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Research Article

The Usage of *Marsdenia condurango* 30C, a Homeopathic (Potentized) Drug Demonstrates Some Cancer Inhibiting Outcomes in *In Vitro* Assessment of Human Cervical Cancer Cell Lines

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Abstract

An ailment in which the proliferation of the cells is uncontrollable it is called as Cancer. Mutation i.e. change in the Genome causes the alteration of normal cells into cancerous cells. As a result of this mutations the Oncogenes are turned on and the TSG i.e. Tumor Suppressor Gene are turned off. Genes are also delimited epigenetically. Methylation and Demethylation are mechanisms by which genes are controlled epigenetically devoid of every variation in the DNA sequence. Cervical Cancer is maximum occurring cancer in women in world affecting the Cervix and around 80% of the cervical cancers are squamous cell cancer and rest are Adenocarcinomas. Risk factors include HPV infections, smoking, destabilized immune coordination, administration of oral contraceptive pills for more than 5 years, having more than 5 children or having at an early age. Due the rising of this terminal illness, there is a crucial need for the development of Drugs which has the anti-cancerous effects. The emphasis of research is shifted towards the plant based phytomedicines. These Medicines are considered for the reason that they do not have any austere side effects. Homeopathy is now considered as the alternative medicine in cancer research. For this project a commercially available phyto based medicine called Condurango was taken. It's an ethanolic preparation of plant based compound or extract. The potency of the drug taken was 30c for evaluating its effect in HeLa Cells. It has given away some worthy effects in different types of Cancers. Condurango has displayed anti-cancerous plus apoptotic activities in Cancer Cell Lines. In this project, analysis of the anti- cancerous and demethylating effects of Condurango was done.

The objectives of the project were to study the cytotoxic effects of the drug and evaluate its dosage in the HeLa cells, to study the morphological changes in treated and untreated cells and to study the Methylation patters of the genes. The methodologies adopted for the project were MTT assay, flow Cytometry, PCR, RT PCR, Methylation specific PCR, RNA and DNA isolation. MTT showed that there were cytotoxic effects of the drug, and the dosage was deduced to be 1.5% concentration. Flow Cytometry showed increased sub G1 population and apoptotic effects of the drug. All these results conclude that the drug, Condurango, has some anti-cancerous effects.

Keywords: Cervical Cancer; DNA Methylation; Epigenetics; Homeopathy; Condurango; Tumor Suppressor; Genes; Cell Cytotoxicity

Introduction

Cancer is the disease in which malignant (progressively worse) transformation of the normal cells happens. In this process, damage occurs to the genome resulting in a sequential acquisition of mutations. Several endogenous processes like unwanted replication of DNA, free radicals can cause this damage. can be the result

of endogenous processes such as errors in replication of DNA and chemical instability of certain DNA bases. Other than these factors, interactions with exogenous agents such as ionizing radiation, UV radiation, and chemical carcinogens, may result in DNA damage. Cells have the capability to overcome with such damage, but under certain conditions, permanent changes in the genome get introduced that is known as mutation. Some inactivating mutations occur in genes responsible for maintaining genomic integrity, thus facilitating the acquisition of additional mutations [1]. In normal condition, there are certain genes that maintain homeostasis in cell proliferation and cell death. When mutational damage happens, this homeostatic condition gets disturbed. The imbalanced homeostasis activates the genes (known as oncogenes) promoting cell proliferation or cell survival. Simultaneously, this also inactivates the genes which would normally inhibit proliferation, the Tumor Suppressor Genes (TSG). Cervical cancer is the cancer of a cervix in females, in which inferior and tapered part of the uterus is exaggerated. There are 2 main types of cancers: Squamous Cell Carcinomas (SCC) and Adenocarcinomas (AC).

In early stages, Cancer of the cervix is often symptom less. Globally, cervical cancer is the cancer that is considered at fourth place most frequent in women, with an estimated 570,000 new cases in 2018 representing 6.6% of all female cancers. Cervical cancer is the 2nd most common cancer in India accounting to almost 22.86% of all cancer cases in women. The probability aspects of cervical cancer include Human Papilloma Virus (HPV) infection, smoking, having a weakened immune system, Chlamydia infection, long term use of oral contraceptives, use of intra-uterine devices, having manifold pregnancies, Diethylstilboestrol (DES), and having a family history of cervical cancer [2,3].

Development of new-fangled cancer hostile drugs as an effectual treatment strategy is of chief significance in drug discovery and clinical therapy. The focus needs to shift towards alternative medicines to explore their potential as anti-cancer agents. The kingdom of plants "Plantae" epitomizes the enormous reservoir of biologically active compounds, and yet only a petite portion of plants with medicinal properties have been tested. Significant numbers of drugs are from natural resources and more than 50% of drugs are plant sources. This percentage increases in case of anticancer drugs.

In this project, an ethanolic preparation of a homeopathic medicine was used to test its effects on HeLa cells, which is a cervical cancer cell line. The drug of choice is *Marsdenia cundurango* which is a plant of Apocynaceae family and is commonly known Condurango. Condurango contains conduritol, a cyclitol or cyclic polyol. Parts of the plant used for medicinal purposes are barks of the tree. I have used a commercially available ethanolic extract of Condurango of potency 30C. The concentrations that was taken for the treatment of the HeLa cells for various experiments was 0.5%, 1%, 1.5% and 3%.

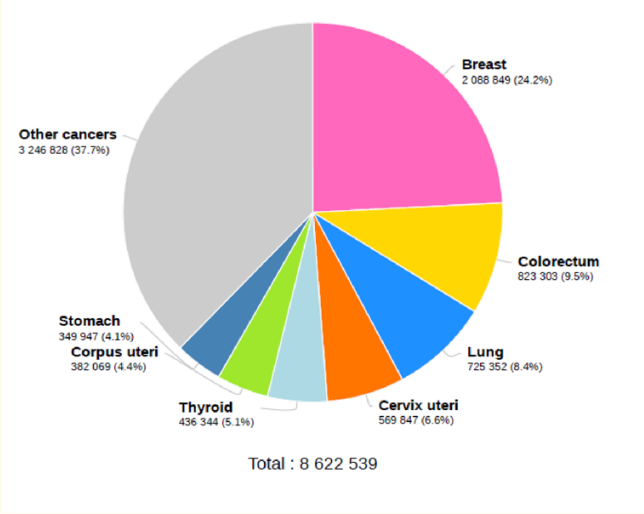

Figure 1: Estimated number of new cases in 2018, worldwide, all cancers, females, all ages.
Data source: Globocan 2018 Graph production: Global Cancer Observatory (http://gco.iarc.fr).

Condurango has reportedly been used historically to treat various types of inflammatory conditions and stomach related issues. Condurango also has anti-cancer effects. Although conducted some *in vivo* studies in mice and showed anticarcinogenic properties of Condurango based on enzymatic studies [4]. In this publication, it was tried to uncover and understand the possibilities of epigenetic effects of drug on cancer cell lines, whether the drug causes its effect on mere cellular level or epigenetic and genetic level in cancer cells.

Materials and Methods

Reagents used in project

For different experiments carried out for completion of my project mentioned reagents were used to support the reactions

in those experiments. All the reagents were provided by NICPR-ICMR Noida molecular genetics and oncology laboratory where most of the reagents were purchased online. In cell culture, 10% Foetal Bovine Serum (FBS) and 1% Penicillin- Streptomycin (PS) antibiotic solution to DMEM media was used. Along with Cryopreserving Solution containing 10% DMSO + 20% FBS+ 70% Media for cryopreserving the cell lines. In assessment of cell lines, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] Reagent (7.5 mg/ml) for cytotoxicity assay and Propidium Iodide Master Mix for cell profiling was used. For SDS-PAGE and AGE, Acrylamide Solution (40%) with 38g of Acrylamide and 2g of Bi-sacrylamide, PAGE Gel (6%), Sodium Dodecyl Sulphate (10%) and Agarose solution (1X), Ethidium Bromide (10 mg/ml), Loading Dye (6X) with 0.25% Bromophenol Blue, 0.25% XylneCynol and 30% glycerol, TAE (50X) was used respectively.

Cell culture

The HeLa- human cervical cell lines were provided by National Centre for Cell Science (NCCS, Pune, India). The cell lines were maintained in a culture growth media- DMEM that was supplemented with the 10% Foetal Bovine serum (FBS) and 1% penicillin and streptomycin. This cell culture was incubated at 37°C, 5% CO_2 and 95% humidity. Sub culturing of the cells was carried out when cells in T-25 (25 cm^2) or T-75 (75 cm^2) flask reaches the confluence of 80 - 90% at 1:5 ratios.

Seeding and treatment of the HeLa cells for MTT assay

Cell trypsination is done by adding 1.5 ml of Trypsin to the T-25 Flask to detach the cells (before performing seeding the confluency of the cells should be 80 - 90%). Before adding Trypsin, the old media is discarded. After adding Trypsin, incubation is done for 5 minutes. In Cell suspension preparation 1.5 ml media was added. Then the media was collected in a given 15 ml falcon tube and then centrifuged at 15000 rpm for 5 mins. The supernatant is then discarded and 3 ml new media is added to the falcon tube. After the cells are suspended, 10 µl of the cell suspension was added to Haemocytometer for cells counting. 8000 cells/well were seeded. Seeding was done in a 96 well plate. Altogether, 5 conditions for the treatment were taken Control, 0.5%, 1%, 1.5%, 3% (Table 1). Then 0.3 ml media/well was added for the treatment. After preparing the drug aliquots for treatment 0.3 ml is added in each well and it was then incubated for 24hrs and 48hrs.

Concentrations	Drug	Ethanol (90%)	Media
Control	No drug	45µl	1.5ml
0.5%	7.5	37.5	1.5ml
1%	15	30	1.5ml
	22.5	22.5	1.5ml
1.5%	45	No ethanol	1.5ml
3%			

Table 1: Drug Treatment Conditions.

Cell viability analysis by MTT Assay

MTT (3-(4, 5-dimethylhiazol-2-yl)-2, 5-diphnyl tetrazolium bromide) colorimetric assay was performed on the cells to check their viability after treating them with proposed drug. For this cytotoxicity testing cells were trypsinised and were seeded in 96 well plates with approximately 8000 cells/well by counting the cells with haemocytometer. Five different concentration of the drug: 0.5% (0 µM), 1% (0 µM), 1.5% (0 µM), 3% (0 µM) along with 90% ethanol + 10% media control was used for the HeLa cells cytotoxic experiment. Four replicates of each concentration of drug were set. After seeding cells in well plates (96 wells), the cells were permitted to attach and grow with 200 µl DMEM media for 24 hours before administering drug to them. After overnight incubation, old media was replaced by fresh 200 µl DMEM media and 20 µl of freshly prepared MTT 5 mg/ml was added to the seeded plates. After MTT was added, cells were incubated in CO_2 incubator for 2 - 4 hours. Cells taken out of incubator when purple colour formazan crystals were observed at bottom of the wells. Media along with MTT was removed from the wells and added 100 µl DMSO. Then, absorbance of each well was taken at 570 nm wavelength in microtiter plate reader. The increased intensity of purple colour indicates low cell mortality. The graph of cell viability (OD vs. Drug concentration) was plotted using Graph pad prism software.

Cell cycle analysis by flow cytometry

A flow cytometry type of profiling of cells was performed to investigate the consequence of drug in arresting of cells into various phases of cell cycle. Trypsinization was done for harvesting the cells and thorough washing was done using 1x PBS. Fixation of the cells was done by adding 70% of the chilled ethanol in drop

by drop manner and mixing the cells simultaneously to prevent the unwanted clumping of cells. Then cells were with added 70% ethanol were incubated at 4°C for approximately 1 hour. Propidium iodide staining solution (PI Mix) was prepared with Propidium Iodide (5 mg/ml stock) - 8 µl/sample, RNase (10 mg/ml stock) - 10 µl/sample, 1X PBS - 982 µl/sample. The sample with PI mix was incubated for around 50 - 60 minutes at RT (Room Temperature) in dark. Samples were analyzed by BD Accuri™ C6 Plus personal flow cytometer for determining the cell cycle arrest.

DNA isolation from HeLa cell lines (drug treated and untreated cells)

DNA isolation from treated and untreated HeLa cells was carried out by GSURE GCC Biotech Blood and Cell culture DNA Isolation Kit. Centrifugation at 13000 rpm was done to settle down the pellet in micro centrifuge tube for 5 mins. Harvested cell was re-suspended in 250 µl GDBC1 buffer under vigorous vortexing. The tubes were then incubated for 15 mins at 70°C pursued by similar incubation after adding 250 µl GDBC2 buffer. Added 350 µl of GDBC3 buffer along with vigorous shaking (avoid Vortexing). After centrifugation, supernatant was transferred into Gmini Spin Column and was given another spin. Membrane wash buffer was further added to wash spin column and this process was repeated twice. To elute out DNA, the spin column was kept into a 1.5 ml centrifuge tube and 50 µl of nuclease free water was added. After incubation for 1 minutes at RT, the sample was given a quick spin before collecting the eluted DNA.

Bisulphite conversion of isolated DNA sample from HeLa drug treated and untreated cells

The bisulphite conversion was done by using EZ DNA Methylation- Gold TM Kit. From this kit, 130 µl of Conversion reagent was added to 20 µl of DNA followed by incubation as instructed in kit guide steps. Then, 600 µl M- Binding Buffer added to Zym-spin IC column followed by loading of sample from earlier step into column. Solution was centrifuged for 30 sec. After that addition of 100 µl of M-Wash buffer was followed by centrifugation. Added 200 µl M-Desulphonation buffer to Colum and incubated for 15 - 20 minutes at room temperature. After that, washing was done twice by using 200 µl M-Wash buffer to column along with centrifugation at full speed for 30 seconds. After washing, the column was kept into 1.5 ml micro-centrifuge tube and 10 µl of M-Elution buffer was added to the column matrix. At last, centrifugation at full speed was done for 30 seconds to elute out the Bisulphite converted DNA that was further processed for Methylation Specific (MS) PCR.

Methylation specific PCR

Nested PCR or external PCR for Methylation Specific (MS) PCR performed on the Bisulphite converted DNA sample. In total, 8 genes were used for doing Nested PCR of MS-PCR. Working concentration of the master mix or reaction mix for one reaction of 25 µl along with NTC (Non Template control - contains water instead of DNA sample) was prepared with Bisulphite converted DNA - 3µl (50 to 200ng), Forward primer and Reverse primer - 1 µl (2.5 to 12.5pm) of 8 genes (p14, p15, p16, GST, MGMT, E-CAD, RARB, and HMLH1), 2.5 mM DNTPs - 1 µl (50 µM to 200 µM), 1X Buffer - 2.5 µl, Enzyme (Taq DNA polymerase) - 0.5 µl (0.3 to 0.5 units) then lastly Water according to remaining volume left to make up 25 µl of master mix for 1 reaction. The reaction PCR tubes in the Thermal cycler or PCR machine was kept for 30 cycles (Table 2).

Temperature	Run time
95°C (denaturation)	5 minutes
95°C (annealing- repeated 30 times)	30 seconds
56°C (annealing- repeated 30 times)	30 seconds
72°C (annealing- repeated 30 times)	1 minutes
72°C (elongation)	7 minutes
4°C (storage)	∞

Table 2: Nested PCR setup.

After obtaining the PCR product of the bisulphite converted DNA sample, a Methylation specific PCR or internal PCR was carried out on PCR product. The sample was divided for two different setup conditions: Methylated and Un-methylated. Two genes p15 and p16 were taken under both conditions with different reaction mix of 25 µl each. The Methylation specific PCR (MS-PCR) was a Hot Start PCR where enzyme (aliquot in water) was added after heating the sample at 98°C for 3 minutes in PCR machine. Therefore, 4 reactions were setup for P16 Methylated, P16 Unmethylated, P15 Methylated, P15 Un-methylated. Working concentration of the master mix or reaction mix for 4 reactions of 25 µl each along

with NTC (Non Template control - contains water instead of DNA sample) was prepared with External PCR product – 2 µl (50 to 200 ng), Forward primer and Reverse primer - 1 µl (2.5 to 12.5 pm) of 1 genes (p16), 2.5 mM DNTPs - 1µl (50 µM to 200 µM), 10 X Buffer - 2.5 µl then lastly Water was added according to remaining volume (excluding the enzyme and aliquot water) left to make up 25 µl of master mix for 1 reaction, Enzyme (Taq DNA polymerase) - 0.5 µl (0.3 to 0.5 units). The reaction PCR tubes in the Thermal cycler or PCR machine was kept for 30 cycles (Table 3).

Temperature	Run time
98^{o}C (denaturation)	3 minutes - pause - added enzyme-(Taq DNA polymerase)
95^{o}C (annealing- repeated 30 times)	
61^{o}C (annealing- repeated 30 times) For P16 M, P15 M and P15 UM	30 seconds
59^{o}C (annealing- repeated 30 times) For P16 UM	30 seconds
72^{o}C (annealing- repeated 30 times)	1 minutes
72^{o}C (elongation)	7 minutes
4^{o}C (storage)	∞

Table 3: MS-PCR setup.

Results

Morphological study of drug treated and untreated HeLa cervical cell line

The seeded HeLa cells were permitted to nurture for 5 days till they reach the confluence of 70% in 6 well plates. The cells were given treatment after a required confluence of cells per well. The conclusion from three different conditions can be drawn as the positive effect of drug Condurango on the morphological and biological aspects of HeLa cell lines. According to the experiment, under required conditions, the untreated cells show no or little sign of cell death or cell mortality in well whereas a decrease or disoriented pattern of cell growth and cell death can be seen in the sample with Placebo (ethanol control) treatment. The Drug Condurango treated cells show more sign of cell death or cell mortality in well as compared to normal and placebo condition. The decrease in cell growth and increases in cell death implies the effect of drug on cells when used in different concentration.

MTT assay to study the cell viability and cytotoxicity in placebo (control) and drug treated cells

The proposed drug was found to be showing the cytotoxicity effect on the HeLa cancer cell lines. In the present study, the cell viability and cell toxicity was studied and examined via MTT assay where the cells were treated and incubated for 24 hours and 48 hours' time intervals. The results for both 28 hours and 48 hours' drug treatment shows a likely uniform increase in cell death as the concentration of drug was increased from.

FACs analysis for cell cycle arrest in drug treated and untreated cells maintained for 48 hours and 6 days

FACs (Fluorescence- activated cell sorting) analysis was done to examine and determine the phase of cell cycle in which the cells were getting arrested after getting the drug treatment. This analysis also helps to determine the level of apoptosis the cells have gone through after the drug administration. For FACs analysis, IC50 (Inhibitory Concentration at 50%) value of drug was determined by the MTT cytotoxicity assay. The cells were treated with IC50 drug concentration and the consequence of drug on the cell death and cell cycle arrest was analysed by using flow Cytometry. The drug shows the cell succession apprehension in phase and also persuade cell mortality in cancer cells which can proves that the drug is effective against cancer.

MS-PCR for examining the methylation and demethylation pattern in drug treated and untreated HeLa cell lines

MS-PCR (Methylation specific polymerase chain reaction) was done to examine the Methylation and Demethylation pattern in the CpG islands, i.e. where there are abundant CpG sites, on the genome of the individual. This analysis was done for understanding the effect of drug in reversal of epigenetic alterations that occurred in the two specific genes that is p15 and p16 genes which are known to harbour the Methylation on the CpG islands in their sequences of promoter regions. The isolated DNA samples of drug treated and untreated were subjected to bisulphite conversion and were later used for Nested PCR for 8 genes. Two different batches for methylated and unmethylated analysis were set up. Those batches were subjected to the SDS-PAGE gel electrophoresis.

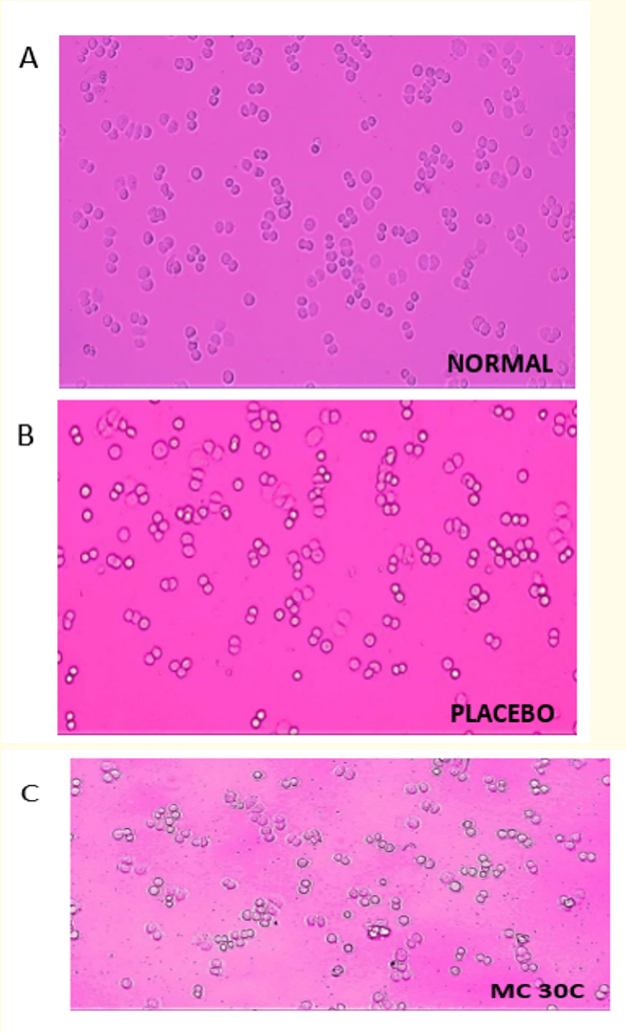

Figure 2: (A) The growths of HeLa cells were uniformly equal as they attained morphology after attaching at the bottom surface of the well. Without any drug and placebo treatment the confluence of about 70-80% in each well can be seen having an identical pattern that shows the normal growth of cell under required condition. (B) The growths of HeLa cells were found to be decreasing as they attained morphology after attaching at the bottom surface of the well. With Placebo (ethanol control) treatment the confluence of about 70-80% in each well can be seen having a decrease pattern that shows the cell growth and cell death under required condition as compared to normal one. (C) The growths of HeLa cells were found to be consistently decreasing as they attained morphology after attaching at the bottom surface of the well. With Drug Condurango treatment the confluence of about 70-80% in each well can be seen having a standardized decrease pattern.

Discussion

Various reports are there on the biological activities of Condurango in literature. Conduritol which is the active compound of Condurango has been studied in different in vivo and in-vitro studies [5]. Condurango [4] has cytotoxic effects in cancers cells. Cervical Cancer is spreading globally and it is a malignant ailment which is

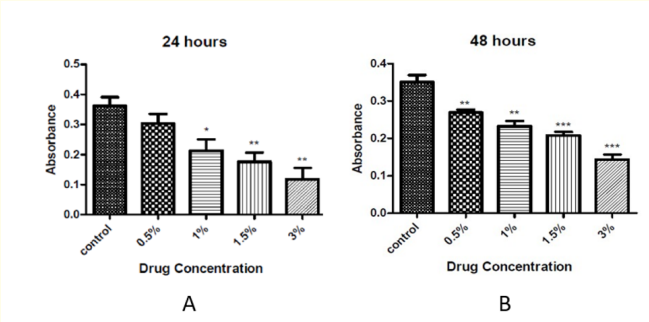

Figure 3: (A) Incubation of 24 hours after drug administration shows increase in cell death in drug treated wells 0.5%, 1%, 1.5%, 3% DC (Drug concentration) when compared to placebo (ethanol control) in all four replicates. (B) Incubation of 48 hours after drug administration shows increase in cell death, similarly to the earlier incubation, in drug treated wells 0.5%, 1%, 1.5%, 3% DC (Drug concentration) when compared to placebo (ethanol control) in all four replicates.

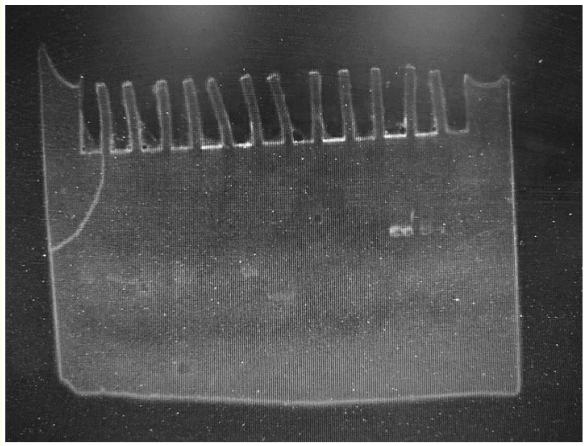

Figure 5: The experiment shows that drug have no effect in the methylation and demethylation pattern of the epigenetic alteration of the cervical cancer.

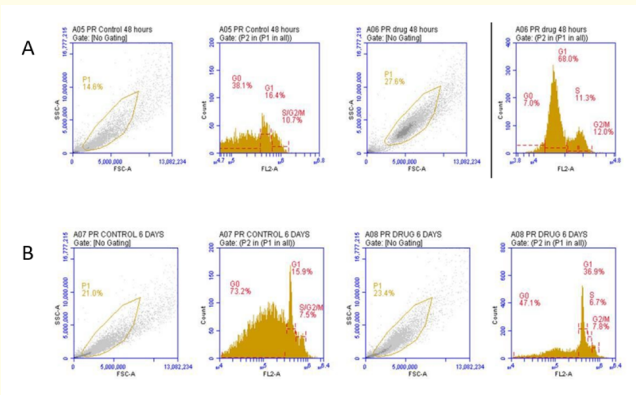

Figure 4: (A) The sample taken for the FACs analysis was incubated for 48 hours under Placebo (ethanol control) and Drug treatment. The drug treated cells shows more apoptosis and cell cycle arrest in specific cell cycle phase as compared to the placebo (ethanol control) of the experiment. (B) The sample taken for the FACs analysis was incubated for 6 days under Placebo (ethanol control) and Drug treatment. The drug treated cells shows more apoptosis and cell cycle arrest in specific cell cycle phase, similarly to the 48-hour incubation, as compared to the placebo (ethanol control) of the experiment.

urgently required to be controlled. Cancer researchers [4] have uncovered anti- cancerous properties in the plant-based medicines. Condurango is one of them. The reason why this Drug was chosen because not many researchers have explored this drug, hence it created an immense interest in evaluating its effect on the Cervical cancer cell lines. To adding surprise, the results were quite positive which gave a further boost to move forward with this drug. A range of concentrations of this Drug was taken out of which 1.5% concentration showed quite promising results. Condurango induced apoptosis into the sub G1 stage of the cell succession phase. The morphological changes of the treated and untreated cell were quite distinct. The number of cells in the drug treated flask was distinctly low as compared with untreated one. Adaptation of programmed cell mortality or death (PCD) mechanism is very complex [4]. But as hypothesized in the project the drug showed no significant effect in the epigenetic modification like DNA Methylation (p15 and p16 genes) involved in cancer progression of cervical cancer.

There was not much evidence on the effects of the homeopathy medications; however, it has shown some promising results fulfilling the objectives of my project.

Despite, a long debate, whether homeopathy is just placebo or more, it has been considered safe and cost effective. A number of

Human diseases are treated with Homeopathy then why not Cancer when it is showing positive effects [3]. However, the appropriate selection of the medicine against a disease is a tedious task. One has to keep total spectrum of symptoms in frame so as to get all the possible benefits from the drug.

Data suggest that homeopathy has the competence to not only to treat diverse types of cancer but also shows zero to minimum side effects which on the other hand is caused by therapeutic modalities like chemotherapy, radiotherapy or surgery [6-18].

Conclusion

According to the phytomedicines drug "Condurango" used in the project showed some effective results in increasing cell cytotoxicity of the cancer cell lines, inducing apoptosis in the cells and cell cycle arrest leading to halt in the cell division, also initiating programmed cell death but was unable to show effect in the epigenetic modification like DNA Methylation (in the promoter region of p15 and p16 genes) involved in Tumorigenesis of Cervical cancer. Thus it can be said that drug does not have any epigenetic reversal properties for treating and preventing cancer progression.

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Conflict of Interest

Declare if any financial interest or any conflict of interest exists.

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