



***In-vitro* Anti-cancer Activity of Endophytic Fungi Isolated from *Ziziphus mauritiana* in Cervical Cancer Cell Line**

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Authors' contributions

The complete work was done in teamwork among all authors. Author HS performed the whole experiment, wrote the procedure, did the statistical analysis, wrote and edited the final draft of the manuscript. Authors MSA and VA designed the study, managed the analysis of the study and literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The fungal crude extract of *Trichoderma viride* isolated from the medicinal plant of *Ziziphus mauritiana* was investigated for anti-cancer activity against HeLa cell line.

Place and Duration of Study: The entire research work was done in the Department of Biotechnology, Mohammed Sathak College of Arts and Science, Chennai, India and Stellixir Biotech Pvt Ltd, Bangalore, Karnataka, between April 2019 to June 2019.

Methodology: In MTT assay, cells were seeded in 96 well plates with concentrations of 2.5, 25, 50, 100, 200 µl/ml of fungal bioactive compound and the absorbance was measured at 570 nm. Apoptosis was carried out via flow cytometry using control Camptothecin and fungal extract concentration. Subsequently, cell cycle arrest was determined by 400 µl PI/RNase staining buffer

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and further analyzed using PI solutions by flow cytometry. Finally, the damage of the nucleus observed under a Fluorescent microscope using DAPI staining.

Results: MTT assay revealed an obtained fungal compound showed IC50 concentration against the HeLa cells at 23.57 µg/ml. Apoptosis analysis suggested under the M1 population the viable cells are low in mean Annexin V-FITC while M2 is the apoptotic cells have taken up the Annexin V-FITC and high in mean Annexin V-FITC fluorescence intensity. The cell cycle arrest has been noted by decreasing the percentage of arrest in the G0/G1 phase and increased in S and G2/M phase compared to controls. In the course of DAPI staining, it has been understood that IC50 23.57 µg/ml has an efficient nuclear dent after the treatment.

Conclusion: *In-vitro* studies revealed that *Trichoderma viride* has promising cytotoxicity effect against the HeLa cell line. Hence the bioactive compounds of 3-beta-hydroxy urs-12-en-28-oic acid which is responsible for the treatment of cervical cancer. However *in-vivo* experiments need to be conceded out for further studies.

Keywords: Anti-cancer; cytotoxic; HeLa; MTT; apoptosis; DAPI.

1. INTRODUCTION

Cancer is the world's second leading cause of death in humans. Among them, cervical cancer is the most common type of cancer affects females globally. More than 500,000 women are diagnosed every year, even though the mortality rate was highly increased in worldwide. Existing cervical cancer treatment such as radiotherapy, hysterectomy, and surgery, but these treatments induce several side effects including fatigue, weight loss, loss of hair, tiredness and so on. To overcome this fact, scientists paid more attention to novel compounds from natural sources in specific endophytic fungi [1].

An endophytic fungus belongs to a fungal microorganism that colonizes in the living cells of healthy plant tissues by protecting themselves without causing any apparent effects. Endophytes show suspicious mechanisms against insects and herbivores. Also, endophytic fungi in the plants extend biotic and abiotic stress tolerance. Million of endophytic fungi exist in the various plant kingdoms. On considering this fact, researchers investigated endophytes due to its several secondary metabolites obtained from medicinal plants. Due to the evolution, several endophytes can produce bioactive compounds similar to the plant hosts [2]. In association with the plant and endophyte, adapt the secondary metabolite production in the host plant. The secondary metabolites isolated from plant endophytic fungi exhibit an extensive range of pharmacological properties including antiviral, antibacterial, anticancer, antifungal, anti-malarial, anti-diabetic activity and also possible application in agriculture and food industry [3].

Trichoderma viride is an asexual endophytic fungus found especially in the soil. It is a rapidly

growing fungus, spore former, cell wall degrading enzymes with significant antibiotic properties [4]. *Trichoderma viride* also has effective antagonistic activity among other soil-borne pathogens thereby it protects the plant from infectious diseases. Researchers investigated that these endophytes produce many bioactive compounds namely terpenoids, alkaloids, quinones, steroids, phenols, lactones, gliotoxin, enzymes such as L-Lysine α -oxidase, etc [5,6]. Hence these metabolites act as potential therapeutic agents against cancer and infectious diseases. The entire investigation was carried out from *Trichoderma viride* isolated from *Ziziphus mauritiana*. Though it is a medicinal plant that has major phytochemical compounds like triterpenoids, saponins, alkaloids, quercetin, sitosterol, flavonoids, stigmasterol, ursolic, betulinic, oleanolic and maslinic acid [7].

The present work aimed to determine the effect of 3-beta-hydroxy urs-12-en-28-oic acid obtained from *Trichoderma viride* against cervical cancer cell line. Further *in-vitro* anti-cancer assays were carried out namely MTT assay, apoptosis cell study by flow cytometry, cell cycle study and DAPI staining method using the HeLa cell line to understand the effect the bioactive compounds of fungal extract.

2. MATERIALS AND METHODS

2.1 Isolation, Cultivation and Extraction of Secondary Metabolites from Endophytic Fungi

Trichoderma viride was isolated from *Ziziphus mauritiana* and further, it has been mass cultivated in 500 ml of (PDB) potato dextrose broth medium incubated for 21 days. After the incubation period, the culture was filtered and

extracted using ethyl acetate with an equal volume of obtained filtrate and solvent. After evaporation, the metabolites were dissolved via DMSO to yield the final *Trichoderma* extract and stored at 4°C [8].

2.2 MTT Assay

The fungal crude extract was separated using column chromatography and the bioactive compound namely 3-beta-hydroxy urs-12-en-28-oic acid was purified and further determined for their cytotoxic effect by MTT assay. The HeLa cell lines were maintained in DMEM medium. Around 20,000 cells per well were seeded in a 96 well plate, without test agent. The cells were allowed to grow for about 24 hours. After 24 hours the cells were treated with 5 different concentrations such as 12.5, 25, 50, 100, 200 µl/ml of fungal crude extract and further incubated at 37°C in a 5% carbon dioxide atmosphere for 24 hours. After incubation, the spent medium was removed from the wells and MTT reagent was added to a final concentration of 0.5 mg/mL of total volume. The plates were covered with aluminum foil to avoid exposure to light and further incubated for 3 hours. The MTT reagent was removed and then 100 µl of solubilization solution (DMSO) was added to the corresponding wells. Further, it is kept in a gyratory shaker for gentle stirring for complete dissolution of the MTT formazan crystals especially in dense cultures and the absorbance was measured Elisa reader at 570 nm used as reference wavelength [9,10].

2.3 Apoptosis Cell Study by Flow Cytometry

Cells were seeded in 6-well plates at a density of 3×10^5 cells/2 ml and incubated overnight at 37°C for 24 hours. The spent medium was aspirated and the cells were treated with a concentration of fungal compounds along with positive control Camptothecin, in 2 ml of culture medium and incubated for 24 hours. After incubation, the medium was removed from all the wells and PBS wash was given. The PBS was removed and 200 µl of the Trypsin-EDTA solution was added and kept under 37°C for 3-4 minutes. The cells were harvested directly into 12 x 75 mm polystyrene tubes by adding 2 ml of culture medium. The tubes were centrifuged at 300 x g at 25°C for five minutes. The supernatant was decanted. Further, the cells were washed twice with PBS. The PBS was removed completely and 5 µl of FITC Annexin V was

added. The cells were vortex and incubated at room temperature under dark conditions. Then it is further treated with 5 µl of PI and 400 µl of 1X Binding Buffer was added to each tube and mixed gently. Finally, it was analyzed using flow cytometry immediately after the addition of PI [11-13].

2.4 Cell Cycle Study

The HeLa cultured cells at density of (2×10^5 cells/2 ml) were incubated in a CO₂ incubator at 37°C for 24 hours under overnight condition. The spent medium was aspirated and the cells were treated with the concentration of fungal bioactive compounds and controls, in 2 ml of culture medium and further incubated for 24 hours. After 24 hours the medium was removed from the wells and PBS wash was given. Hence the PBS was removed and 200 µl of trypsin-EDTA solution were further added and incubated for 3-4 minutes at 37°C. After incubation, a 2ml culture medium was added and the cells were harvested directly into 12 x 75 mm polystyrene tubes and centrifuge at 300 x g at 25°C for 5 minutes. Then the supernatant was removed and washed with PBS and finally, PBS was completely decanted. Later it was fixed in 1 ml of cold 70% ethanol by adding drop wise to the pellet while vortex and fixed at least 30 minutes on ice. It ensures the fixation of cells and minimizes clumping. After vortex, the supernatant was aspirated the pellet cells at a higher speed compared to live cells for 5 minutes. It should be noted that ethanol fixed cells require higher centrifugal speeds to pellet compared to unfixed cells since they become more buoyant upon fixation. Followed by PBS wash twice, pellet cells were treated with 400 µl PI/RNase Staining buffer solution to ensure that only DNA is stained (PI stains all nucleic acids). After this, cells were incubated for 10-20 minutes under room temperature. Finally, the samples were analyzed by flow cytometry in PI solution [14-17].

2.5 DAPI Staining Method

The sterile cover slips coated with Poly L-Ornithine Solution at a density of 2×10^5 cells/2 ml culture cells in a 6-well plate were placed and incubated overnight at 37°C for 24 hours under CO₂ incubator. The spent medium was aspirated and the cells were treated with 2 ml of culture medium with a required concentration of experimental compounds and controls and incubated for 24 hours. At the end of the treatment, the medium was removed from all the

wells and washed with PBS. Later PBS was completely removed from the wells. Finally, the cover slips from the six-well plates were removed and washed with 1 ml 1X DPBS. The cells were stained with 200 μ L of DAPI Staining solution (Dilute the DAPI stain solution (TCL087) with 1X PBS Solution in 1:9 proportions) for 10 minutes under the absence of light and cover slips were washed with 1X DPBS 2-3 times and drop of mounting medium were fixed before imaging and observed under fluorescence microscope with filter cube with Excitation 358 nm and Emission 461 nm for DAPI [18,19]. Images were captured and analyzed using Cell Sens Software.

3. RESULTS AND DISCUSSION

3.1 Cytotoxic Effect of Fungal Crude Extract on HeLa Cancer Cells

The 3-beta-hydroxy urs-12-en-28-oic acid from *Trichoderma viride* was treated with HeLa cell lines at different concentrations for the efficacy of anticancer activity. The HeLa cell lines showed major responsibility towards the fungal metabolites. Hence the above compound obtained from *Trichoderma viride* has significant anti-cancer activity against the HeLa cell line at the concentration of 23.57 μ g/ml. Further, the cell lines were treated with different concentrations of extracts and the morphology was identified under the microscope. It was observed that compared to the positive control Camptothecin, the test crude extract has significant cytotoxic potential

properties with IC50 concentration at 23.57 μ g/ml. The fungal crude extract can kill 50% of viable cells against HeLa cells 23.57 μ g/ml respectively after the treatment of 24 hours of incubation at 37°C (Figs. 1 and 2). Therefore it has suggested that the test fungal compound having an effective cytotoxic effect against human cervical cancer cells.

3.2 Apoptosis of HeLa Cells

In general cell death occurs by apoptosis or necrosis which can be differentiated using the coloring agent Propidium Iodide (PI). In apoptotic cells, the phospholipid phosphatidylserine (PS) membrane is trans-located from the inner to the outer leaflet of the plasma membrane, which exposes PS to the external cellular environment. Since the externalization of PS occurs in the earlier stages of apoptosis, FITC Annexin V staining can identify apoptosis at an earlier stage than assays based on nuclear changes such as DNA fragmentation. Therefore, staining with FITC Annexin V is typically used in conjunction with a vital dye such as Propidium Iodide (PI) or 7-Amino-Actinomycin (7-AAD) which identifies the early apoptotic cells (PI negative, FITC Annexin V positive). From the result, it has been shown that the lower-left quadrant population represents the viable cells 92.60%, 54.72% and 57.60% of cells were found in untreated, standard and test compounds namely fungal crude extract-treated cells respectively in lower left quadrant. The upper left quadrant represents cells debris or necrotic cells 2.26%, 0.25% and

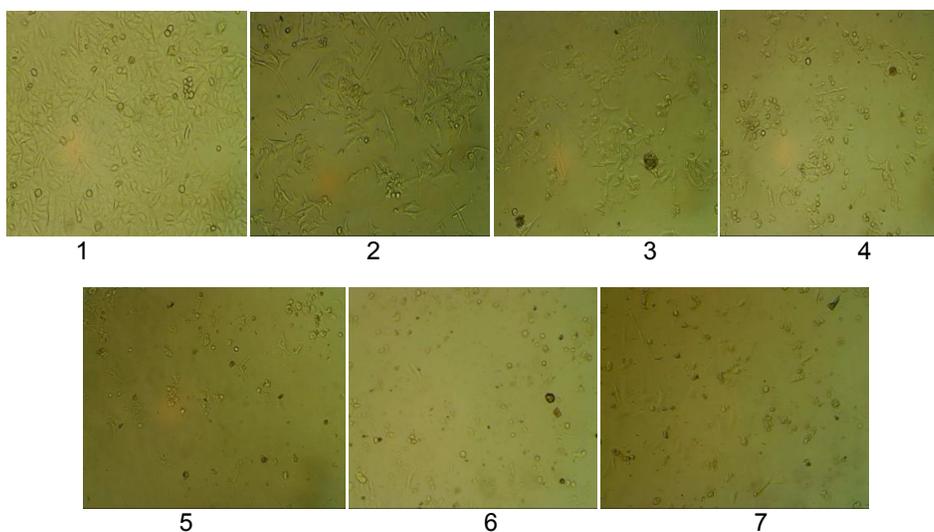


Fig. 1. Microscopic identification of cancer cell line at different concentrations of fungal crude extract (1. HeLa Cell Control; 2. 12.5 μ g; 3. 25 μ g; 4. 50 μ g; 5. 100 μ g; 6. 200 μ g; 7. Standard Control-Camptothecin)

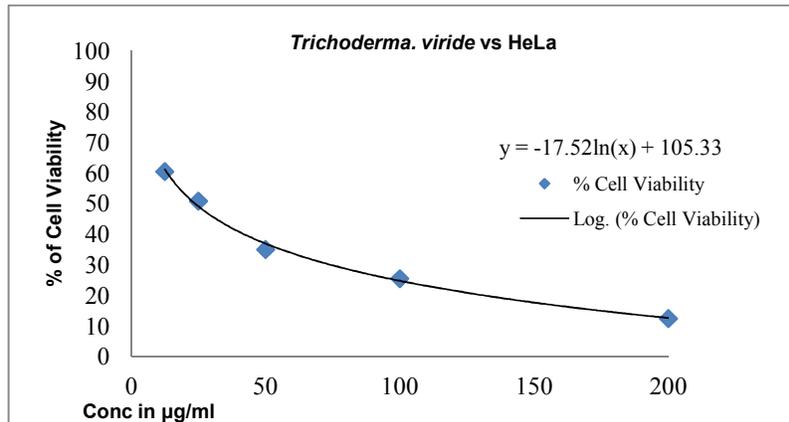


Fig. 2. The cytotoxic effect shows the percentage of cell viability of HeLa Cell line against crude extract and the Inhibitory Concentration (IC50 Value) observed is 23.57 µg/ml

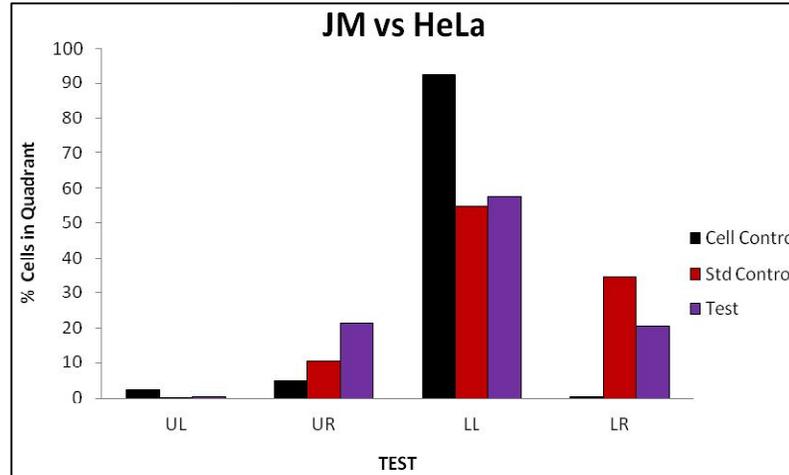


Fig. 3. Percentage of necrotic cells, late apoptotic cells, viable cells and early apoptotic cells gated in the different samples like untreated, standard drug and fungal compound treated cells. TV- *Trichoderma viride*

0.52% of cells were found in untreated, standard and test compounds namely fungal crude extract-treated cells respectively in upper left quadrant. The upper right quadrant represents late apoptotic cells 4.82%, 10.38% and 21.24% of cells were found in untreated, standard and test compound namely fungal crude extract-treated cells respectively in the upper right quadrant. Lower right quadrant represents early apoptotic cells 0.32%, 34.65% and 20.64% of cells were found in untreated, standard and test compounds namely fungal crude extract-treated cells respectively in lower right quadrant (Figs. 3 and 4). The M1 populations are the viable cells that are low in mean Annexin V-FITC fluorescence intensity While M2 populations are

the apoptotic cells that have taken up the Annexin V - FITC and are high in mean Annexin V-FITC fluorescence intensity.

3.3 Cell Cycle Analysis

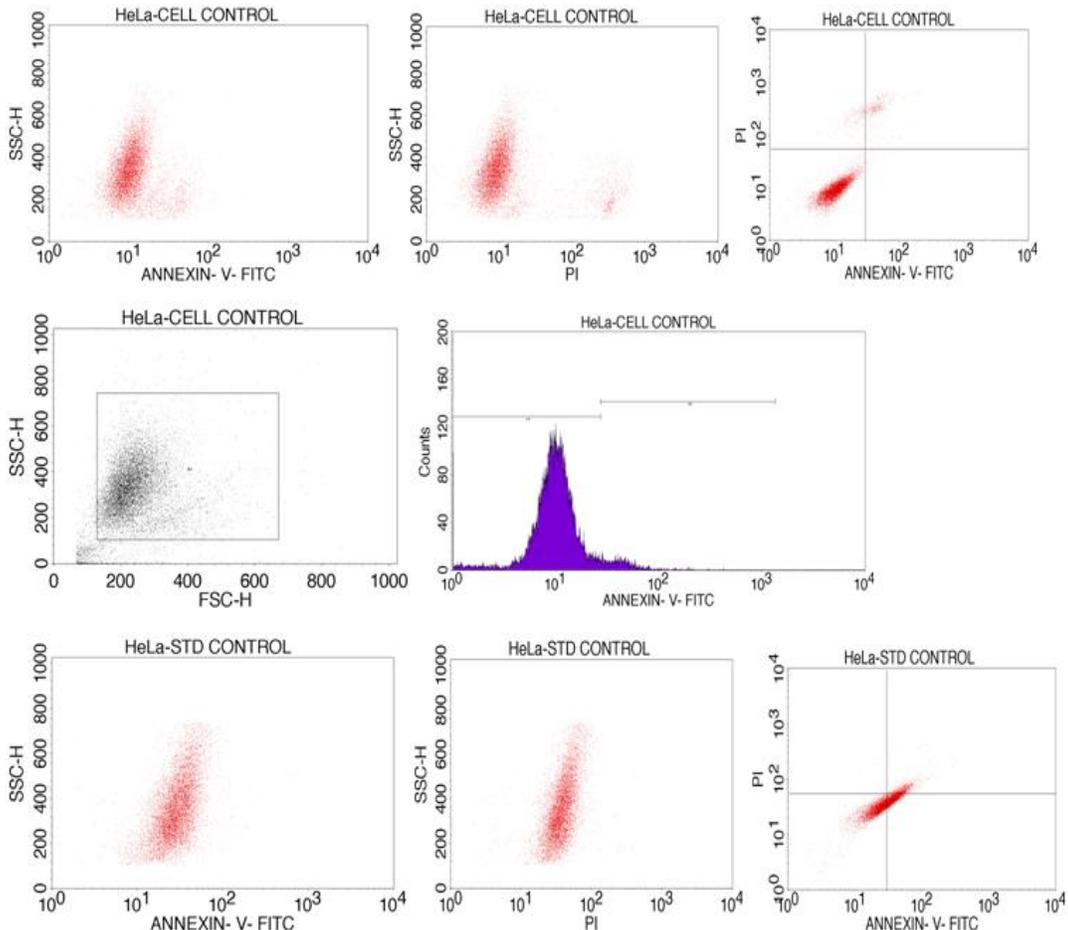
The HeLa cells were treated with different concentrations of fungal crude extract and it was stained with propidium iodide for determination of cell death or undergoing apoptosis by flow cytometry. In these study different cell cycle phases such as Sub-G0/G1, G0/G1, S, and G2/M, cell population distribution can be identified. In the G0/G1 phase, 70.23%, 46.93% and 55.73% of cells get arrested in untreated, standard control and test compound respectively.

In S phase, 4.24%, 9.79% and 13.53% of cells get arrested in untreated, standard control and fungal crude extract respectively and on the other hand, in G2/M phase, 21.83%, 30.20% and 23.92% of cells get arrested in untreated, standard control and fungal crude extract respectively (Table 1). 10,000 singlet cells were selected for the analysis for all samples including cell control. The Observations in statistical data of cell cycle study by flow cytometry against HeLa cells suggesting that compared to cell control, after the drug treatment of fungal crude extract with the IC50 Concentration, it is showing that number of cells get arrested in the G0/G1 phase (55.73%) and in G2/M phase (23.92%) of cell cycle stages of Human Cervix Cancer cells and standard drug used in this study is Camptothecin with the concentration of 15 μm arresting the HeLa Cells at G0/G1 phase and G2/M phase at 46.93% and 30.20% respectively (Figs. 5 and 6. The fungal crude extract showing considerable cell cycle arrest by decreasing the percentage of cell cycle arrest in the G0/G1

phase compared to the positive controls and also increasing the % of cell cycle arrest in S and G2/M phase compared to the controls. It is confirmed that the fungal crude extract showing good cell cycle arrest against the HeLa Cells. The number of HeLa cells gets arrested in G2/M and S Phase compared to the standard control and the compound has good therapeutic potential against the human cervix derived diseases.

3.4 DAPI Staining Method

The DAPI is a nucleic acid stain that binds to A-T rich regions of DNA along the minor groove. It is one of the hallmarks of apoptotic cells. In this study *T. viride*, a bioactive compound with IC50 23.75 $\mu\text{g/ml}$ concentration is used to study nuclear damage after drug treatment. After treatment, the slides were observed under 20X and 40 X fluorescent microscope and images were captured and analyzed using Cell Sens Software (Fig. 7).



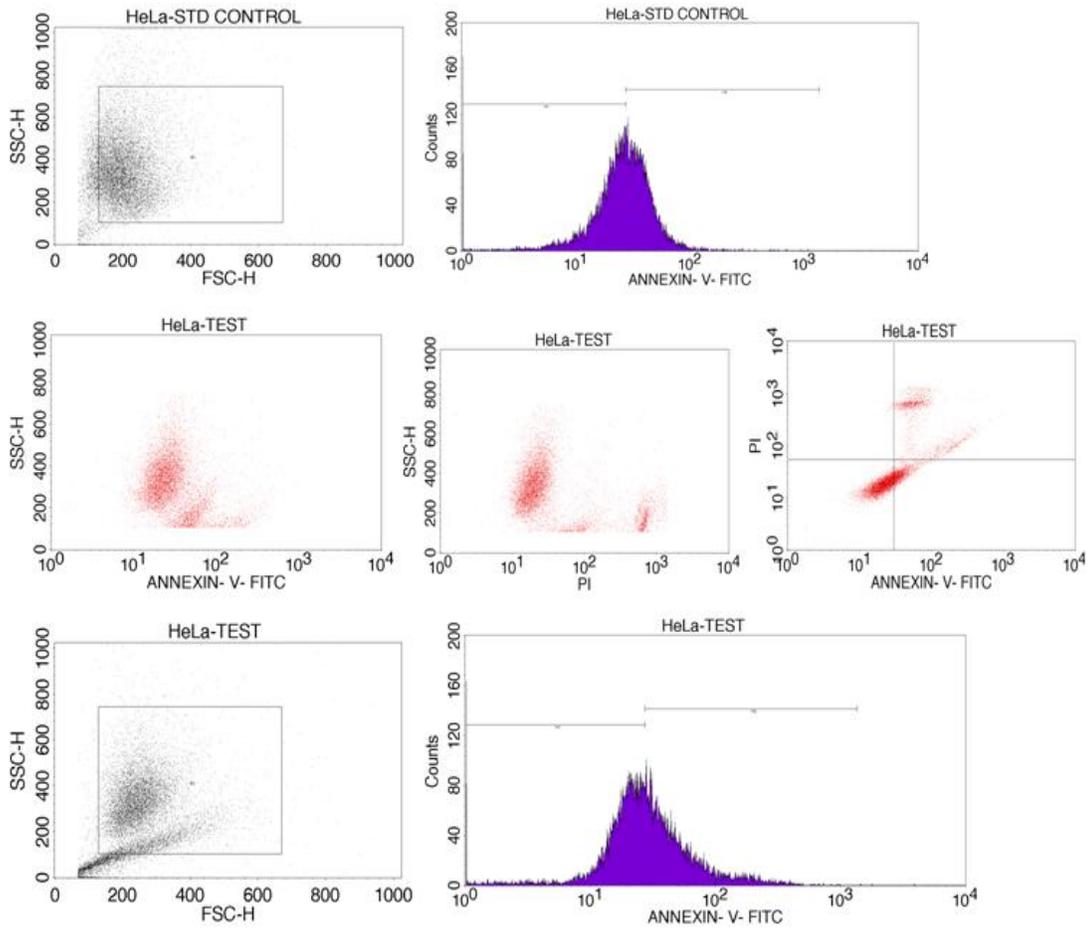


Fig. 4. Induction of apoptosis by *Trichoderma viride* extract and authorization by Annexin V-FITC/PI dual staining against HeLa cells using BD FACS Calibur, Cell Quest Pro Software (Version: 6.0)

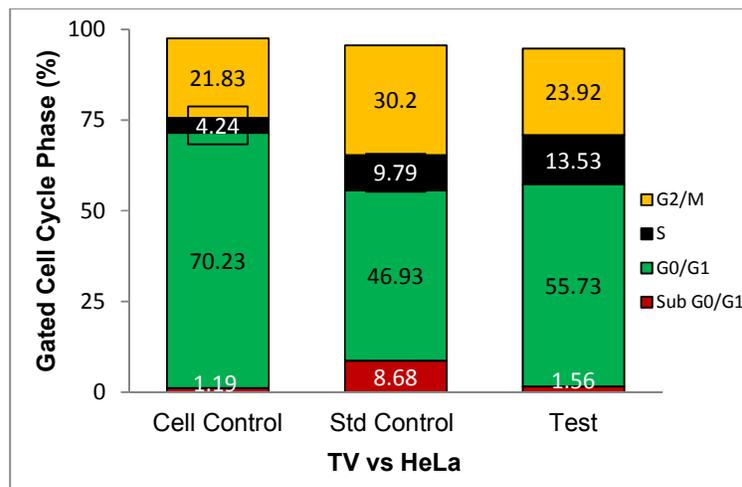
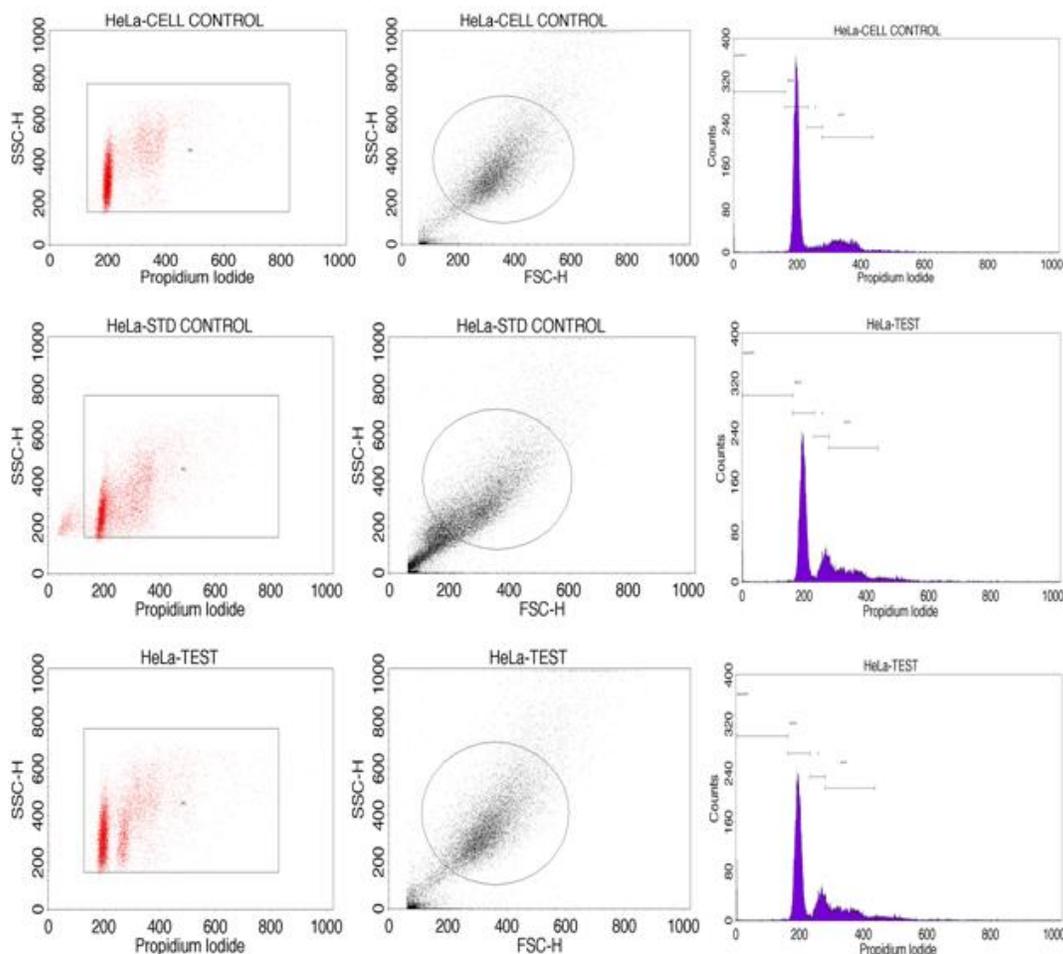


Fig. 5. Percentage of cell cycle analysis after the treatment of *Trichoderma viride* extract along with cell control and standard control

Table 1. Details of drug treatment to respective cell line used for the study

S. No	Test compounds	Cell line	Concentration treated to cells
1	Untreated	HeLa	No treatment
2	Standard (Camptothecin)	HeLa	15 μ m
3	Test Compound-1 (TEST)	HeLa	23.57 μ g

**Fig. 6. Cell cycle analysis of the HeLa cell line treated with a bioactive compound of *Trichoderma viride* at IC50 concentration along with cell control and standard control**

In ancient history, many natural products have been used for the treatment of various diseases in mankind. Among them, medicinal plants have vital therapeutic properties with effective treatment in cancer. Novel natural compounds can be obtained from potential sources of microorganisms, especially in endophytic fungi. Many researchers and organizations have investigated and proved that this endophytic fungus produces novel known and unknown new chemical compounds called secondary metabolites which have remained the matter of

frequent investigation groups throughout the world [20]. Many novel drugs have been isolated from various endophytic fungi especially one of the novel compounds is Taxol produced from the various endophytic fungus from multiple medicinal plants and has been well documented with the effect of anti-cancer activity.

Similarly, secondary metabolites such as camptothecin, podophyllotoxin, and vinblastine have also been obtained from the endophytic fungi. Initially many novel anti-cancer drugs have

been produced from the numerous medicinal plants, especially to obtain large quantities of drugs most the host plants has to be destructed, and due to this, there is an increasing demand of drugs in the pharmaceutical industry which cannot expect hope in the lack of unconventional sources [21,22]. Comparing to plant sources, many researchers suggested that endophytic fungi from the host plants which produces effective anti-cancer drugs with non-toxic, cost-effective, instant expenditure and high yield [23].

Kusakabe et al. investigated new antitumor agents in the aqueous extract of a wheat bran culture of *T. viride* isolated from soil. Similarly, *Trichoderma viride* produces active enzyme L-lysine oxidase [24]. Also, many species of *Trichoderma* act as a potential biocontrol agent in plant diseases but recent studies suggested that *Trichoderma* species have various pharmacological properties. Therefore, the cytotoxic activity of the metabolites isolated from *Trichoderma viride* has been investigated against HeLa cell lines. The cytotoxicity of many natural fungal extracts besides human cervical and breast cancer cell lines (MCF-7 and HeLa) has been investigated in several studies. Cytotoxicity assay is the method primarily used for screening

novel substances within a petite time to establish cytotoxicity on cancer cells. Trichoderone is a cytotoxic cyclopentenone isolated from *Trichoderma* species and the cytotoxic activity was evaluated against various cell lines [25].

Traditional methods are used for the treatment of cervical cancer are chemotherapy, radiation, and surgery. Through continuous exposure to radiation leads to damage DNA along with healthy cells, therefore it inhibits normal cell growth. Hence all this existing cancer treatment has many side effects in woman's thereby losing their confidence throughout their life span. Therefore in the present study, the fungal crude extract of *Trichoderma viride* from *Ziziphus mauritiana* was investigated for assessment of potential anti-cancer activity in the HeLa cell line. Hence the results have proved the obtained fungal crude extract has potent bioactive compounds especially pentacyclic triterpenoids, flavonoids, quinones which has anti-cancer activity against human cervical cancer in a contingent manner. The bioactive compound 3-beta-hydroxy urs-12-en-28-oic acid specifically present in *Ziziphus mauritiana* due to the biotransformation this compound is transformed in the fungal crude extract of *Trichoderma viride*.

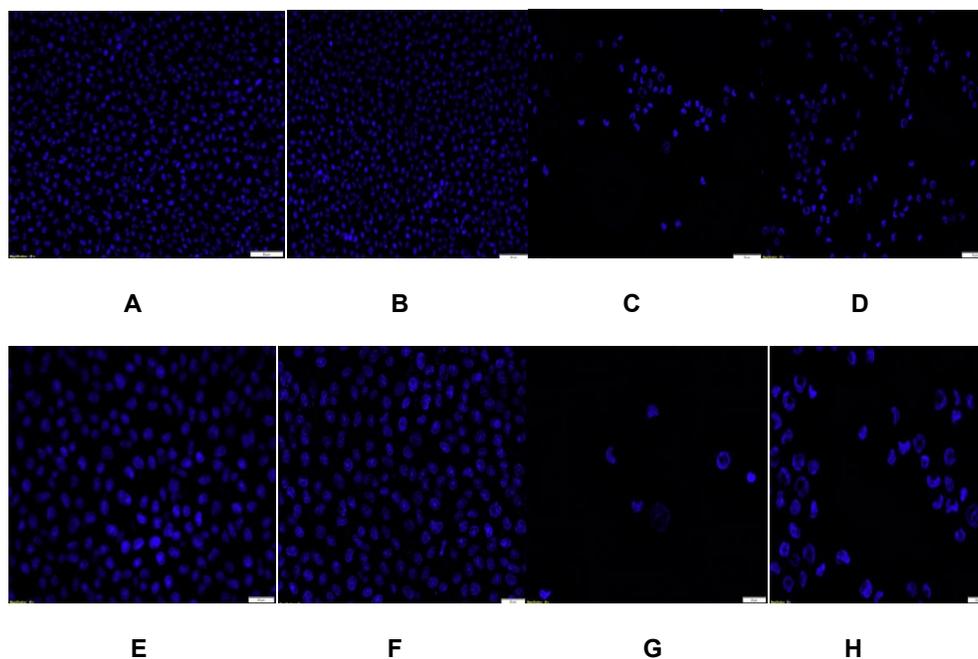


Fig. 7. Schematic representation of the fluorescent microscope examination of the HeLa cell line (20 X and 40 X magnification). 20 X Magnification – A and B: HeLa Control cells; C and D: HeLa cells treated with the fungal compound. 40 X Magnification E and F: Control cell-HeLa; G and H: HeLa cells treated with the fungal extract

Therefore in the early apoptosis cells are FITC Annexin V positive and PI negative, and cells that are in late apoptosis or already dead are both FITC Annexin V and PI positive. Investigation denotes the viable cells from M1 populations are low in mean Annexin V-FITC while M2 are apoptotic cells taken up the Annexin V-FITC with high in mean Annexin V-FITC fluorescence intensity. The fungal extract showing substantial cell cycle arrest by decreasing the percentage in the phase of G0/G1 cell cycle arrest comparing to controls but increasing the percentage of cell cycle arrest in S and G2/M phase compared to controls. Therefore it has been noted that the *Trichoderma viride* extract showed effective cell cycle arrest against the HeLa cell line. Through the DAPI staining method, it has been clearly understood the test compound with IC₅₀ 23.57 µg/ml concentration has effective nuclear damage after the treatment.

4. CONCLUSION

The experimental reports suggested that the bioactive compound namely 3-beta-hydroxy urs-12-en-28-oic acid from *Trichoderma viride* harbor potential anticancer activity in the human cervical cell line by inhibiting the proliferation of cancer cells by inducing apoptotic cells. Hence this compound belongs to pentacyclic triterpenoids which has many pharmacological properties, especially in anti-tumor. Therefore it has efficient therapeutic activities for the treatment of cervical cancer. However, further work needs to be conceded out via in-vivo studies.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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