Letrozole, an anti-proliferative agent from Glycosmis pentaphylla (Retz.) DC. downregulates the expression of cyclin D1, PCNA and Bcl – xL in human neuroblastoma cell lines

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Research Article

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Abstract

Background

To evaluate the effect of letrozole, an anti-proliferating agent from *Glycosmis pentaphylla* (Retz.) DC. on regulating the proliferation, cell cycle distribution, apoptosis and key mechanisms in human neuroblastoma cell lines.

Methods

The human neuroblastoma cell lines, IMR 32 were treated with different doses of letrozole. The effects of letrozole on cell viability were measured by MTT assay, and the cell cycle distribution was determined by flow cytometry. The expression change in mRNA of proliferating cell nuclear antigen (PCNA), cyclin D1 and Bcl-xL were taken from real-time PCR analysis and the protein levels were detected by Western blotting.

Results

The results of the present study showed that letrozole could cause significant inhibitory effect on proliferation of IMR 32 cells in a dose dependent manner. Cell arrest was obtained at S phase with the treatment of letrozole. Apart from this, the expression of PCNA, cyclin D1 and Bcl-xL were decreased both at mRNA and protein levels for the same treatment.

Conclusion

Letrozole can inhibit proliferation, induce cell arrest and cause apoptosis in IMR 32 cell lines. The decreased expression of PCNA, cyclin D1 and Bcl-xL induced by letrozole contributes to the above effects *in vitro*.

Introduction

Neuroblastoma is a most commonly occurring tumor and is a major cause of death for children below five years of age. It is frequently classified as an embryonal neuroendocrine tumor that originates from neural crest progenitor cells, thereby occurring anywhere in along the sympathetic nervous system [1]. Because of its high variability in presentation, clinical signs and symptoms range from benign to malignant. Hence, the impetus for development of targeted therapeutics in the treatment of neuroblastomas is inevitable. New targets and strategies for therapeutic treatments have been disclosed as a result of recent advancements in our understanding of the cellular, molecular, and genetic foundation of cancer. An emerging concept in this scenario is the selectively targeted terminal differentiation, thereby leading to eventual elimination of cancerous cells and finally restoring the cellular homeostasis. Additionally, natural remedies for cancer have emerged as a complementary therapy or as a standalone option to traditional cancer treatment regimens. Several plant based compounds, that are readily available in the form of ayurvedic formulations have been reported to have anti-cancerous

properties [2, 3]. *Glycosmis pentaphylla* (Retz.) DC. belonging to the family Rutaceae has been traditionally known for its immense medicinal properties. Strong antiproliferative and anti-metastatic effects of this plant have been demonstrated in both in vitro and in vivo settings [4, 5]. The plant has been used in India by Ayurvedic and other traditional medical practitioners to treat a variety of conditions, including cough, rheumatism, anaemia, arthritis, jaundice, and skin irritation [6]. Though research on this plant's extracts against health-related issues has been conducted, little is known about the pharmacological compounds obtained from *G. pentaphylla* [7]. In this study, an attempt has been made to examine the effect of letrozole (4,4'-(1H-1,2,4-Triazol-1-ylmethylene)dibenzonitrile) obtained from the leaf ethanolic extract of *G. pentaphylla* against neuro-oncogenesis. Being a third-generation aromatase inhibitor, letrozole reversibly inhibits aromatase, which may result in growth inhibition of estrogen-dependent breast cancer cells. Additionally, letrozole has been shown to play a function in neuroprotection [8, 9].

Numerous malignant cell types have been found to differentiate in vitro through various routes induced by pharmacological and physiological treatments. This study was aimed in investigating the cytostatic and differentiation inducing ability of letrozole obtained from ethanolic leaf extract of *G. pentaphylla* by different chromatographic and spectroscopic methods on IMR 32 human neuroblastoma cell lines. Cell cycle is a tightly orchestered process that have profound impact on signalling pathways. Stress and growth factor responses in the cell cycle regulatory proteins may lead a cell for indefinite division, thereby leading to cancer. As cancer is a serious health burden and being the second leading cause of death world wide, severe therapeutics have to be adapted to bring this under control. Cyclins are enzymes that gradually stimulate the cell from its resting state to cell division. A number of sequentially activated cyclin/cyclin dependent kinase (CDK) complexes and cyclin dependent kinase inhibitors (CDKI) regulate the cell cycle. These processes are generally balanced to guarantee proper gene replication and adequate cell proliferation. Increased cell proliferation can pay way to oncogenesis. Among the important regulators in the G1/S checkpoint, cyclin D plays a key role in driving the cell forward into S phase. It has been shown that, overexpression of cyclin D by binding with CDK4 or CDK6, can ultimately result in cell proliferation [10, 11]. Proliferating cell nuclear antigen (PCNA), a non-histone nuclear acidic protein functions as a cofactor for DNA polymerase. The protein is expressed differentially during cell cycle and the its rate of synthesis is correlated with the arte of proliferation [12]. Thus, PCNA and its binding partners, such as cyclins and cyclin dependent kinases (CDKs) complexes, are overexpressed DNA clamp sliding family proteins that cause uncontrolled cell proliferation [13]. Apoptosis is an inevitable process that maintains a balance between cell proliferation and death. It consists of two main pathways which are regulated mainly by the Bcl-2 family of proteins. Thus, in this study, the effect of letrozole on the proliferation, cell cycle progression, apoptosis and the expression of related molecules including PCNA, cyclin D1 and Bcl-xL of IMR 32 cell lines have been explored in vitro.

Materials And Methods

Fresh leaves of *Glycosmis pentaphylla* were collected from the district of Thiruvananthapuram (Latitude- 8.54ºN, Longitude- 76.91ºE and Altitude 18.00 m), Kerala, India. The botanical identities were done by

Curator, Department of Botany, University of Kerala, Thiruvananthapuram, Kerala, India. A voucher material was deposited at the Herbarium of the Department with an accession code KUBH 6043. The leaves so obtained were washed under running tap water, rinsed with distilled water, dried under shade at room temperature and was subjected to cold extraction using ethanol as solvent. The previous study found that the ethanolic extract has high pharmacological activity [14]. Hence, this extract was subjected to column chromatography and letrozole obtained from the extract was further subjected to anti-proliferation studies (unpublished data).

Cell Culture and measurement of cell viability by MTT assay

The human neuroblastoma cell line, IMR 32 were obtained from Rajeev Gandhi Centre for Biotechnology, Thiruvananthapuram, Kerala, India. The cells were kept alive in Dulbecco's modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS), 1% streptomycin, non-essential amino acid, L-glutamine (2 mM) and were maintained in a CO_2 incubator. Cells after attaining a confluence of 60 % were treated with letrozole ranging from concentrations 6.25 to 100 µg/ml in 96 well plates and incubated for 24 h. Further, MTT solution was added and the cells were again incubated for 4 h. Further, the media containing MTT was discarded and the insoluble formazan crystals were dissolved using 100 µl DMSO and absorbance was measured spectrophotometrically at 570 nm with Multiskan PLUS reader (Thermo Scientific) [15].

Cellular and Nuclear Morphology Determination

The neuroblastoma cells were treated with different concentrations of letrozole, viz., 20 and 30 µg/ ml after dose fixation by MTT assay and were observed under phase contrast microsocopy (Leica DFC 280, Cambridge, UK) for morphological changes and nuclear morphology was observed by 4',6-diamidino-2-phenylindole (DAPI) staining in fluroscent microscope (Leica DFC 280 CCD camera).

Cell cycle analysis

To determine the effect on the cell cycle, IMR 32 cells were initially seeded and incubated with letrozole for 72 h. Further, the cells were harvested by trypsinization and washed twice with PBS, fixed with 70 % ethanol for 2 h at 4°C. Finally, staining of cells was done with propidium iodide (PI) and thereafter, the distribution of cell cycle was analysed by BD Accuri C6 Flow cytometer (BD Biosciences).

Immunofluroscence Study

IMR 32 cells at a suitable cell density were cultured on coverslips in a multi well plate and were allowed to settle. After the attachment of cells, they were treated with both control and indicated concentrations of letrozole. After this, the cells were washed and fixed with 4% paraformaldehyde solution and was permeabilized using 0.2 % Tritox -X for 10-15 min at room temperature. This was then blocked with 3% BSA in PBS for 30 min. Later, cells were incubated with cyclin D1, PCNA and Bcl-xL antibody from Sigma-Aldrich (1:250 dilution) at 4°C for 12 h. After washing with PBS-T for three to four times, cells were incubated with secondary antibody (goat anti-mouse/rabbit IgG/IgM Alexaflour 488/543 from Invitrogen,

Carlsbad, CA, USA) for 2 h at room temperature. Visualization of the fluorescent images was done using confocal laser scanning microscope (Leica TCS SP8). Experiments were done in triplicates.

RT-PCR Studies

Trizol method was used for the extraction of total cellular RNA and the cDNA was generated with the help of iScript cDNA synthesis Kit (Biorad). Real-time PCR was performed using SYBR Green Master Mix (2X, Biorad). PCR reactions were done in triplicates on 1 µl of cDNA and amplification condition comprised of 40 cycles with denaturation at 95°C for 15 s, further annealing and extention at 60° C for 1 min. The endogenous control used was 18S ribosomal RNA and relative gene expression was calculated using the comaparative Δ Ct method.

Protein Assay and Western blotting Analysis

For the total extraction of proteins, IMR 32 cells were grown, treated and harvested with PBS-EDTA. The pellets were homogenized in RIPA buffer and the protein concentration was determined by Bradford method. The protein samples containing lysate were loaded into the well to separate them on SDS polyacrylamide gel, thereafter, transferred on PVDF membrane. This was further blocked using 3 % BSA solution for 2 h at room temperature and incubated overnight with primary antibodies of cyclin D1, PCNA and and Bcl-xL (1:2,000) (Sigma-Aldrich) at 4° C. Further, the membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibody (1:20,000) and visualized using Fusion-FX chemiluminescence imager (Vilber Lourmat, France). α-Tubulin was used as an endogenous control for normalizing the expression of the protein of interest and the experiment was done in triplicates.

Statistical Analysis

Values were expressed as Mean±SE and analyzed using SPSS version 22.0 software. ANOVA was used to determine statistical significance of differences of treated samples. P value less than 0.01 was considered as significant.

Results

Letrozole decreased cell viability in a dose dependent manner

In order to check whether letrzoloe has effect on IMR 32 cell proliferation, cells were treated with different concentrations of the drug ranging from 6.25 to 100 µg/ml for 24, 48 and 72 h and the cell viability was analyzed as the mean of the different time intervals. The half maximal inhibitory concentration (IC_{50}) was 24.64 µg/ml. Based on this preliminary results, a dose of 20 µg/ml, a lower dose of the IC_{50} and 30 µg/ml, a higher dose of IC_{50} was selected for further studies on IMR 32 cells (Fig. 1).

Effect of letrozole on cellular and nuclear morphology

The cellular morphology of IMR 32 cells, treated with letrozole was visualized using phase contrast microscope and observed that, at different concentrations, letrozole arrested growth and normal differentiated cell-like morphology with multiple elongate process (Fig. 2). DAPI staining was done to check the nuclear morphology, where DAPI stained A-T rich regions (Fig. 3).

Effect of letrozole on cell cycle distribution in IMR 32 cells

Cell cycle analysis revealed that, letrozole treatment arrested the cells in G0/G1 phase, which was more pronounced in the higher dose group ($p<0.05$) with a simultaneous decrease in S phase cells ($p<0.05$) as compared to control group (Fig. 4). As can be seen from the figure, there were only 17.6% of cells in 20 µg/ml and 14.35 % in 30 µg/ml letrozole treatment group, in S phase as compared to 25.6 % of cells in control group. Further, 20 µg/ml treatment showed 62.65 % cells in G0/G1 phase and 30 µg/ml treatment showed 67.2% cells in G0/G1 and 18.6 % in G2/M phase. Whereas, control group showed 55.2 % of cells in G0/G1 and 19.3% of total population in G2/M phase as compared to letrzole treated groups.

Inhibitory effect of letrozole on the mRNA and protein expression of PCNA, cyclin D1 and Bcl-xL

Letrozole treatment was observed to inhibit cyclin D1, PCNA and Bcl-xL expression at both transcriptional and translational levels, which was more pronounced in lower treatment group ($p<0.01$) as compared to control group. The changes in mRNA levels of PCNA, cyclin D1 and Bcl-xL were shown in Fig. 5. A reduced cyclin D1 expression in letrozole treated neuroblastoma cells was followed by a cell cycle arrest at G0/G1 and G2/M phase, it seems that letrozole, being a multi-component system may be targeting multiple cell cycle check points simultaneously. The higher percentage of cells in G0/G1 and G2/M phase and lower population in S phase in letrozole treated group as compared to control group, explains these findings. This was substantiated with a decreased expression profiling of Bcl-xL suggesting that, letrozole maintains a balance between cell survival and death inducing signaling cascades. The decreased expression profiling at translation level was also observed and was authenticated with the results of Western Blotting (Fig. 6). Immunostaining results using the corresponding antibodies also confirmed the aforesaid results (Fig. 7).

Discussion

Children with neuroblastoma frequently have severe side effects as well as long-term consequences from standard therapies like radiotherapy and chemotherapy. An understanding into the molecular mechanisms behind the cellular proliferation and apoptosis have helped to develop new therapeutics. With respect to this, as, natural therapies aims in selectively eliminating cancer with less side effects, the present work has been done with an objective to evaluate the effect of letrozole from leaves of *Glycosmis pentaphylla* on regulating the proliferation of cell cycle distribution, apoptosis and relevant mechanism in human neuroblastoma cell lines, IMR 32. The present study elucidates the effect of letrozole on anti-proliferation on IMR 32 cells in a dose-dependent manner. Letrozole (4,4'-(1H-1,2,4-Triazol-1-ylmethylene)dibenzonitrile), a third generation aromatse inhibitor obtained from the leaves of *G. pentaphylla*, selectively and reversibly inhibits aromatase, that result in growth inhibition of estrogen

dependent breast cancer cells. It has also an effect in neuroprotection induced by GABA metabolism [17]. Different doses of letrozole (6.25 to 100 µg/ml) was used in IMR 32 cells to determine its viability through MTT assay. Preliminary data on this, resulted in fixing the dose of letrozole as 20 and 30 µg/ml. Result shows that IMR 32 cells treated with low concentration of letrozole (20 µg/ml) found terminal differentiation. Through the present study, it was concluded that, letrozole supresses the proliferation of IMR 32 cells in a dose-dependent manner and induced cell arrest at the G1/S phase. Relevant to this, letrozole changed PCNA and cyclin D1, the molecules that are closely associated with cell proliferation and cell cycle. A decrease in both the mRNA and protein levels was observed with the treatment of letrozole, especially at the lower dose of 20 µg/ml. For many forms of aggressive cancers, targeting cells with high rates of proliferation is a crucial concern. One such protein, that can participate in a variety of processes of DNA metabolism and cell survival is Proliferating Cell Nuclear Antigen (PCNA). This is a nuclear protein with a molecular weight of 36 kD and is synthesised in the late stages of G1 and S phase [18]. Apart from this, it was documented that, D type cyclins plays an inevitable role in the progression of cells from the G1 to S phase [19]. Since, both PCNA and cyclin D1 in cells is important for the S-phase execution and G0/G1 phase progression, a downregulation of these regulatory proteins is essential for the inhibition of cell proliferation. As apparent from the present study, letrozole is one such compound, that can inhibit the proliferation in neuroblastoma cells. Research findings have substantiated that, regulatory proteins have the these regulatory proteins were over expressed in patients suffering from lung cancers [20]. At the moment, methods for treating cells with drugs that target PCNA and cyclin D1 rely on the use of tiny molecules or peptides that either bind to these regulatory proteins or compete with its interaction partners [21]. Our results suggest that PCNA and cyclin D1 play important roles in cell proliferation and inhibition induced by letrozole, furthermore, letrozole might induce IMR 32 cell arrest at the G1/S phase partly *via* reducing the expression of PCNA and cyclin D1.

In the present study, it was found that, letrozole could induce apoptosis in IMR 32 cells. The notion that apoptosis might influence the malignant forms of cancer dates back to 1970s [22]. Apoptosis is characterized by chromatin condensation, cell shrinkage, DNA fragmentation, mitochondrial swelling and the generation of apoptotic bodies in the form of blebs [23]. The two major pathways in apoptosis include, the death receptor pathway and the mitochondrial pathway, mediated mainly by the Bcl-2 family of proteins. In fact, it is currently believed that errors in apoptotic pathways have a role in a variety of human diseases, from cancer to neurodegenerative disorders [24]. In the present study, an attempt was done to check the expression of Bcl-xL in IMR 32 cells. Bcl-xL, a recently discovered member of Bcl-2 family is a potent death suppressor that is upregulated in some forms of tumour. The expression of Bcl-xL, an anti-apoptotic molecule was observed to be decreased after the treatment of letrozole. This was more pronounced with the lower dose. Bcl-xL can block cell apoptosis as similar to Bcl-2, whereas, some others of the family, such as Bax have the opposite effect of induction of apoptosis [25]. In neuroblastomas, the expression of Bcl-xL is closely relevant in cell survival [26]. In line with it, letrozole treatment resulted in declined Bcl-xL expression both at mRNA and protein levels. This can be co-related with the fact that, letrozole was partly responsible for the increased apoptosis in IMR 32 cells.

Neuroblastoma is enigmatic and unpredictable because of its distinct clinical behaviour [27, 28]. It frequently occurs in toddlers and children and pathogenesis behind its proliferation is unclear [29, 30]. According to the findings of the present work, the effect of letrozole in IMR 32 cells were as follows: the inhibitory effect of letrozole in IMR 32 cell proliferation was in a dose-dependent manner; letrozole induced the cell cycle arrest at the G1/S transition partly *via* decreasing the expression of cyclin D1 and PCNA; letrozole induced apoptosis partly via down regulating the expression of anti-apoptotic protein Bcl-xL. These results provided a strong rationale for the therapeutic application of letrozole in the treatment of neuronal cancers. Nevertheless, the application of letrozole in clinical needs has to further investigated by conducting *in vivo* assays.

Conclusion

The present study demonstrates that, letrozole has an anti-metastatic effect on human neuroblastoma cell lines, IMR 32. Different doses of letrozole were used and based on MTT assay, standard dose for treating the cells were selected. The mRNA expression of proliferating nuclear antigen (PCNA), cyclin D1 and Bcl-xL were determined from real-time qPCR and the protein levels were detected by Western blotting analysis. Immunostaining was also done using appropriate antibodies. From the results, it was found that, letrozole could induce an inhibitory effect on proliferation of IMR 32 cells in a dose-dependent manner. Cell arrest at G1/S phase were also detected on treatment with letrozole. Also, the expression of PCNA, cyclin D1 and Bcl- xL were decreased both at mRNA and protein levels. Thus, it was concluded that, letrozole can inhibit cell proliferation, induce cell cycle arrest and can cause apoptosis in IMR 32 cells, which was in line with the decreased expression of PCNA, cyclin D1 and Bcl-xL. Hence, the present findings suggest letrozole as a potential therapeutic strategy against neuroblastomas without compromising the anti-cancer effect of letrozole.

Declarations

Acknowledgements

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Conflict of Interest

None

Compliance with Ethical standards

This article does not contain any studies with human participants or animals performed by any of the authors.

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Figures

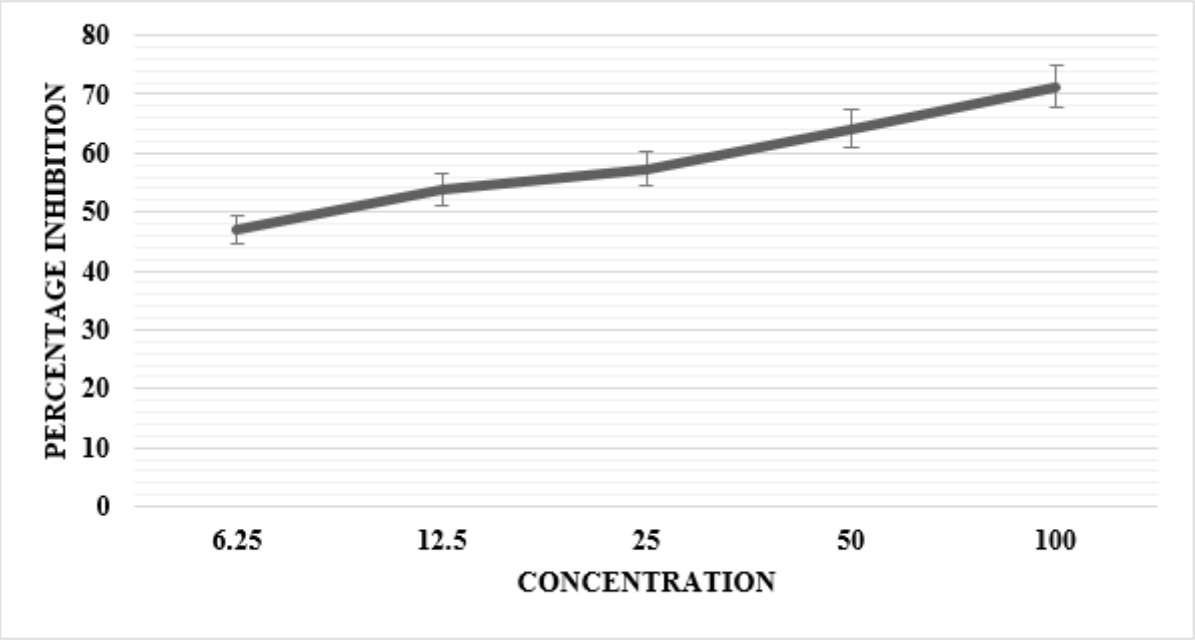

Figure 1

The inhibition effects of letrozole on proliferation of IMR 32 cells in a dose dependent fashion.

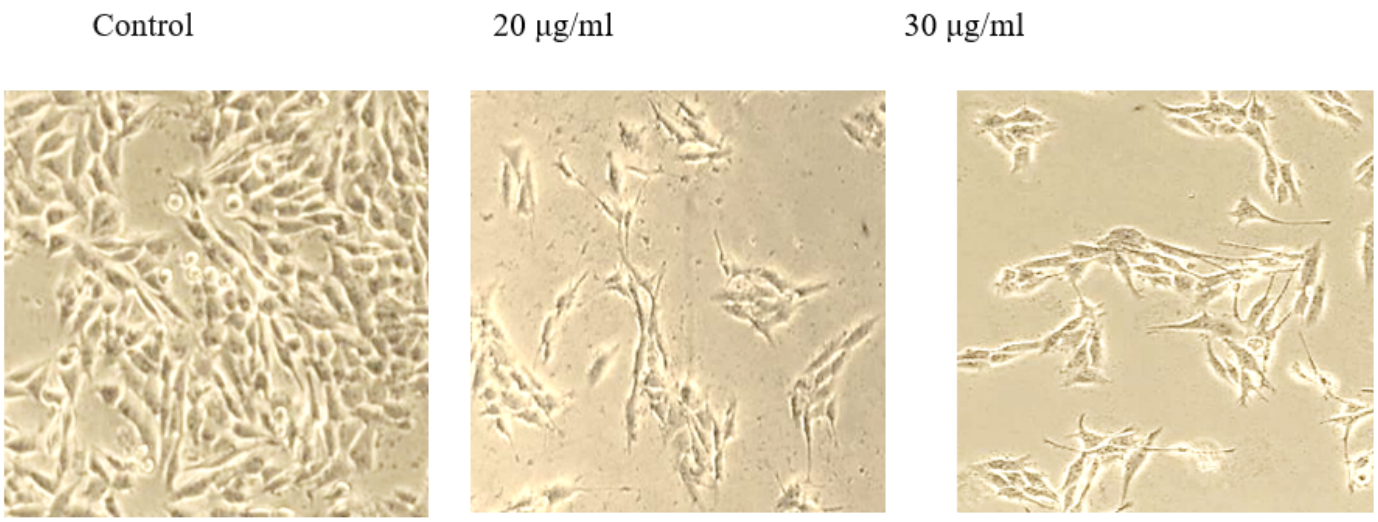

Figure 2

Effect of letrozole on cellular morphology visualized under phase contrast microscope

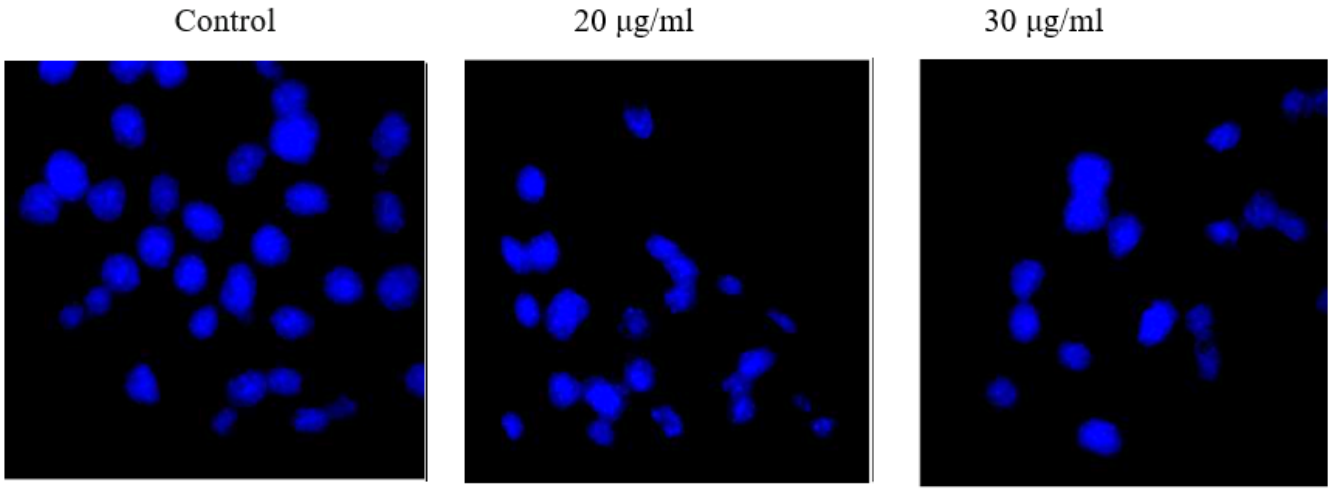

Figure 3

Effect of letrozole on nuclear morphology

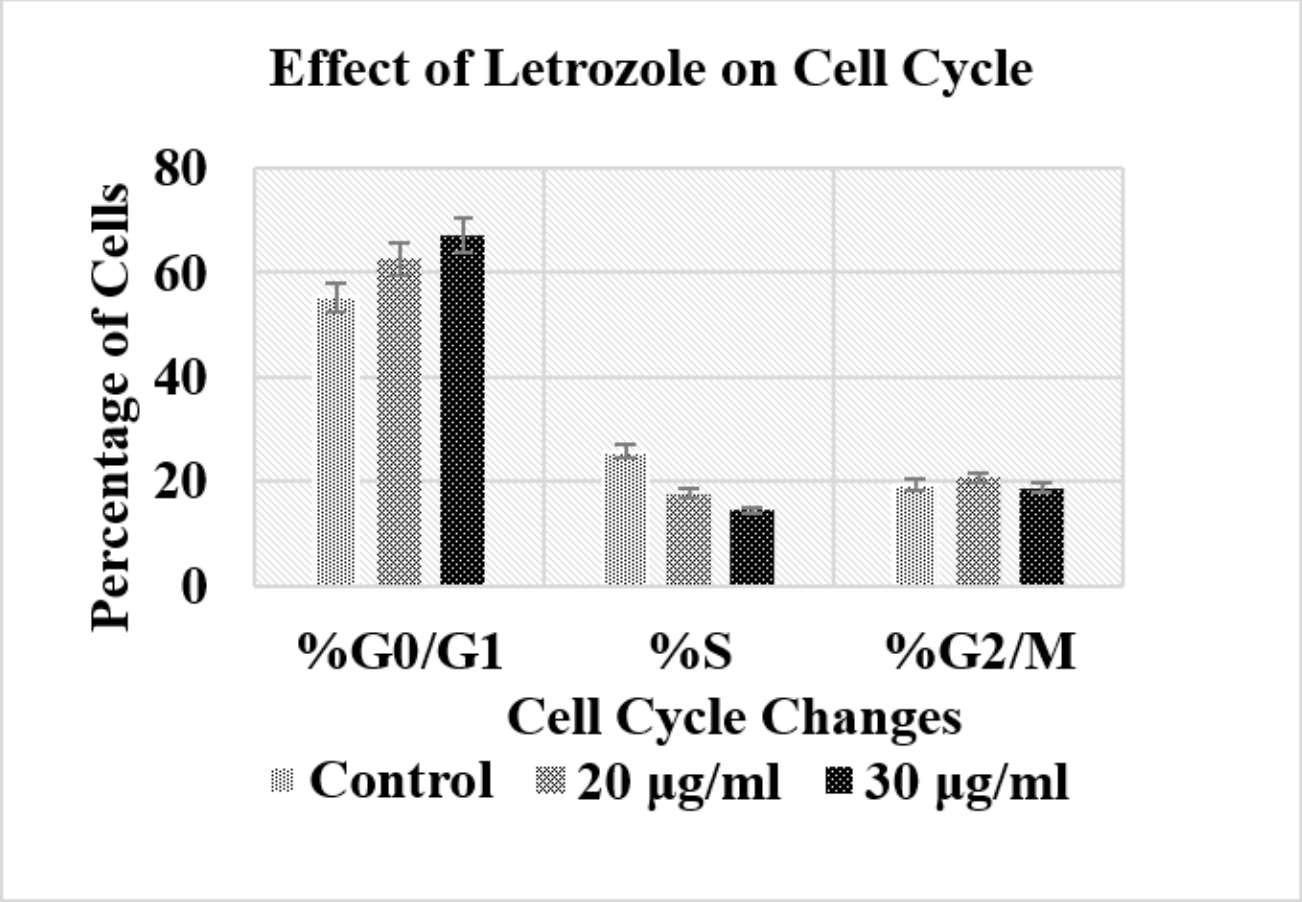

Figure 4

Bar diagram representing the distribution of cells in G0/G1, S and G2/M phase of cell cycle analysed by PI stain using flow cytometer

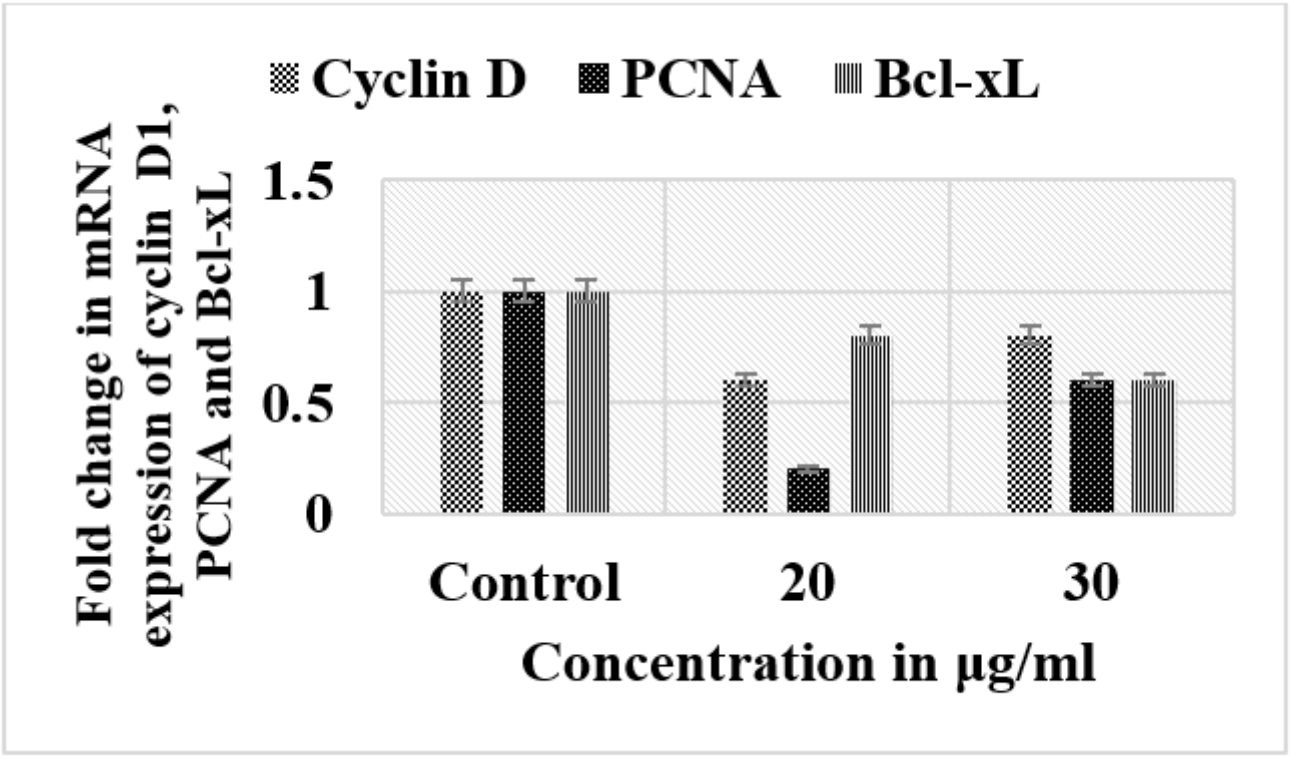

Figure 5

Bar diagram representing the mRNA expression of letrozole treated IMR 32 cells on cyclin D1, PCNA and Bcl-xL

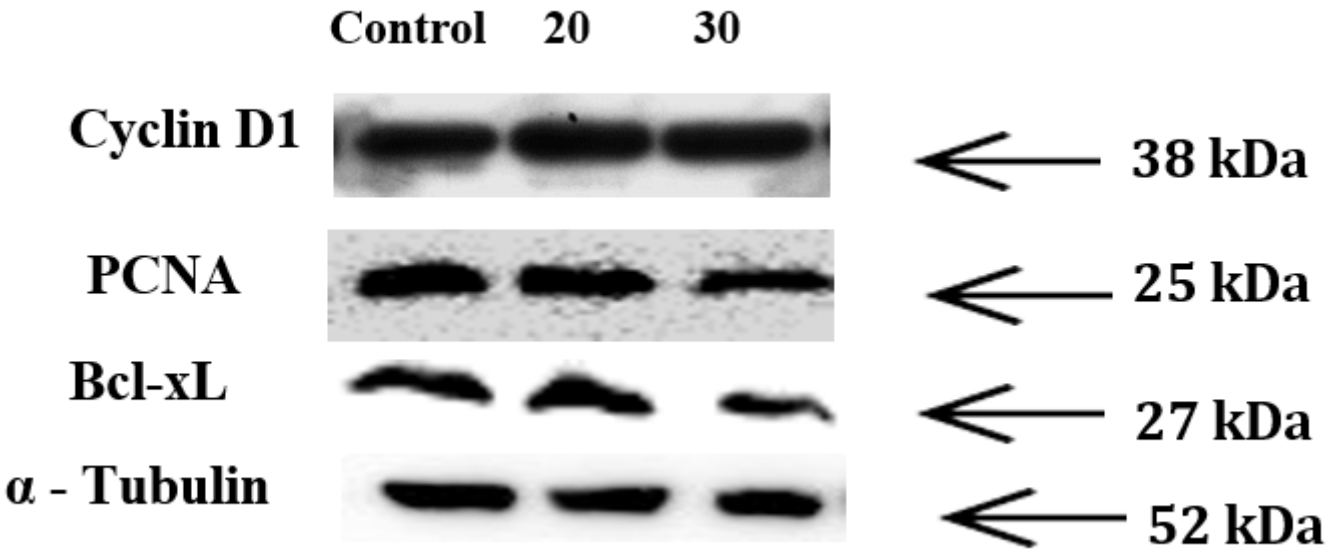

Figure 6

Western blot image of cyclin D1, PCNA and Bcl-xL treated with letrozole on IMR 32 cells with α -Tubulin as internal control.

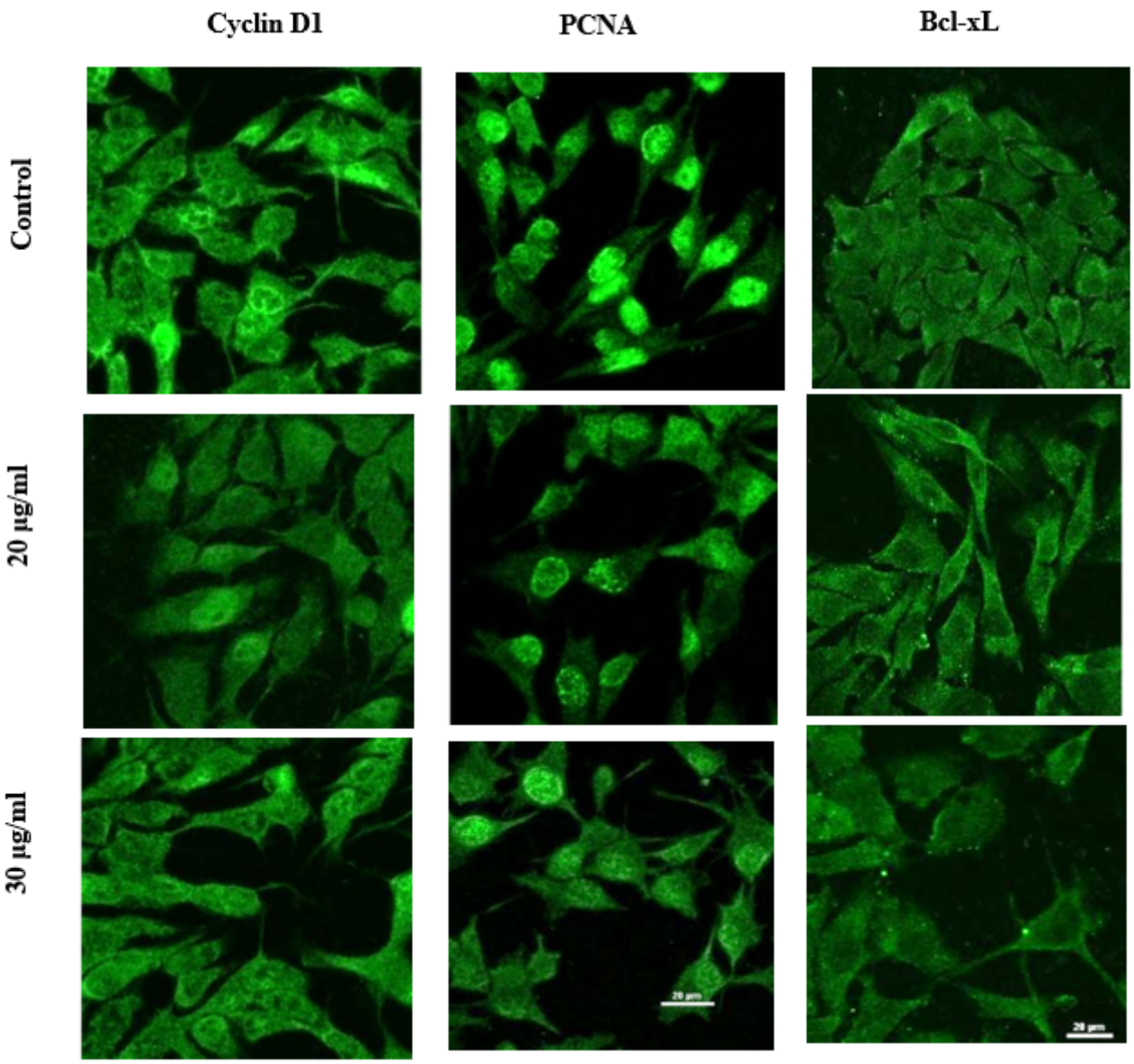

Figure 7

Immunostaining of IMR 32 cells with anti cyclin D1, anti PCNA and anti Bcl-xL treated with letrozole