

Evaluation of Anticancer Property of Claviceps Purpurea Secondary Metabolites on Different Cancer Cell Lines

Lokesh ST

Dept. of Microbiology, Kuvempu University

Thippeswamy Basaiah (✉ thippeswamyb272@yahoo.in)

Dept. of P.G. Studies and Research in Microbiology, Bioscience Complex, Kuvempu University, JnanaSahyadri, Shankaraghatta-577451, Shivamogga District, Karnataka, INDIA <https://orcid.org/0000-0002-6436-0289>

Sowmya HV

Dept. of Microbiology, Kuvempu University

Vijaya Kumar ML

National Education Society

Research

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Abstract

The present study deals with the secondary metabolites of *Claviceps purpurea* extract, which has been confirmed as an empirically potential cure for cancer. The purpose of this research was to prove scientifically the anticancer potential of ethyl acetate extract as its effects on the cell cycle, membrane apoptosis, DNA fragmentation and nuclear staining of lung cancer (A549) cell lines. The MTT method was used to measure cytotoxic effects, ethyl acetate extract of *Claviceps purpurea* showed IC_{50} value of 56.38 $\mu\text{g/ml}$ compare to other cancer cell lines like breast cancer (MCF-7) cell lines (90.04 $\mu\text{g/ml}$) and cervical cancer (HeLa) cell lines (84.97 $\mu\text{g/ml}$) and normal cell lines fibroblast (3T3L1) does not show IC_{50} values due to lesser percentage of inhibition. The effect of inhibition of cell cycle and induction of cell apoptosis was measured by the flow cytometry method. DNA fragmentation studied by the gel electrophoresis method. The results showed, the ethyl acetate extract of *Claviceps purpurea* gives an induction of apoptosis effect on A549 cancer cell through inhibition of cell cycle in the G0-G1, Synthesis, and G2/M phases. In necrotic cell regions apoptosis was found. In DNA fragmentation 80 $\mu\text{g/ml}$ concentration of extract showed significant results against A549 cell lines. In the nuclear staining study, membrane blebbing will be found. It indicates the extract was very active against cancer cell lines and it occurs in late apoptotic phase. In this study of all parameters, Colchicine was used as standard for cell cycle studies, for membrane apoptosis studies vinblastine and DNA fragmentation studies hydrogen peroxide was used.

Introduction

The *Claviceps purpurea* (Fr.) Tul. (Hypocreaceae) is probably the best-known species of the *Claviceps* genus due to its pharmacological activities. The fungus produces different ergot alkaloids (ergoline and clavine type) with a wide range of biological activities. The broad spectrum of ergot alkaloids effects is mostly based on their interactions with adrenergic, serotonergic or dopaminergic receptors, as well as on their interference with some cellular and molecular processes. The ergoline alkaloids are used in the treatment of uterine atonia, postpartum bleeding, migraine, orthostatic hypotension, cerebral insufficiency, hyperprolactinemia and Parkinson disease. The antitumoral effect observed in the case of some clavine-type alkaloids reinforced the interest for *Claviceps purpurea* as a possible source of new oncotherapeutical agents (Craita *et al.*, 2011). The therapeutic application of microbial metabolites provided the opportunity for the discovery of antibiotics (e.g., Penicillin, Erythromycin, Streptomycin, Amphotericin etc.) and anticancer agent (e.g., Daunorubicin, Doxorubicin, Bleomycin and Pentostatin). Chemotherapy is one of the potential treatments for prolonging the patient's life. Almost 60% of anticancer drugs are of natural origin, such as plants (i.e., Vincristine, Irinotecan, camptothecines) and microorganisms (i.e., Doxorubicin, Dactinomycines, Mitomycin and bleomycin) (Senthilraja *et al.*, 2015).

Microorganism produce secondary metabolites such as enzyme, growth hormone, antimicrobial and anticancer substances. The secondary metabolites were potential drugs for treatment of newly developing diseases in humans, plants and animals. Lack in advance knowledge of fungi and their medicinal activity has encouraged researchers to investigate the importance of fungal microorganisms

particularly the secondary metabolites produced by these microorganisms as potential anticancer agents (Kumala *et al.*, 2007). The antibiotics have been useful in our battles against infectious bacteria and fungi for over 50 years. However, many antibiotics are used commercially, or are potentially useful, in medicine for activities other than their antibiotic action. They are used as antitumor agents, immunosuppressive agents, hypocholesterolemic agents, enzyme inhibitors, antimigraine agents, and antiparasitic agents (Demain, 1999).

Traditionally, the *in vitro* determinations of toxic effects of unknown compounds have been performed by counting viable cells after staining with a vital dye. Alternative methods used are measurement of radioisotope incorporation as a measure of DNA synthesis, counting by automated counters and others which rely on dyes and cellular activity. The MTT system is a means of measuring the activity of living cells via mitochondrial dehydrogenases. The MTT method is simple, accurate and yields reproducible results. The key component is (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) or MTT, is a water-soluble tetrazolium salt yielding a yellowish solution when prepared in media or salt solutions lacking phenol red.

The two most obvious features of the cell cycle are the synthesis and duplication of nuclear DNA before division, and the process of division itself -mitosis. These two components of the cell cycle are usually indicated in shorthand as the synthesis phase (S phase) and mitosis (M). When the S phase and M phase of the cell cycle were originally described, it was observed that there was a temporal delay or gap between mitosis and the onset of DNA synthesis, and another gap between the completion of DNA synthesis and the onset of mitosis. These gaps were termed G1 and G2 respectively (Joany *et al.*, 1998). The use of fungus in the regression of certain forms of cancer has been recognized for more than a century. Important advances have been made to study and develop live fungal products such as proteins, enzymes, immunotoxins and secondary metabolites which specifically target cancer cells and cause tumor regression through growth inhibition, cell cycle arrests or apoptosis induction (Vijaya *et al.*, 2014).

Apoptosis and necrosis are two distinct forms of cell death in mammals. Unlike necrosis, which can invade large populations of cells, apoptosis normally triggers the death process only in a single cell. Furthermore, necrosis is an accidental cell death that occurs as a result of severe physical or chemical changes. while apoptosis not only occurs during natural fetal development but can be also induced by various stimulators, including drug treatment and other stress conditions. Since mis regulation of apoptosis, including both excessive or reduced cell death, normally leads to various human diseases such as cancer, apoptosis-based therapies are considered new biological approaches for the treatment of such abnormalities (Azadeh *et al.*, 2014).

Acridine orange/ethidium bromide (AO/EB) staining is used to visualize nuclear changes and apoptotic body formation that are characteristic of apoptosis. Cells are viewed under a fluorescence microscope and counted to quantify apoptosis. The aim of the study was to screen metabolites from *Claviceps purpurea* for study of *in vitro* anticancer properties against human cancer cell lines by nuclear staining studies, DNA fragmentation analysis, cell cycle analysis and membrane apoptosis studies.

Materials And Methods

Cell lines and culture medium

Human lung carcinoma (A549), MCF-7 (breast adenocarcinoma cells), Hela (cervical adenocarcinoma cells) and nonmalignant fibroblast 3T3L1 were used for anticancer activity. The stock cells were cultured in DMEM/RPMI supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) in a humidified atmosphere of 5% CO₂ at 37°C until confluent. The cell was dissociated with cell dissociating solution (0.2 % trypsin, 0.02 % EDTA, 0.05 % glucose in PBS). The viability of the cells is checked and centrifuged. Further, 50,000 cells /well was seeded in a 96 well plate and incubated for 24 hrs at 37°C, 5 % CO₂ incubator.

Cytotoxicity studies by MTT assay

The monolayer cell culture was trypsinized and the cell count was adjusted to 5.0×10^5 cells/ml using respective media containing 10% FBS. To each well of the 96 well microtiter plate, 100 µl of the diluted cell suspension (50,000 cells/well) was added. After 24 hrs, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different test concentrations of drugs were added on to the partial monolayer in microtiter plates. The plates were then incubated at 37°C for 24 hrs in 5% CO₂ atmosphere. After incubation the test solutions in the wells were discarded and 100 µl of MTT (5 mg/10 ml of MTT in PBS) was added to each well. The plates were incubated for 4 hrs at 37°C in 5% CO₂ atmosphere. The supernatant was removed and 100 µl of DMSO was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 590 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (IC₅₀) values is generated from the dose-response curves for each cell line. The IC₅₀ value is determined by using the formula i.e. % Inhibition = (OD of Control – OD of sample)/OD of Control) x 100 (Vijay *et al.*, 2014).

Cell cycle Analysis

The number of cells (1×10^6) were seeded and cultured for 24 hrs in a 6 well plate containing 2 ml of media. Cells were then treated with desired concentrations of given samples were prepared in media and incubated for another 24 hrs cells were then harvested and centrifuged at 2000 rpm for 5 minutes at room temperature and supernatant was discarded carefully retaining the cell pellet. Cell pellet was washed by resuspending in 2 ml of 1XPBS. The washing was repeated another time with the same conditions. Supernatant was discarded retaining the pellet. Cells were fixed by resuspending in 300 µl of Sheath fluid followed by addition of 1 ml of chilled 70% EtOH drop by drop with continuous gentle shaking and another 1 ml of chilled 70% EtOH added at once. The cells were then stored at 4°C for or overnight. Post fixing, the cells were centrifuged at 2000 rpm for 5 minutes. The cell pellet was washed twice with 2 ml of cold 1XPBS. Cell pellet was then resuspended in 450 µl of sheath fluid containing

0.05mg/ml PI and 0.05 mg/ml RNaseA and incubated for 15 minutes in dark. The percentage of cells in various stages of cell cycle in fungal extracts treated, untreated and control (colchicine) populations were determined using FACS Caliber (BD Biosciences, San Jose, CA) (Ramesh *et al.*, 2011).

Membrane Apoptosis Studies

The day before induction of apoptosis, plated 1×10^6 cells per well for a 6 well plate using DMEM cell culture medium. After 18 hrs, the wells for floating (dead) cells and removed by pipette. Replaced with new culture medium to the original volume. Treated cells to induce apoptosis with samples at different concentrations, and incubate for 24 hrs Later, collected cell culture medium into 15 ml tubes. Using policeman, the cells were detached from the dish and added 1 ml of medium to each well and transferred the contents to the 15 ml tubes. Centrifuged and discarded the supernatant. Washed the cells twice with cold PBS and then resuspend cells in 1 ml 1X binding buffer at a concentration of 1×10^6 cells/ml. 500 μ l of cell suspension is aliquoted and 10 μ l of Propidium Iodide and 5 μ l Annexin V is added. The suspension is incubated for 15 minutes at RT in the dark. Post incubation, the cells were analyzed by flow cytometer as soon as possible (within 1 hour) (Seamus *et al.*, 1995).

DNA Fragmentation Studies

Cells were seeded at a concentration of 1×10^6 per 35 mm dish incubated at 37°C/5% CO₂ incubator for 24 hrs. The confluent cells grown after 24 hrs of incubation were treated with sample concentration. After treatment, cells were trypsinized, and both adherent and floating cells were collected by centrifugation at 2000 rpm for 5 minutes. The cell pellet was suspended in 0.5 ml lysis buffer (pH 7.8) [Tris-HCl 10 mM, pH 8; EDTA 20 mM, pH 8.0; TritonX-100 0.2%], vortex vigorously and incubated at 50°C for 5 minutes. To the lysate, 0.5 ml of phenol chloroform isoamylalcohol was added and mixed for 2-3 minutes. It was centrifuged at 10000 rpm for 15 minutes at 4 °C. The upper aqueous layer was taken in a new tube, to which double the volume of cold 100% ethanol was added and 3M sodium acetate was added (Final concentration of sodium acetate 0.3 M). It was incubated for 5-10 minutes at room temperature and centrifuged at 10000 rpm for 15 minutes (Saboo *et al.*, 2012).

Acridine Orange/Ethidium Bromide (AO/EB) Staining method

Approximately 25 μ l (1×10^5 cells) of treated and untreated cells were taken separately in a micro centrifuge tubes and was stained with 5 μ l of AO-EtBr (Acridine orange and Ethidium Bromide) for about 2 minutes followed by gentle mixing. The 10 μ l of cell suspension was placed onto a microscopic slide and covered with a glass coverslip and examined in a fluorescence microscope using a fluorescein filter (Shailaja *et al.*, 2006).

Statistical analysis

The differences observed between the control and treated groups for cell proliferation. Comparison between two groups were made using one-way ANOVA to assess the IC₅₀ value. The results were

expressed as the Mean \pm SEM (standard error of the mean) from three different replicates and a value of $p < 0.05$ was considered statistically significant.

Results And Discussion

Cytotoxicity studies by MTT Assay

In the present study, the *in vitro* cytotoxic effects of ethyl acetate extract of *Claviceps purpurea* on several cell lines was evaluated by micro-culture tetrazolium assay (MTT). MCF-7, HeLa, A549 cell lines and normal cell lines (3T3L1) were evaluated. The cytotoxicity of the fungal extracts was also investigated against the normal Fibroblast cell line. The ethyl acetate extract of *Claviceps purpurea* showed an exponential increase in cytotoxic activity against both the cells are MCF-7, A549, and HeLa. In A549 cell lines the ethyl acetate extract showed IC₅₀ value is 56.38 μ g/mL (Table 1). Standard vinblastine showed 20.73 μ M on MCF-7, 24.86 μ M on A549 and 21.23 μ M on HeLa cellines. For further activity based on IC₅₀ values A549 for ethyl acetate extract of *Claviceps purpurea*. Normal cellines of fibroblast (3T3L1) showed lesser % inhibition.

The increasing number of cancer cases around the world is demanding for more improved strategy for prevention as well as treatment of this dreaded disease. Approaches, which are more than just a treatment mode rather which may be included as a nutritional supplement in our regular diet, can be a better strategy to prevent carcinogenesis (Arunchand *et al.*, 2018).

MTT assay is a commonly believed *in vitro* method for screening the drugs having anticancer activity. *In vitro* anticancer activity against ethyl acetate extract of *Claviceps purpurea* on A549 cell line at different concentrations was evaluated. Standard vinblastine showed IC₅₀ values are in MCF-7, 20.73 μ M/mL, A549, 24.86 μ M/mL, HeLa 21.23 μ M/mL, and normal cell line Fibroblast (3T3L1) showed no IC₅₀ values in all the cancer cell lines.

Cell cycle studies

Ethyl acetate extract in cell cycle studies, the results were found to be as followed in order to determine the cell cycle pattern of A549 cells, vinblastine and ethyl acetate extracts were treated to cells were analyzed in flow cytometric method. The cell cycle phase distribution was quantitated from 3 independent sets of measurements that showed relatively similar pattern to ethyl acetate extract which was different than standard Colchicine. The results were showed in figure 1-5 and table 2.

The treatment of cells at the concentration of 40 μ g/ml of ethyl acetate extracts showed on G0/G1 phase 77.11%, synthesis phase 11.56% and on G2 phase 7.98% of cells were arrested. The concentration of 80 μ g/ml 19.75% of cells were arrested on G2 phase of lung cancer (A549) cells when compared to standard *Colchicine* at 25 μ M showed 44.22% of cells were arrested on G2 phase. According to (Angel *et al.*, 2011) the cell cycle analysis revealed that the proportion of cells in the G2/M phase, this indicated that doxorubicin induced cell cycle arrest at the G2/M phase. The ethyl acetate extract of F 21- E

(*Aspergillus* sp) induced an accumulation of cells showing prominent G2-M phase arrest. in the Sub-G0 phase (90.92 %).

The ability of anticancer agents to suppress the growth of cancer cells can also be associated with blocking cell-cycle progression. Based on the significant inhibition of A549 cell proliferation by ethyl acetate extract, it was postulated that the extract might modulate the cell-cycle progression of these cancer cells. Cell-cycle distribution analysis showed this extract caused a reduction in S phase population, which was associated with the accumulation of cells in the G0/G1 phase at a concentration of 40 µg/ml. A secondary metabolite of Epanorin (EP) exhibited a G1 cell cycle arrest which was similar in magnitude to that shown by TXM, a clinically used anti proliferative drug for estrogen positive breast cancer cells. Moreover, the effects on cell cycle progression were comparable to the reported for coumetarol on the MCF-7 Cells (Juan *et al.*, 2019).

Apoptosis detection using PI Annexin V-FITC staining of A549 cells by flow cytometry

Apoptotic and necrotic cell populations in both control and treated cancer cells were studied by FACS using Annexin V-FITC and Propidium Iodide. The concentration of ethyl acetate extract 40 µg/ml and 80 µg/ml has induced necrosis in A549 cells with 27.54% and 35.54% cells respectively; and Standard Vinblastine showed 11.06% and 10.22% early and late apoptotic cells when compared to control cells with 56.11% also has shown necrosis of 22.61%. The results were showed in figure 6-10 and table 3.

Apoptosis is a programmed cell death characterized by cleavage of chromosomal DNA into oligonucleosomal fragments. Apoptosis is an active form of cell death that occurs in response to several agents, including chemotherapeutic drugs. Apoptosis was initially described by its morphological characteristics, including cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation. Analysis of nuclear morphology is highly essential to examine the induction of cell death (Ramya *et al.*, 2017).

DNA fragmentation studies on A549 cell line

DNA fragmentation is an important feature in cells undergoing apoptosis. To confirm that ethyl acetate of *Claviceps purpurea* makes cell death in A549 cells via apoptosis and not necrosis, DNA fragmentation patterns in treated cells were analyzed. The activation of endogenous endonucleases results in the cleavage of chromatin into inter-nucleosomal fragments of approximately 180–200 bp; it is believed that DNA fragmentation is carried out by Caspase-activated DNase (CAD), leading to the cleavage of nuclear DNA into multiple fragments with low molecular weights. A549 cells treated with two different concentrations of ethyl acetate extract for approximately 2hrs showed inter-nucleosomal DNA fragmentation in the form of a DNA ladder, typical of apoptotic DNA fragmentation (Fig 11). The exact DNA fragmentation and the absence of a DNA smear due to non-specific DNA degradation show the apoptosis-inducing ability of ethyl acetate extract. In this study, A549 cells were treated with Sample 2 (Ethyl acetate extract) and it was found that Sample has induced comparatively more DNA damage at 80 µg/ml when compared to Standard H₂O₂ with 200 µM concentrations.

The fragmentation of genomic DNA was confirmed by DNA laddering assay to support the apoptosis induction by the extract in the cancer cells. It was observed that the treated cells showed DNA laddering pattern and a single genomic DNA band was in untreated cells. The cells treated with control positive had also DNA laddering pattern.

In ethyl acetate extract of *Claviceps purpurea* showed significant result of DNA fragmentation studies in A549 cell lines. The concentrations of 40 µg/ml and 80 µg/ml of treated cells were showed significant DNA fragmentation compare to control cells these treated cells showed better results. The standard H₂O₂ also showed more DNA damaged in cells and this activity may be contributed by the extract constituents. The fragmentation was not found in distinct, but increased DNA damage was observed, which provide evidence for apoptotic cell death.

Cancer chemotherapy drugs usually exert cytotoxic effects on malignant cells by inducing apoptosis. Major apoptotic events include cell cycle arrest and the formation of reactive oxygen species, leading to loss of mitochondrial membrane potential and DNA fragmentation. It is believed that caspase 3, a major downstream effector of apoptosis, mediates proteolytic cleavage of poly (ADP ribose) polymerase (PARP), leading to DNA fragmentation. These apoptotic events, accompanied by the potent growth inhibition of HeLa cells, were detected when the cells were treated with partially purified VCR from *T. radicus*—CrP20 (Padmini *et al.*, 2015).

AO/EtBr Staining

Dual staining was examined under a fluorescent microscope. Normal cells are seen with circular nucleus uniformly distributed in the center of the cell which is seen in the control figure 12.

In treated sample 40 µg/ml show in the early stage apoptotic cells, where cell's nucleus is showing yellow-green fluorescence by Acridine orange (AO) staining and concentrated into a crescent or granular that located in one side of cells. Staining was localized asymmetrically within the cells and membrane blebbing was seen in figure 13.

In treated sample 80 µg/ml show in the nucleus of cells were orange fluorescence by EtBr staining and gathered in concentration and located in bias, this is late apoptotic phase. Cells that have taken up complete EtBr are the necrotic cells as in Figure 14. In standard Vinblastine at 25 µM/ml showed on A549 cell lines in figure 15.

Ethyl acetate extract of *Claviceps purpurea* showed at 40 µg/ml concentration yellow-green fluorescence by Acridine orange (AO) staining and concentrated into a crescent or granular that located in one side of cells. Staining was localized asymmetrically within the cells. At 80 µg/ml concentration orange fluorescence by EtBr staining and gathered in concentration and located in bias. This is late apoptotic phase in A549 cell lines. Membrane blebbing will be found. It indicates ethyl acetate extract of *Claviceps purpurea* is very active against cancer cell lines.

According to Tayyaba *et al.*, 2018 state that the results of acridine orange (AO) and ethidium bromide (EB) staining of cells treated with AHC. The results of acridine orange (AO) and ethidium bromide (EB) staining of cells treated with AHC. AO is a cell permeable fluorescent dye and stains nuclear DNA in both live and dead cells, whereas EB is a fluorescent dye that only stains nuclear DNA in cells that have lost their membrane integrity. We observed that after AO/EB staining viable cells were uniformly stained green, early apoptotic cells were stained greenish yellow or displayed green yellow fragments, late apoptotic cells were stained orange or displayed orange fragments, and necrotic cells showed orange to red fluorescing nuclei with no indication of chromatin fragmentation.

Conclusion

In cytotoxicity studies of fungal extract of *Claviceps purpurea* showed significant anticancer activity during preliminary study by MTT Assay. In MTT assay there are 3 cancer cell lines were used against ethyl acetate extract of *Claviceps purpurea*. This extract showed significant IC₅₀ values on A549 cell lines (IC₅₀ 56.38 µg/ml). This is used for further assays of anticancer activity. In cell cycle study, ethyl acetate extract is increased and showed significant results in synthesis and G2M phases these studies were evaluated by flow cytometric method also studied membrane apoptosis studies. In membrane apoptosis study, ethyl acetate extract acts as a cell arrest in necrotic cells region. DNA fragmentation study, ethyl acetate treated extract showed significant damaged DNA bands on A549 cell lines. In Nuclear staining method ethyl acetate extract showed early stage apoptotic cells to found in DNA damage and membrane blebbing will be occur on A549 cell lines. Ethyl acetate extract of *Claviceps purpurea*, some of the secondary metabolites acts as a significant anticancerous activity against different cancer cell lines.

Abbreviations

FBS: Fetal Bovine Serum

DMEM: Dulbecco's Modified Eagle Medium

PBS: Phosphate buffer solution

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

OD: Optical density

EtOH: Ethyl alcohol

FACS: Fluorescence-activated cell sorting

AO/EB: Acridine Orange/Ethidium Bromide

ANOVA: Analysis of variance

FITC: Fluorescein isothiocyanate

PI: Propidium iodide

CAD: Caspase-activated DNase

Declarations

Conflict of Interest

The authors declare they have no conflict of interest.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

Not applicable

Competing interests

The authors declare that they have no competing interests

Funding

Not applicable

Authors' Contributions

Lokesh S.T, Sowmya H.V and Vijaya Kumar M.L. provided substantial contributions in the analysis of anti-cancer studies. Thippeswamy B contributed to the designing and supervision of the work along with drafting the article. Lokesh S.T supported in critically evaluating the work and drafting the article. All authors read and approved the final manuscript. All authors are the guarantors.

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References

1. Arunchand Rayaroth, Rajesh Singh Tomar, Raghavendra Kumar Mishra (2018) Cytotoxicity Studies of *Mortierella alpina* AIB RR2 Extracts on Hela and A549 Cell Lines. *Asian Journal of Pharmaceutics*. 12, 1084-1088.
2. Craita rosu, Ana clara Aprotosoia, Pincu Rotinberg, Daniela gherghel, Cosmin Mihai, Zenovia Olteanu, Anca Miron, Stefania Surdu, Oana Cioanca, Monica Hancianu (2011) The biochemical investigations of some *Claviceps purpurea* bioproducts and their *in vitro* citostatic potential. *Farmacia*. 59, 713-720.
3. Demain AL (1999) Pharmaceutically active secondary metabolites of microorganisms. *Applied microbiology and biotechnology*. 52, 455-463.
4. Dian Handayan (2018) Cytotoxic Activity Screening of Fungal Extracts Derived from the West Sumatran Marine Sponge *Haliclona fascigera* to Several Human Cell Lines: Hela, WiDr, T47D and Vero. *Journal of Applied Pharmaceutical Science*. 8, 055-058.
5. Joany Jackman, Patrick MO, Connor (1998) Methods for Synchronizing Cells at Specific Stages of the Cell Cycle. *Current Protocols in Cell Biology*. 8.3.20.
6. Juan Palacios-Moreno (2019) Epanorin, a lichen secondary metabolite, inhibits proliferation of MCF-7 breast cancer cells. *Biological Research*. 52, 01-12.
7. Kathamuthu Gokul Raj, Shanmugam Sambantham, Ramar Manikanadan, Chinnansamy Arulvasu, Mohan Pandi (2014) Fungal Taxol Extracted from *Cladosporium oxysporum* Induces Apoptosis in T47D Human Breast Cancer Cell Line. *Asian Pacific Journal of Cancer Prevention*. 15, 6627-6632.
8. Kumala S, Utji R, Sudarmono P, Kardono LBS (2007) Cytotoxic secondary metabolites from fermentation broth of *Brucea javanica* endophytic fungus 1.2. 11. *Res. J. Microbiol.* 2, 625-631.
9. Padmini PC, Palem Gini C, Kuriakose Chelliah Jayabaskaran (2015) An Endophytic Fungus, *Talaromyces radicus*, Isolated from *Catharanthus roseus*, Produces Vincristine and Vinblastine, Which Induce Apoptotic Cell Death. *Plos one*. DOI: 10.1371/journal.pone.0144476.
10. Ramesh Thylur P, Subramanian Senthivinayagam, Edward M. Campbell, Velusamy Rangasamy, Nithyananda Thorenoor, Gautam Sondarva, Suneet Mehrotra, Prajna Mishra, Erin Zook, Phong T. Le, Ajay Rana, Basabi Rana (2011) Mixed Lineage Kinase 3 Modulates Beta-Catenin Signaling in Cancer Cells. *Journal of biological chemistry*. 286, 37470 –37482.
11. Ramya N, Priyadharshini Prakash R, Dhivya R (2017) Anticancer activity of *Trachyspermum ammi* against MCF-7 cell lines mediates by p53 and Bcl-2 mRNA levels. *The Journal of Phytopharmacology*. 6, 78-83.
12. Saboo Shweta S, Khadabadi S, Tapadiya Ganesh G (2012) *In vitro* Evaluation of Antimitotic, Antiproliferative, DNA fragmentation and Anticancer activity of Chloroform and Ethanol extracts of *Revia hypocrateriformis*. *Asian Pacific Journal of Tropical Disease*. 503-508.
13. Seamus J Martin, Chris EM Reutelingsperger, Anne J McGahon, James A Rader, Rob CAA van Schie, Drake M LaFace, Douglas R Green (1995) Early Redistribution of Plasma Membrane Phosphatidylserine Is a General Feature of Apoptosis Regardless of the Initiating Stimulus: Inhibition by Overexpression of Bcl-2 and Abl. *The Rockefeller University Pres*. 182, 1545-1556.

14. Senthilraja P, Kathiresan K (2015) *In vitro* cytotoxicity MTT assay in Vero, HepG2 and MCF-7 cell lines study of Marine Yeast. J Appl Pharm Sci. 5, 080-084.
15. Shailaja kasibhatla (2006) Acridine Orange/Ethidium Bromide (AO/EB) Staining to Detect Apoptosis. Cold Spring Harbor Protocols. doi:10.1101//pdb. prot4493.
16. Tayyaba Afsar, Janeen H Trembley, Christine E Salomon, Suhail Razak, Muhammad Rashid Khan & Khalil Ahmed (2018) Growth inhibition and apoptosis in cancer cells induced by polyphenolic compounds of *Acaciahydasypica*: Involvement of multiple signal transduction pathways. Scientific Reports. DOI: 10.1038/srep23077.
17. Uzma Faridi, Fahad Al-Mutairi, Humaira Parveen, Sahar Khateeb (2020) An *in-vitro* and *in Silico* Anticancer Study of FDA Approved Antidiabetic Drugs Glimepiride and Empagliflozin. International Journal of Life science and Pharma Research. doi Ipr.2020.10.2. L52-57.
18. Vijaya Kumar ML, Thippeswamy B, Vasanth Raj P (2014) Cytotoxicity and Anticancer Studies of *Bacillus cereus* and *Bacillus pumilus* Metabolites Targeting Human Cancer Cells. Applied Biochemistry and Microbiology. 50, 619–623.
19. Angel Treasa Thomas (2011) *In vitro* anticancer activity of microbial isolates from diverse habitats. Brazilian Journal of Pharmaceutical Sciences.47, 280-287.
20. Azadeh Haghighitalab, Maryam M Matin, Ahmad Reza Bahrami, Mehrdad Iranshahi, Morvarid Saeinasab, Fereshteh Haghighi (2014) *In vitro* Investigation of Anticancer, Cell-Cycle-Inhibitory, and Apoptosis-Inducing Effects of Diversin, a Natural Prenylated Coumarin, on Bladder Carcinoma Cells. Z. Naturforsch. 99 -109.DOI: 10.5560/ZNC.2013-0006.

Tables

Table:1. IC₅₀ values of ethyl acetate extracts of *Claviceps purpurea* and standard Vinblastine on different cancer cell lines and normal cell lines obtained from MTT Assay

Samples	IC50			
	MCF-7	A549	HeLa	3T3L1
Ethyl acetate extract (Sample 2) in µg/ml	90.04	56.38	84.97	No activity
Vinblastine in µM	20.73	24.86	21.23	No activity

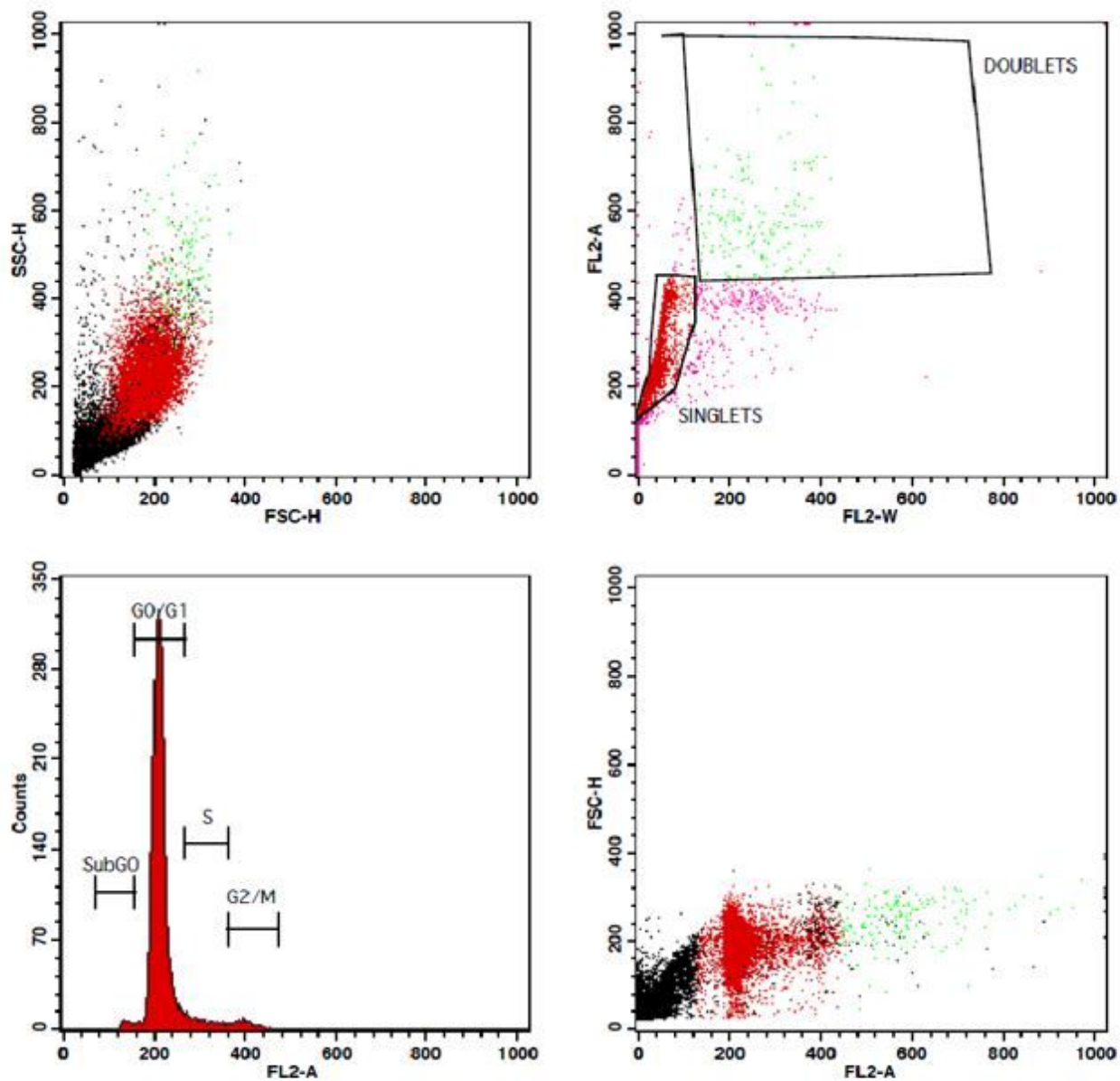
Table: 2. Flow cytometry analysis of cell cycle arrest in A549 cells

FACS analysis of Cell cycle arrest in A549 cells					
Cell line	Sample µg/ml	SUBG0	G0/G1	S	G2M
A549	Control	1.49	90.91	4.88	2.92
	Ethyl acetate 40 µg/ml	3.97	77.11	11.56	7.98
	Ethyl acetate 80 µg/ml	1.84	66.39	12.67	19.75
	Colchicine 25µM	1.61	44.22	10.26	44.38

Table: 3. Flow cytometry analysis of Apoptosis detection of A549 cells

FACS analysis of Apoptosis detection in A549 cells				
Sample µg/ml	Viable cells	Early Apoptotic	Late Apoptotic	Necrotic cells
Control	94.21	0.56	1.45	3.78
Ethyl acetate 40 µg/ml	71.48	0.13	0.85	27.54
Ethyl acetate 80 µg/ml	63.72	0	0.74	35.54
Vinblastine 25µM	56.11	11.06	10.22	22.61

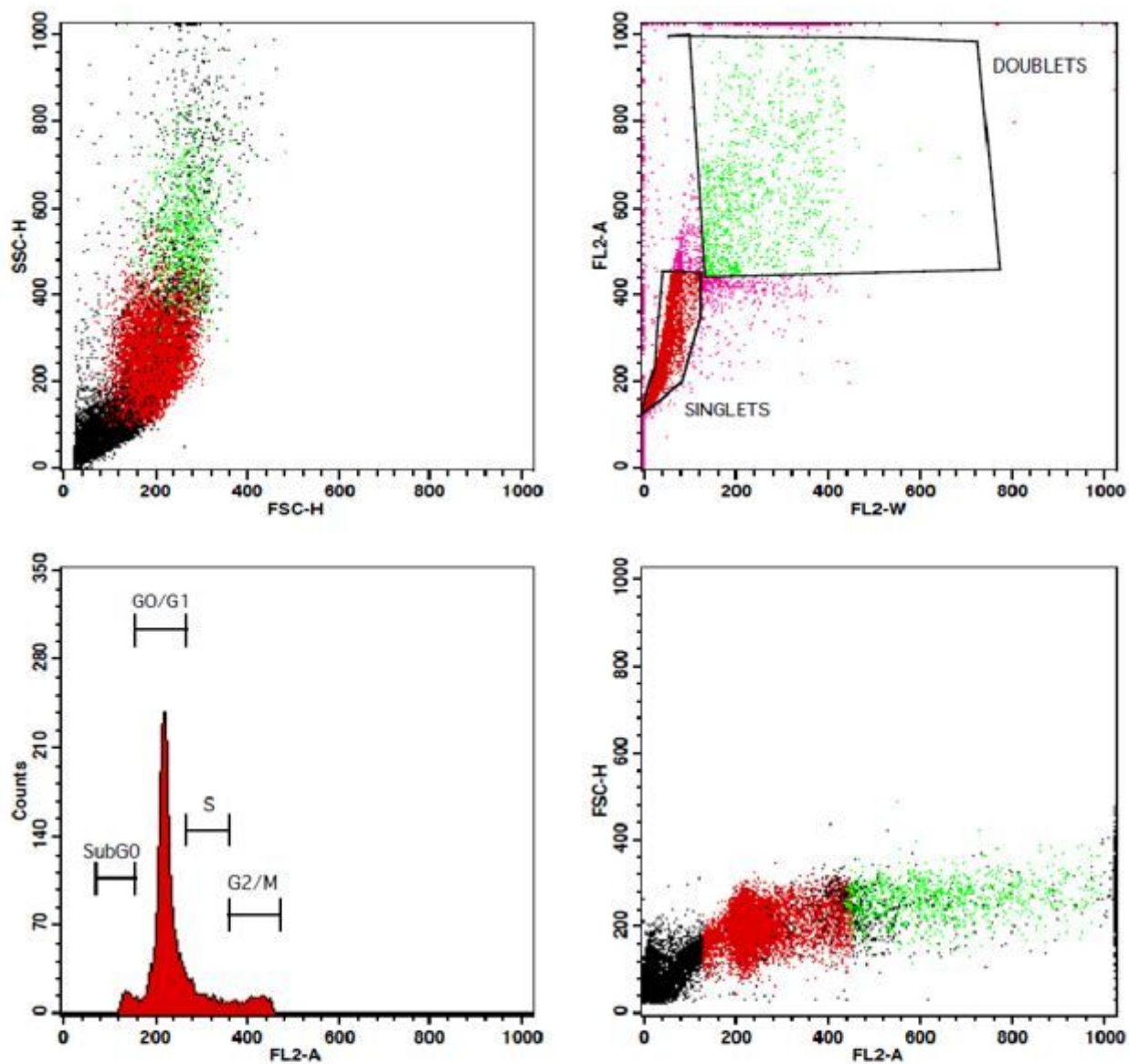
Figures



Histogram Statistics						
Marker	Left, Right	Events	% Gated	% Total	Mean	CV
All	0, 1023	10000	100.00	40.16	221.12	18.41
SubG0	72, 158	149	1.49	0.60	141.40	5.97
G0/G1	156, 268	9091	90.91	36.51	212.26	6.83
S	268, 362	488	4.88	1.96	305.52	9.00
G2/M	362, 474	292	2.92	1.17	397.91	5.25

Figure 1

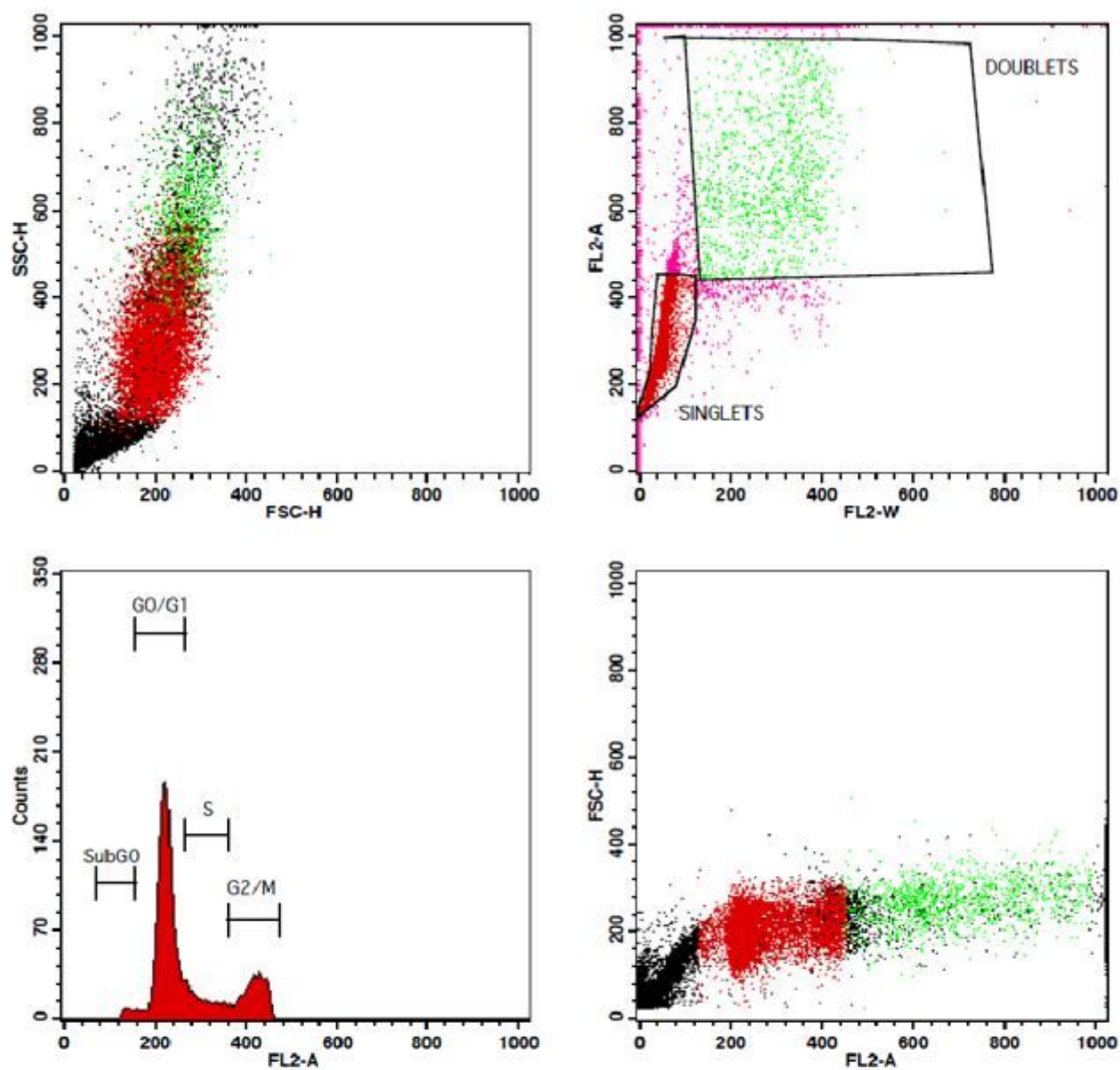
Flow Cytometry Control plots of A549 cells



Histogram Statistics						
Marker	Left, Right	Events	% Gated	% Total	Mean	CV
All	0, 1023	10000	100.00	36.86	242.53	25.43
SubG0	72, 158	397	3.97	1.46	142.03	6.13
G0/G1	156, 268	7711	77.11	28.42	221.10	8.77
S	268, 362	1156	11.56	4.26	303.61	8.76
G2/M	362, 474	798	7.98	2.94	409.43	6.25

Figure 2

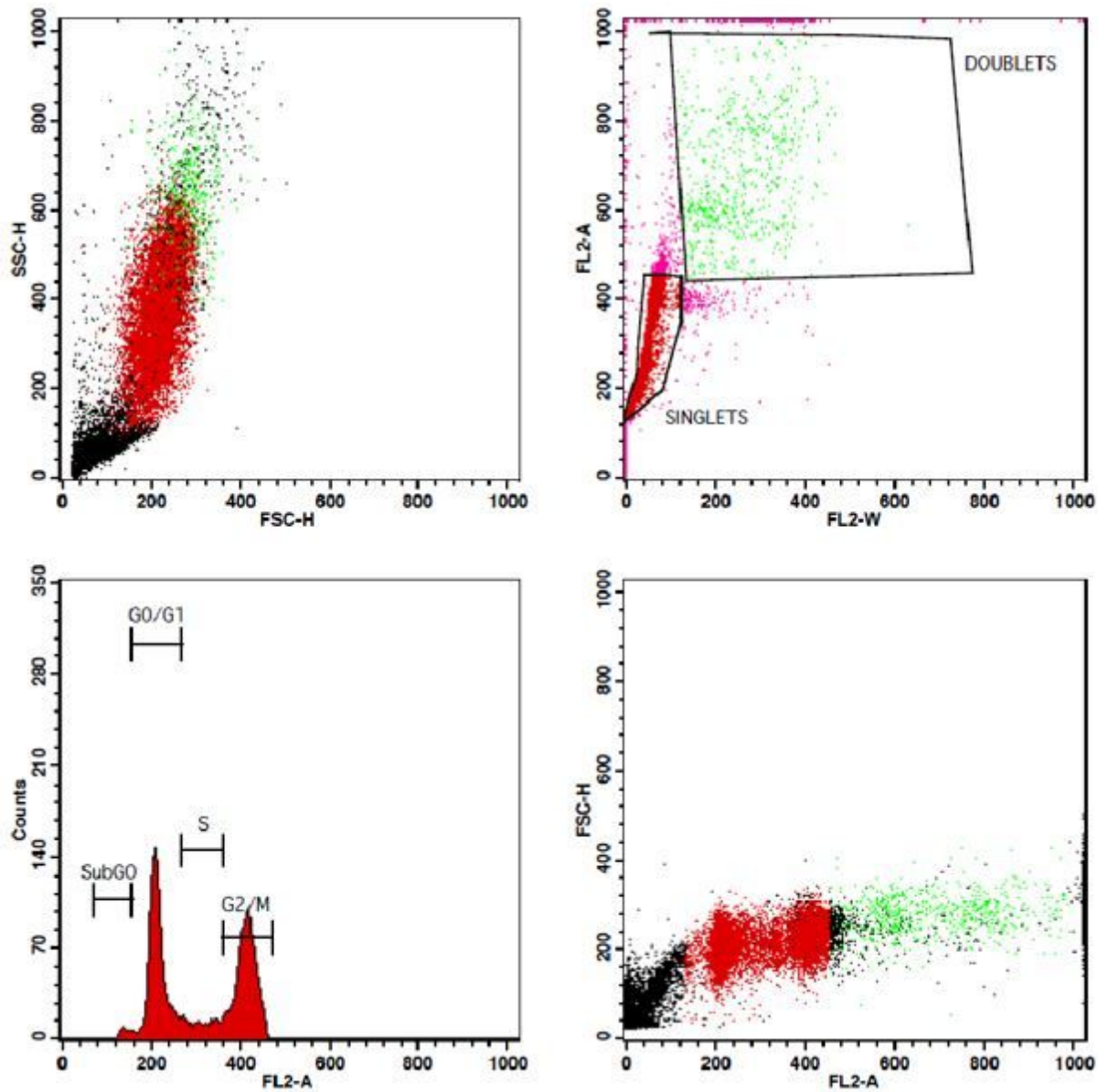
Flow Cytometry plots of A549 cells treated with 40µg/ml of ethyl acetate extract of *Claviceps purpurea*



Histogram Statistics						
Marker	Left, Right	Events	%Gated	%Total	Mean	CV
All	0, 1023	10000	100.00	44.50	270.72	29.73
SubG0	72, 158	184	1.84	0.82	143.07	6.06
G0/G1	156, 268	6639	66.39	29.54	223.98	8.09
S	268, 362	1267	12.67	5.64	306.94	9.08
G2/M	362, 474	1975	19.75	8.79	415.98	5.58

Figure 3

Flow Cytometry plots of A549 cells treated with 80µg/ml of ethyl acetate extract of *Claviceps purpurea*



Histogram Statistics

Marker	Left, Right	Events	% Gated	% Total	Mean	CV
All	0, 1023	10000	100.00	53.40	311.00	31.32
SubG0	72, 158	161	1.61	0.86	143.06	5.95
G0/G1	156, 268	4422	44.22	23.61	214.29	8.85
S	268, 362	1026	10.26	5.48	314.84	9.03
G2/M	362, 474	4438	44.38	23.70	411.99	4.85

Figure 4

Flow Cytometry plots of A549 cells treated with 25µM of Standard- Colchicine

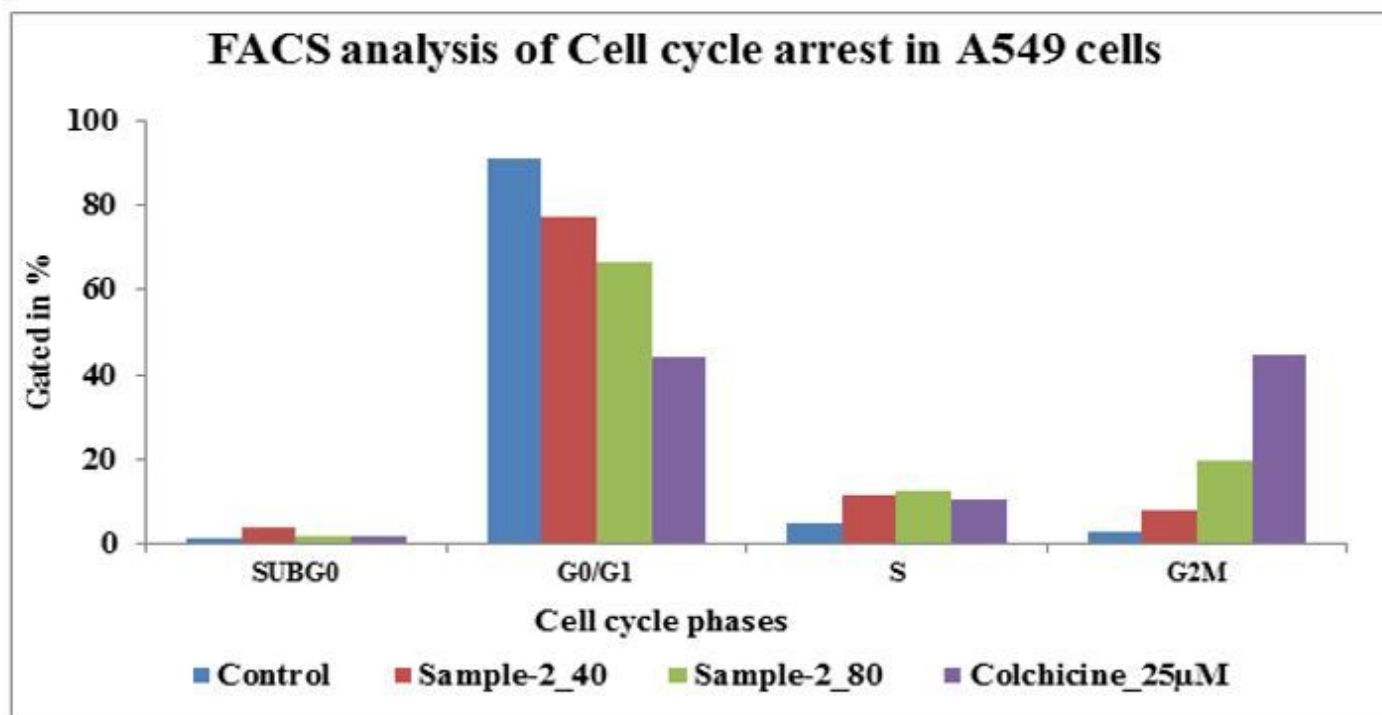
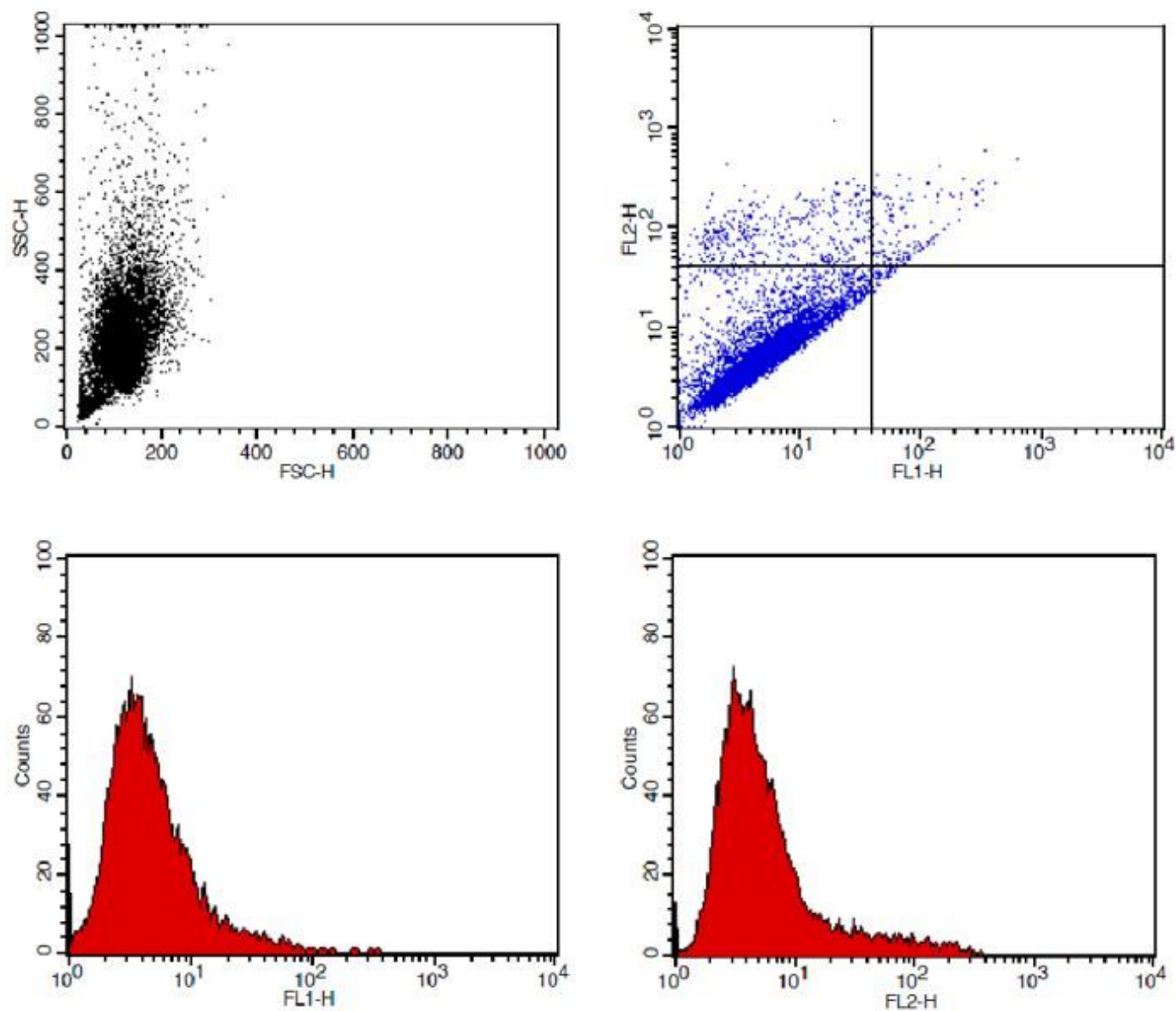


Figure 5

Flow cytometry analysis of cell cycle arrest in A549 cells



Quadrant Statistics

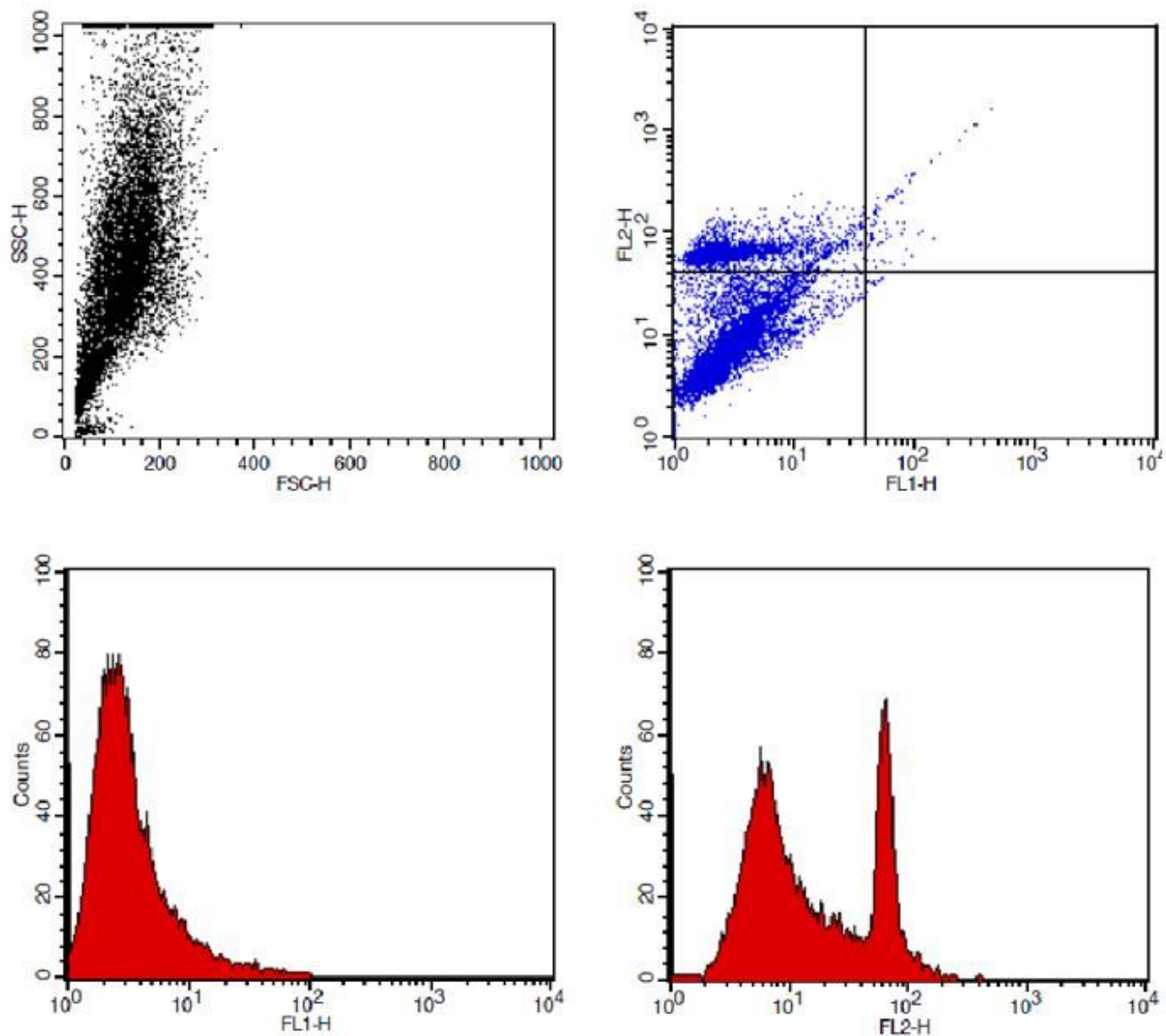
Gated Events: 10000

Total Events: 10000

Quad	Events	% Gated	% Total	X Mean	Y Mean
UL	378	3.78	3.78	10.62	102.91
UR	145	1.45	1.45	100.67	127.02
LL	9421	94.21	94.21	5.43	5.89
LR	56	0.56	0.56	48.24	33.21

Figure 6

A549 untreated cells



Quadrant Statistics

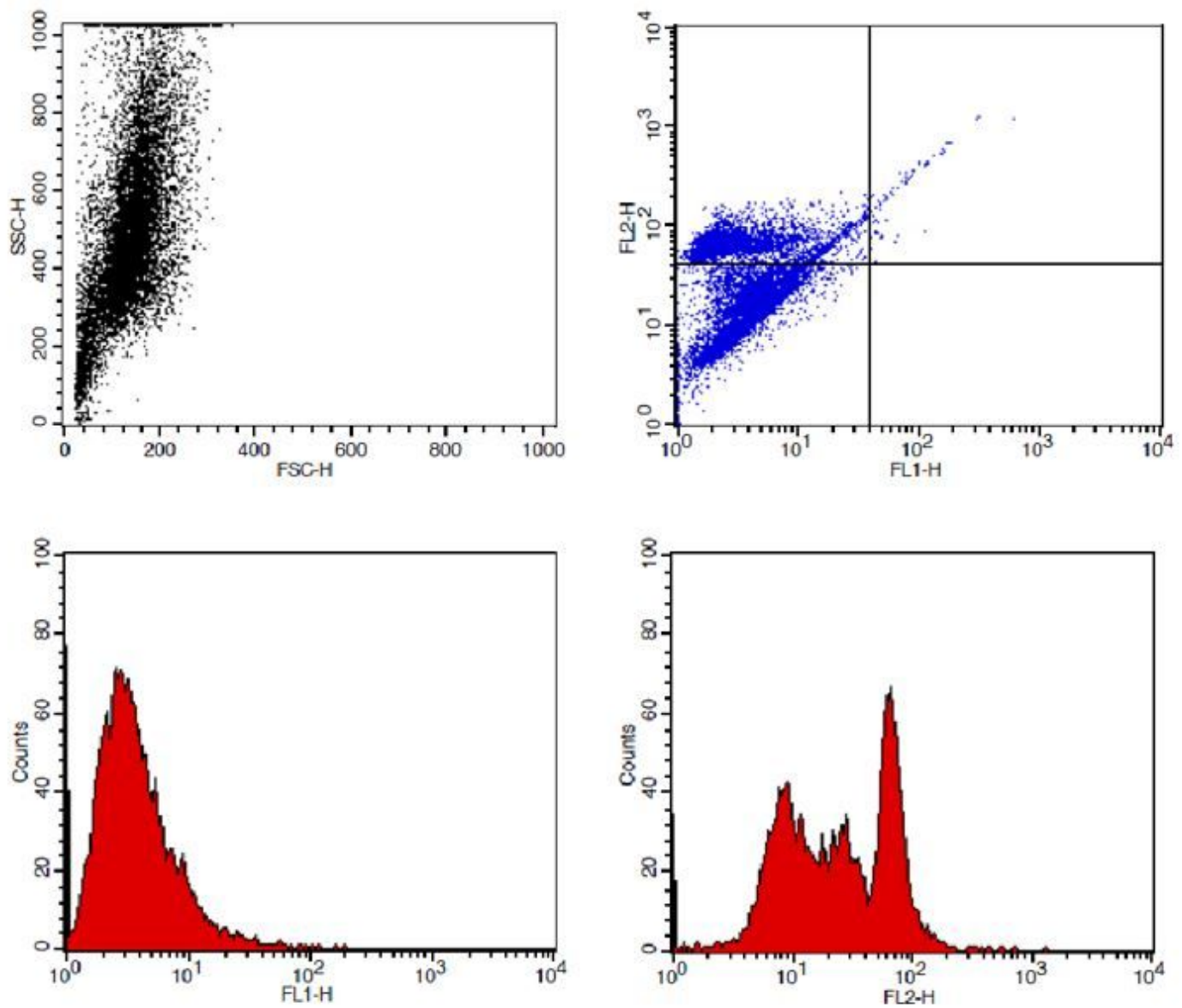
Gated Events: 10000

Total Events: 10000

Quad	Events	% Gated	% Total	X Mean	Y Mean
UL	2754	27.54	27.54	4.76	66.84
UR	85	0.85	0.85	79.25	221.61
LL	7148	71.48	71.48	3.65	9.85
LR	13	0.13	0.13	45.83	32.81

Figure 7

A549 cells treated with ethyl acetate extract of *Claviceps purpurea* (40µg/ml)



Quadrant Statistics

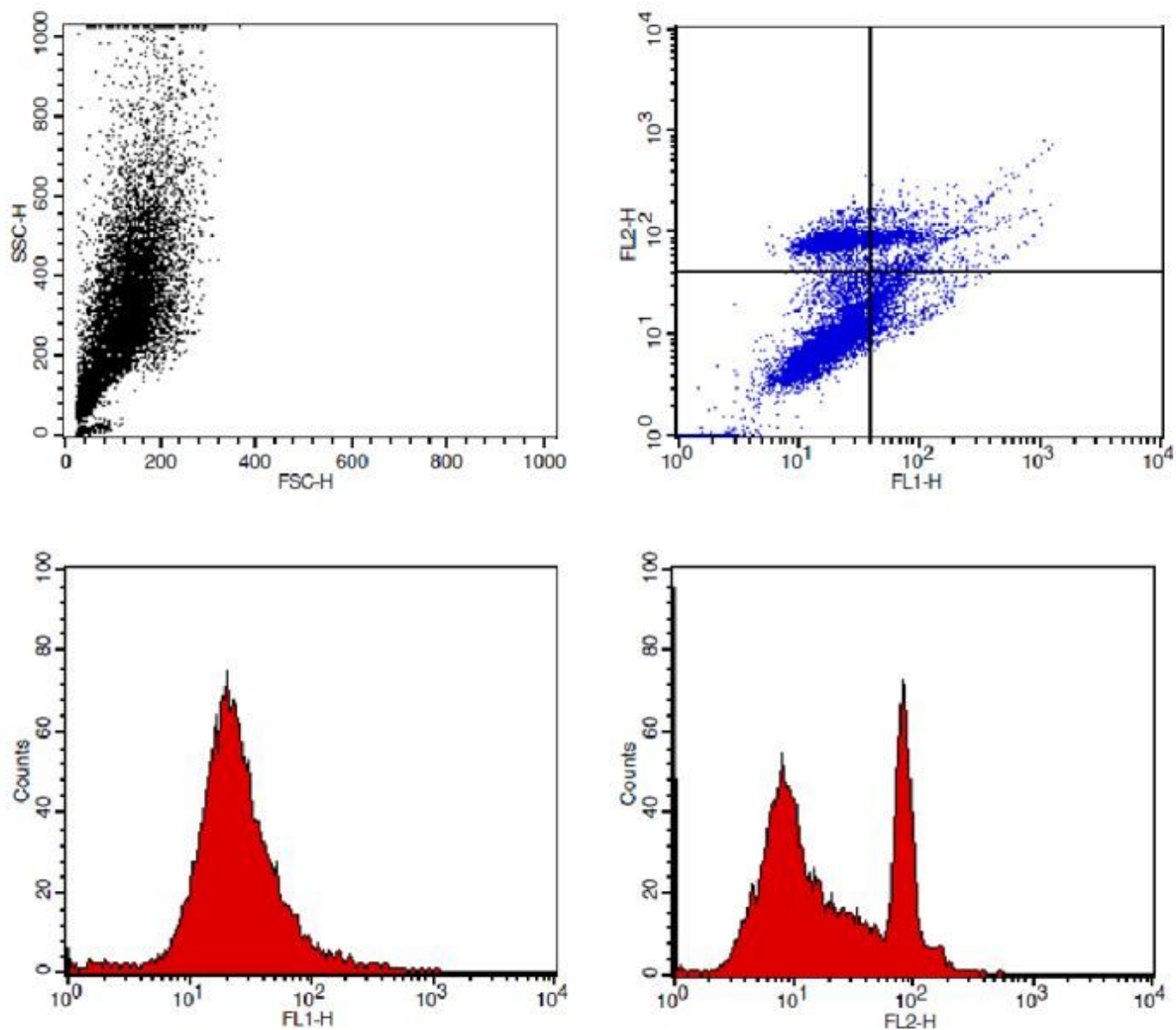
Gated Events: 10000

Total Events: 10000

Quad	Events	% Gated	% Total	X Mean	Y Mean
UL	3554	35.54	35.54	5.10	68.91
UR	74	0.74	0.74	89.44	297.86
LL	6372	63.72	63.72	4.19	15.17
LR	0	0.00	0.00	***	***

Figure 8

A549 cells treated with ethyl acetate extract of *Claviceps purpurea* (80µg/ml)



Quadrant Statistics

Gated Events: 10000			Total Events: 10000		
Quad	Events	% Gated	% Total	X Mean	Y Mean
UL	2261	22.61	22.61	21.11	82.00
UR	1022	10.22	10.22	122.69	99.76
LL	5611	56.11	56.11	19.33	9.74
LR	1106	11.06	11.06	63.86	23.00

Figure 9

A549 cells treated with Standard Vinblastine (25μM)

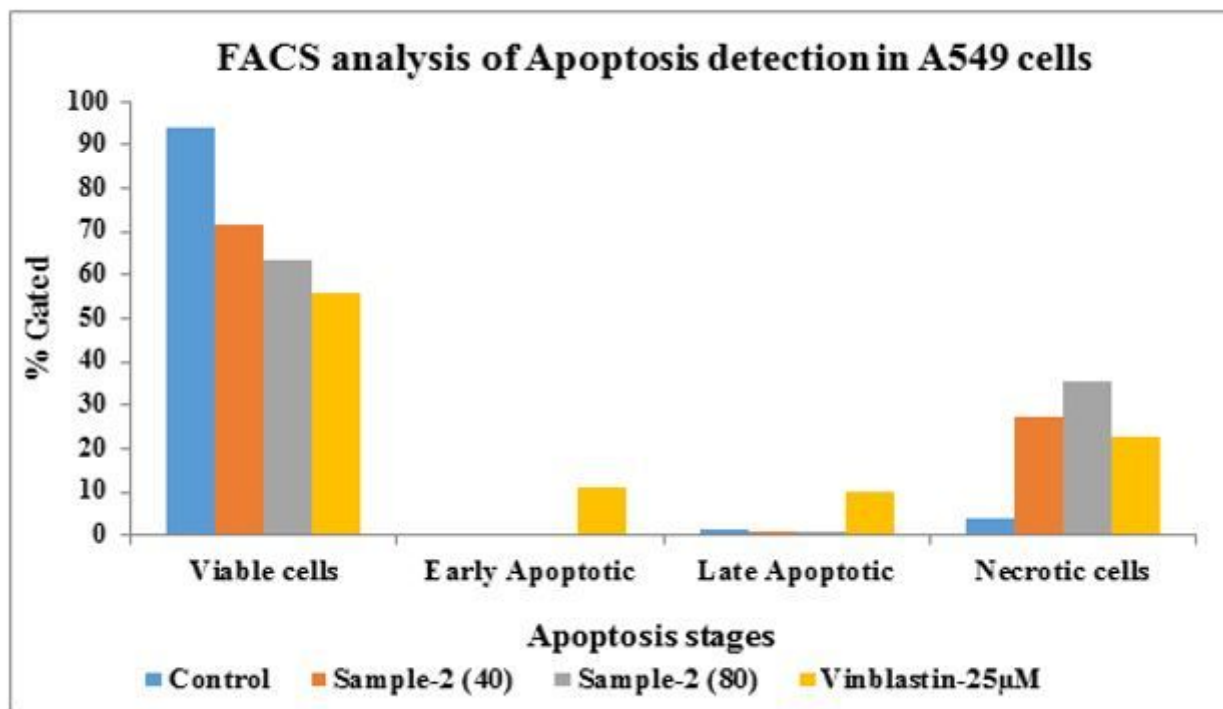


Figure 10

Flow cytometry analysis of Apoptosis detection of A549 cells

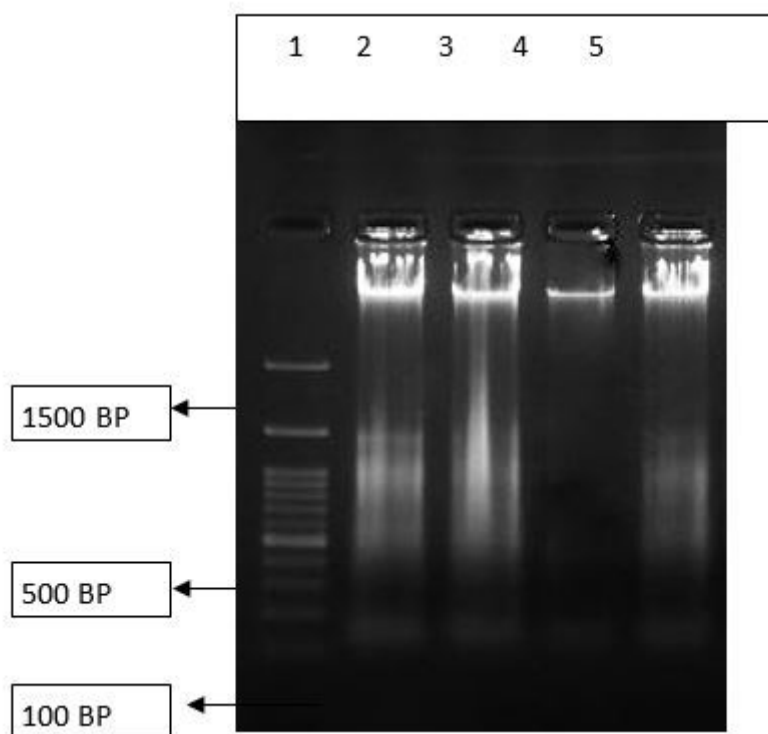


Figure 11

DNA Fragmentation studies using ethyl acetate extract of *Claviceps purpurea* on A549 cells. Lane 1- ladder; lane 2- Sample-80µg/ml; lane 3- Sample-40µg/ml; lane 4- control; lane 5- H2O2 200µM

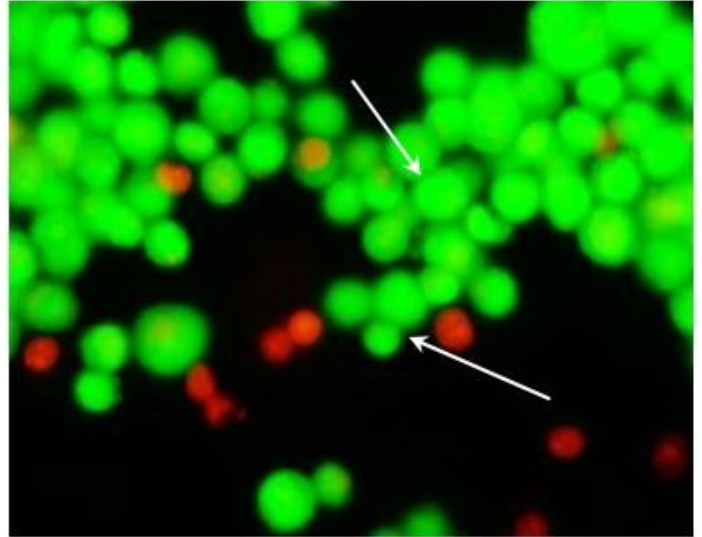
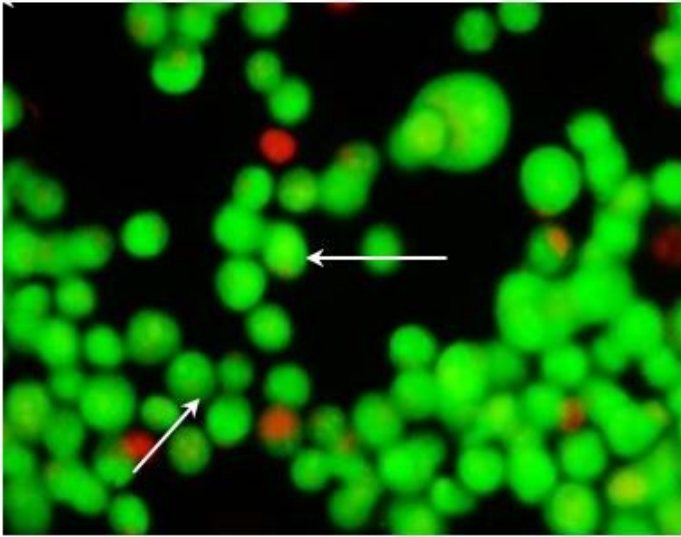


Figure 12

Nuclear staining of A549 cells using acridine orange under fluorescent microscope on Control cells and arrows indicate the membrane blebbing.

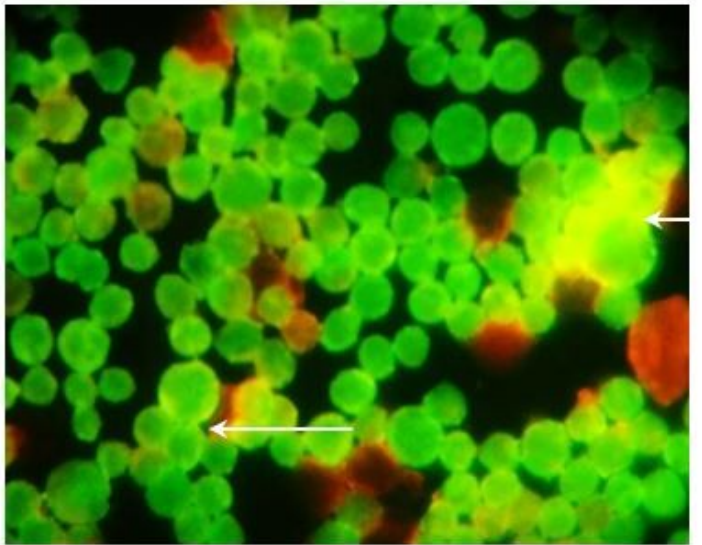
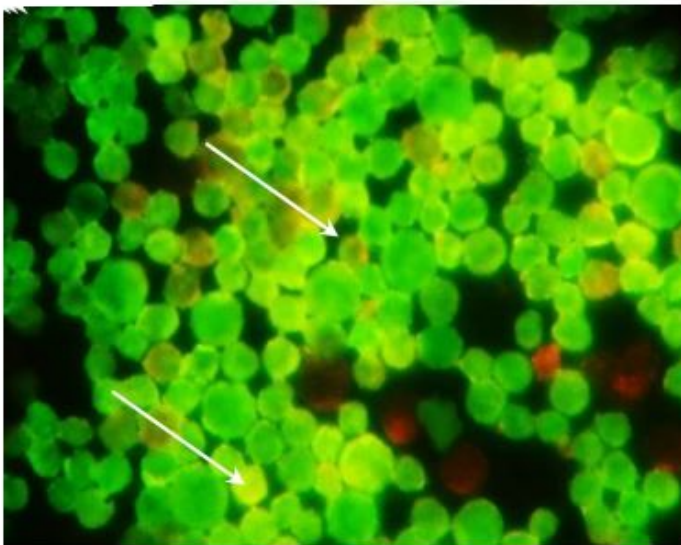


Figure 13

Apoptotic cell morphology of cells treated with fraction 40 µg/ml of ethyl acetate extract on A549 cells and arrows indicate the membrane blebbing.

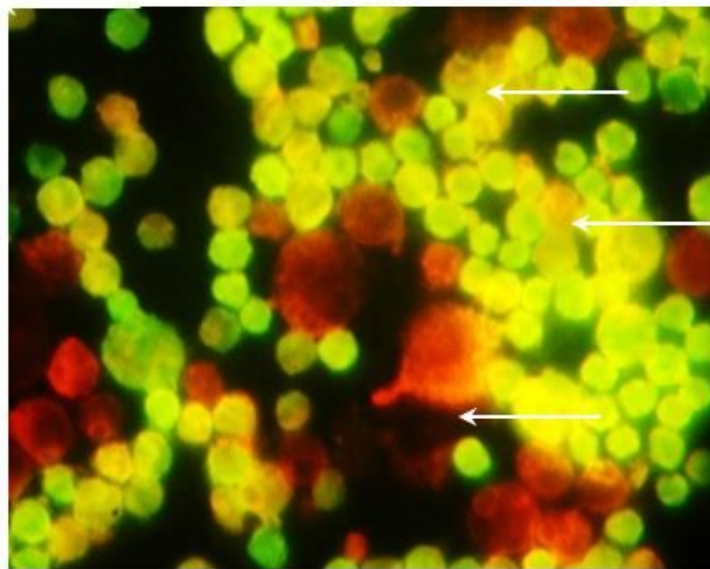
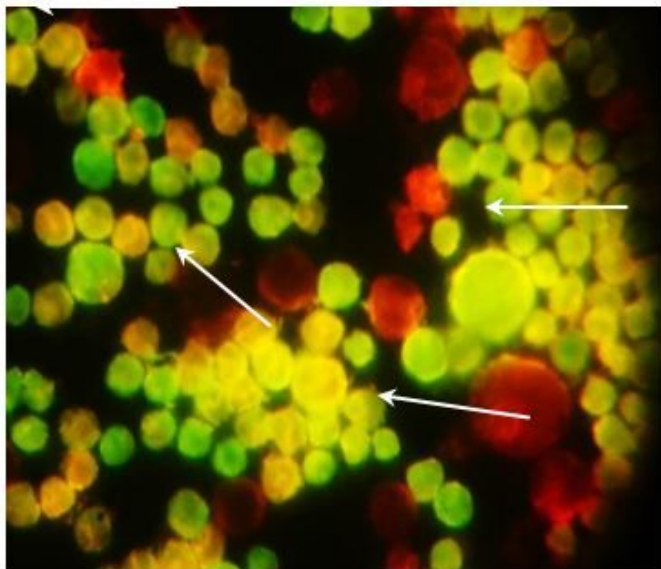


Figure 14

Apoptotic cell morphology of cells treated with fraction 80 $\mu\text{g/ml}$ of ethyl acetate extract on A549 cells and arrows indicate the membrane blebbing

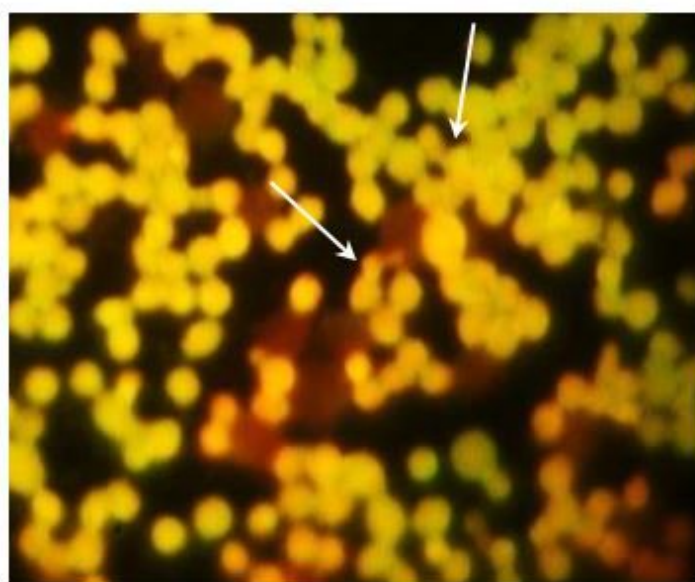
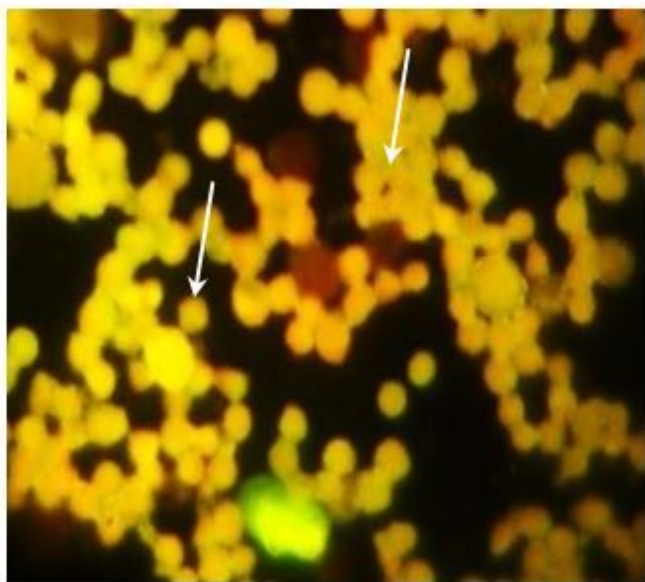


Figure 15

Apoptotic cell morphology of cells treated with standard Vinblastine 25 $\mu\text{M/ml}$ on A549 cells and arrows indicate the membrane blebbing.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Graphicalabstract.docx](#)