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Anticancer Activity of *Cissus quadrangularis*: An *in vitro* 2D Model Based Study

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Abstract

Cissus quadrangularis (CQ) is a perennial rambling shrub of the grape family commonly known as “Hadjora” (in Hindi) probably native to India or Sri Lanka. It is one of the valuable medicines in the Indian Traditional Systems of Medicine because of the presence of several bioactive compounds. However, in the present study we have checked its anticancer activity along with its safety profile on normal skin cells. Apart from this we have generated the spheroid HeLa culture *in vitro* model for analyzing the CQ extract response on the growth of HeLa tumoroid. From the present findings we have observed that the CQ selectively induces cytotoxicity, ROS liberation and G1 phase cell cycle arrest only in HeLa cancer cells without affecting the normal skin cells at similar dose. CQ also significantly inhibits the growth of tumoroid and finally leads to cell death as revealed by phase contrast microscopy. Therefore, it can be concluded from the present findings that CQ extract shows targeted anticancer activity, though the mechanism of action in support of its exhibited activity needs to be further explored.

Keywords: ROS; Anticancer; Tumoroid.

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1. Introduction

Cancer prevalence in India is estimated to be around 2.5 million with over 800,000 new cases and 550,000 deaths each year. It's usually treated with chemotherapy, radiation therapy and surgery. Chemotherapy is an effective treatment against cancer either singly or in combination with surgery and/or radiotherapy. In chemotherapy, drugs like cisplatin, carboplatin, cyclophosphamide, doxorubicin, mephalan, mitomycin C, gemcitabine, etc. have been used for treatment of cancer [1]. However, therapeutic efficacies of most of them are limited due to development of various side effects. In an attempt to abate these side effects and as a better remedy against various malignancies many plant derivatives have been used with varying degrees of success. Thereby the search of natural products with anti-tumor properties which could be used for treatment with least side effects is the hot area of research in the field of biomedical science and cancer therapy [2, 3, 4].

Tissue engineering literature suggests that 3-dimensional structures formed by cultured cells *in vitro* are better models of *in vivo* tissue than planar cultures of cell lines, particularly in mimicking some of the cell-cell, cell-matrix interactions found *in vivo*. Culture parameters like cell types, seeding densities, medium compositions, and static or stirring growth determine cell aggregation efficiency and uniformity of spheroid size and shape. Spheroids generated by this method usually display a broad size distribution. Cancer research studies performed on monolayer culture conditions cannot account for limitations caused by diffusion, cell-cell interactions, and factors such as hypoxia, apoptosis, and proliferation [5].

In Indian systems of medicine, a large number of drugs of either herbal or mineral origin have been implicated in various diseases and other pathological conditions in human. Recently, considerable research has been carried out in the search for natural or synthetic compounds as a means of chemopreventive agents against cancer [6]. *Cissus quadrangularis* is a perennial rambling shrub of the grape family. It is commonly known as Veldt Grape or Devil's Backbone and as "Hadjora" (in Hindi). It is probably native to India or Sri Lanka, but is also found in Africa, Arabia, and Southeast Asia. It is one of the valuable medicines in the Indian Traditional Systems of Medicine. The aerial parts of *Cissus quadrangularis L.* contain nearly thirty bioactive chemical compounds. Predominantly acid derivatives, fatty acid, fatty acid ester, alcoholic compounds and hydrocarbons, the existence of n-hexadecanoic acid, ethan-1,1-diethoxy, 9,12,15-octadecatrienoic acid-methyl ester (Z,Z,Z) as major constituents. These different active phytochemicals possess a wide range of biological applications [7]. Taking in to consideration the medicinal properties exhibited by this genus, it was decided to investigate the correlation between ROS generation and induction of apoptosis by CQ ethanolic extract obtained from Soxhlet extractor using, HeLa (Cervical epidermoid carcinoma, human) and HaCaT (normal skin) cells. We have also evaluated the CQ treatment response by phase contrast microscopy using HeLa spheroid culture.

The outcome of the present study shows that the HeLa cells are significantly and specifically targeted by CQ extract without affecting the normal skin cells at similar doses. CQ extract significantly inhibited the growth of Tumoroid which finally leads to cell death as revealed by phase contrast microscopy. In conclusion it can be deduced from the present data that CQ extract shows targeted anticancer activity, though the mechanism of its action still needs to be further explored.

2. Materials and methods

2.1 Cell Culture, Chemicals and reagents

EMEM and DMEM/F12, fetal calf serum (FCS), penicillin G and streptomycin were obtained from Himedia. Dimethylsulfoxide (DMSO), ribonuclease (RNase), and propidium iodide (PI), DAPI, Acridine orange and EtBr were purchased from Sigma-Aldrich (St. Louis, MO). Cervical cancer cells (HeLa) and normal keratinocytes skin cells (HaCaT) were obtained from the National Centre for Cell Sciences Pune, India. Cells were maintained in EMEM and DMEM/F12 medium supplemented with 10% fetal calf serum, and antibiotics at 37°C in a humidified atmosphere of 5% CO₂.

2.2 Plant Material

The *Cissus quadrangularis L* were taken from State Ayurvedic medical college of Lucknow. The plant material was authenticated by the Department of Botany, Lucknow University where voucher specimen was submitted. The plant material was shade dried and powdered.

2.3 Preparation of Plant Extract

The powder of *Cissus quadrangularis L*. (20 gm) was extracted with 250 ml ethanol by soxhlet extraction for 8 hrs. The extract was concentrated on water bath at 60°C. The obtained dark brown thick liquid was stored in a glass vial in refrigerator [8].

2.4 Cytotoxicity analysis by MTT assay

The cell viability was assessed by MTT assay, which determines the metabolically active mitochondria of intact cells. The assay was carried out using MTT cell assay kit, following the protocol described by the manufacturer's (HiMedia, India). Briefly cells were seeded in 96-well plates, with 5×10^3 cells/100 μ L and incubated for 24 h at 37°C. The cells were then treated with CQ (5 μ g/ ml to 400 μ g/ ml) and incubated for another 24 h at 37°C in a 5% CO₂ atmosphere. The assay was performed by the addition of premixed MTT reagent, to a final concentration of 10% of total volume, to culture wells containing various concentrations of the test substance and incubated for further 4 h [9].

2.5 Morphological Analysis by Haematoxylin/Eosin Staining

For Hematoxylen/Eosin staining, cells (20×10^3 cells per well) were placed in DMEM by using 24-well plates. After treating with the CQ extract at different concentrations for 24 h period, the medium was removed, the cells washed with distilled water and fixed in ethanol, and stained with Hematoxylen/Eosin. After staining, the cells were observed by light inverted microscope (Nikon). By this way, cellular and nuclear morphology is shown in cultured cells stained with Hematoxylen/Eosin [10].

2.6 DNA Damage Assessed by DAPI Staining

DAPI staining was performed according to the procedure with minor modifications. Cells were cultured in a 24-well tissue culture grade plate for 24 h. After incubation with IC₅₀ concentrations of CQ (200µg/ml) of ethanolic extract for 24 h, cells were washed in phosphate buffered saline (PBS), fixed with 2% paraformaldehyde for 15 min and were treated with 0.2% triton X-100 in PBS for 15 min at room temperature. Cells after washing with PBS were stained with DAPI (1 µg/ml) and incubated in dark for 30 min. The cells were then examined and photographed using a fluorescence microscope (Nikon) [11].

2.7 Apoptosis Detection by Acridine Orange (AO) /Ethidium Bromide (EtBr) Dual Staining

Apoptosis detection by AO/EtBr dual staining procedure was performed. AO is a vital dye that stains both live and dead cells; EtBr only stains cells that have lost membrane integrity. Early apoptotic cells stain green and contain bright dots in the nuclei. Late apoptotic cells also incorporate EtBr and show condensed and often fragmented nuclei. Cells were washed in phosphate buffered saline (PBS), fixed with 2% paraformaldehyde for 15 min and were treated with 0.2% triton X-100 in PBS for 15 min at room temperature. 10µl of 1mg/ml AO and EtBr mixture was added to each well. Nuclei were visualized and photographed under the fluorescent microscope [12]. The cells were then examined and photographed using a fluorescence microscope (Nikon)

2.8 Quantification of Apoptosis and Cell cycle arrest by flow cytometry.

After cells were treated with or without extract for 24 h, were harvested by centrifugation for determining the percentage of apoptosis and cell cycle distribution by staining with PI (5 µg/ml) analyzed by flow cytometry (Becton-Dickinson, San Jose, CA, USA) as previously described (30,31). For cell cycle distribution and apoptosis determinations, the adherent cells were washed with PBS, and 300µl trypsin was added for 5 min at room temperature to detect the cells and harvested the cells by centrifugation. Cells were fixed gently in 70% ethanol at 4°C overnight and then re-suspended in PBS containing 40 µg/ml PI and 0.1 mg/ml RNase and 0.1% Triton X-100 in a dark room for 30 min at 37°C. Cells were analyzed with a flow cytometer. The results were analyzed by Mod Fit LT 3.0 software [13].

2.9 DCFDA Staining

The effect of extract on ROS production was measured in treated normal, control as well as cancer cells using 2',7'- dichloro fluorescein diacetate (DCFDA). Briefly, cells were exposed to the CQ extract IC₅₀ dose for 24 h. Cells were then washed with PBS and were resuspended in PBS containing 10µM DCFDA for 30 min at 37°C in incubator. Relative amount of intracellular ROS was subjected to evaluation by Fluorescence microscopy. The pictures were taken by Blue FITC filters of Fluorescence Inverted Microscope (Nikon) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm [14].

2.10 Generation of culture model and CQ treatment response analyzed by phase contrast microscope

Regular ELISA 96-well plates coated with agarose thin films were used for generating spheroids. HeLa cells

aggregated homogenous tumoroids that were similar in shape and size to tumors reported in the literature [15] as shown in Figure.6, after 72-h post seeding. Phase contrast microscopy clearly shows the formation of spheroid culture aggregated in groups or singles. Tumoroids composed of HeLa cells forms small aggregates of 50 to 100 μm , 24 h post seeding. Subsets of these aggregates develop into steadily growing tumoroids by doubling their size by day 6. However Tumoroid composed of HeLa cells exhibit a rapid aggregation phase, particularly after day 3. Phase contrast images of cell aggregates show that the tumoroid becomes more compact and dense day after day by exhibiting a steady exponential growth, reaching a plateau of about 1 mm in diameter by day 6. The time course of tumoroid formation depends on the composition of the tumoroids. Only a fraction of these initial aggregates, however, develops to become larger tumoroids at later time points. A viable rim of cells approximately 100 μm is visible in the tumoroid periphery. On 21st day the tumoroid was subjected to the treatment of CQ extract, which is of same dose as given to the monolayer culture i.e. 200 $\mu\text{g/ml}$.

2.11 Statistical analyses

All values were presented as the mean \pm SE. Statistical analyses were performed by using SPSS 11.0 software. Representative data were analyzed for statistical significance by one-way ANOVA. A P-value of less than 0.05 was considered as statistically significant.

3. Results

3.1 Cytotoxicity Assay by MTT

Cell viability was assessed by MTT assay, which is a colorimetric assay for measuring the activity of cellular enzymes that reduce the tetrazolium dye, MTT, to its insoluble formazan, giving a purple colour. For the evaluation of inhibition of cell viability of cancer cells, CQ at different doses (5-400 $\mu\text{g/ml}$) was administered for 24 hrs. It was found that the number of cells decreases as the dose increases and at approximately 200 $\mu\text{g/ml}$ dose of extract, 50% of the cells (HeLa cells) were less as compared to normal (HaCaT cells) treated cells as shown in figure 7. The percentage of cells viability was determined by calculating the O.D of treated against the Blank.

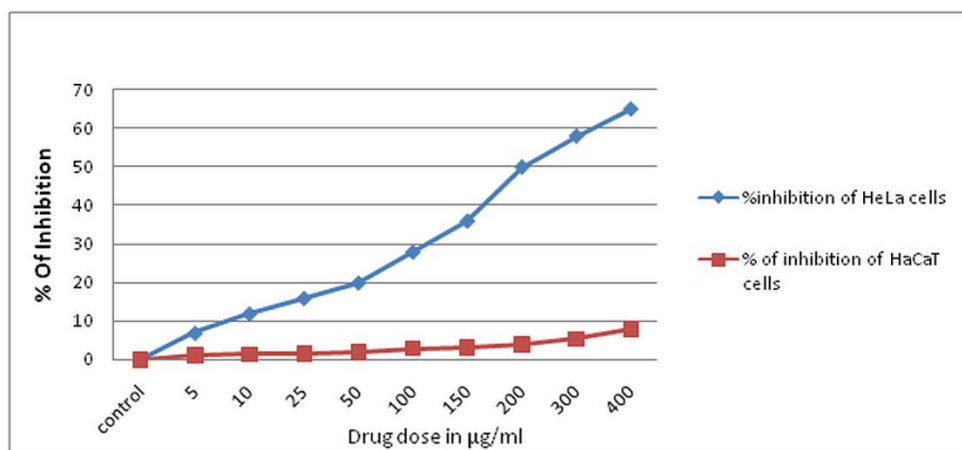
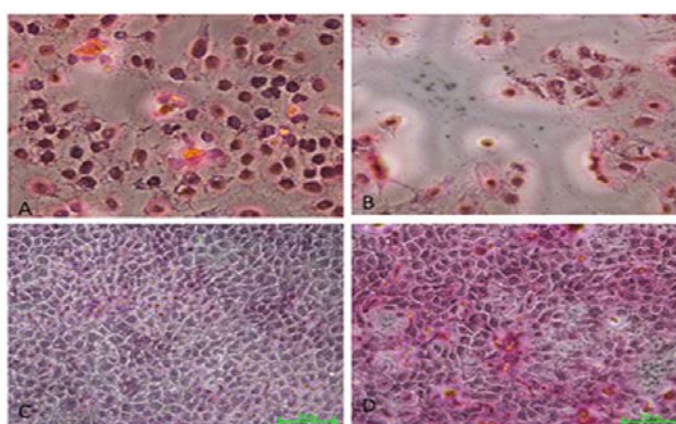


Figure 7: Drug dose % Inhibition of HeLa and HaCaT cells after the CQ extract treatment

Graph 1 show the Drug dose % Inhibition of HeLa and HaCaT cells after the CQ extract treatment. It can be observed by the result of MTT assay that the IC₅₀ dose of CQ is 200µg/ml whereas CQ does not inhibit the growth of HaCaT cells significantly. As the dose increases the HeLa cell viability decreases whereas at the same amount of CQ dose and treatment time there is no such inhibition of normal cells (HaCaT).

3.2 Analysis of Membrane Morphological Characteristics by Haematoxylin /Eosin (H/E) Staining

Morphological changes such as changes to the cell membrane, loss of membrane asymmetry and cell shrinkage, are the early stage of apoptosis was analyzed by H/E staining. The IC₅₀ dose (200µg/ml) treated cancer cells show features of apoptosis whereas treated with same amount of dose, to normal treated cells appeared without any significant changes (Figure 1).



A- Cancer control, B- Cancer Treated, C-normal control, D- normal treated

Figure 1: CQ significantly altered the morphology of HeLa cancer cells (Figure 1B) as compared to control; however at the same dose of CQ extract, normal skin cells (HaCaT) does not show morphological alteration (Figure 1D). (Figure 1A) is HeLa control and (Figure 1C) are HaCaT control cells. Simultaneously the cell density is also low in HeLa treated section Figure.1B in comparison to normal treated, normal control and cancer control section Figure 1D, 1C and 1A respectively.

3.3 Assessment of DNA Damage by DAPI Staining.

The present study found that CQ-extract induced dose-dependent apoptosis in HeLa cervical cells as determined by the DAPI nuclear staining assay (Figure 2). DNA strand cleavage induced by extract was observed in cervical cancer cells which suggested that 200 µg/ml dose of CQ extract treatment could induce apoptosis in cervical cancer cells. It can be observed from the Figure 2B that the bright fluorescent cells marked by arrows indicate nuclear changes that are characteristic of apoptotic cells.

3.4 Apoptosis detection by Acridine orange and Ethidium Bromide dual staining

After staining with AO/EtBr control, normal treated cells appeared green in colour with intact nuclei. After CQ extract treatment, early apoptotic cells showed condensed nuclei and appeared bright green in colour whereas

late apoptotic cells appeared in red colour with condensed and fragmented nuclei. From the observation of AO/EtBr dual staining, number of apoptotic and dead cells increased as compared to control (Figure 3).

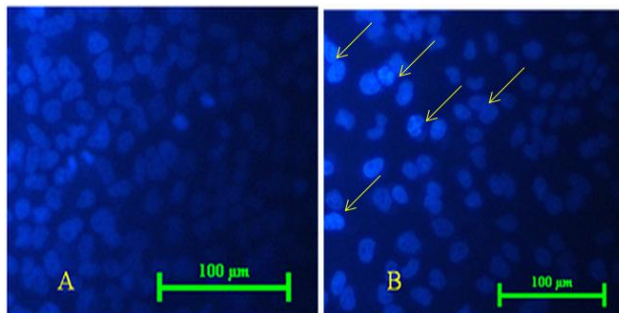


Figure 2: CQ treatment shows genotoxic effect as revealed by DAPI staining. DAPI staining is a reliable apoptotic assay which is used to detect the nuclear morphology of the cells by fluorescent microscopy. It can be observed from the Figure 2; Control-A & Treated B that the bright fluorescent cells marked by arrows indicate nuclear changes that are characteristic of apoptotic cells. The test was done after the 24 h administration of CQ extract (200 µg/ml i.e IC₅₀ dose) to HeLa cell.

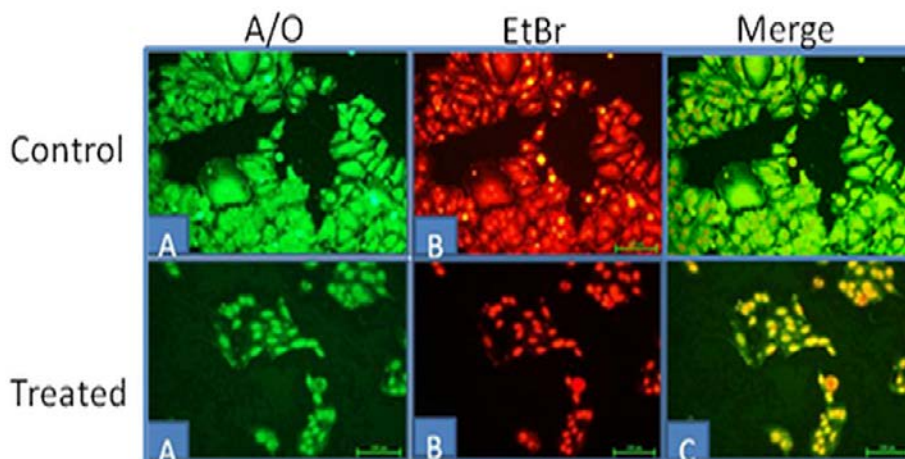
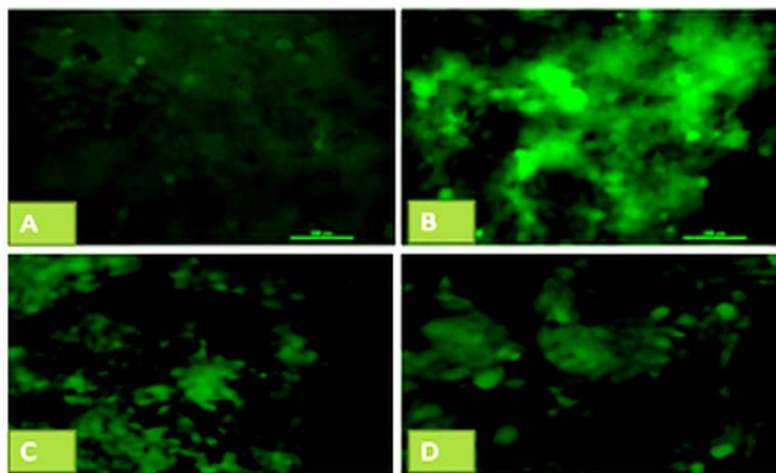


Figure 3: Dual staining by Acridine orange and Ethidium Bromide shows the apoptotic activity of CQ extract. The CQ induces the apoptosis in HeLa cells at 200µg/ml, the images were taken after 24 h of administration of CQ extract to HeLa cells. As compared to control the number of cell also decreases in treated HeLa after CQ treatment. Figure 3C (merge) shows both early and late apoptotic cells whereas Figure 3A in AO (early apoptosis) and in Figure3B EtBr (late apoptosis) respectively in treated section.

3.5 Evaluation of Reactive Oxygen Species generation by DCFDA Staining

DCFDA, a non-fluorescent cell-membrane permeable probe, was used to penetrate the cells, react with cellular esterase and ROS, and then metabolize into fluorescent DCF that can be detected by fluorescence microscopy

(Figure 4). The image shows a significant increase in fluorescence in HeLa cancer cells when treated with CQ extract, as compared to the untreated control, normal untreated control and normal treated.



A-Cancer control, B-Cancer treated,C-Normal control D-Normal Treated

Figure 4: Higher level of Reactive oxygen species detected by DCFDA staining after treatment of CQ.

Increased level of ROS is indicated by the green fluorescent cells in section B of Figure 4.

Figure 4B as compared to cancer controls Figure 4A, whereas no significant changes of fluorescence were observed in normal treated Figure 4C and normal control Figure 4D cells. This test indicates the selective targeting of CQ extract as it liberates ROS only in Cancer cells and not in normal cells.

3.6 Analysis of induction G1 Arrest by Flow Cytometry

Mechanism of HeLa cell growth inhibition by CQ extract was observed and resolved by its effect on cell cycle distribution by flow cytometer. Flow histograms represent cell cycle distribution in cancerous and normal cells at 24 h exposure of 200 $\mu\text{g/ml}$ dose of CQ extract as shown in (Figure 5). CQ extract exposed cells resulted in the 64% arrest of HeLa cancer cells at G0-G1 fraction with 46 % of apoptosis, which was accompanied by a decrease in both S phase 35.17% and G2-M phase cells 0.02% (Figure 5.2). The CQ extract treated normal cells were in the phase of the cell cycle in a normal manner (Figure 5). Thus, CQ extract mediated growth inhibition of HeLa cervical cancer cells. Normal skin HaCaT cells treated with the same concentration of CQ extract did not show any effect or cell cycle arrest (Figure 5.4).

3.7 CQ treatment inhibit growth of HeLa tumoroid

The formation of tumoroid starts from 3rd day (A) to 6th day (B) and at 9th (C) day it grows and takes proper shape of spheroid. As the days increase the size also increases the pictures were taken at an interval of three days (shown in Figure 6 12th day (D), 15th day (E), 18th day (F) and 21st day (G)). However the treatment of CQ was given at 22nd day and after 24h it starts shrinking Figure. H is of 23rd day, Figure I is of 24th day (after 48h of treatment), Figure J is of 25th day (after 78 h of treatment).

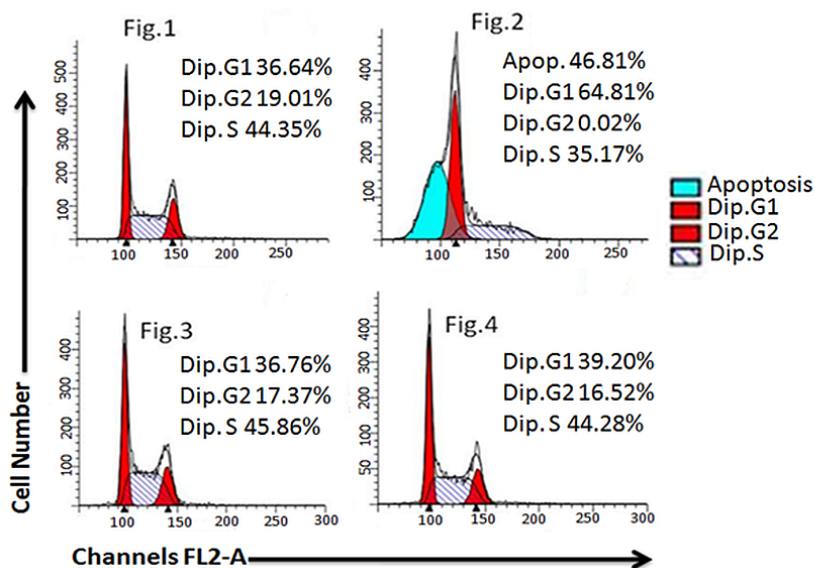


Figure 5: Flow histogram for cell cycle analysis: CQ 200µg/ml dose of extract inhibits the HeLa cancer cell cycle as it arrests the treated HeLa cancer cells at G1 phase with 46.81% of apoptosis as analyzed by FACS (Figure 5.2). Whereas in normal treated cells (HaCaT) CQ does not halt or arrest the cells (Figure 5.4). It can be observed that the CQ shows its efficacy and selectivity in induction of cancer cell growth arrest.

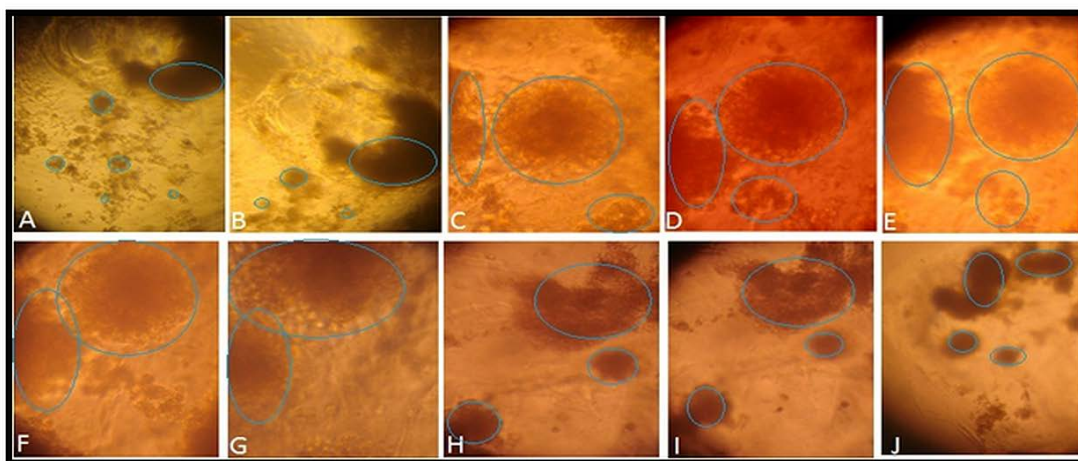


Figure 6: For the establishment of three-dimensional cultures in cancer drug discovery and target validation, we standardized agarose coated microplate method with the following steps and condition (i) 96-well adherent culture; (ii) pelleted cells were seeded onto the agarose coated wells (iii) Phase contrast microscopic analysis. We used low EEO agarose for attachment on 96-well flat-bottomed plates. Pelleted HeLa cell formed a three-dimensional structure within 72 h. 5×10^3 cells pelleted found as optimal seeding densities for HeLa cells. Cultures were maintained by replacing the medium at every three days.

4. Discussion

Most of the plant extracts or its derivatives have been shown to be effective in human cervical cancer cell lines however, plant-based drugs with potent anticancer effect will add to the efforts to find a cheap drug with least clinical side effects. Hence we have evaluated the cytotoxicity of ethanolic extract of CQ and it showed cytotoxic effects on HeLa cervical cancer cells, whereas CQ shows its non-toxicity against normal skin cells (HaCaT) as revealed by Hematoxylin and Eosin staining. Other cervical cancer cell lines where plant extracts or its derivatives have been found effective are KB3-1, SiHa, C33a and CaSki [16]. Some of the plant products such as topotecan, a topoisomerase inhibitor, have been shown to be more effective than antineoplastic drugs-taxol for cervical and other cancers as well in clinical trials [17]. Others such as the extracts of *A. confertifolia*, has indicated significant toxic effects in cervical cancer cells in concentration comparable to those of the FDA-approved anti-cancer drug Onxol® especially at highest doses [18]. In general, the plant extracts or its constituents, cause cellular toxicity in cervical cancer cells and result in cell death by two primary pathways-cell cycle arrest and apoptosis. We have evaluated the cell cycle arrest induced by CQ and it was found that, extract arrests 64% cancer cells in G1 phase, 35% cells in S phase followed by approximately 0.02 cells in G2 phase with 46.81% apoptosis whereas at the same dose of CQ extract the normal skin cells have been found to be unaffected. Cell cycle arrest results into fragmentation or degradation of DNA, a hallmark of induction of apoptosis. So, we observed the DNA damage induced after the treatment of CQ extract on HeLa and HaCaT cells by DAPI staining. In recent years, an increasing number of experimental results indicated that the increase of ROS is involved in apoptosis induction by chemotherapeutic anticancer agents [19]. Gallic acid (3, 4, 5-trihydroxybenzoic acid, GA), a polyhydroxy phenolic compound, is abundant in natural plants such as gallnut, grapes, sumac, oak bark, green tea apple peels, grapes, strawberries, pineapples, bananas, lemons, and in red and white wine. Its antioxidative DNA-damage action has been well documented [20]. However, gallic acid induces apoptosis in several cancer cell lines by increasing ROS level and GSH depletion [21]. Several ROS inducing compounds previously investigated as antimicrobial agents, pesticides, or natural products of vegetables, have been demonstrated to possess anticancer activity in a number of cancer models. Whereas several experimental results demonstrated that the increase of ROS in cancer cells may play an important role in the initiation and progression of cancer [22, 23] such that intrinsic oxidative stress is often viewed as an adverse event. However, excessive levels of ROS stress can also be toxic to the cells: cancer cells with increased oxidative stress are likely to be more vulnerable to damage by further ROS insults induced by exogenous agents [24]. As it was observed by different staining that the CQ extract has discriminating property against cancer and normal cells, then we have done the DCFDA staining to correlate the ROS mediated apoptosis in cancer and normal cells. From the DCFDA staining result it was observed that the no. of green fluorescent cells in HeLa indicating ROS generation were more in comparison to control cells, whereas after the treatment in normal cells there was no significant increase in fluorescent cells as compare to the normal control cells. Therefore, manipulating ROS levels by redox modulation is a way to selectively kill cancer cells without causing significant toxicity to normal cells [25]. Apart from this we have primarily established an in vitro 2D model or tumoroid model and found that the CQ inhibits the growth of tumoroid within 72 h. This observation is in conflict with previously reported literature wherein it was demonstrated that cells grown in three-dimensional culture systems showed reduced susceptibility to chemotherapeutic agents when compared with those grown on

two-dimensional surfaces [26]. However increased ROS liberation along with selective G1 cell cycle arrest may act as factors in inducing tumoroid growth inhibition.

5. Conclusion

It can be concluded by the findings that CQ liberates ROS which mediates apoptosis and induces G1 phase cell cycle arrest unambiguously only in cancer cells. Generation of tumoroid culture as an *in vitro* 2D model is an extensive tool for screening the drug mechanism of action in detail without limitations such as increased duration of experimentation, ethical constraints and costs.

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