In vitro comparative cytotoxic effect of Nimbolide: A limonoid from *Azadirachta indica (*Neem tree) on cancer cell lines and normal cell lines through MTT assay

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Abstract: The present study was conducted to find the cytotoxicity *in vitro* of nimbolide, limonoids derivative of flowers and leaves from *Azadirachta indica* (neem tree) on the selected cell lines of cancer (Du-145, PC-3, A-549) and normal fibroblast cell lines (NIH3T3, CCD-18Co) using MTT assay. The cells were seeded in 96 multi-well tissue plate using different concentrations of nimbolide for 24hrs and 48hrs. The percentage of viability of cell lines was calculated by optical density obtained by micro plate reader and cytotoxic effect in term of IC50 value was determined by using linear regression analysis. The percentages of viability of cells treated with different concentrations of nimbolide were significantly lower ($P<0.05$) than the untreated cancer cell lines while in normal cell lines no significant difference ($P>0.05$) between treated and the non-treated cells was observed. Nimbolide exerted time and dose dependent cytotoxic effect on the cancer lines and mild effect on the normal cell lines. It was further confirmed through PKH 26. Results of the present study suggested nimbolide as a potent chemotherapeutic and chemopreventive agent as it exerted a more cytotoxic effect on cancer cell lines as compared with the normal cell lines. Nimbolide may be a new hope as an anticancer drug in future.

Keywords: Nimbolide, MTT assay, cancer cell lines, normal cell lines, cytotoxic effect.

INTRODUCTION

In developed countries, cancer is number one cause of death and the second most common cause of death in developing countries. According to a global survey, 14.1 million people were diagnosed and 8.2 million died of cancer in 2012 (Torre *et al*., 2015). Medicinal plants have been used to treat various diseases (including cancer) ever since the early days of human civilization (Cruz-Vega *et al*., 2009). The use of medicinal plants to treat diseases is illustrated in different religions including Hinduism, Christianity and Islam. Various parts of plants and their products were used and recommended by our Prophet Muhammad (PBUH) in the treatment of different diseases (Al-Bukhari, 1976).

Presently, in most parts of the world, various types of local plants and their products are used in the treatment and prevention of various diseases. Previous studies have reported that curcumins, black seed, olive, and dates are effective in cancer treatment and prevention through modulation of antioxidant, anti-inflammatory and antitumor activity (Chattopadhyay *et al.*, 2004; Rahmani *et al*., 2014a; b). The present modes of treatment of cancer including chemotherapy, radiotherapy, and surgery are expensive, toxic and may modify the action of various normal genes, whereas medicinal plants are effective, safe and cost effective as well.

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Azadirachta indica (locally called Neem) is evergreen, fast-growing, resistant to high temperature and drought, native to tropical and semi-tropical climates like in Pakistan, India, and Bangladesh (Hao *et al.*, 2014). Asian countries used this plant as a traditional medicine for centuries to cure multiple human and animal ailments. It has been shown to harbor numerous effects including antibacterial, antiviral, anithelmintic, antifungal, antidiabetic, sedative and contraceptive effects (Juss *et al.,* 2015). Apart from these properties laboratory findings suggested that neem components also possess anticancer efficacy (Elumalai and Arunakaran, 2014). Nimbolide is a tetranortriterpenoid limonoid derivative of seeds and leaves from *Azadirachta indica* (Nair *et al.*, 1997). It is a readily available compound used in various studies and is effective as an anti-feedent (Suresh *et al*., 2002), antimalarial (Elumalai *et al*., 2012), antimicrobial (Farooqui *et al*., 2014) and anti-tumor (Bose *et al.*, 2007). It exhibited cytotoxicity against various cancer cell lines and has attracted extensive research attention as a potent candidate for cancer chemoprevention (Bodduluru *et al*., 2014).

Most chemotherapeutic drugs have lost their therapeutic effect due to drug resistance by microorganism aside from the toxicity to the host cell. Therefore, in the present scenario, there is a dire need to develop anticancer drugs which should have minimal effect on the normal cells. Therefore, the present study was planned (*in vitro*) to compare the cytotoxic effect of nimbolide on the cancer cell lines and normal cell lines with the hypothesis that

nimbolide has a highly cytotoxic effect against the cancer cells with minimum effect on the normal cells.

MATERIALS AND METHODS

Nimbolide solution preparation

Nimbolide of purity ≥98% by TLC was purchased from Bio Vision Incorporated (CA 95035 USA, Molecular weight: 466.52). The stock solution I of nimbolide was prepared by dissolving 5 mg nimbolide in 214ul of Dimethyl Sulfoxide (DMSO) making final concentration of 50mM. Stock solution II was prepared by dissolving 10ul from stock solution I in 990ul of Dulbecco's Modified Eagles Medium (DMEM) making a final concentration of stock solution II 500uM. The stock solution II (prior to use) was diluted in medium to obtain the required final running concentration (0.625uM to 10 uM) of the drug.

Chemicals

Fetal Bovine Serum (FBS), Dulbecco's Phosphate Buffered Saline (DPBS), Penicillin-streptomycin and Trypsin-EDTA, were purchased from Welgene Inc, South Korea, MTT dye (Thiazolyl Blue Tetrazolium Bromide, Amresco®) from Solon Ind. Pkwy, PKH 26 from Sigma-Aldrich, DMEM and RPMI-1640 medium from Capricorn Scientific, DMSO from Junsei, Japan.

Cell lines and Cell culture

Cancerous cell line DU-145 (human prostate carcinoma), PC-3 (human prostate adenocarcinoma), A-549 (human lung carcinoma) and normal cell line NIH3T3 (mouse embryo normal fibroblast), CCD-18Co (human colon normal fibroblast) were obtained from Korean Cell Line Bank, South Korea. RPMI-1640 medium was used for growing cancer cell lines whereas normal cell lines were cultured in DMEM (using 25 ml culture flask). The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and the medium was changed on alternative day. The cells were trypsinized and transferred to 75 ml culture flask after reaching confluence, as per instructions of manufacturers. The medium used was added with 10% FBS and 1% antibiotics. The cells after reaching 80-90% confluence were used in all the experiments.

Determination of proliferation of cell/viability through MTT Assay

MTT assay was used to determine the effect of nimbolide on the viability of cancerous cell lines and normal cell lines. It is based on the principle that dead cells do not reduce the MTT dye, a tetrazolium compound while the live cells convert it into the purple colored product (formazan) by mitochondrial enzyme (Mosmann, 1983). Shortly, cultured cells were trypsinized and after dilution, approximately 8000 cells per well seeded in 96 wells tissue culture plate and treated with different concentrations of nimbolide ranging from 0.625uM to 10 uM and untreated control for 24hrs and 48hrs. Thereafter medium was discarded and added 100ul of MTT solution (0.5 mg/ml) to each well and incubated for 2hrs at 37°C in 5% CO_2 atmosphere. MTT solution was then discarded, 50ul DMSO was added to each well and plates were gently shaken to dissolve the formazan crystals. The micro-ELISA plate reader was used to measure the intensity of developed color at a wavelength of 570nm. The percentage of viability of cells in the treatment groups was calculated with the help of the following formula, % cell viability = {average optical density at 570 nm of individually treated cells/average optical density of 570nm of control cells} × 100, while the cell viability in the control group was considered 100%. IC50 values of nimbolide were calculated for each cell line. All of the experiments were performed in triplicate.

Labeling of cancer and normal cells with PKH 26

PKH 26, a fluorescent dye that is mainly used for labeling of the cell membrane. The cancer cells (Du-145) and normal cell (NIH3T3) were labeled separately with PKH 26 as per manufacturer's instructions. Briefly single cell suspension (1×10^7) was placed in a 15 ml polypropylene tubes, washed once with serum free medium and then centrifuged for 5 minutes (400 xg). Then 2X cell suspension was prepared by addition of 1ml of the diluent C to the cell pellet and dispersed completely by gentle mixing. At the same time, dye solution was prepared by adding 4ul of ethanolic solution of PKH 26 into 1ml of diluent C in other conical polypropylene tubes, then added the cell suspensions to dye solution and mixed the both samples by gently pipetting. It was incubated for 5 minutes by periodic mixing and examined under fluorescent microscope. All the cells got stained. After that, reaction was stopped by adding 2 ml of heat inactivated serum, the sample was centrifuged for 10 minutes at 400 x g, the supernatant was removed and the cells were resuspended in 10 ml of complete medium and the cells were further washed twice with the complete medium.

Co-culture of labeled and unlabeled cells

To further confirm the better cytotoxic effect of nimbolide on cancer cells, we co-cultured the stained cancer cells with unstained normal cells and stained normal cells with the unstained cancer cells in equal number using 6-well tissue culture plate and divided into two groups, i.e. cells not treated with nimbolide (control) and cells treated with nimbolide 10uM final concentration of the medium.

STATISTICAL ANALYSIS

The data was represented as means ± standard errors of means. Statistical significance difference between cells treated with nimbolide and untreated cells were estimated by student-Newman-Keuls test using R software version 3.2. The differences in the viabilities of cells considered statistically significant at $P<0.05$. The linear regression analysis was used to calculate IC50 values of nimbolide.

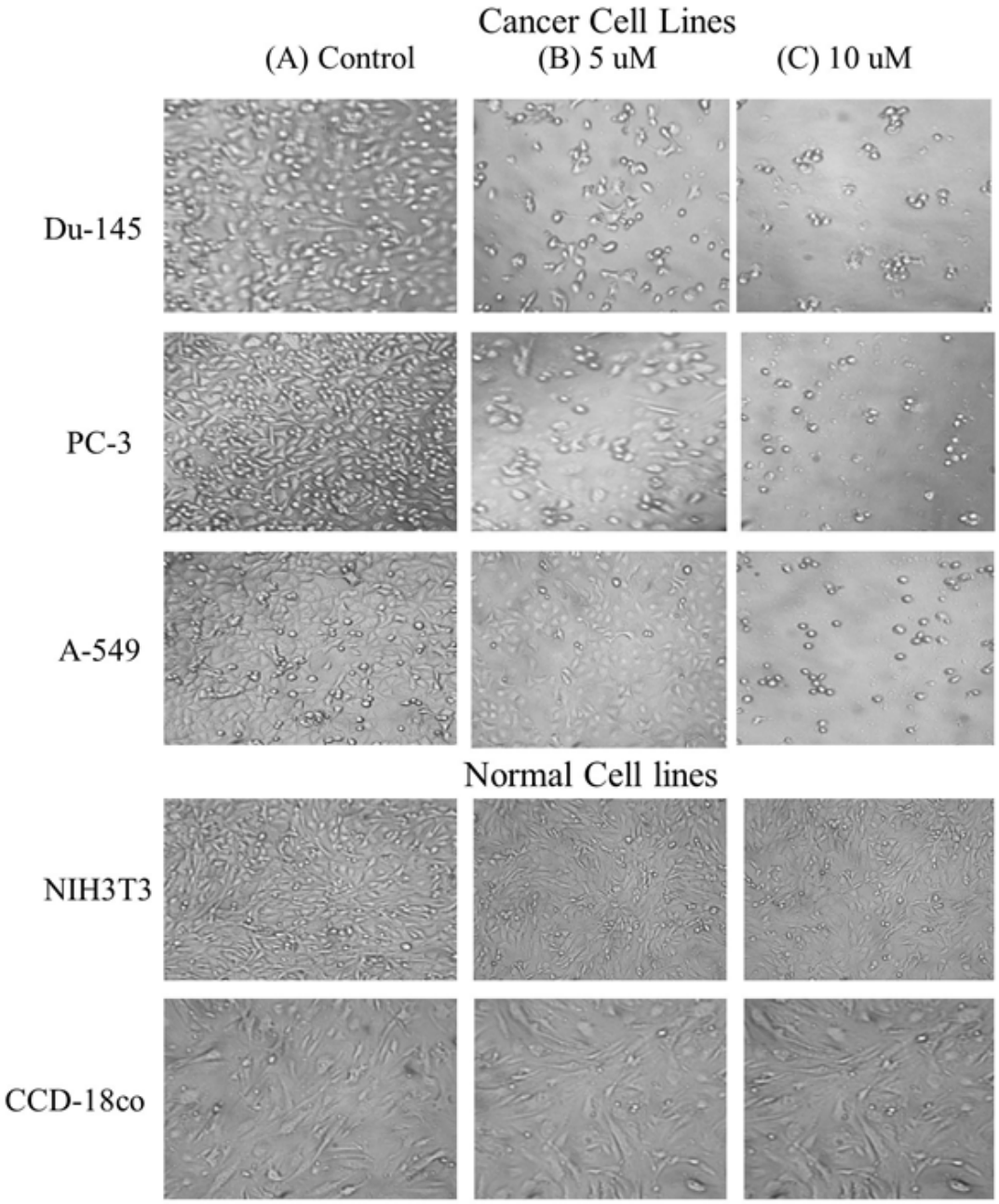

Fig. 1: Morphological alterations observed under phase contrast microscope at 10x magnification in the cancer cell lines and normal cell lines treated with different concentration of nimbolide and control after incubation for 24hrs. (A) Control (B) Treated with 5uM nimbolide, (C) Treated with 10uM nimbolide.

Table 1: IC50 is the concentration of nimbolide (uM) at which 50% cell death occurred and was calculated using linear regression analysis after 24hrs and 48hrs. Data are shown as means ± standard errors of means derived from three independent experiments.

	Cancer Cell lines			Normal Cell lines	
	Du-145	PC-3	A-549	NIH3T3	CCD-18co
After 24hr	6.86 ±0.53	8.01±0.44	11.16±0.84	39.43±3.72	74.01±5.33
After 48hrs	4.97±0.72	5.83±0.33	7.59±0.34	36.92±1.42	59.13±5.68

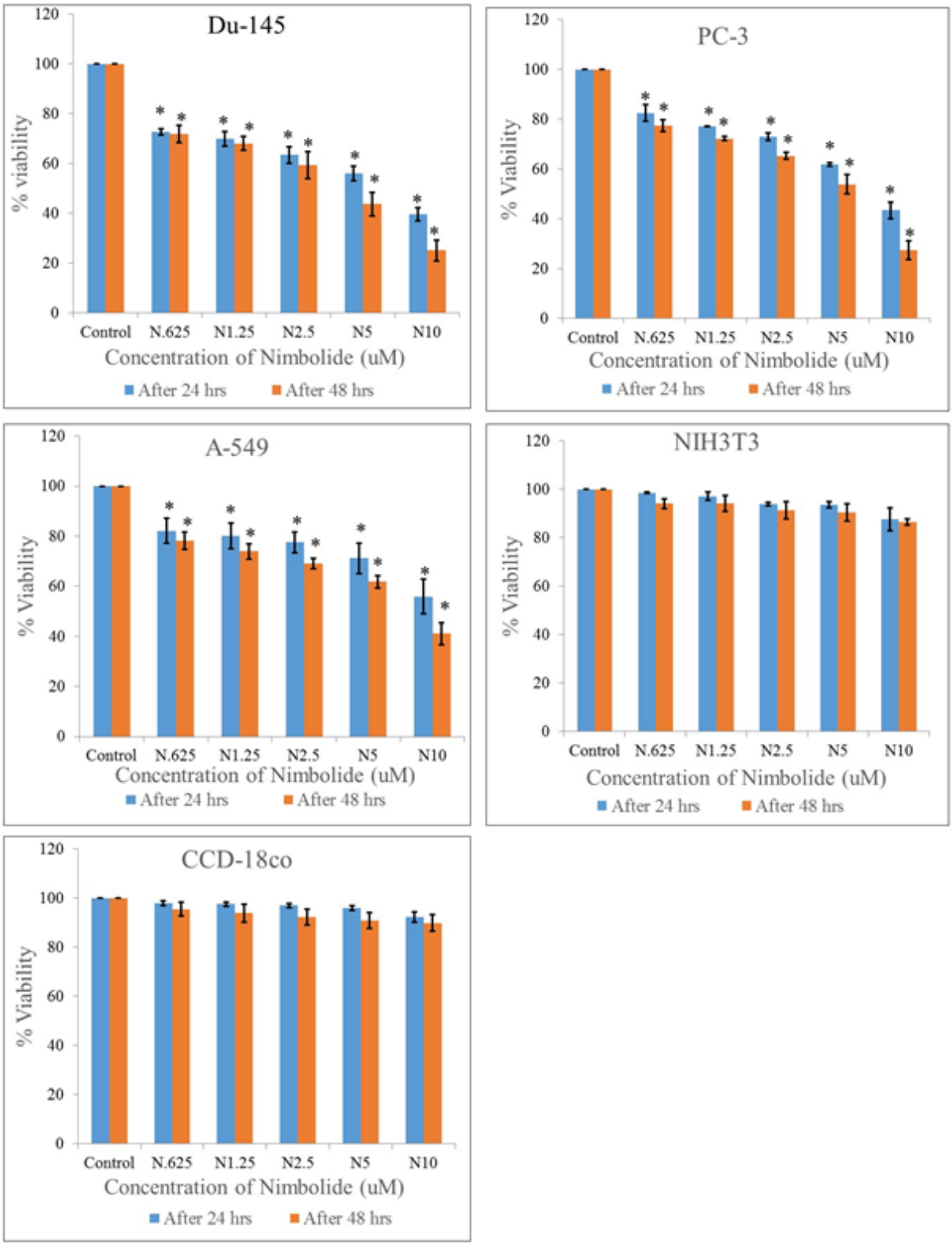

Fig. 2: Effect of nimbolide on the cell viability. Cell viability was measured by MTT assay using different concentrations of nimbolide and control for 24hrs and 48hrs. Each bar represents means ± standard errors of means of three independent experiments. *represented the significance difference between the cells treated with different concentration of nimbolide and control using student-Newman-Keuls test at $P<0.05$.

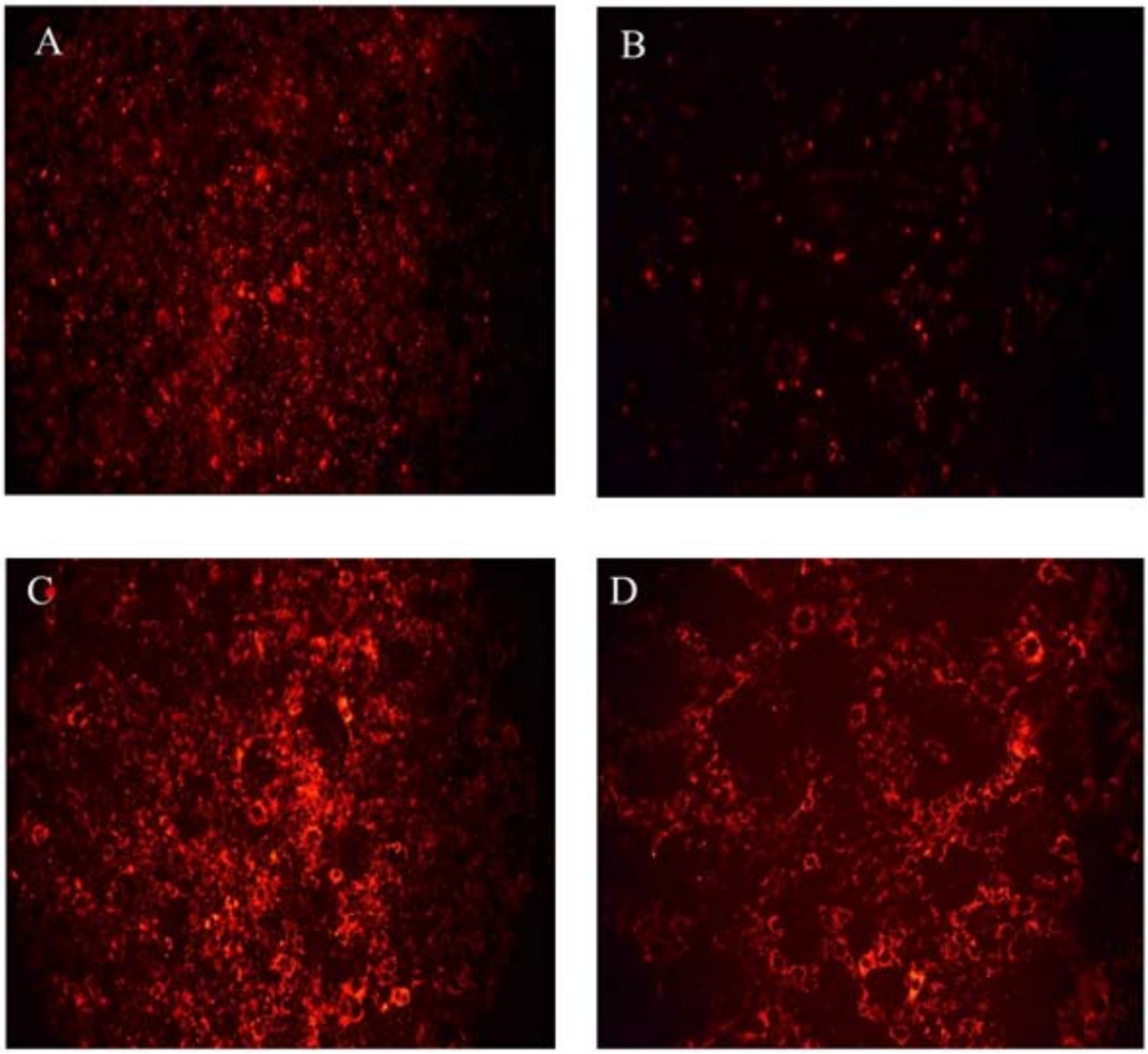

Fig. 3: Cytotoxic effect of nimbolide in co-culture of cancer cells and normal cells labeled with PKH 26 after 48hrs. Labeled cancer cells and unlabeled normal cells co-cultured without nimbolide treatment and labeled cancer cells appear under the fluorescence microscope (A). Labeled cancer cells and unlabeled normal cells co-cultured were treated with 10uM nimbolide, labeled cancer cells were inhibited by nimbolide and therefore few cells appear under fluorescence microscope (B). Labeled normal cells and unlabeled cancer cells co-cultured without nimbolide treatment and labeled normal cell appeared under the fluorescence microscope (C). Labeled normal cells and unlabeled cancer cells co-cultured were treated with 10uM nimbolide, labeled normal cells were not inhibited by nimbolide, therefore, appear under the fluorescence microscope (D).

RESULTS

Morphological changes in cancer and normal cell lines were observed under phase contrast microscope. In cancer cell lines after (24hrs and 48hrs) incubation of cells with different concentration of nimbolide, dramatic changes were observed in treated cells such as cell detachment, condensed cell, decreased cell density and loss of spindle formation as compared with the untreated cells which exhibited normal shapes with a clear outline and intact nuclei. In the normal cell lines, treated cells and untreated cell showed normal shape. The images of the cytotoxic effect of nimbolide against different cell lines are shown in fig 1.

Nimbolide reduced the viability of cells and exerted time and dose dependent cytotoxic effect on the cancer cell lines and the little effect on the normal cell lines. It significantly decreased ($P<0.05$) the viability of cancer cell line treated with nimbolide as compared with untreated cells whereas in normal cell lines no significant difference ($P>0.05$) was observed in nimbolide treated and untreated cells. Among the cancer cell lines, higher efficacy was shown in Du-145 cell line followed by PC-3

and A-549 while in normal cell lines only highest concentration exerted a mild effect. There was a different viability of each cell line by different concentrations of nimbolide after 24hrs and 48hrs (depicted in fig 2).

The derived IC50 values of nimbolide (uM) for the Du-145, PC-3, A-549 (cancer cell lines) were 6.86±0.53, 8.01±0.44, 11.16±0.84 after 24hrs and 4.97±0.72, 5.83±0.33, 7.59±0.34 respectively after 48hrs whereas in NIH3T3 and CCD-18Co (normal) cell lines the values were 39.43±3.72, 74.01±5.33 after 24hrs and 36.92±1.42, 59.13±5.68 respectively after 48hrs (table 1).

Nimbolide inhibited the stained cancer cells in co-cultured stained cancer cells and unstained normal cells as few stained cells were seen in nimbolide treated group as compared to the control under the fluorescent microscope. It exerted not much cytotoxic effect on the normal cells in co-culture of stained normal cells and unstained cancer cells as in treatment group stained cells appear same as in control group under fluorescent microscope shown in fig. 3.

DISCUSSION

Several studies have been conducted *in vitro* on different cancer cell lines that showed antitumor activity of the neem tree extracts and nimbolide. Neem extract preparations and its limonoids exerted inhibitory effects *in vitro* on the growth of tumor cells (Bose *et al.*, 2007; Mandal-Ghosh *et al.*, 2007) and ethanolic extract of neem reduced the viability of cells and induced apoptosis in prostate cancer cells (Gunadharini *et al.*, 2011; Kumar *et al*., 2006). Nimbolide exerted cytotoxicity against NIE-155 and 143B TK cell lines with IC50 values 4.75uM and was suggested as most potent among all the neem limonoids (Cohen *et al*., 1996). It also exhibited a cytotoxic effect on various human cancer cell lines with IC50 values ranging from 4.17uM to 15.56uM with mean value 8.31 (Sastry *et al*., 2006). The antiproliferative effect of nimbolide on B16 (melanoma) and THP-1, HL-60 and U937 (leukemic) cell lines with IC50 value ranging from 1.12uM to 1.74uM was observed by Roy *et al*., (2007). The cytotoxicity of nimbolide against BeWo cells through induction of apoptosis with IC50 value was 2.016uM and 1.93uM after 7hrs and 24hrs respectively (Kumar *et al*., 2009). Nimbolide exhibited a more cytotoxic effect on cancer cell lines (HT-29, HL-60, SW-480, A-549, HeLa) as compared to the untreated cells through induction of apoptosis (Gupta *et al.*, 2013; Chen *et al.*, 2011).

The cytotoxic effect of nimbolide on the normal cells has not been yet reported. Therefore, in the present study, we for the first time have compared cytotoxicity of nimbolide on the selected cancer cell lines (Du-145, PC-3, A-549) and normal cell line (NIH3T3 and CCD-18Co) through MTT assay. Results of the present studies showed that nimbolide reduced the viability of cells and exerted duration and concentration-dependent cytotoxicity on all the cancer cell lines. However, Du-145 cells were more sensitive to nimbolide with IC50 value of 6.86uM and 4.97uM for 24hrs and 48hrs, respectively. Nimbolide also induced a cytotoxic effect on PC-3 cell line with IC50 values of 8.01uM and 5.83uM and A-549 with IC50 values of 11.16uM and 7.59uM for 24hrs and 48hrs, respectively. The differences in IC50 values in different cancer cell lines in the present study and previous studies might be due to the duration of exposure and the different sensitivities of cancer cells to the cytotoxic effect of nimbolide. On the other hand, in normal cell lines, a little viability was reduced as compared to the untreated cells and exhibited a mild cytotoxic effect in term of IC50 values of ranging 39.43uM and 36.92uM for 24hrs and 48hrs, respectively in NIH3T3 cell lines and 74.01uM and 59.13uM for 24hrs and 48hrs, respectively in CCD-18Co cell lines. Higher IC50 value represented low cytotoxic effect. It suggested that nimbolide effects only the cancer cells with little effect on the normal cells.

PKH 26 (an extremely stable fluorescent dye) is a dye of choice as a cell linker used *in vitro* and *in vivo* cell tracking studies and cytotoxic assays (Jr *et al*., 2011; Kusumbe and Bapat, 2009). In a previous study after labeling with PKH 26, no difference was observed in the morphology of cells and no significant difference of antitumor activity of CIK labeled with PKH 26 and CIK unlabeled (Du *et al*., 2013). Therefore, in the present study, we labeled separately both cancer cell and normal cell with PKH 26. Nimbolide inhibited the stained cancer cells but did not effect the stained normal cells in co-culture.

CONCLUSION

It is suggested that nimbolide may be a potential chemotherapeutic or a chemo-preventive agent as it exerted a more cytotoxic effect on the cancer cells as compared to the normal cells. Further clinical trials and *in vivo* studies are needed to develop it as an anticancer drug. In future, nimbolide may be new hope and a candidate as an anticancer drug.

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