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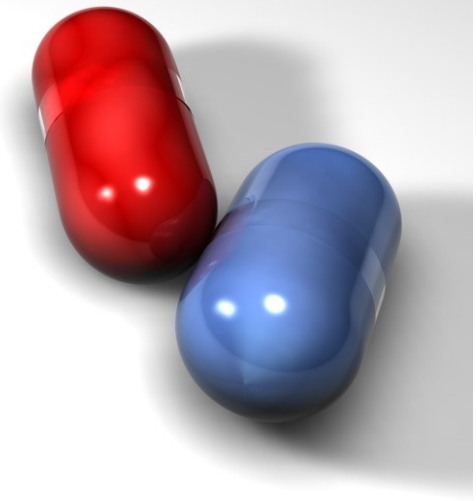

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Letter to the Editor

Anti-cancer effect of naringin in human lung carcinoma cell line

Sir,

Lung cancer is the most common cancer and the leading cause of cancer death in men. The use of chemotherapeutic agents and/or ionizing radiation (combination therapy) is the major choice to treat cancer.

Currently, cisplatin has been the cornerstone of most combination regimens in advanced non-small cell lung cancer. Taxanes, gemcitabine, topotecan, and berberine are some of the compounds that have been used to treat lung cancer (Bao and Chan, 2011).

Naringin, a flavonoid, is present in the citrus fruits. It has the inhibitory potential against numerous cancer types, including breast, lungs, liver, prostate, pancreatic, brain, throat, skin, colorectal, bladder, and mammary carcinosarcoma cancer both *in vivo* and *in vitro*. Naringin alone or in combination with other polyphenols, were proven to have efficacy and safety for cancer patients (Rauf et al., 2022).

The effect of naringin is not studied with respect to DNA fragmentation. To delineate the biological hallmarks of apoptosis, a DNA fragmentation assay is performed. Also pro-apoptotic and anti-apoptotic proteins' expression pattern are analyzed in the present study on A549 lung cancer cell lines *in vitro*.

The sources of chemicals are as follows: 3-(4,5-dimethyl -thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), naringin, Dulbecco's modified eagle medium (DMEM) and DMSO were purchased from Sigma-Aldrich (USA). All the chemicals used were of the highest grade commercially available. All the plastic wares were of cell culture grade and obtained from SPL, Korea.

Lung cancer (A549) cell line was obtained from National Centre for Cell Science, India. The cells were grown in T25 culture flasks containing DMEM supplemented with 1 mM sodium bicarbonate, 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotics. The cells were maintained in a humidified incubator at 37°C with 5% CO_2.

Cell proliferation of A549 cancer cell line was assessed using MTT. Cells were seeded at density of 2.5 × 10^4 cells per well in a 24-well plate, incubated overnight at 37°C in a 5% CO_2 incubator and treated with various concentrations of naringin (5, 10, 25, 50, 75, 100, 125, 150, 200 and 250 µM) or vehicle alone (DMSO) for 24 hours. After treatment, MTT solution (5 mg/mL in 1x PBS) was added followed by incubation for 3 hours at 37°C in the dark. The formazan crystals formed were solubilized by incubating cells with 500 µL of DMSO. Cell absorbance was read by an ELISA reader at 550 nm (Sirios, Seac Radim Group, Italy). The growth inhibition was determined by the formula (Rubinstein et al., 1990):

Growth inhibition (%)=Control OD - Sample OD/Control OD× 100

Percentage inhibitions were calculated and plotted against the concentrations and used to calculate the IC_{50} values

For DNA fragmentation assay DNA was isolated with little modification following DNA extraction protocol (Rogakou et al., 2000). Briefly, untreated and naringin-treated A549 cells incubated for 24 hours were harvested and were lysed with cell lysis buffer for 30 sec at room temperature. The supernatant was collected after centrifugation at 3,000 rpm for 5 min followed by incubation at 56°C for 2 hours after adding 10% SDS solution and RNase A. Proteinase K (25 mg/mL) was added and incubated overnight till complete lysis at 37° C. After adding saturated NaCl and absolute ethanol to the samples, the mixture was incubated at -80°C for precipitation. Centrifugation for 20 min at 12,000 rpm followed by washing the white pellet with 80% ice cold ethanol and air-dried at room temperature. The obtained pellets were dissolved in 1x TE buffer. The total DNA solutions were then subjected to 1.5% agarose gel electrophoresis at 100 V for 45 min at room temperature. Then, the gel was stained with ethidium bromide and viewed under UV-Transilluminator (UVP white/ultra violet transilluminator, USA) and photographed.

For reverse transcriptase-polymerase chain reaction (RT -PCR), total RNA was isolated from cells using total RNA isolation reagent from Invitrogen. The concentration and purity of RNA were determined spectrophotometrically at A260/280 nm. A ratio of absorbance of >1.8 was considered as good quality RNA. Total RNA was used for the synthesis of complementary DNA (cDNA). The specific oligonucleotide primers were used for the generation of complementary DNAs (Table I).

Statistical analysis was performed using one-way

Table I

Reverse transcriptase-polymerase chain reaction and primer sequences

Gene	Primer sequence (5′→3′)	Product/ Amplicon size
BAD	F- CCTCAGGCCTATGCAAAAAG R- AAACCCAAAACTTCCGATGG	120 bp
BAX	F- GCTGGACATTGGACTTCCTC R- CTCAGCCCATCTTCTTCCAG	168 bp
BCL-$_{XL}$	F- GGCTGGGATACTTTTGTGGA R- AAGAGTGAGCCCAGCAGAAC	131 bp
BCL$_2$	F- TTGTTCAAACGGGATTCACA R- GAGCAAGTGCAGCCACAATA	176 bp
FADDR	F- AGATGAACCTGGTGGCTGAC R- AGGACGCTTCGGAGGTAGAT	120 bp
FASL	F- CCATGTGAAGAGGGAGAAGC R- AAGACAGTCCCCCTTGAGGT	146 bp
CYC-A	F - GTGGTGTTTGGCAAAGTGAA R -TCGAGTTGTCCACAGTCAGC	116 bp

analysis of variance (ANOVA) followed by Duncan's multiple range test for post hoc comparison by SPSS software version 16. Statistical significance was set at $p<0.05$. All the data that were collected from at least three individual experiments were presented as mean ± SD.

In order to assess the potential antiproliferative activity of naringin on A549 cancer cells, MTT assay was conducted. The antiproliferative effect of naringin was found to be dose- and time-dependent (Figure 1). The treatment with different doses of naringin (5 µM - 250 µM) exhibited inhibition of cell proliferation. Besides, the IC_{50} value was found to be 150 µM at 24 hours.

The apoptotic effect of naringin on cancer A549 lung cancer cell lines was investigated by qualitative and quantitative analyses of DNA fragmentation, one of the key biochemical hallmarks of apoptosis (Hanahan and Weinberg, 2011). As shown in Figure 2 naringin induced DNA fragmentation on lung cancer cell lines.

The expression patterns of six genes *viz.* BAX, BCL$_{XL}$, BCL$_2$, BAD, FASL and FADDR were analyzed with CYC-A as an internal control. Naringin significantly down-regulated the expression of anti-apoptotic BCL$_{XL}$ and BCL$_2$. Conversely, the expression of pro-apoptotic BAX, BAD, FASL, BCL$_{XL}$ and FADDR were up-regulated (Figure 3). The expression of *BAX* was significantly increased on treatment with naringin by two fold and six fold at 100 µM and 150 µM concentrations of naringin, respectively (Figure 3A). Likewise, the expression of *BAD* was significantly increased on treatment with naringin by 5.7 fold and 11.2 fold when compared to control at 100 µM and 150 µM concentrations of naringin, respectively (Figure 3D). Similarly, the expression of FASL (Figure 3E) and FADDR (Figure 3D) were significantly increased on treatment with naringin

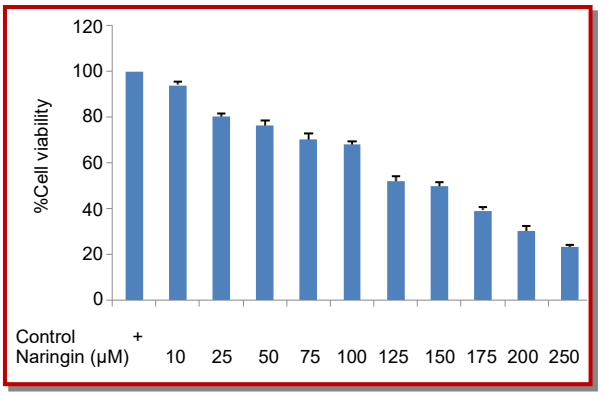

Figure 1: Growth inhibitory effect of naringin on A549 cell proliferation. Incubation time was 24 hours. Each value is expressed as mean ± SD from minimum of three independent experiments

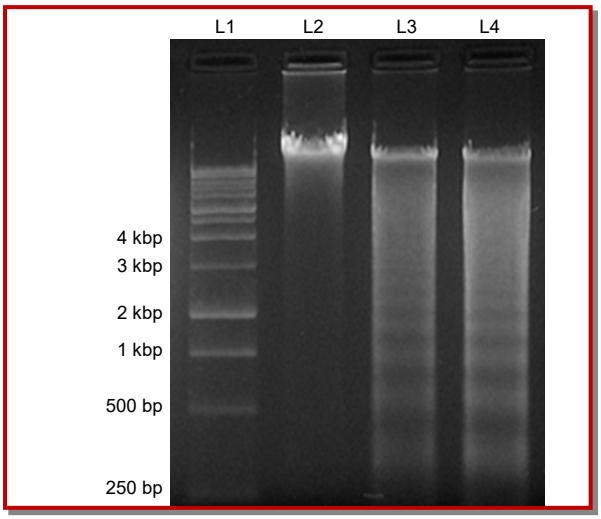

Figure 2: Effect of naringin on DNA fragmentation in control and naringin-treated A549 cells. Lane 1: 1 kb DNA ladder; Lane 2: Control A549 DNA; Lane 3: A549 cells treated with 100 µM of naringin; Lane 4: A549 cells treated with 150 µM of naringin

by 10.7 fold and 5.1 fold, respectively at 100 µM concentrations. In contrast, the expression of *BCL$_{XL}$* was significantly decreased on treatment with naringin by 0.69 fold at 100 µM concentration (Figure 3B). The expression of *BCL$_2$* was decreased significantly on treatment by 0.17 fold at 150 µM concentrations of naringin (Figure 3C).

From these results, it can be implied that naringin exhibits strong anti-cancer properties by inhibiting cell proliferation and inducing programmed cell death through intrinsic and extrinsic pathways of apoptosis. Taken together, it seems that naringin has the potential to be a promising anti-cancer agent for treating lung cancer.

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Ethical Issue: The development, acquisition, authentication, cryopreservation, and transfer of cell lines between labora-

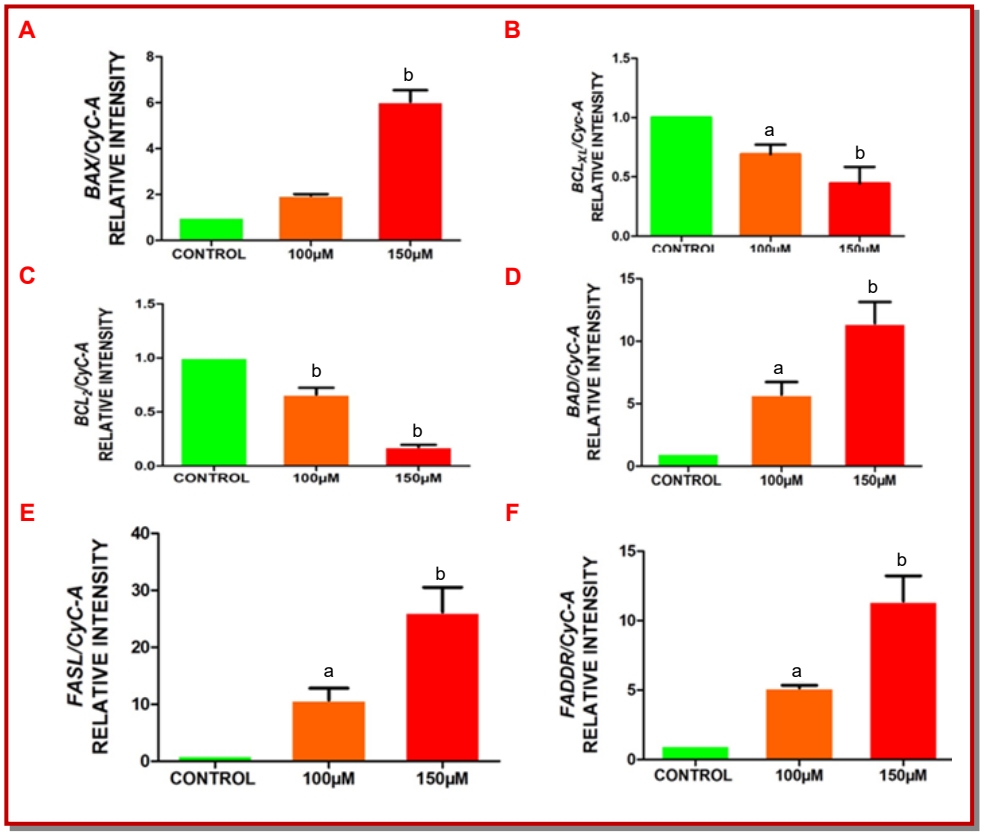

Figure 3: Effect of different concentrations of naringin (100 and 150 μM) on expression of BAX (A), BCL_{XL} (B), BCL_2 ©, BAD (D), FASL (E), and FADDR (F); Statistical significance [a]p<0.05, [b]p<0.01

tories were followed according to the guidelines published in British Journal of Cancer, 2014.

Conflict of Interest: The authors declare that they have no conflict of interest.

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