
5	62.5	1:8	0.692	41.58
6	31.2	1:16	0.804	48.31
7	15.6	1:32	0.918	55.16
8	7.8	1:64	1.052	63.22
9	Cell control	-	1.664	100



Graph-1: The IC50 value of standard camptothecin on HeLa cell line was found to be 31.2µg/ml.

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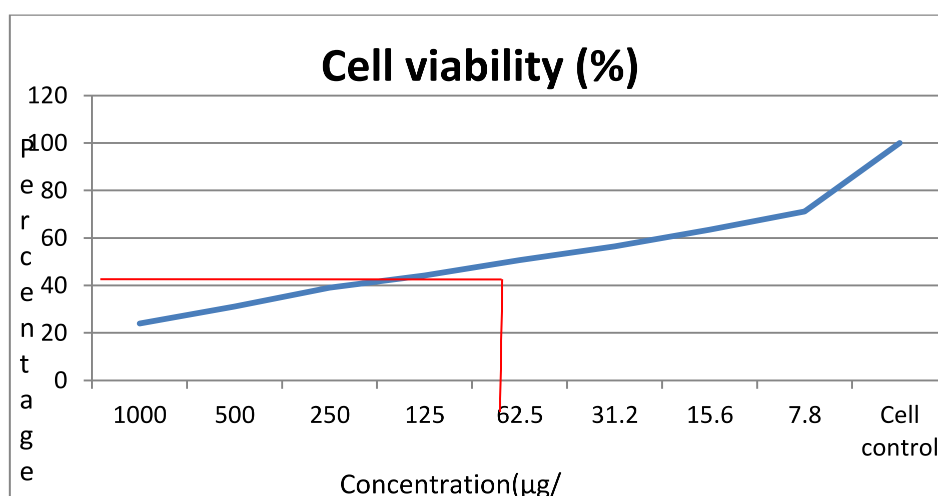
Table 2: Anticancer effect of Camptothecin *on HeLa* cell line

S.No	Concentration (µg/ml)	Dilutions	Absorbance (O.D)	Cell viability (%)
1	1000	Neat	0.399	23.97
2	500	1:1	0.518	31.12
3	250	1:2	0.650	39.06
4	125	1:4	0.734	44.11
5	62.5	1:8	0.842	50.60
6	31.2	1:16	0.938	56.37
7	15.6	1:32	1.056	63.46
8	7.8	1:64	1.184	71.15
9	Cell control	-	1.664	100



Graph-2: The IC50 value of the extracted camptothecin was found to be 62.5µg/ml.

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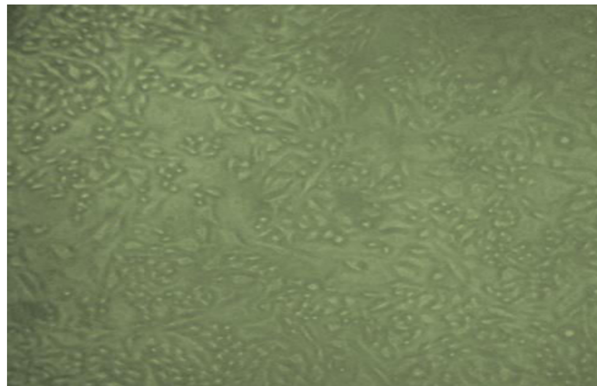
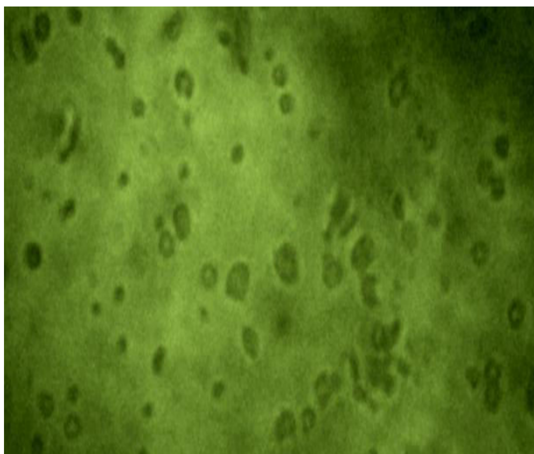
Figure 3: Anti-cancer effect of Camptothecin Standard on *HeLa cell* line

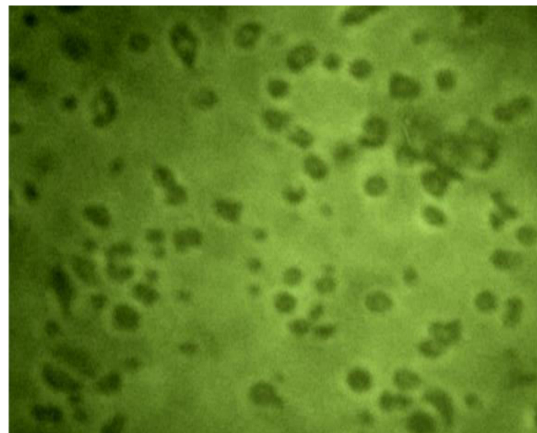
Normal *HeLa* cell line



Toxicity – 1000 µg/ml



Toxicity – 31.2µg/ml



Toxicity – 7.8 µg/ml

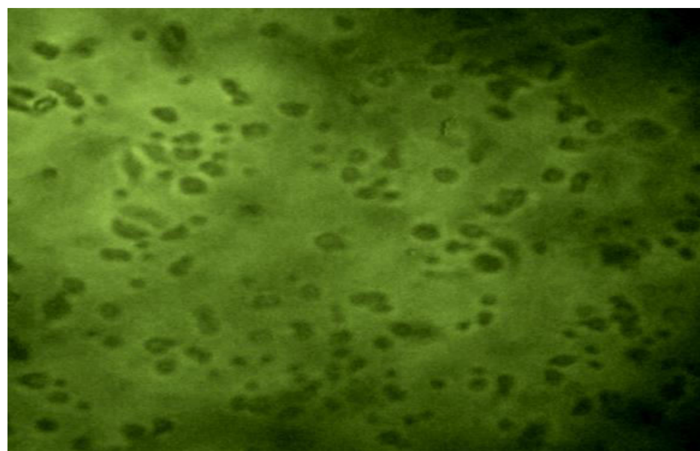
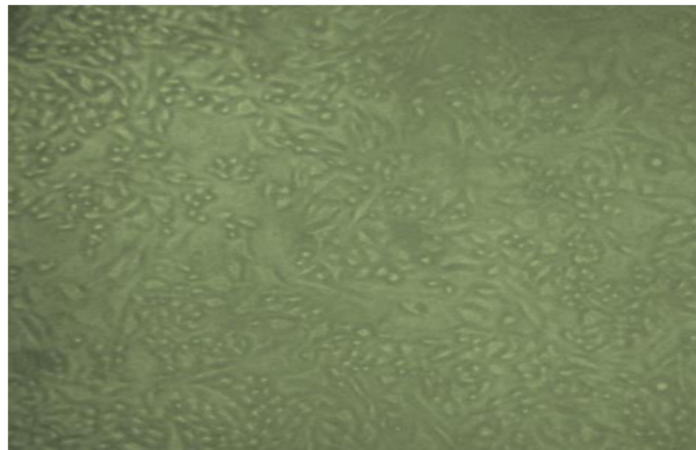
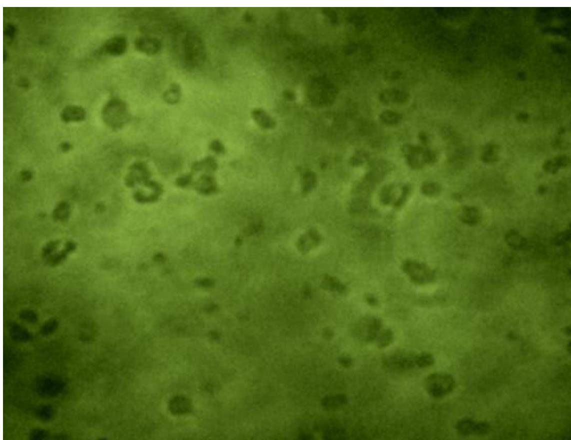


Figure 4: Anticancer effect of Camptothecin on *HeLa cell* line

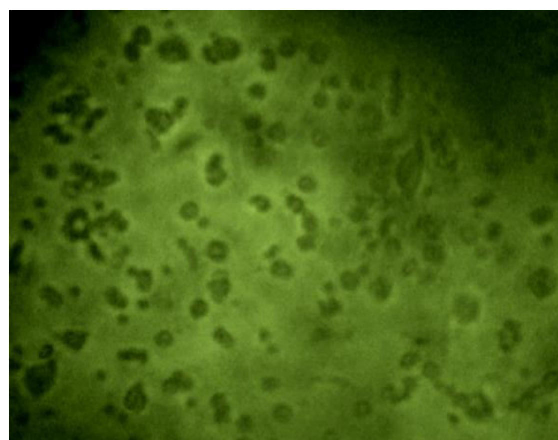
Normal *HeLa* Cell line

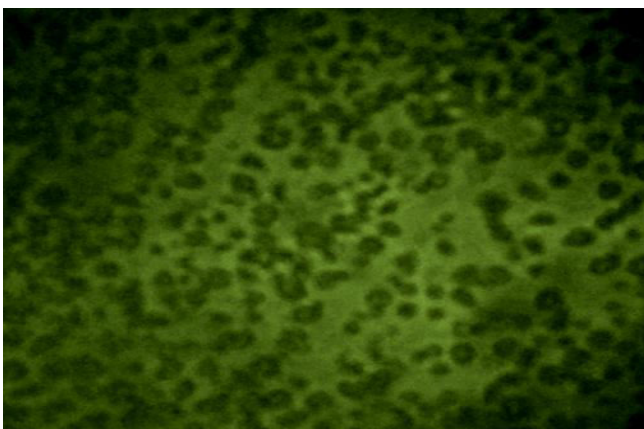


Toxicity – 1000 µg/ml



Toxicity – 62.5 µg/ml





Toxicity – 7.8 µg/ml

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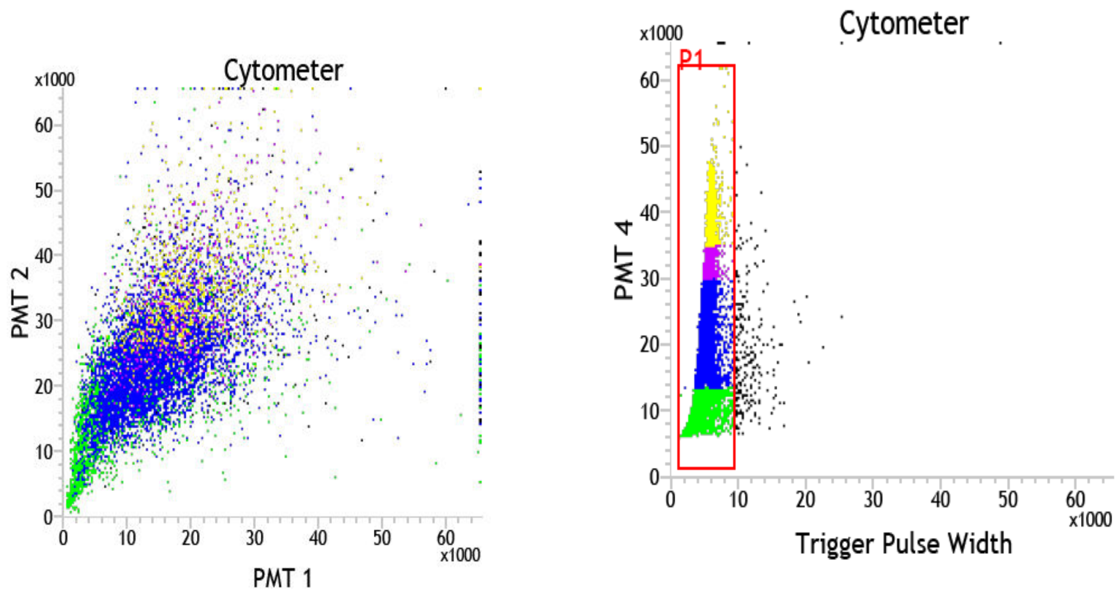
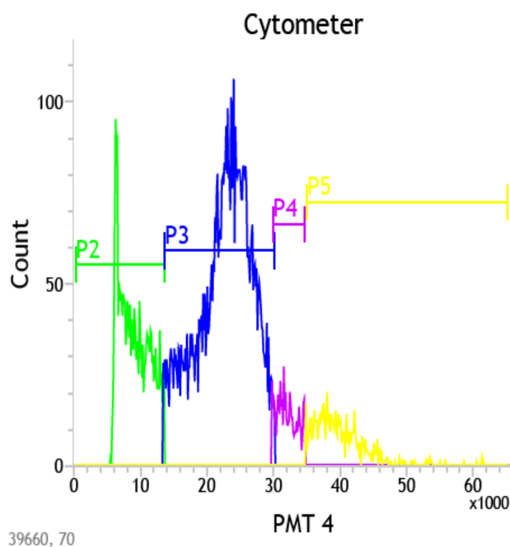
The anti-cancer effect of camptothecin on MCF7, HCT 8 and A549 cells have been studied by Chu *et al.*, 2014 and found to be effective. The results of this study are found to be in accordance with it.

Cell cycle analysis was done by flow cytometry. Cell lines were treated with and without samples and were taken as control and treated respectively. The cell cycle was found to be normal for the control while it was arrested for the camptothecin treated cells.

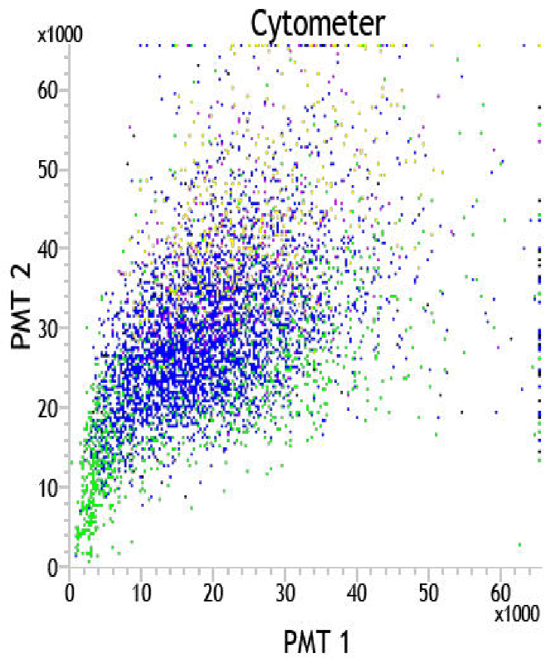


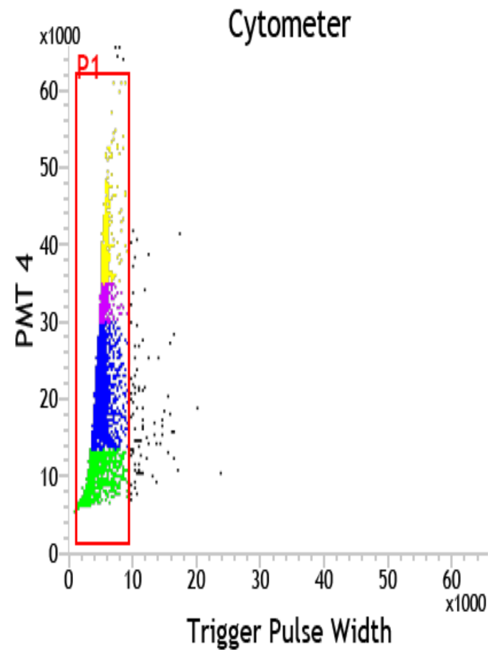


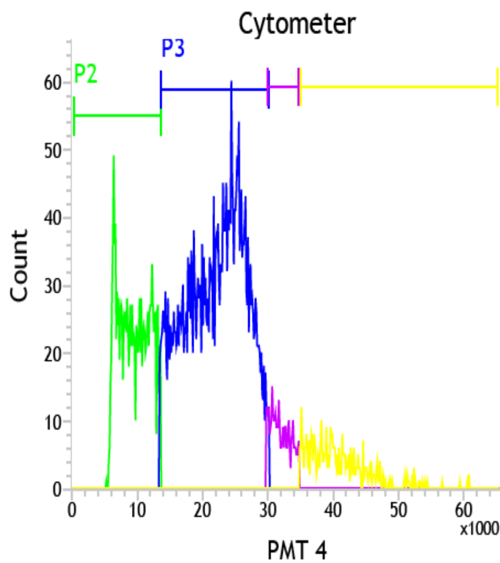
Statistics: Cytometer

Populations	Events	% Total	% Parent	PMT 4 Mean	Time 1 Mean
All Events	10,000	100.00%	####	21,371	32,968
P1	9,747	97.47%	97.47%	21,384	33,001
P2	2,258	22.58%	23.17%	9,024	33,580
P3	6,267	62.67%	64.30%	22,592	32,831
P4	565	5.65%	5.80%	32,019	33,430
P5	785	7.85%	8.05%	39,890	32,815

Control







Statistics: Cytometer

Populations	Events	% Total	% Parent	PMT 4 Mean	Time 1 Mean
All Events	6,082	100.00%	####	20,829	32,647
P1	5,971	98.17%	98.17%	20,824	32,659
P2	1,483	24.38%	24.84%	9,464	32,955
P3	3,790	62.32%	63.47%	21,963	32,675
P4	337	5.54%	5.64%	31,949	30,914
P5	447	7.35%	7.49%	40,738	33,085

HeLa Cell Lines

SUMMARY AND CONCLUSION

Camptothecin was extracted from the endophytic fungi *Aspergillus niger*. The extract was then used to treat the HeLa cell lines. Anticancer activity of the extracted camptothecin was studied against the standard. The IC50 value of the standard was 31.2µg/ml while that of the sample was found to be a little higher at 62.5µg/ml. Nevertheless it can be seen that

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camptothecin acts as an effective anti-cancer agent against the HeLa cell line. To confirm the anti-cancer activity, cell cycle analysis was carried out. It can be concluded that the control had normal cell cycle while the sample did not. Thus camptothecin can be used as an effective anti-cancer agent. With further research, it can be used as a lead compound in the pharmacological industry, due to its beneficial biological properties.

Conflict of interest: NIL

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