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APOPTOSIS AND ANTI-PROLIFERATIVE PROPERTIES OF LUPEOL ON HUMAN TUMOR CELLS

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ABSTRACT

Lupeol, a triterpene found in plant source like fruits and vegetables which selectively induced apoptosis of cancer cells. It also regulates several apoptosis signaling pathways in cancer cells. It has downregulated the proteolysis activity of secreted MMP-2 protein suggesting the efficacy of Lupeol against the spread of Cancer cells. Its role in induction of pro-apoptotic protein indicates that Lupeol rectifies the errors in apoptotic machinery of cancer cells. Lupeol treatment showed cell growth inhibition, anti-inflammatory effects, and tumor regression using FACS for cell cycle analysis, apoptosis, ROS. Furthermore, Lupeol showed antiproliferative action towards U87MG cells at the Inhibitory concentration of 33.76µM. On cell cycle analysis, Inhibition of cells at various stages by lupeol was observed between control and treated. The vast difference in peak count revealed with treated sample count at 300(peak 1) & 240(peak 2) whereas control count at 440(peak 1)& 300(peak 2). The apoptosis inducing activity of lupeol on U-87MG cells shows mean difference treated at 64.982 compared to control at 80.823. Lupeol also regulated the reactive oxygen species in the cells in which excessive distribution occurred at the range of 50-150. These studies conclude that lupeol has the ability to inhibit and induce the Apoptosis, ROS. Thus, confirming anti tumor properties of lupeol.

KEYWORDS: Lupeol, cell cycle analysis, apoptosis, ROS.

INTRODUCTION

Glioblastoma multiforme accounts for 60% of all glial tumors at a frequency rate of 5 cases per 100 000 people which is the most common as well as malignant.[1] Glioma is one of the most lethal and aggressive type of human cancer and it is also a type of primary brain tumor containing ligand groups expressed by *glioblastoma multiforme* and linked to *glioblastoma multiforme* invasion.[2,3] Since gliomas through downregulation of Bcl-2 family, these are much sensitive to apoptosis. In U87 GSLCs, apoptosis was induced by some natural compounds or sources which showed reduction in Akt phosphorylation with inactivation of antiapoptotic protein Bcl2 followed by up regulating the apoptosis-promoting protein Bax and finally cleaving PARP.[4] Lupeol found in fruits and vegetables is a triterpene and phytosterol and researches revealed its various important pharmacological activities. It is said to have a potential to depict as an anti-microbial, anti-poliferative, antiangiogenic, anti-invasive, antiprotozoal, anti-inflammatory through several in vitro and preclinical animal studies.[5] To understand the Efficiency of Lupeol on BCL2 and BCL XL expression level, Post treatment with lupeol suggested that significant downregulation of BCL 2 expression observed in mRNA analysis. Bcl-2 and Bax are the major apoptotic proteins. Therefore, these act as decisive factors and alterations in their expression levels highly influence the cell apoptosis.[6] By suppression of the EGFR/MMp-9 signaling pathway, lupeol induce apoptosis and inhibition of gallbladder carcinoma GBC-SD cells invasion(7).Lupeol also involves in inactivation of Wnt–β-catenin signaling pathway and down regulation of cMYC, CCND1, CCNA2, and CLDN1 by inhibting proliferation, migration and disruption of *TNF-α-VEGFR-2 axis* involving capillary network formation.[7,8] Apoptosis is an important mechanism to maintain homeostasis balance between cell death and cell proliferation which can be achieved by two pathways as intrinsic and extrinsic. In glioblastoma formation, various factors are involved like NFκB, loss of PTEN.[9,10] ROS in cancer cells are predominantly produced by high rate metabolism in cell membranes, endoplasmic reticulum and mitochondria. The tumor cells differ from normal tissues by metabolic phenotypes and these ROS involves in different developmental stages of cancer as initiation, promotion and progression. In cancer cells, the increase in intracellular ROS may lead to diversity of mechanism and its intrinsic mechanism may result from inactivation of tumor

suppressor genes, activation of oncogenes, high metabolism and mitochondrial dysfunction.[11]

MATERIALS AND METHODS

Chemical reagents

Lupeol, DMEM (Dulbecco's Modified Eagle Medium),fetal bovine serum, Annexin V& PI Apoptosis Detection Kit(BD Bioscience cat no:556547), 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2',7'-Dichlorofluorescein diacetate (DCFH-DA) are used.

Cell culture

The U87MG cells were purchased From NCCS, Pune, India, which is a Human brain tumor cell line (glioblastoma) and were maintained in DMEM with 10% fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin under humidified atmosphere with 5 % CO2 at 37 °C.

Cell viability assay

The MTT assay was used to measure the viability of lupeol treated U87MG cells. The cells were cultured overnight by seeding into 96 well plates at a density of 8x103 cells per well after trypsinization. After overnight, the cells were replaced with fresh medium at various concentrations of lupeol (5-100µg) for 24, 48h in comparison with untreated cells as control. Proceeding that, 20 µL MTT (5 mg/mL) was added to each well and incubated for 4hrs then medium was discarded. To dissolve the resulting formazan crystals, 150 µL DMSO was added to each well and the absorbance value were measured at 492nm. For all the three time points, data were collected separately and the effect of growth inhibition was assessed as percent cell proliferation inhibition wherein vehicle-treated cells were taken as 0 % inhibition.

Apoptosis assay

The Annexin V-FITC kit was used to quantify the percentage of cells undergoing apoptosis. In six-well plates at 3x105 cells/well, U87MG cells were seeded followed by exposure to lupeol (IC50) concentration for 24h. By using Annexin with propidium iodide (PI) double fluorescence apoptosis detection kit(BD Bioscience cat no:556547) procedure, staining was carried out after collection of cells and then incubation at room temperature in the dark, the analysis was made with flow cytometry within 30 mins hrs after the staining.

Cell-cycle analysis

The cell cycle analysis of lupeol was determined by methods of Mohammudkhan, et al (12)With normal culture medium, Cells (2×105) were cultured in each well of six-well plates till it reaches 60% confluent. Cells were synchronized by replacing the medium containing 0.1% FBS for 12 hours to arrest them in the G0 phase of the cell cycle, followed by treatment with presence or absence of lupeol for 24 hours in DMEM complete media. Cells were trypsinized thereafter, washed twice with cold PBS, and centrifuged at 1,500 rpm. Cells were fixed in pre-chilled 70% ethanol overnight at 4°C for cell cycle analysis and then centrifuged at 1500 rpm for 5 minutes. The pellet obtained was washed twice with cold PBS and finally suspended in 500 µL PBS, and incubated with 5 µL RNase () at 37°C for 30 minutes. Cells were ice chilled for 10 minutes and then stained with PI staining solution (PI 50 mg/mL, RNase A 10 mg/mL, and 0.1% Triton X-100 for 30 mins and the cells were analyzed by using FACS verse cytometer (BD). Data were analyzed by FACS verse software.

ROS analysis

The ROS generation in U87MG by lupeol was performed using method of Sahdeo Prasad, *et al.*[13] For ROS generation using FACS verse flow cytometer (BD) of 10,000 cells, the untreated as well as treated U87MG cells were monitored. For 1h, U87MG cells were suspended in PBS supplemented with 50 mM glucose for ROS detection and incubated with 10 µM DCF-DA at 37°C. The fluorescence increases due to the hydrolysis of DCF-DA to dichlorofluoroscein (DCF-DA) by some nonspecific cellular esterases and its subsequent oxidation by peroxides was measured.

RESULTS

Effect of lupeol on viability of U87MG cells

Lupeol is a established as important anti cancer drug for various tumors and has shown its efficiency. The effects of lupeol on viability of U87MG cells was investigated using MTT assay. It was found that decrease in viability of cells treated with lupeol with proportion to dose dependent manner at 24 hrs and 48 hrs(Figure 1). The half inhibitory concentration (IC50) of lupeol at 24hrs and 48 hrs was 33.76µM.

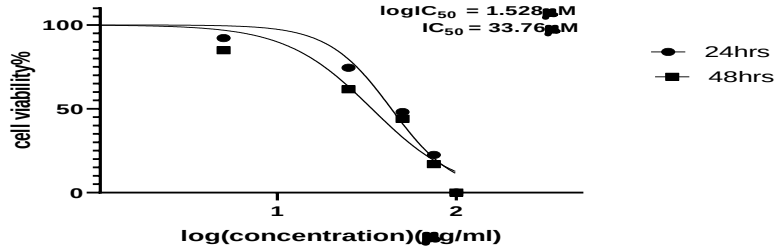


Figure 1: Cell viability assay.Inhibition of U87MG proliferation by Lupeol with different concentration (5-100µg) for different time period (24,48hrs). The cell viability percentage was calculated by using MTT assay.

Analysis of U87MG cell apoptosis Induction on the treatment with lupeol

Based on the view of above inhibitory effect, we observed that lupeol was able to induce apoptosis in U87MG cell. The study resulted in identifying the apoptosis in treated cells with comparision to untreated. The percentage of apoptotic cells increased on comparision of control cells to cells treated with lupeol.

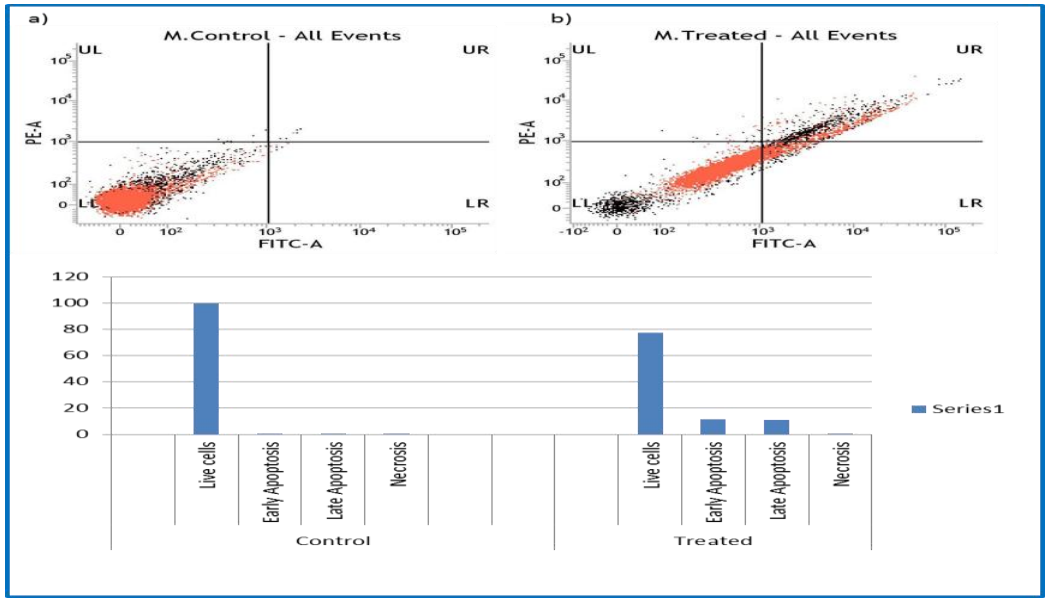


Figure 2: lupeol induces apoptosis in brain tumor cells. Flowcytometric analysis of U87MG cells by lupeol at concentration 33.76µg. Representative figures shows lower left is normal cells,lower right shows early apoptosis,upper right shows late apoptosis

Analysis of cell cycle on U87MG treated with lupeol

Further analysis involved possible identification of growth inhibition by cell cycle arrest at specific check point. So the effect of lupeol on cell-cycle perturbation was assessed(fig.2).The low confluent cell culture were treated with lupeol (33.76µM), th concentration same as apoptosis assays. After 24 hrs, cells were tagged with PI and analyzed by flow cytometry. In order understand the distribution of actively dividing cells before the induction of apoptosis, we harvested cells at 24hr.

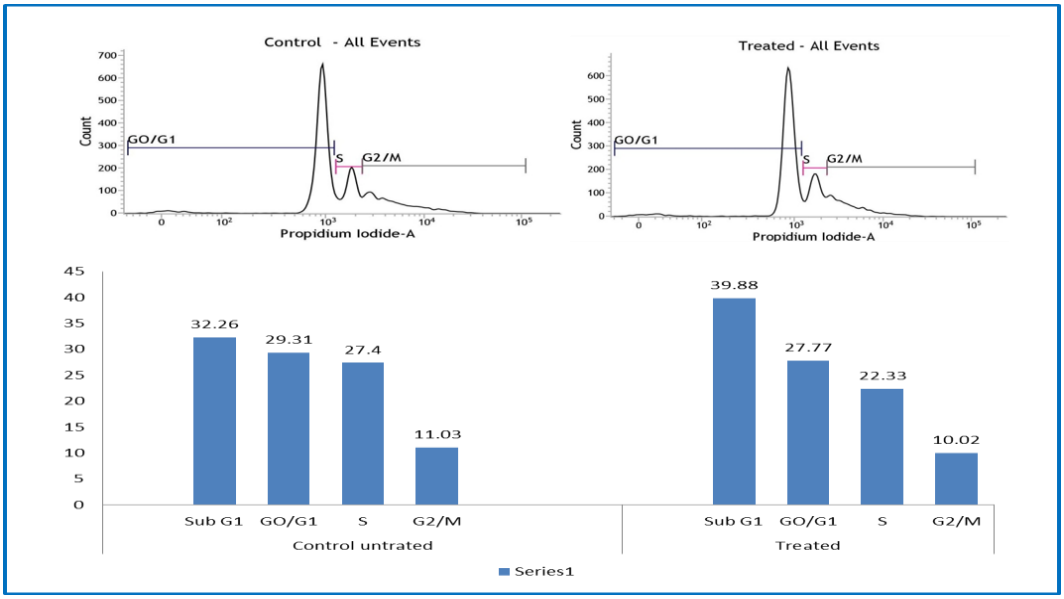
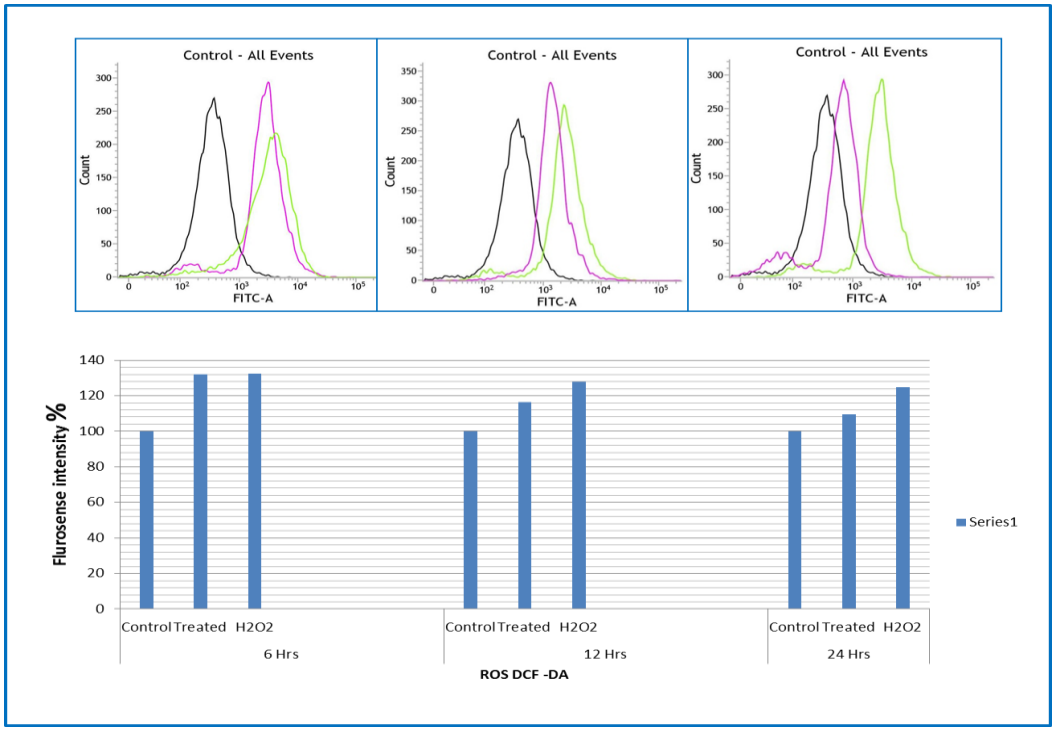


Figure 3: Cell cycle analysis of lupeol treated U87MG in comparison with control. Histogram peaks shows variation in various stage of cell.

Analysis of ROS on U87MG cells treated with lupeol

The ROS levels were calculated in terms of MFI values. On treatment of cells with Lupeol (33.76µM) for 24 h resulted in increase in ROS level (MFI) in comparison to untreated cells (MFI). Thus increase in ROS generation by treated cells confirms the direct induction of apoptosis.



Figures 4: ROS analysis of lupeol treated U87MG in comparison with control at 3 time points as 6 hrs, 12 hrs and 24 hrs. The difference in intensity was observed between control(Black), treated (Lavender), H_2o_2(Green).

DISCUSSION

The cytotoxic effects of conventional cancer therapies such as radiotherapy and chemotherapy is by damaging the DNA cancer cells but there exist limitation with these treatment as the deregulation of several cell signaling cascades and when used as single modalities due to the high heterogeneity in solid tumors. GBM due to its heterogeneity of tumor, the presence of blood barrier and its highly aggressive infilteration into surrounding tissue, thus it is difficult to treat.[14] Lupeol belonging to triterpene family(wide spread group of natural compounds) of phytochemicals has a practical significance which is produced by arrangement of squalene epoxide in a chair-chair - chair-boat arrangement followed by condensation.[15]

Lupeol is a well established as anti cancer drug for various tumors and has shown its efficacy against many types of cancer which includes head, neck, prostate, pancreatic, hepatocellular and skin cancers.[16] However, mode of action and antitumor efficiency of lupeol on brain tumor is unknown. According to recently available literature, lupeol has shown to be anti proliferative and induce apoptosis in brain tumor cells.[17] In our current study, lupeol revealed its cytoxic ability against U87MG cells by decreasing cell viability with increasing concentration. The MTT data proves lupeol has tendency to penetrate mitochondria of the cells and distrupt its activity. Thus,it inhibits the cell proliferation and growth. Lupeol arrested cell cycle at S phase in PCNA cells by inducing various molecular mechanism.[7] The current results proved that lupeol induced apoptosis and cell cycle arrest was observed at various stages. The U87MG cells treated with Lupeol at concentration (33.76µl) arrested S phase and G2 phase. From the apoptosis studies, we determined the lupeol induces cell apoptosis by activating various proapoptosis factors, by interfering with function of antiapoptotic factors.

To destroy the malignant cells driving the formation of intracellular ROS, anticancer drugs were developed recently. A large number of studies revealed that there are various agents available to induce ROS activation in cancer and many dysregulating signaling modulators are also found to be associated with an increase in ROS levels. Based on the cytotoxic power, it is considered that ROS can inactivate cancer cells through their selective modulation against tumors.[18] From our experimental data, we understood that lupeol has potential to generate ROS which in turn induces cell apoptosis. FACS analysis shows variation in peak formation in histogram in treated tumor cells.

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