

## Original Research Article

# Anticancer Activity of Linalool Terpenoid: Apoptosis Induction and Cell Cycle Arrest in Prostate Cancer Cells

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## Abstract

**Purpose:** To evaluate the anticancer activity of linalool against human prostate cancer (DU145) cells.

**Methods:** The anticancer activity of linalool against DU145 cancer cells was evaluated by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Flow cytometry, using propidium iodide and Annexin V-FITC, was applied to study apoptosis and cell cycle phase distribution. Inverted light microscopy was used to study the effect of linalool on cell morphology and apoptotic body formation in DU145 cells while gel electrophoresis was employed to evaluate the effect of linalool on DNA fragmentation.

**Results:** Linalool induced a dose-dependent as well as time-dependent growth inhibitory effect on DU145 prostate cancer cells. It induced sub-G1 phase growth arrest which led to increase in sub-G0/G1 cell population after treatment with increasing doses of linalool. DNA ladder appeared to be more evident with increasing linalool concentration. However, no DNA fragments were observed in the control groups. It was observed that 4.36, 11.54, 21.88 and 15.54 % of the cells underwent early apoptosis after treatment with 0 (no linalool treatment), 20, 40, and 80  $\mu$ M of linalool, respectively. Compared to control cells, linalool treatment resulted in the appearance of cell shrinkage along with membrane blebbing which are characteristic features of cell apoptosis.

**Conclusion:** The findings of this study indicate that linalool can be developed as a plant-based chemotherapeutic agent against prostate cancer

**Keywords:** Prostate cancer, Linalool, Chemotherapy, Cell cycle, Apoptosis, DNA fragmentation, Sub-G1 phase growth

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## INTRODUCTION

Prostate cancer (PC) is the principal cause of new cancer cases and the second most common cause of cancer-related deaths in men in United States of America [1]. Prevalence rate for clinical prostate cancer in Western men are 35 - 45 times higher than those for Asian men. In the United States, prostate cancer accounts for about 30 % of all cancer cases in men whereas in China, prostate cancer accounts for less than

2 % of all cancers in men. The survival rate for advanced prostate cancer has not improved much during the past 10 years [2,3].

The standard treatment options for prostate cancer include surgery, radiotherapy and hormonal therapy, but these treatment options are not without serious side-effects like urinary incontinence and sexual dysfunction among others. Traditionally, chemotherapy and radiotherapy have not provided important

survival benefits to patients with advanced prostate cancer and most treatment options available are only palliative. Recent studies on taxane derivatives alone and in combination with other chemotherapeutic agents have revealed some limited benefit [4], but the need for more effective and less toxic means to target and/or thwart this disease still exists. Complementary and alternative medicine may provide better treatment options by inhibiting or slowing down disease progression. According to a recent survey, around 30 – 40 % of patients suffering from prostate cancer use complementary and alternative medicine as an additional therapy [5-7].

Keeping in view the limited treatment options available for prostate cancer along with the side-effects of known treatment strategies, the aim of this study was to identify a plant-based drug molecule (linalool, a monoterpene) that will be capable of inhibiting prostate cancer cell growth.

## EXPERIMENTAL

### Chemicals and biological reagents

Linalool was purchased from Sigma Chemical Company (St Louis, MO, USA). The cells were cultured in Dulbecco's modified Eagle's media supplemented with 10 % fetal bovine serum (Lonza Biologics, Singapore) and 100 U/mL penicillin and 100 µg/mL streptomycin (Vega Pharma Limited, Zhejiang, China). MTT kit was obtained from Roche (USA). Annexin V-FITC-Propidium Iodide Apoptosis Detection Kit was purchased from Bestbio, Shanghai, China. All other chemicals and solvents used were of the highest purity grade. Cell culture plastic ware was bought from BD Falcon (USA).

### Cell line and cell viability assay

Human prostate carcinoma (DU145) cells were procured at the China Center for Type Culture Collection (Wuhan, China) and grown in a humidified 5 % CO<sub>2</sub> atmosphere at 37 °C in an incubator, and cultured in RPMI-1640 medium supplemented with 10 % heat-inactivated newborn calf serum, 100 IU/mL penicillin and 100 µg/mL streptomycin. MTT assay (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) was used to measure the inhibition of cell proliferation. MTT was added in cells exposed to different concentrations (5, 10, 20, 40 and 80 µM) of linalool for 24 and 48 h. Three hours later, the formazan precipitate was dissolved in 100 µL dimethyl sulfoxide, and then the absorbance was measured in a microplate

reader (Bio-Rad). The cell viability was calculated as in Eq 1.

$$\text{Inhibition (\%)} = \{(Ac - At)/Ac\}100 \dots\dots\dots (1)$$

where Ac and At are the absorbance of control and treated cells, respectively.

### Cell cycle phase distribution analysis

Human prostate carcinoma (DU145) cells ( $1 \times 10^5$ ) were seeded in 60-mm dishes and subjected to various concentrations (0, 20, 40 and 80 µM) of linalool for 48 h. The cells were collected by trypsinization and washed twice with PBS (Sigma Chemical Co.). Cells were incubated in 50 % ethanol at -20 °C overnight and treated with 40 µg/mL RNase A (Guangzhou Geneshun Biotech Ltd. China), and stained with 10 µg/mL of propidium iodide (PI) (Guangzhou Geneshun Biotech Ltd. China). Finally, the stained cells were analyzed by FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

### DNA fragmentation analysis

After subjecting DU145 cells to linalool treatment at various concentrations (0, 20, 40 and 80 µM), both adherent and floating cells were collected and washed with PBS. Pellets were then lysed with DNA lysis buffer (20 mM EDTA, 100 mM Tris, pH 8.0, 0.8 % SDS) at room temperature for 25 min. After centrifugation for 10 min at 12 000 × g, the supernatants were collected and treated with RNase A (final concentration, 500 µg/mL) for 20 min at 37 °C, followed by digestion with proteinase K (final concentration 500 µg/ml) for 3.0 h at 55 °C. The DNA was extracted using the phenol/chloroform/isoamylol (25:24:1), precipitated with ethanol, dissolved in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA), and subjected to 2 % agarose gel electrophoresis for DNA fragmentation analysis.

### Annexin V-FITC assay for cell apoptosis

Apoptosis detection was performed using the Annexin V-FITC and PI apoptosis kit (Bestbio, Shanghai, China). DU145 cells were plated at a density of  $2 \times 10^5$  cells/well into 12-well plates and incubated overnight. Apoptosis was induced by treating cells with linalool at various concentrations (0, 20, 40 and 80 µM). Cells grown in media containing an equivalent amount of DMSO without any drug served as control. After 48 h of incubation, the cells with different treatments were harvested and the final samples measured on a FACS Calibur flow cytometry (Becton Dickinson, San Jose, CA, USA).

### Morphological assessment

DU145 cells were seeded in 6-well plates at  $1.5 \times 10^5$  cells per well in 3 mL of complete growth medium, incubated for 24 h and treated with linalool at various concentrations (0, 20, 40 and 80  $\mu\text{M}$ ). Control cells treated with 0.3 % DMSO alone were also included. The morphological changes, characteristic of apoptosis or necrosis, were observed and the images were captured under an inverted light microscope (Olympus, PA, USA) after 48 h. The same spot of cells was marked and captured.

### Statistical analysis

The experiments were performed in triplicate. Data were expressed as the mean  $\pm$  standard deviation (SD). Statistical correlation of data was checked for significance by ANOVA and Student's t test.  $P < 0.05$  was taken to indicate statistically significant difference.

## RESULTS

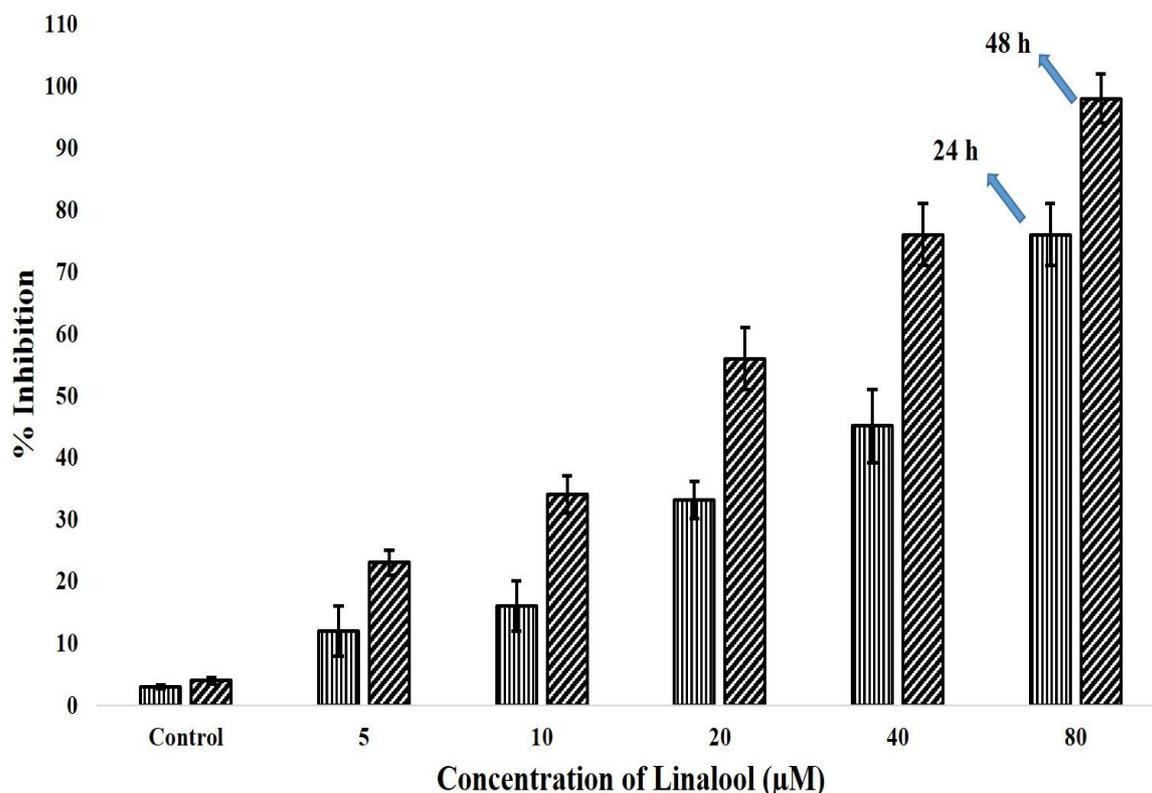
### Linalool induced strong cytotoxicity in human prostate cancer (DU145) cells

The cancer cells showed considerable and dose-dependent susceptibility to the treatment of

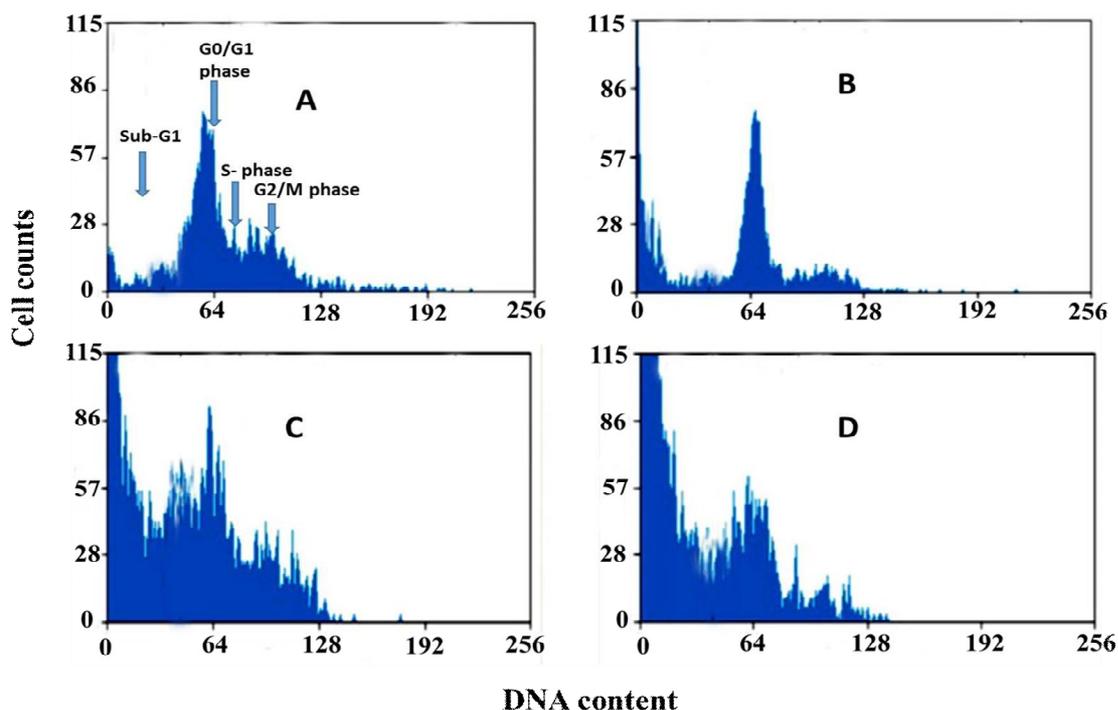
different concentrations of linalool. The compound also showed time-dependent inhibition of the cancer cell growth as shown in Figure 1 at 24 h and 48 h time intervals. The number of viable human prostate cancer cells exposed to linalool treatment reduced considerably. The  $\text{IC}_{50}$  value of the compound after 12 h and 24 h time intervals was found to be 28.3 and 10.5  $\mu\text{M}$  respectively.

### Linalool induces sub-G1 cell cycle arrest and increases fraction of apoptotic cells in DU145 cells

Linalool induced sub-G1 cell cycle arrest with a significant increase of apoptotic cells. The results showed that treatment with different concentrations of linalool for 48 h led to an increase in the population of cells in the sub-G0/G1 phase (apoptotic population) ( $p < 0.01$ ) (Figure 2, A-D). This increase in sub-G1 population was accompanied by a corresponding decrease of the cells in s-phase and G2/M phase of the cell cycle. As compared to the control (Fig2-A), linalool treated (Fig 2, B, C, D) cells showed a significant proportion of cells in apoptosis phase.



**Figure 1:** Cancer cell growth inhibition by linalool at various concentrations and at two different time intervals

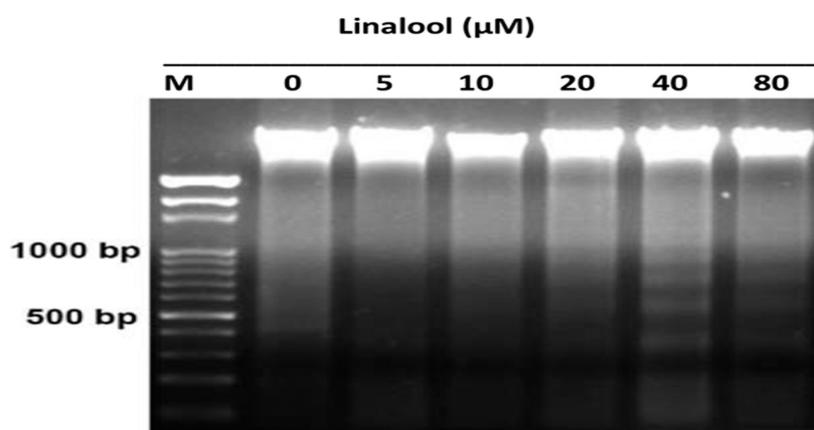


**Figure 2:** Effect of linalool on the cell cycle arrest in human prostate cancer (DU145) cells. Cells (DU145) were treated with (B) 20  $\mu$ M emodin, (C) 40  $\mu$ M and (D) 80  $\mu$ M of linalool for 48 h. (A) shows the control (untreated) group. The treatment affected the cell cycle distribution and induced apoptosis. The percentage of cells in the sub-G1 phase increased significantly with increase in the linalool dose from 0 Mm (A) to 20 (B), 40 (C) and finally to 80 Mm (D). The DNA histogram shows the distribution and the percentage of cells in phases of the cell cycle. Results are the mean  $\pm$  SD of 3 independent experiments. Each DNA histogram represents one of the three independent experiments

**Effect of linalool on DNA fragmentation in DU145 human prostate cancer cells**

As shown in Figure 3, DNA ladder appeared to be more evident with the increasing linalool concentration. However, no DNA fragments were observed in the control groups (Figure 3, left

panel). Even lower doses of linalool (5  $\mu$ M) also did not induce DNA fragmentation. However, 40 and 80  $\mu$ M doses of linalool resulted in a substantial increase in DNA fragmentation (Figure 3, right panel).



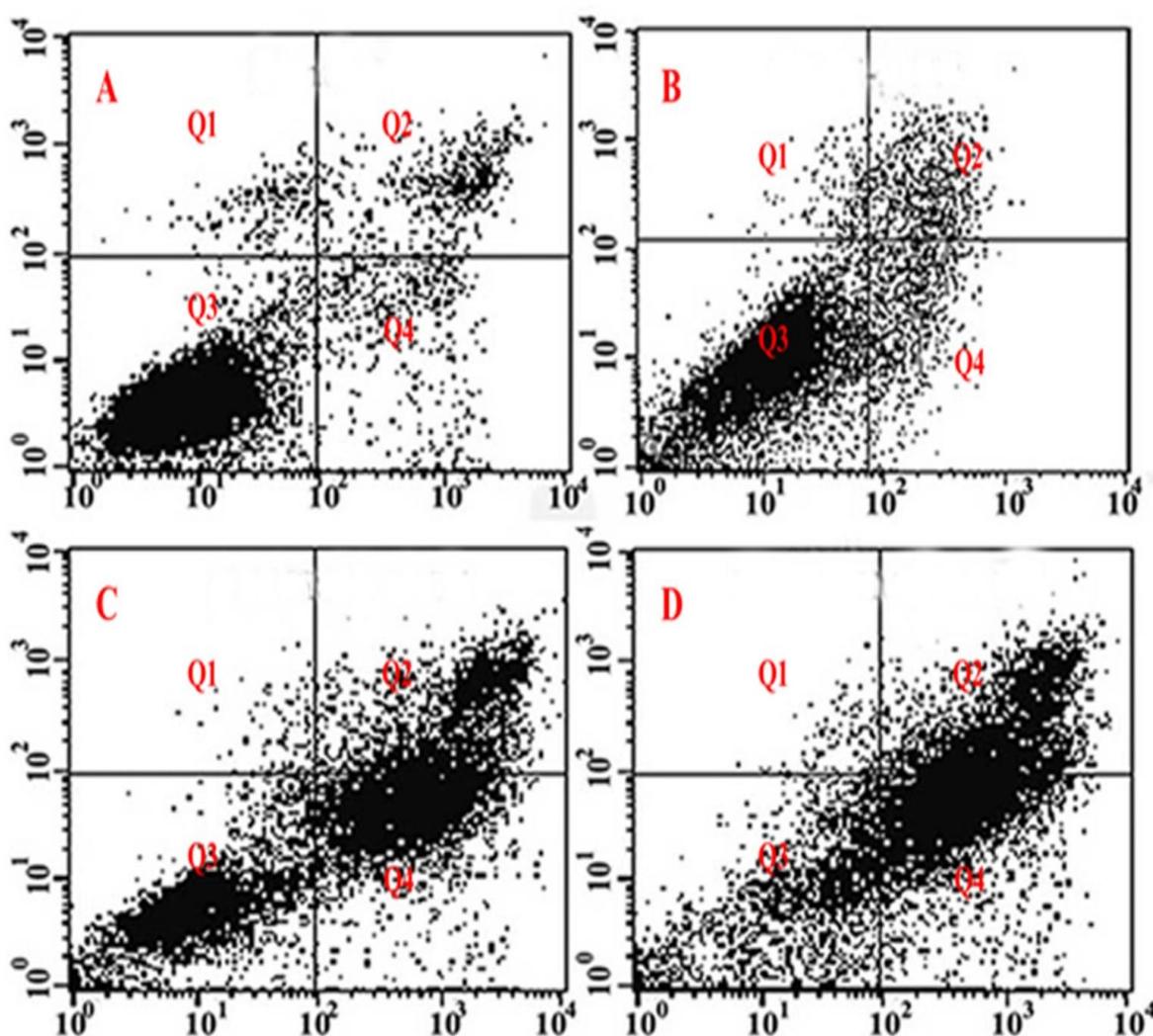
**Figure 3:** Linalool induces DNA fragmentation in human prostate cancer cells (DU145). Prostate cancer cells were treated with 0, 5, 10, 20, 40 and 80  $\mu$ M linalool for 48 h. Cells from each sample were harvested for DNA gel electrophoresis as described in Materials and methods. Each point is the mean  $\pm$  SD of three experiments

### Effect of linalool on DU145 cell apoptosis

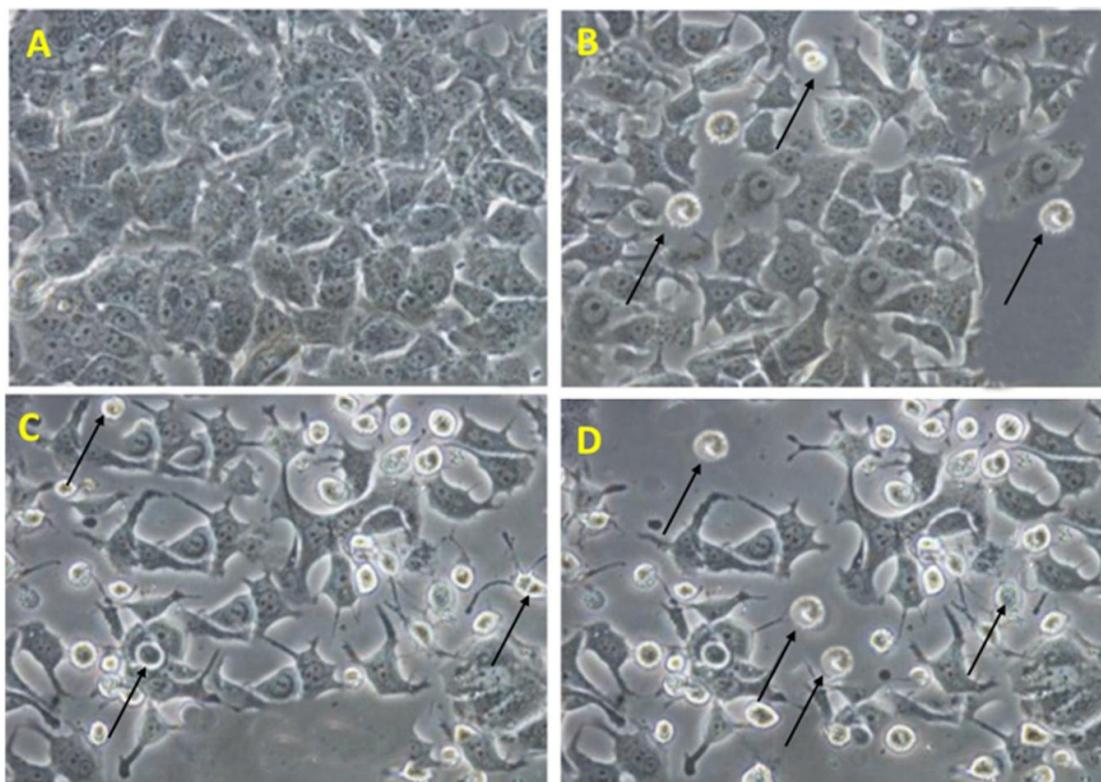
The results of Annexin V assay are shown in Figure 4A-D. The results of the flow cytometry study with Annexin V/FITC and PI showed that within 48 h of incubation, approximately 4.36, 11.54, 21.88 and 15.54 % of the cells underwent early apoptosis after treatment with 0 (no linalool treatment), 20, 40 and 80  $\mu\text{M}$  of linalool respectively. Similarly, 6.07 %, 25.11 %, 29.34 % and 43.14 % of the cells underwent late apoptosis after treatment with 0 (no linalool treatment), 20, 40 and 80  $\mu\text{M}$  of linalool respectively (Figure 4).

### Morphological characteristics of apoptosis

Morphological study using inverted light microscopy revealed that linalool induced growth inhibition and apoptosis in DU145 prostate cancer cells. As shown in Figure 5A-D, the number of cells in the control and the ones treated with different concentrations of linalool increased from 0, 20, 40 and 80  $\mu\text{M}$  dose. The cells with higher doses revealed that cellular shrinkage and blebbing occurred. This effect was shown to be related to linalool dose. The number of cells with shrinkage increased with linalool concentration.



**Figure 4:** Induction of apoptosis by linalool evaluated by Annexin V-FITC/PI dual staining. DU145 prostate cancer cells were treated with 20  $\mu\text{M}$  (B), 40  $\mu\text{M}$  (C) and 80  $\mu\text{M}$  (D) of linalool for 48 h and analyzed using FACS Calibur flow cytometer as described in "Materials and Methods". A, represents experiment control of untreated cells alone. Q1, Q2, Q3 and Q4 quadrants show percentage of normal healthy, early apoptotic, late apoptotic and necrotic cell populations respectively



**Figure 5:** Linalool inhibited the growth of DU145 prostate cancer cells as revealed by inverted light microscopy. Morphological changes of DU145 cells after treatment with linalool (magnification 400X). Cellular shrinkage was observed in linalool-treated cells (arrows). A represents control (untreated cells), B,C and D represent effect of 20, 40 and 80  $\mu\text{M}$  of linalool on cell morphology of DU145 cells. The number of cells with shrinkage increase with increase in the dose of linalool

## DISCUSSION

Today, plant-based drugs continue to play an essential role in health care. It has been estimated by the World Health Organization that 80 % of the population of the world rely mainly on traditional medicines for their primary health care [8]. Natural products have contributed significantly to the development of anticancer drugs. According to a recent review, among the 79 FDA approved anticancer drugs and vaccines from 1983 - 2002, 9 were directly from the isolation of natural products and 21 were natural product derivatives [9].

Linalool has been reported to exhibit good inhibitory effects against breast, colorectal and liver cancer cells [10]. Cell cycle analysis also confirmed that linalool can lead to apoptosis. By using flow cytometry, it was reported that treatment with linalool significantly increased the sub-G1 phase and that there were more cells concentrated in the G1 phase. Linalool has also been reported to stimulate IFN- $\gamma$ , IL-13, IL-2, IL-21, IL-21R, IL-4, IL-6sR and TNF- $\alpha$  secretion [11,12]. Linalool has also been investigated for its ability to induce apoptosis and differentiation

in human leukemia HL-60 cells [13,14]. Linalool reportedly possesses strong activity against U937 histiocytic lymphoma cells and P3HR1 Burkitt lymphoma cells [13]. Moreover, several recent reports have revealed that linalool reverses doxorubicin resistance in human breast adenocarcinoma cells [15] and exhibits anti-proliferative activity against certain solid tumor cells, such as melanoma and renal cell adenocarcinoma cells and HepG2 [16]. Particularly, linalool induces apoptosis in human leukemia cells without affecting normal hematopoietic cell growth [17]. These terpenoid components become ligands in nuclear receptors, regulate expression of various genes and ameliorate conditions related to these diseases [18].

In this study, linalool was found to exert anticancer effects against prostate cancer cells by inducing apoptosis and sub-G1 cell cycle phase arrest. Linalool even induced potent DNA fragmentation in these cells. It caused both early and late apoptosis with cell shrinkage and membrane blebbing which are characteristic features of apoptosis.

## CONCLUSION

The findings of this work indicate that linalool exhibits potent dose-dependent as well as time-dependent anticancer activity in human prostate cancer (DU145) cells by inducing apoptosis, DNA fragmentation and cell cycle arrest. These findings may pave way for future research on linalool in the hunt for novel and less toxic chemotherapeutic agents for prostate cancer.

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