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Original Research

Elucidation of *in-vitro* Anticancer Effects of Lutein on HeLa Cells

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ABSTRACT:

Introduction: Since antiquity plants are considered as therapeutic agents in traditional medicine. Several clinical trials and research are being conducted on phytocompounds to validate their medicinal effects. It has been observed that these compounds may combat inflammation, mutagenicity, cancer, bacterial and viral diseases to a mild or severe extent. The carotenoids have been gained much attention due to their antioxidant properties and played a therapeutic role in cancer suppression and cardiovascular diseases. Lutein and zeaxanthin are only carotenoids present in the macula and lens of the human eye. They filter high energy blue light as well as act as an antioxidant. The role of lutein as anticancer agent in various cancers have been explored by several researchers but a very few studies have been conducted on cellular models. Based on the anticancer properties of lutein, a study was designed to elucidate its anti-proliferative and apoptotic properties on cervical cancer cells (HeLa) against a standard drug tamoxifen. **Methods:** Cultured HeLa cells (conditions: DMEM 5ml+10% FBS+1% penicillin, streptomycin in a CO_2 incubator containing 5% CO_2 at 37°C) were treated with nine different concentrations each of lutein and tamoxifen. MTT assay was performed to measure cell proliferation. Statistical analyses were done by Two-way independent ANOVA and Tukey Post Hoc with Bonferroni correction. The anti-proliferative potency of lutein on HeLa cells was evaluated by determining IC_{50} values. Further, annexin-V/FITC-PI staining coupled with flow-cytometry was used to mechanistically evaluate the concentration-dependent apoptosis induced by the drugs. **Results:** Lutein showed better potency after 48 hr incubation when compared to tamoxifen. **Conclusion:** Lutein was found as potent as tamoxifen in inhibiting cervical cancer cell proliferation in vitro.

Keywords: Apoptosis, Cancer, Carotenoid, Tamoxifen

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INTRODUCTION

Lutein (Ln), a carotenoid was isolated from the marigold flower, *Tagetes Erecta*. It is present in the macula region of the retina along with zeaxanthin where they act as antioxidants.[1] Lutein is a nonprovitamin A carotenoid present in broccoli and spinach.[2] It has been shown with its chemopreventive activity in a mouse model of murine breast and colon cancer.[3] Dietary Ln is found to be associated with decreased breast cancer risk. [4] An animal-based study focusing on the platelet-derived growth factor (PDGF), a potent stimulator of growth and motility of vascular smooth muscle cells (VSMC) was performed to evaluate the anticancer properties of Ln. Abnormalities in PDGF

may cause malignancy and vascular disease. Overexpression of the PDGF receptor is traced in carcinoma of the brain and diverse malignancies. Lutein blocked the PDGF- induced signaling and significantly blocked the migration of VSMC as compared to ZXN.[5] Based on prior research on anticancer effects of lutein, a study was designed to elucidate its anti-proliferative and apoptotic properties on cervical cancer cells (HeLa) against a standard drug tamoxifen.

MATERIAL AND METHODS

Cell culture

Cervical cancer cell lines (HeLa) were obtained from the National Centre for Cell Science (NCCS), Pune, India to carry out the experiments. Lutein (purity ≥95%) and Tx [6] (purity 98%, analytical grade) were procured from Sigma Aldrich, India. HeLa cells were cultured (DMEM 5ml+10% FBS + 1% penicillin, streptomycin) and maintained at 37°C in a humidified atmosphere of 5% CO_2. After trypsinization, the cells were pelleted and the supernatant was discarded followed by resuspension of cells in fresh assay media (2ml) to achieve 1 million cells / ml. We added 10,000 cells in 100 µl medium / well into 96 well plates a day before performing the assay and incubated for 24 hrs. After 24 hrs, cells were inoculated with 100 µl of each concentration of serially diluted drug (Ln and Tx) preparation. Cancer cells were treated with 9 different concentrations (20, 10, 5, 2.5, 1.25, 0.625, 0.3125 and 0.078 µM) of Ln, and Tx in triplicates for 24 and 48 hrs.

Cell proliferation assay

MTT Assay:

5mg MTT reagent was prepared in 1ml of PBS. 20 µl MTT was added to all the wells and the plate was incubated for 4 hrs at 37°C. Thereafter, each well was fed with 100µl of dimethyl sulfoxide to dissolve the formazan crystal formed during the reaction. Further, the 96-well plate was incubated in dark at room temperature for one hr. A microplate reader (BioRed Lab, Model 3550) was used to read the absorbance at 570 nm with a reference wavelength of 650 nm.

Half maximal inhibitory concentration (IC_{50}):

The absorbance (measured in MTT assay) was used as a proxy for cell viability. The data set from each cell-line type and drug treatment pair were fitted with a sigmoid curve, separately at 24 and 48 hrs time points. Consequently, IC_{50} estimates were obtained from each dataset which was then used to make inference about their anti-proliferative potencies.

Measurement of apoptosis using Annexin V-FITC/PI staining:

FITC annexin V apoptosis detection kit was procured from BD Pharmingen, India and the recommended protocol was followed. Cancer cells were treated with 20, 10, and 5 µM (in triplicates) concentration of each of Ln, and Tx in 6-well plates for 24 hr. Thereafter, cells were suspended in 100 µl 1X binding buffer. 100 µL solution of cell suspension ($5x\ 10^4$ cells) + 5 µL FITC annexin V + 5 µL PI was added in each well and labelled as: one well each for 3 concentrations of 3 drugs; control, FITC, PI, FITC+PI and incubated for 15 min at room temperature in the dark. Further, 400 µl of 1x binding buffer was added in each well to analyze the reading within one hour using flow cytometry (BD Accuri C6).

Statistical Analysis

A two-way grouping between ANOVA was applied to evaluate the effect of time points and treatment levels (nine different concentrations of a drug) as well as the effect of treatment types and treatment levels on percent viability of HeLa cells. Bonferroni adjusted simple main effects analyses were carried out to evaluate the difference between two levels of time points and drug types at each level of treatment. Further, Tukey HSD post hoc analyses were done separately at respective time points as well as drug types. For all purposes, $p>0.05$ was considered to indicate statistical significance. IC_{50} values were estimated from the modified Hill-equation curve fitted to the cell viability data, using non-linear regression analysis by Graph Pad Prism 8 software. Kruskal Wallis one way ANOVA was done to measure the apoptosis of three different doses of Ln, and Tx each. All values of Flow cytometry to quantify the different stages of cell death were analysed by MATLAB R2015a.

RESULTS

Cell Proliferation Assay

Effects of Ln on HeLa cells: (Figure: 1)

For Ln, there was a statistically significant interaction between the factors of time points and treatment levels on the effect of % viability, $F_{(9,\ 40)}=5.491$, $p<0.001$, partial $\eta^2=0.553$. The mean % viability of 48 hrs incubation were consistently lower and the differences of % viability between 24 hrs and 48 hrs were significant at 20 µM to 1.25 µM ($p<0.001$), 0.625 µM ($p=0.001$), 0.3125 µM ($p<0.001$), 0.15625 µM ($p<0.001$) and 0.078 µM ($p=0.010$), respectively. The mean difference between control and Ln was significant at two concentrations (20 µM: $p<0.001$; 2.5 µM: $p=0.001$) at 24 hrs time points. All the concentrations at 48 hr were significant with control.

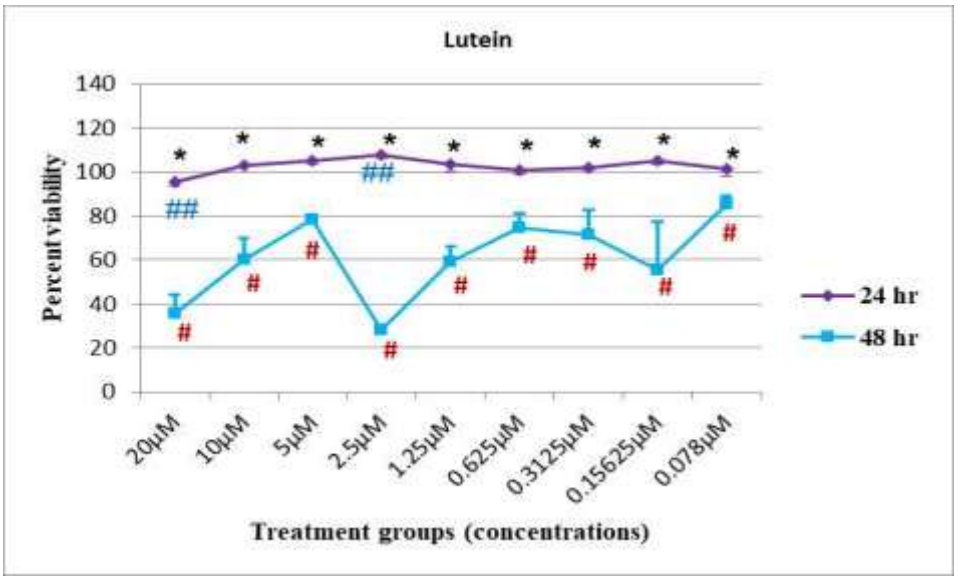

Figure 1: Significant difference b/w 24 and 48 hrs*
Significant difference b/w 24 hrs and control ##
Significant difference b/w 48 hrs and control #

Effect of Tx on HeLa cells: (Figure: 2)
A statistically significant interaction between the factors of time points and treatment levels on % viability, $F_{(9, 40)} = 5.37$, $p < 0.01$, partial $\eta^2 = 0.55$. Bonferrroni adjusted simple main effects analyses were performed to evaluate the differences of two levels of time points (24 hrs and 48 hrs) at each level of treatment (control and 9 different concentrations of drug). The mean differences of % viability between 24 hrs and 48 hrs were significant at 20 μM ($p = 0.001$), 2.5 μM ($p < 0.01$), 0.625 μM ($p = 0.001$), 0.3125 μM ($p< 0.01$), 0.15625 μM ($p < 0.01$), 0.078 μM ($p < 0.01$) respectively. Tukey HSD post hoc showed after 24 hrs the mean differences of % viability were significant between control and Tx concentrations at 20 μM ($p < 0.01$), 10 μM ($p = 0.003$) and 0.078 μM ($p = 0.002$) respectively. Similarly, after 48 hrs the differences between control and Tx groups were significant at 20 μM ($p < 0.01$), 10 μM ($p = 0.003$) and 0.078 μM ($p = 0.001$) respectively.

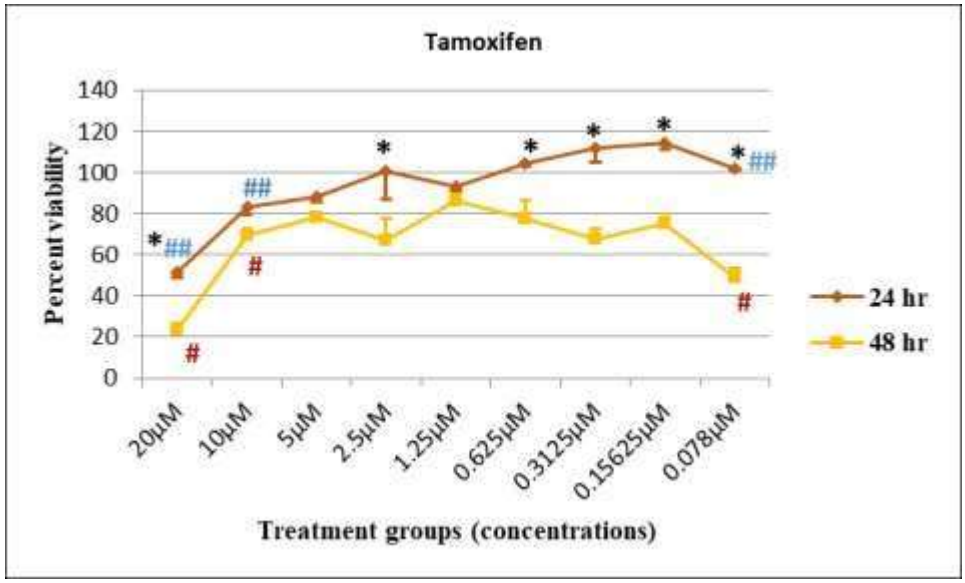

Figure 2: Significant difference b/w 24 and 48 hrs*
Significant difference b/w 24 hrs and control ##
Significant difference b/w 48 hrs and control #

Comparative effects of Ln and Tx on HeLa cells at 24 hrs: (Figure: 3)
For Ln vs Tx at 24 hrs, there was a statistically significant interaction between the factors of drug type and treatment levels on the effect of % viability, $F_{(9, 40)}=8.520$, $p<0.001$, partial $\eta^2=0.657$. The % viability of HeLa cells with Tx was much lower than Ln for few low drug concentrations where the mean differences were significant at 20 µM ($p<0.001$), 10 µM ($p=0.001$), 5 µM ($p=0.004$), respectively but the difference nullified for rest of the drug concentrations (2.5 µM and above).

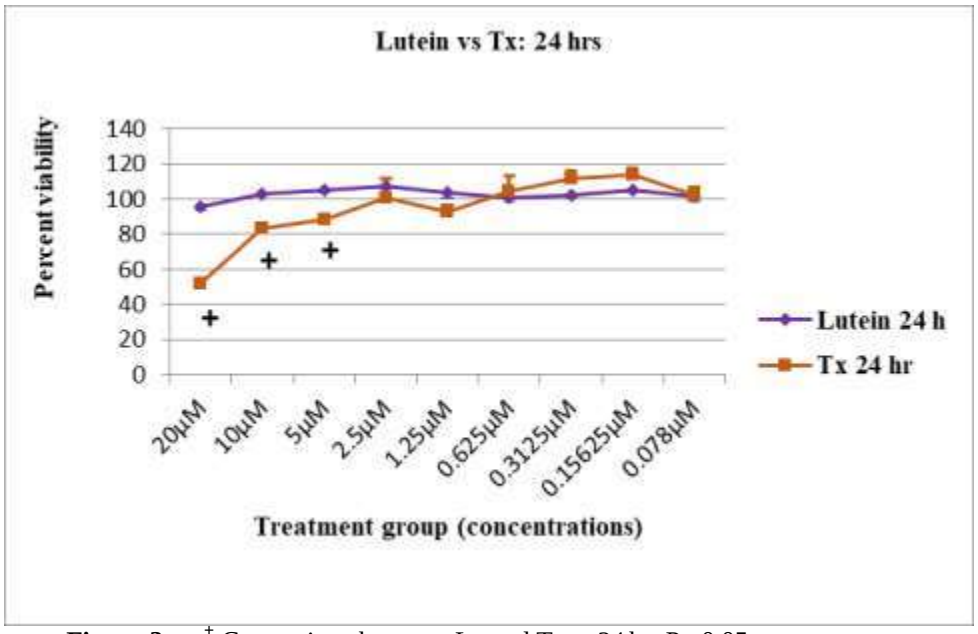

Figure 3: + Comparison between Ln and Tx at 24 hr, P< 0.05

Comparative effects of Ln and Tx on HeLa cells at 48 hrs: (Figure: 4)
For Ln vs Tx at 48 hrs, there was a statistically significant interaction between the factors of drug type and treatment levels on the effect of % viability, $F_{(9, 40)}=3.707$, $p=0.002$, partial $\eta^2=0.455$. The % viability due to Ln was significantly lower than Tx at 2.5M ($p=0.001$) whereas Tx produced significantly lower % viability at 0.078 µM ($p=0.002$). The effects on HeLa cells were not significantly different in the rest of the concentrations.

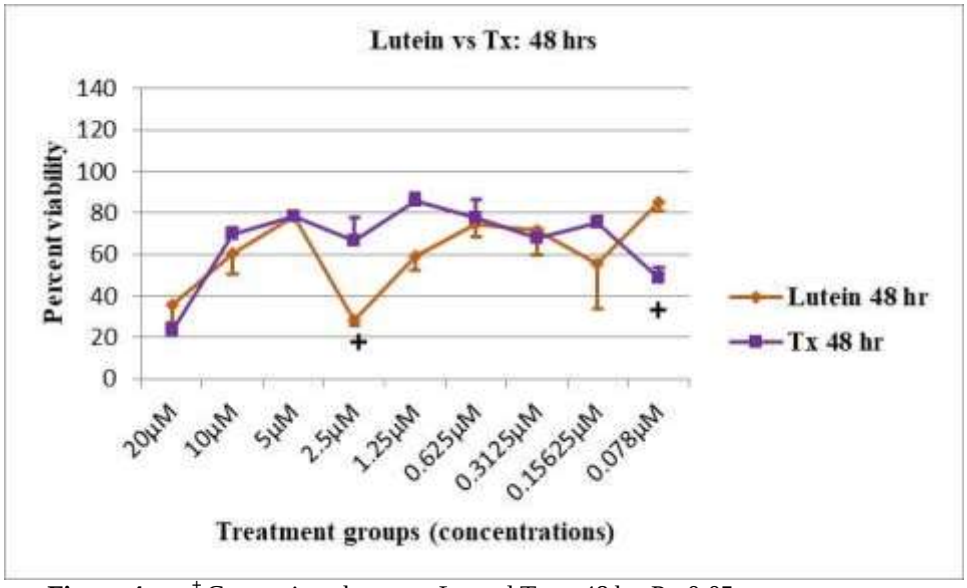

Figure 4: + Comparison between Ln and Tx at 48 hr, P< 0.05

Potency of carotenoids:

Lutein showed better potency after 48 hr incubation (IC_{50} = 2.93µM, Fig.5b) when compared to Tx (IC_{50} = 8.85µM, Fig. 6b;) while Tx was more potent (IC_{50} = 5.63 µM, Fig.6a) than Ln (IC_{50} = 14 µM, Fig.5a) after 24 hr incubation.

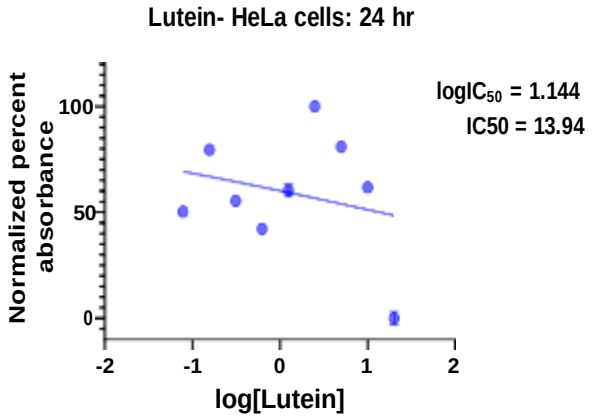

Figure 5a: Potency of Ln on HeLa cells at 24 hr

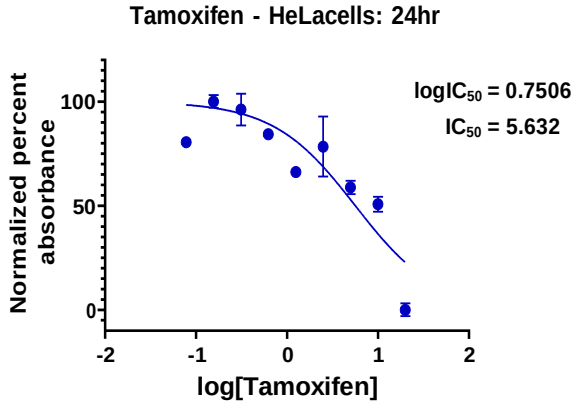

Figure 6a: **Potency of Tx on HeLa cells at 24 hrs**

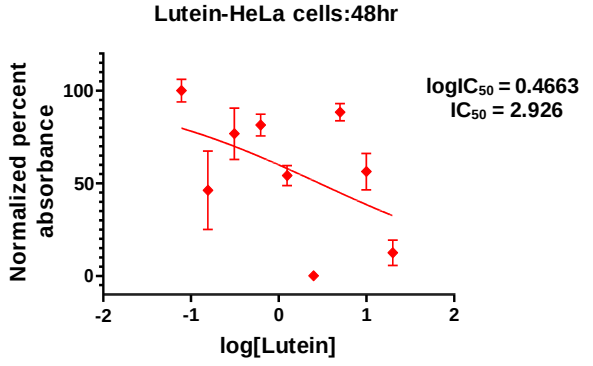

Figure 5b: **Potency of Ln on HeLa cells at 48 hrs**

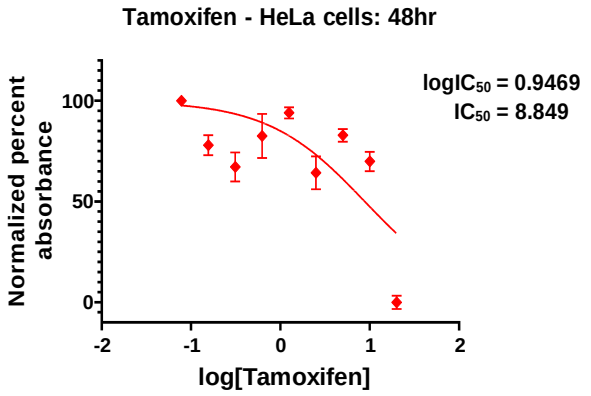

Figure 6 b: **Potency of Tx on HeLa cells at 48 hrs**

Apoptosis assay:

Annexin-V/FITC-PI staining coupled with flow-cytometry was used to mechanistically evaluate the concentration-dependent (three concentrations here) stages of cell death induced by different carotenoids.

Treatment of HeLa cells with Ln:

HeLa cells were treated with three different concentrations (in triplicates) of Ln for 24 hr, the distribution of cells in various stages of cell death was estimated. The cells distribution of LL quadrant (unstained cells) was 48.23±0.51%, 42.22±0.45%, and 52±0.55% after treatment with Ln (L1= 20µM, L2= 10µM, and L3= 5µM respectively). The cells distribution in early apoptosis was 39.1±0.41%, 48.5±0.51%, and 36±0.38% after treatment with 20, 10 and 5µM concentration of Ln respectively. The distribution of late apoptotic cells was 11.4±0.12%, 9±0.09%, and 10.24±0.10% respectively after treatment with 20, 10 and 5µM Ln respectively. The distribution of necrotic cells was 1.65±0.02%, 0.81±0.009% and 2.21±0.02% after treatment with Ln (20, 10 and 5µM respectively). **(Fig: 7, 7a, 7b, 7c)**

Treatment of HeLa cells with Tx:

HeLa cells were treated with three different concentrations (in triplicates) of Tx for 24 hr, the distribution of cells in various stages of cell death was estimated. The cells distribution of LL quadrant (unstained cells) was 51±0.53%, 54.7±0.6%, and 27.6±0.29% after treatment with Ln (L1= 20µM, L2= 10µM, and L3= 5µM respectively). The cells distribution in early apoptosis was 15.34±0.16%, 35.65±0.38% and 44.6±0.47% after treatment with 20, 10 and 5µM concentration of Ln respectively. The distribution of late apoptotic cells was 34.02±0.36%, 7.22±0.08% and 27.11±0.28% respectively after treatment with 20, 10 and 5µM Ln respectively. The distribution of necrotic cells was 0.08±0.0008%, 2.85±0.03% and 1.18±0.01% after treatment with Ln (20, 10 and 5µM respectively). **(Fig: 8, 8a, 8b, 8c)**

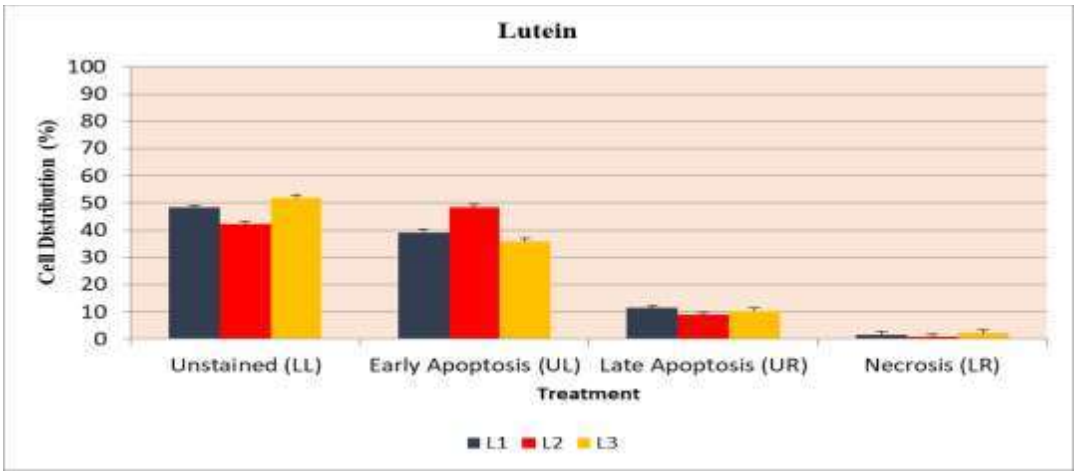

Figure 7: HeLa cells distribution upon Ln treatment in various stages of apoptosis at 24 hr

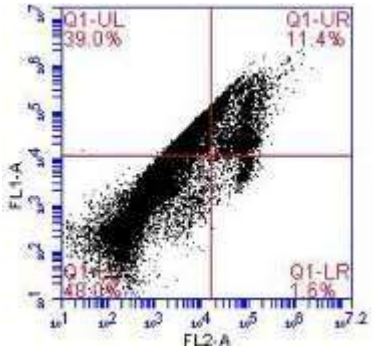

Figure: 7a

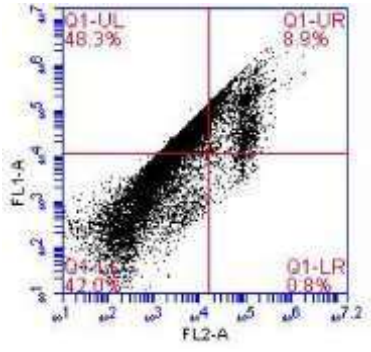

Figure: 7b

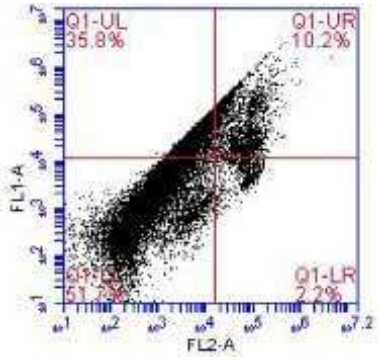

Figure: 7c

Flow cytogram representing percentages of cells in various stages at 24 hr. LL -unstained cells, UL- early apoptosis, UR- late apoptosis, LR- necrosis. HeLa cells were treated with Ln (in triplicates); L1= 20µM (Fig. 7a), L2= 10µM (Fig. 7b), and L3= 5µM (Fig. 7c)

Upon Post Hoc comparison, L3 of necrotic cells was significantly greater as compared to T1 (p=0.0327). Upon Post Hoc comparison T2 of unstained cells was significantly greater as compared to T3 (p=0.0384); T1 of late apoptotic cells was significantly higher as compared to T2 (p=0.0116), and T2 of necrotic cells was significantly higher than T3 (p=0.0066). All other concentrations were statistically insignificant.

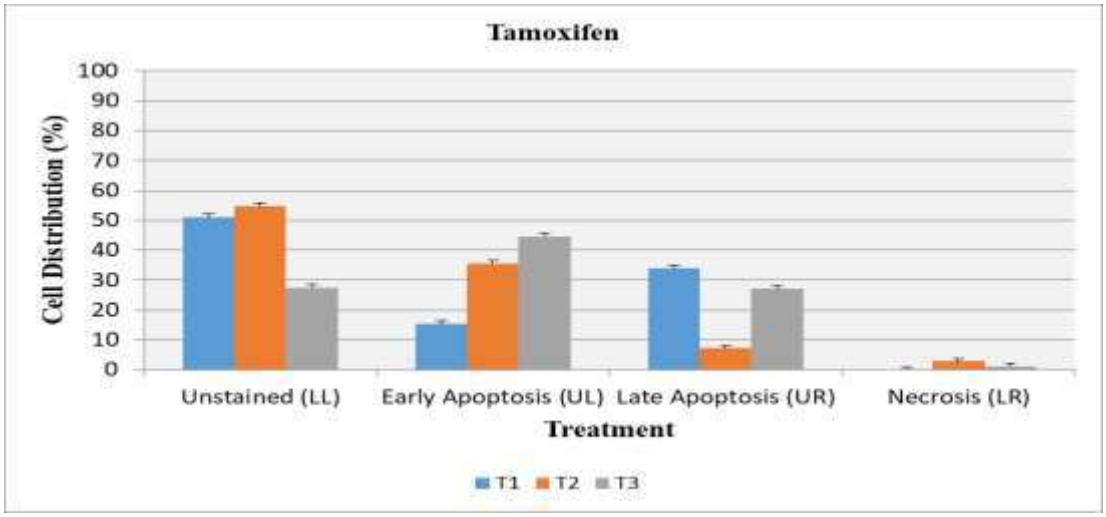

Figure 8: HeLa cells distribution upon Tx treatment in various stages of apoptosis at 24 hr

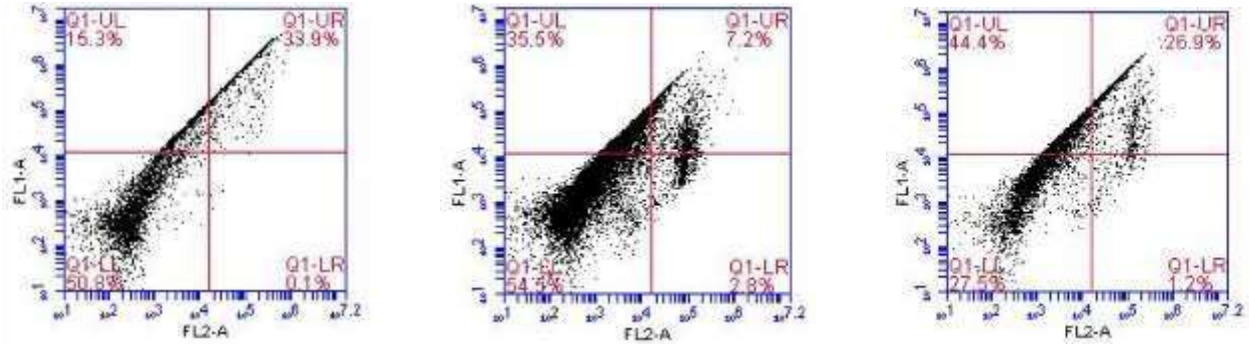

Figure: 8a **Figure: 8b** **Figure: 8c**

Flow cytogram representing percentages of cells in various stages at 24 hr: LL (lower left) -unstained cells, UL (upper left)- early apoptosis, UR (upper right)- late apoptosis, LR (lower right)- necrosis. HeLa cells were treated with Tx (in triplicates); T1= 20µM (Fig. 8a), T2= 10µM (Fig. 8b), and T3= 5µM (Fig. 8c)

DISCUSSION

A study was conducted on HeLa cells to evaluate the cellular and molecular mechanism involved in the anticancer effects of Ln. Cancer cells were treated with different doses of Ln (50, 10, 1, 0.1 µM) for 24 and 48 hrs to evaluate the cell viability using SRB (sulforhodamine B) assay. Cancer cell proliferation was inhibited by Ln up to 62.85% and 84.85 % at 24 and 48 hrs respectively. Lutein was also found to increase ROS levels in cervical cancer cells at 1 µM and 10 µM concentrations. Gene expressions related to apoptosis were also analysed and the results showed upregulation of Bax by 1.65±0.21 fold and downregulation of Bcl-2 by 0.7±0.3 fold with 1 µM Ln treatment. HeLa cells treated with 1 µM and 10 µM concentration increased caspase -3 expression by 1.91±0.2 and 2.1±0.3 fold. These findings suggested the Ln as anticancer agent against HeLa cells. [7]

In the present study, the mean % viability of HeLa cells at 48 hrs incubation was consistently lower and the differences of % viability between 24 hrs and 48 hrs were significant at all concentrations. The mean difference between control and Ln was significant at two concentrations (20 µM: $p<0.001$; 2.5 µM: $p=0.001$) at 24 hrs time points (Fig. 1). We also compared the anti-proliferative activity with that of Tx. Although Tx performed better than Ln to inhibit the proliferation of cervical cancer cells in-vitro in lower concentrations at 24 hrs, Ln achieved similar anti-proliferation properties as Tx at mid to higher concentrations (Fig. 3). Lutein showed further improvement as an anti-proliferating agent after 48 hrs where it matched the properties of Tx for almost all experimental drug concentrations (Fig. 4). In another study liposomes were prepared using phosphatidylcholine (20 µMol) in 2 ml of Tris–HCl buffer with Ln (200 µmol). Thereafter, this mixture was exposed to sunlight for 100 min. Antioxidant and apoptotic activities of photo- oxidized Ln products were studied in terms of cell viability, glutathione, and malondialdehyde levels. These products were identified by LC-MS (APCI+) techniques. Free radical scavenging activity was augmented to 45.9% (IC50, 3.71 µg), 44.1% (IC50, 5.28 µg), and 31.1% by oxidized lutein, Ln and butylated hydroxyl toluene (IC50, 5.53µg) respectively. Oxidized Ln lowered the lipid peroxidation, cell viability, glutathione and malondialdehyde levels by 20.7%, 64%, 40%, and 18% respectively than Ln. It was concluded that oxidized Ln showed high antioxidant property than Ln resulting in cytotoxicity of cervical cancer cells. [8]

In present study, Ln was found to be more potent (IC_{50} = 2.93µM, Fig.5b) as compared to Tx (IC_{50} = 8.85µM, Fig. 6b) after 48 hr incubation. HeLa cells were also treated with three different concentrations (in triplicates) of Ln for 24 hrs to measure apoptosis in early and late stages (Fig, 7a, 7b, 7c). After treatment with 20, 10 and 5 µM of Ln the distribution of apoptotic cells was 39.1±0.41%, 48.5±0.51%, and 36±0.38% in early apoptotic stage and 11.4±0.12%, 9±0.09%, and 10.24±0.10% respectively in late apoptotic stage (Fig.7). Upon Post Hoc comparison, L3 of necrotic cells was significantly greater as compared to T1 ($p=0.0327$). All other concentrations were statistically insignificant.

CONCLUSION

Previous researches have suggested few plausible mechanisms causing inhibition and apoptosis of cervical cancer cells. The P13/AKT pathway serves a key role in cervical cancer cell proliferation and cell cycle regulation.[9] NF-kappa B is activated in many cancers which inhibits apoptosis and promotes the growth of tumor cells. [10] Lutein triggers ROS generation and induced apoptosis of HeLa cells mediated by the intrinsic pathway. [7] In present experiments, efficacy of Ln as an anticancer agent against HeLa cells was profound at 48 hrs. There were certain limitations of present study since it was focused to elucidate the anticancer effects of Ln on HeLa cells irrespective to the underlying mechanism. Further, it is

required to determine the mechanisms and pathways contributing to cell cycle regulation, inhibition of cell proliferation, and apoptosis.

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