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EFFECT OF SMALL MOLECULE INHIBITOR Fisetin OVER PROLIFERATION OF HUMAN NON-SMALL CELL PULMONARY CARCINOMA

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KEYWORDS

Fisetin

Lung carcinoma

A549

NCI-H460

Apoptosis

Proliferation.

ABSTRACT

The percentage of cancer-related deaths attributable to diet and tobacco globally is reported to be as high as 60–70%. A large number of dietary compounds have gained research interest and tested to determine their chemopreventive / antiproliferative effects on *in-vivo/in-vitro* models. The present study effect of flavonoid fisetin on proliferation of human lung carcinoma cells A549 and NCI-H460 was investigated. The effect of fisetin on cell growth was investigated by MTT, Crystal violet, XTT, LDH release, and colony forming assays. Cell proliferation was assessed by BrdU incorporation test. Further, cell morphology and cytopathology was analyzed by crystal violet test and hematoxylin and eosin staining. Results of study revealed that fisetin treatment exerted a dose dependent decrease in the growth of A549 and NCI-H460 cells with IC₅₀ values of 190µM and 210µM respectively. It also inhibited cell proliferation and colony formation in both A549 and NCI-H460 cells. Cytopathology studies exhibited features such as nuclear fragmentation, cytoplasmic vacuolation, karyorrhexis, chromatin condensation, which were suggestive of apoptotic cell death. Results of the study indicate growth inhibitory, anti-proliferative and pro-apoptotic effects of fisetin on lung carcinoma cells.

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1 Introduction

Chemoprevention and functional foods are new areas of great interest in the prevention of diseases. Further, it has been reported that dietary and environmental factors account about 30% of cancers, suggesting that diet plays an important role in the prevention of cancer (Doll & Peto, 1981). In the past two decades a large number of dietary compounds have been tested to determine their chemopreventive / anti proliferative activity *in vivo/in vitro* model systems. Flavonoids are naturally occurring polyphenolic compounds that have very low molecular weight and form a large group of secondary plant metabolites. These compounds have various interesting biological activities such as antioxidative, antimicrobial, anticarcinogenic and cardioprotective (Khan et al., 2008; Moon et al., 2006). Fisetin a major flavonoid has been reported to possess pharmacological properties like anticancer (Huntington et al., 2004), angiogenesis-inhibition (Hirata et al., 2003), anti-inflammatory (Kemik et al., 2011), pro-apoptotic (Liu et al., 2012) and anti-invasive effects (Behrens et al., 2001). Thus fisetin could confer a valuable advantage in treatment of various types of cancers. Like many other flavonoids, fisetin (3, 3', 4', 7-tetrahydroxy flavone), is widely distributed among fruits and vegetables and is found in plants at a concentration of 160 µg/g (Arai et al., 2000).

Lung cancer is the second most common cancer and is the major cause of cancer related death in both men and women. Instead of great advancements in chemotherapy survival rates for lung cancer patients is still disappointing. Majority of the lung cancer patients are in stages III and IV when diagnosed making curative surgery difficult. Based on cell morphology pulmonary cancer is primarily categorized into Non Small Cell Lung Carcinoma (NSCLC) and Small Cell Lung Carcinoma (SCLC). NSCLC is further classified into adenocarcinoma, squamous cell lung carcinoma, and large cell lung carcinoma and comprises nearly 75 to 80% of all lung cancers. The mean 5- year survival rate for those with NSCLC is approximately 15% (Carbone & Felip, 2011). Recent evidences strongly suggest abnormalities in JAK/STAT signaling pathway is involved in the oncogenesis of several cancers especially lung cancers (Song et al., 2011). STAT3 is persistently activated in about 50% of NSCLC primary tumors and lung cancer-derived cell lines (Mukohara et al., 2003; Seki et al., 2004; Haura et al., 2005). Constitutive activation of the P13K/Akt pathway has also been reported in 90% of NSCLC cell lines, thus promoting cell survival and resistance to chemotherapy or γ -radiation (West et al., 2003).

Exploiting and exploring actions of natural products on suitable model systems has led to development of several life saving drugs available in the market today to treat dreadful diseases. Hence, this study is an attempt to understand the anticancer properties of dietary flavonoid/small molecule inhibitor fisetin on suitable human NSCLC *in vitro* model systems.

2 Materials and Methods

2.1 Chemicals

Fisetin (3,3',4',7-tetrahydroxyflavone) was procured from (sigma, St. Louis, MO, USA). A stock solution of 2.86mg/ml of fisetin was prepared in DMSO and stored at -20°C.

2.2 *In-vitro* model systems

2.2.1 Isolation and culture of lymphocytes

Fresh blood was collected and lymphocytes were separated by following the previously described protocol (Boyum, 1968). Based on the density gradient centrifugation the lymphocyte separation carried out using separation media (Hisep™-LSM 1001) procured from Himedia, India. Lymphocytes were culture in complete DMEM media containing 10% FBS at 37°C with 5% CO₂ and 95% humidity in a CO₂ incubator.

2.2.2 Procurement and maintenance of cell lines

The NSCLC pulmonary carcinoma cell lines A-549 (adenocarcinoma), NCI-H460 (large cell lung carcinoma) and normal lung fibroblast cells WI-26 were procured from National Centre for Cell Science (NCCS), Pune, India. The – A-549, NCI-H460 and WI-26 cells were cultured in Hams F12K medium (AL106A- Himedia, India), RPMI 1640 (AL171A- HiMedia, India) and DMEM (AL007- Himedia India) respectively. The media were supplemented with 10% Fetal Bovine Serum (FBS, RM112, Himedia, India), 1X and Antibiotic and Antimycotic solution (A007, Himdia, India) for growth of cells, and cells were grown under standard growth conditions (95% humidity, 5% CO₂ and temperature 37°C) in a CO₂ incubator.

2.3 Cell proliferation and cytotoxicity assays:

2.3.1 MTT assay

Cells were seeded at 1×10^4 cells / well of 96- well micro titer plates and cultured overnight and treated with fisetin in the dose range of 10 µM to 400 µM concentration and were allowed to proliferate 24/48 h (temperature 37°C, 5% CO₂ and 95% humidity). After proliferation, MTT performed following the previously described protocol (Mosmann, 1983). Plates was read at 570nm using a microplate reader and the concentration of fisetin that inhibited cell growth by 50% (IC₅₀) was calculated.

2.3.2 XTT assay

XTT (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide) assay was performed using a commercially available kit (HiMedia, Cat. No. CCK015)

following the manufacturer's recommendations. Cells after treatment were allowed to proliferate (temperature 37°C, 5% CO₂ and 95% humidity). Activated XTT solution was added to the wells and cells were incubated for 2 to 4 hours and the absorbance was read at 450 nm using ELISA reader.

2.3.3 Bromodeoxyuridine (BrdU) assay

Cell proliferation was measured by performing BrdU (5-bromo-2'-deoxyuridine) incorporation assay using commercially available kit (Calbiochem/Merck, Cat. No. QIA58, Whitehouse Station, NJ, USA) following the manufacturer's guidelines. The absorbance was recorded at 450 nm and measured intensity is proportional to the amount of incorporated BrdU in the cells.

2.3.4 Crystal violet assay

Crystal violet assay was performed to determine the effect of fisetin on cell viability following the protocol previously described (Lena et al., 2009). The treated and untreated control cells were stained with crystal violet (0.05% w/v) for 30 min after that wells were thoroughly washed with distilled water and destained with sorenson's buffer. The absorbance of the extracted stain was measured at 540 nm.

2.4 Assays to determine change in cell morphology and apoptotic cell death

2.4.1 Crystal violet staining

Light microscopy analysis of treated and untreated control cells was carried out by fixing the cells in 10% formalin saline and subsequent staining with 0.05 w/v crystal violet for 30

minutes. Cells were observed under phase contrast microscope and images were captured (4×10X magnification).

2.4.2 Hematoxylin and Eosin staining

Cells after treatment were fixed in 10% formalin and the morphology change in both were analysed by staining the cells with hematoxylin and eosin following the protocol previously described (Fischer et al., 2008). Cells were observed under light microscope and images were captured (4×10 X magnification).

2.5 Soft agar assay for colony formation

A cell suspension (5×10³ cells/well) in 1ml of 0.3% agar with complete media was over laid on 35mm dish containing 0.5% base agar as described previously (Hamburger et al., 1978). The media was changed two times a week and cells were maintained under standard growth conditions (temperature 37°C, 5% CO₂ and 95% humidity) in a CO₂ incubator (Forma Scientific, USA). After 15 days, the colonies formed in the untreated control and the treated groups were fixed with 3.7% paraformaldehyde and stained with crystal violet (0.05%) for 1 hour. The colonies were counted under light microscope and images were captured (magnification 10 X).

2.6 LDH cytotoxicity assay

The release of lactate dehydrogenase from treated and untreated control cells was performed by using LDH – cytotoxicity colorimetric Assay kit 11 (catalog no.K313-500) as per the manufacturer's recommendations. After the treatment of cells, the clear medium (10 µl/ well) from treated, control and high control groups was transferred into a clear 96-well plate.

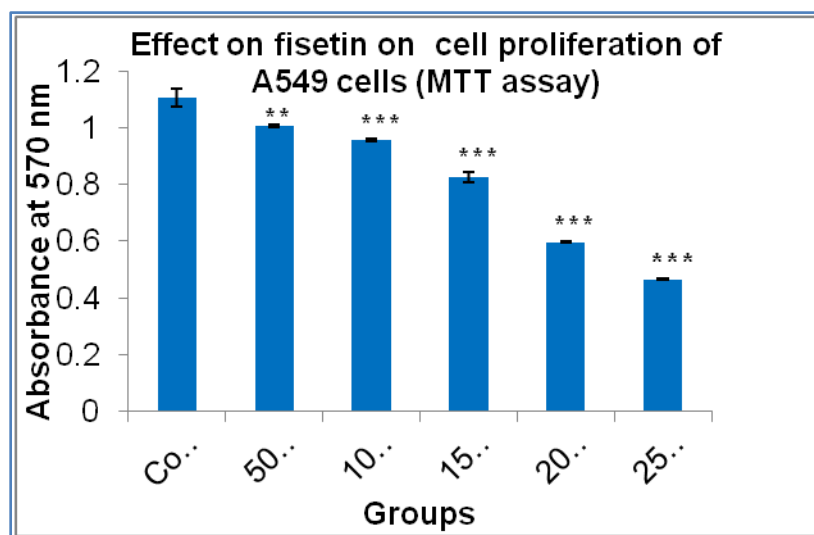


Figure 1(a). Effect of fisetin on proliferation of A549 cells (MTT assay) –pilot studies. Treatment of A549 cells with 50 µM -250 µM concentration showed statistically significant ($p < 0.001$) decrease in the proliferation of A549 cells. Data represents mean \pm SD of six replicates. Intergroup comparisons were made between the cell control and the treated groups. Student's t-test; *** $P < 0.001$, ** $P < 0.01$, versus control.

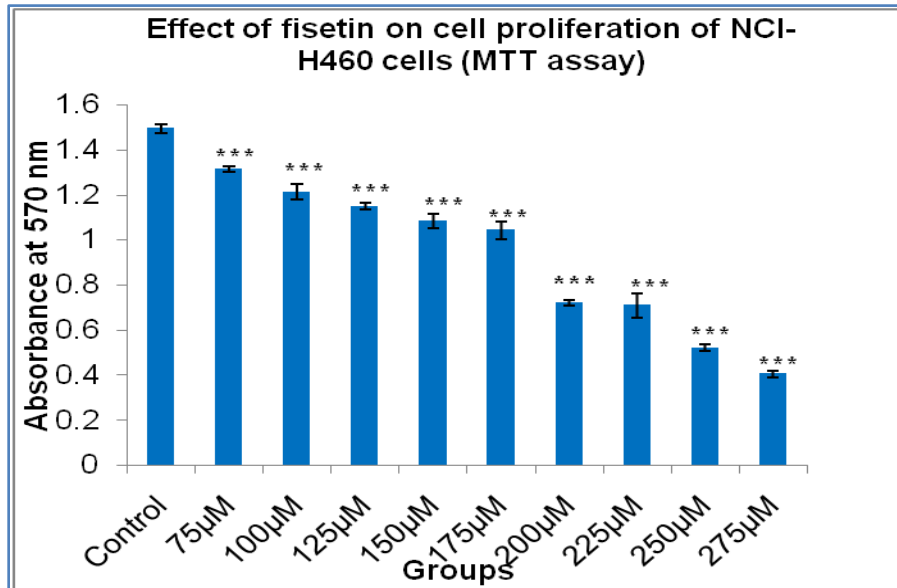


Figure 1(b) Effect of fisetin on proliferation of NCI-H460 cells (MTT assay) –pilot studies. Treatment of NCI-H460 cells with 75µM - 275 µM concentration showed statistically significant ($p < 0.001$) decrease in the proliferation of A549 cells. Data represents mean \pm SD of six replicates. Intergroup comparisons were made between the cell control and the treated groups. Student's t-test; *** $P < 0.001$ verses control.

LDH reaction mixture (100 µl) was added to each well, and after that plates were incubated for 30 minutes at room temp. The reaction was stopped by adding 10 µl stop solution to each well. The red color intensity representing the LDH activity was measured at the absorbance of 450nm using a microplate reader.

2.7 Statistical analysis

Experiments have been carried out in triplicate on at least three different time intervals and the mean of replicate values were taken. All the experimental values were expressed as mean \pm SD. Statistical analysis of the data was determined by Student's t-test and comparisons were made between the untreated control and the treated groups.

3 Results

3.1 Determination of IC_{50} of fisetin on A549 cell line

The Preliminary pilot studies were carried out with various concentrations of fisetin ranging from 10 to 400 µM concentrations. Treatment of A549 cells with 50 µM to 250 µM concentration for 48 h showed a statistically significant ($P < 0.001$) decrease in proliferation of cells however the concentration above 250 to 400 and below 50 µM showed no appreciable decrease in proliferation of cells when compared to 250 µM concentration (data not shown). Based on MTT assay

the concentration of fisetin that inhibited cell growth by 50% (IC_{50}) was determined as 190 µM concentration (Figure 1a).

3.2 Determination of IC_{50} of fisetin on NCI-H460 cell line

Preliminary pilot studies were carried out with various concentrations of fisetin ranging from 10 to 300 µM concentrations. Treatment of NCI-H460 with 10 to 50 µM concentration for 24 h showed no influence on the proliferation of cells as compared to control (data not shown). Whereas concentrations between 75 to 275µM showed statistically significant ($P < 0.001$) decrease in the proliferation of NCI-H460 cells. Based on MTT assay the concentration of fisetin that inhibited cell growth by 50% (IC_{50}) was determined as 210 µM concentration (Figure 1b).

3.3 Effect of fisetin on growth and proliferation of NCI-H460 and A549 cell line

Based on preliminary pilot studies, the IC_{50} doses were further confirmed by performing XTT, BrdU, crystal violet assays (Figure 2, 3, 4). These assays were performed to confirm results obtained from MTT assay and these assays were in agreement. Based on these cell viability and proliferation assays it was observed that treatment with the specified concentrations of fisetin resulted in statistically significant decrease in cell viability, growth, survival and proliferation of A549 and NCI-H460 cells. The IC_{50} doses were confirmed and used for carrying out further assays.

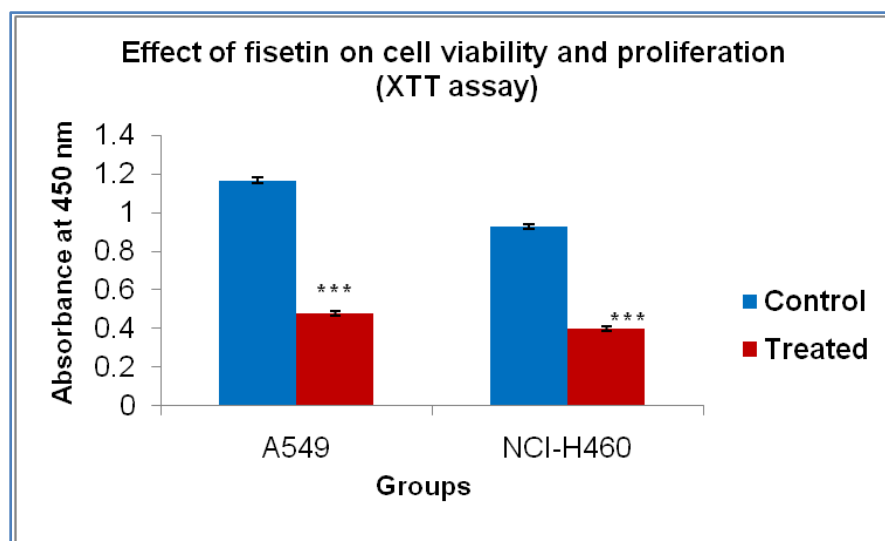


Figure 2 Effect of fisetin on proliferation of A549 and NCI-H46 cells (XTT assay). Treatment of NCI-460 cells with fisetin resulted in a significant decrease in cell proliferation as compared with the control. Data represent mean \pm SD of six replicates. Intergroup comparisons were made between the cell control and the treated group. Student's t-test; ***P< 0.001 verses control.

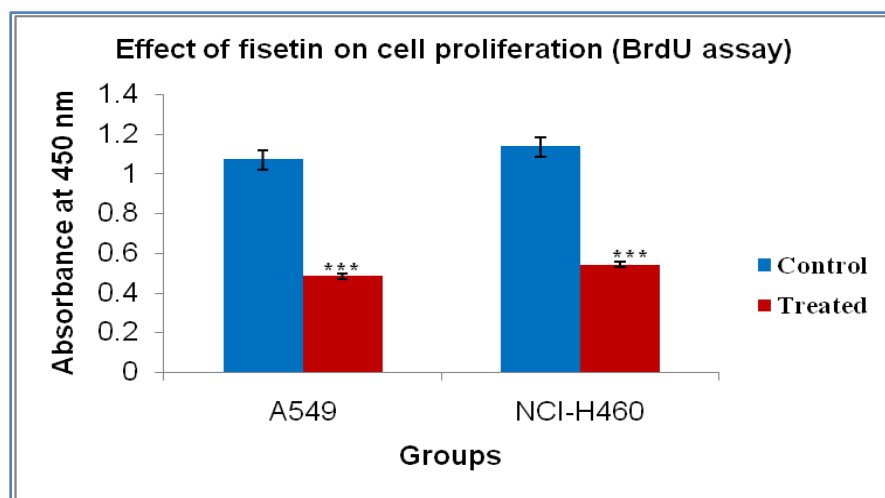


Figure 3 Effect of fisetin on proliferation of A549 and NCI-H460 cells (BrdU assay). Treatment of A549 and NCI-H460 cells with fisetin resulted in a significant decrease in cell proliferation as compared with the control. Data represent mean \pm SD of six replicates. Intergroup comparisons were made between cell control and treated group. Student's t-test; ***P< 0.001 verses control.

3.4 Effect of Fisetin on cell morphology-cytopathological and cytochemical analysis

3.4.1 Crystal violet staining

The fisetin treated A549 and NCI-H460 cells showed the presence of apoptotic bodies, cytoplasmic vacuolization, cell shrinkage, formation of stretched nuclei and nuclear fragmentation after staining the cells with crystal violet (0.05% w/v) for 30 min (Figure 5 A), however these typical features of cell death were not observed in untreated control A549 cells and NCI-H460.

3.4.2 Hematoxylin and eosin Staining

A549 treated cells showed decrease in cell number. The cells were smaller in size and some cells show cell shrinkage and loss of nuclei (cell death) after staining the cells with hematoxylin and eosin whereas they were not evident in the untreated control. NCI-H460 treated cells showed decrease in cell population with many cells showing loss of nuclei, and karyorrhexis was seen which, is a typical feature of apoptosis (Figure 5 B).

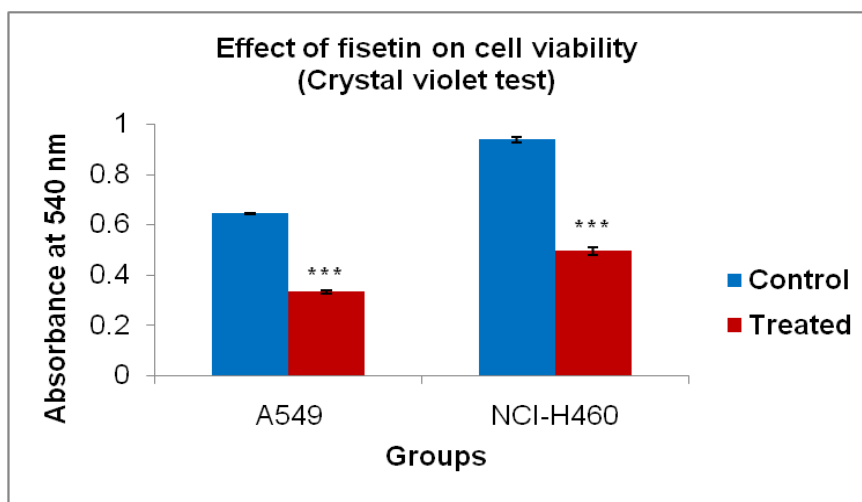


Figure 4 Effect of fisetin on proliferation of A549 and NCI-H460 cells (Crystal violet assay) –pilot studies. Treatment of A549 and NCI-H460 cells with fisetin resulted in a significant decrease in cell proliferation as compared with control. Data represent mean \pm SD of six replicates. Intergroup comparisons were made between cell control and treated groups. Student's t-test; ***P < 0.001 verses control.

3.4.3 Effect of fisetin on cloning efficiency and surviving fraction

Results revealed that A549 control cells showed a cloning efficiency of 0.82 as compared to cloning efficiency of 0.28 observed in treated cells and NCI-H460 control cells showed a cloning efficiency of 1.18 as compared to cloning efficiency of 0.34 observed in treated cells. Thus, it illustrates the significant decrease in the number of colonies and decrease in percentage of survival in fistein treated groups compared to that of untreated control (Table 1). As shown in figure fisetin treatment almost completely blocked the migration of A549 and NCI-H460 cells, and antimetastatic effect was more evident in NCI-H460 treated cells (Fig. 5 C).

3.4.4 Effect of fisetin on release of LDH

Results indicate that the cells treated with fisetin showed marked increase in LDH release in both A549 and NCI-H460 cells which was found to be statistically significant ($p < 0.001$) as compared to untreated control (Figure 6).

4 Discussion

Currently over 50% of anticancer drugs available in the market for therapy werederived directly or indirectly from natural source (Younes et al., 2007). Dietary factors play an important role in prevention of cancer, hence large number of dietary compounds were tested to determine their chemopreventive / antiproliferative ability in *in vivo* / *in vitro* model systems. A549 cells were derived from human alveolar carcinoma cell (Giard et al., 1973) and matched closely with type II alveolar cell phenotype. They share many characteristic features with human primary alveolar epithelial cell (Lieber et al., 1976). Hence they are widely used as models of lung cancer (Wang et al., 2009; Shin et al., 2009) and as human primary alveolar epithelial cells *in vitro* (Tian et al., 2009; Mazzarella et al., 2007). NCI-H460 cells are epithelioid cells isolated from pleural fluid of a patient with large cell carcinoma of lung. Inhibition of cell proliferation is a characteristic feature of majority of anticancer agents.

Table 1 Effect of fisetin on colony efficiency and survival fraction – colony formation in soft agar

Group	No. of Colonies	Colony Efficiency	Surviving Fraction	% Survival
Control NCI – H460	52	1.04	-	100
Treated NCI – H460	11	0.22	0.211	21
Control A549	44	0.88	-	100
Treated A549	13	0.26	0.295	30

Table 1 indicates the cloning efficiency and surviving fraction of the fisetin treated A549 and NCI-H460 cells and untreated control cells. A549 and NCI-H460 control cells showed a cloning efficiency of 0.82 and 1.18 compared to treated A549 and NCI-H460 cells showing cloning efficiency 0.28 and 0.34 respectively. Decrease in percentage of survival was observed in both A549 and NCI-H460 treated cells compared to untreated control cells.

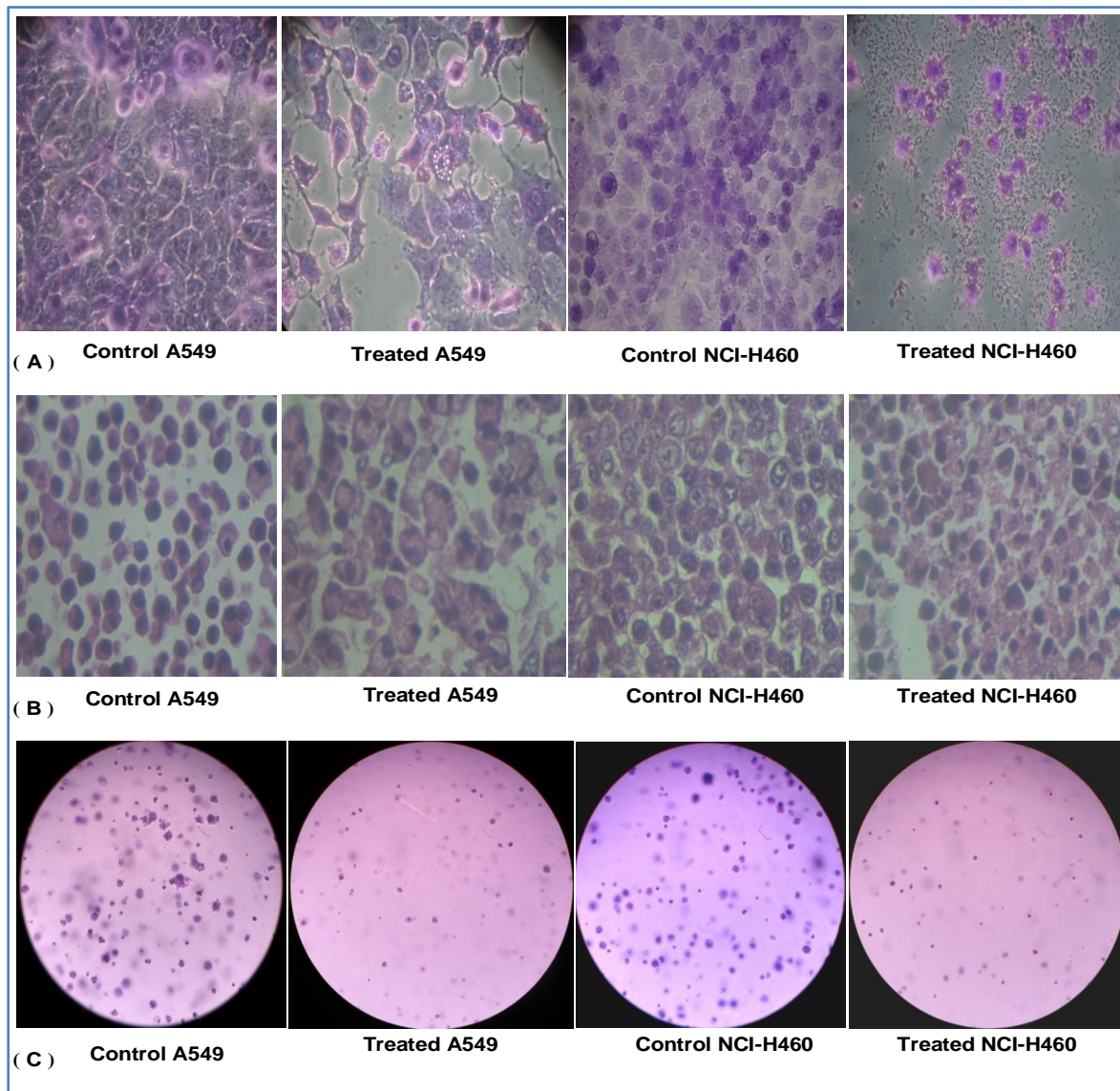


Figure 5 (A) Crystal violet staining revealed the presence of apoptotic bodies, cytoplasmic vacuolization, cell shrinkage, nuclear fragmentation and formation of stretched nuclei in fisetin treated A549 and NCI-H460 cells. Magnification: $\times 4 \times 10$. (B) Hematoxylin – eosin staining revealed that fisetin treated A549 cells were smaller in size and some cells showed cell shrinkage and loss of nuclei (cell death) and NCI-H460 cells showed showing loss of nuclei and karyorrhexis was seen which is a typical feature of apoptosis. However, no such features were evident in A549 and NCI-H460 control cells. Magnification: $\times 4 \times 10$. (C) Figure illustrates the effect of fisetin on colony efficiency and surviving fraction of A549 and NCI-H460 cells. Colonies formed in soft agar after 15 days were stained with 0.05% crystal violet and counted and images were captured. Magnification $\times 10$.

The flavonoid fisetin was reported to inhibit cell proliferation against several *in vivo/in vitro* models of carcinogenesis. The current study also showed significant inhibition of cell growth and proliferation was observed after treatment of pulmonary carcinoma cells with fisetin. In human, the Quadruplex (G_4) forming sequences in telomeric DNA and C-myc promoter regions of DNA are associated with tumorigenesis. Ligands that can help to facilitate or stabilize formation of G_4 quadruplex structures and increase stabilization of G_4 quadruplex can prevent tumor cell proliferation and regarded

as promising candidates as anti-cancer agents (Sen gupta et al., 2013). In the recent years, particular efforts are under way to discover, design and synthesise new ligands that could help to regulate the stability of G_4 quadruplex structures. Fisetin is reported to act as a ligand for G_4 DNA (Sengupta et al., 2013), and hence could have a tight control on the cell proliferation in A549 and NCI-H460 cells. This could be the reason for the observed inhibitory effects of fisetin on cell growth, viability and proliferation of pulmonary carcinoma cell lines.

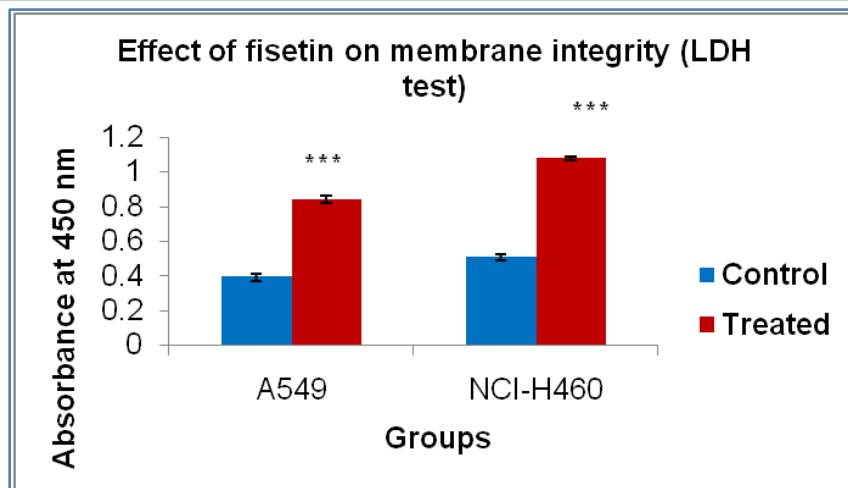


Figure 6 Effect of fisetin on LDH activity of A549 and NCI-H460 cells. Treatment of A549 and NCI-H460 cells with fisetin showed a significant increase in leakage of LDH into culture supernatant as compared to untreated control. Data represent mean \pm SD of six replicates. Intergroup comparisons were made between cell control and treated group. Student's t-test; ***P < 0.001 verses control.

Neoplastic transformation takes place via a series of genetic and epigenetic changes that yield cell population with potential of proliferating independently of both external and internal signals. It is one of the hall marks of malignant transformation and most accurate *in vitro* indication of tumorigenicity (Shin et al., 1975). The decreased number of colonies and inhibition of anchorage independent growth of pulmonary carcinoma cells by fisetin strongly supported its potent antiproliferative effects.

Hematoxylin and eosin staining is a valuable indicator of morphological and tissue architectural changes occurring during pathological conditions. This is an indispensable test to confirm malignant transformation of normal cells or to test efficacy of compounds to prevent pathological changes in cells/tissues. Results of hematoxylin and eosin staining were in line and support observations of cell growth assays, clonogenic assay. This confirmed growth inhibitory effects of fisetin on A549 and NCI-H40 cells. LDH is a cytosolic enzyme that leaks into culture supernatant or serum during membrane damage and injury. Increased leakage of LDH into culture supernatant of cells treated with fisetin implies alteration in membrane permeability and integrity thereby results in increased leakage of enzyme out of cells.

Conclusion

The results of the current study indicate growth inhibitory, antiproliferative and cytotoxic effects of fisetin on human pulmonary carcinoma cells A-549 and NCI-H460. Although the mechanism of action remains unclear at this stage of the study, it is presumed to be due to proapoptotic effects of fisetin and its subsequent implications on multiple cell signaling pathways that mediate cell proliferation and death.

Conflict of interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

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