

ORIGINAL ARTICLE

Limonene terpenoid obstructs human bladder cancer cell (T24 cell line) growth by inducing cellular apoptosis, caspase activation, G2/M phase cell cycle arrest and stops cancer metastasis

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Summary

Purpose: The primary purpose of the current study was to investigate the antitumor activity of limonene which is a plant monoterpene along with evaluating its effects on cell apoptosis, cell cycle phase distribution, cell migration and invasion.

Methods: The cell proliferation of T24 bladder cancer cells was examined by WTS-1 assay. The apoptotic effects induced by limonene were investigated by a combination of fluorescence microscopy and flow cytometry and then confirmed by western blot assay. The effects of limonene on cell cycle in T24 bladder cancer cells were studied by flow cytometry. The effects on cell migration and invasion were examined by wound healing assay and transwell assay using Matrigel.

Results: The results showed that limonene induced cytotoxic effects and reduced cell viability of T24 human bladder cancer cells showing an IC₅₀ value of 9 μ M. Limonene also induced significant apoptosis in bladder cancer cells since it

induced significant nuclear fragmentation, chromatin condensation, and splitting of the nucleus, representative of the apoptotic cascade. The apoptotic cell percentage was 1.95, 5.35, 15.61 and 34.71% at limonene concentrations of 0, 9, 18 and 36 μ M. Further, the apoptotic effects of limonene were also confirmed by Western blot analysis and the results showed increase in the expression of Bax and caspase-3 and decrease of Bcl-2 expression. Limonene also caused G2/M phase cell cycle arrest as well as suppression of cell migration and invasion.

Conclusions: These results indicate that limonene might be used as a potent anticancer agent against human bladder cancer for which further in depth studies are needed, especially over its toxicological studies.

Key words: limonene, apoptosis, bladder cancer, cell migration, cell invasion

Introduction

Bladder cancer constitutes the second most frequent malignancy affecting the urinary tract in USA. It is regarded as one of the most common malignancies categorised as 11th among human cancers in terms of prevalence and is linked

with very high mortality [1,2]. Urinary bladder carcinoma targets men more frequently as compared to women. Among the risk factors, smoking is considered to be a significant cause of bladder carcinoma. In China, urinary bladder cancer has

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shown an increasing trend, especially during the last 10 years in both rural and urban regions. This increasing trend of bladder cancer may be linked with the increased consumption of tobacco, population aging and level of industrial development [3,4]. Bladder transitional cell tumor, which constitutes about 95% of the cases, is the most common type of bladder cancer. It has been reported that about 30% of the bladder cancer patients have an invasive type of bladder malignancy which accounts for a higher risk of cancer metastasis [5]. About 75% of the newly diagnosed bladder cancers belong to non-muscle-invasive bladder cancer (NMIBC). Even though a lower number of patients is diagnosed with muscle-invasive bladder cancer (MIBC), this form of bladder cancer is accountable for a significant number of bladder cancer-related mortality [6,7]. Treatment results of bladder cancer are not satisfactory and lead to significant economic burden due to the fact that the mechanisms and the etiology of this disease remain uncertain [8]. Currently, bladder cancer treatment comprises removal of bladder tumor mass, chemotherapy, radiation therapy and cystoprostatectomy. In China, the main treatment of bladder cancer involves surgery in combination with intravesical chemotherapy [9]. Despite recent developments in bladder cancer treatment, the currently used therapies are linked with important side-effects affecting the patient quality of life. It is important to mention here that various anticancer chemotherapeutic drugs used to treat bladder cancer (doxorubicin, cisplatin, vincristine, methotrexate etc) are very costly along with their serious side-effects [10]. Therefore, there is a pressing need for the development of novel therapeutic agents with lower side-effects and lower cost-effectiveness. Natural products have always played a crucial role in treating a wide range of human diseases, especially cancer. More than 60% of the clinically used anticancer drugs are derived from plants or are semisynthetic/synthetic derivatives of natural products.

In the present study, we investigated the anticancer effects of a naturally occurring plant monoterpene-limonene-along with evaluating its effects on cellular apoptosis, caspase activation, cell cycle phase distribution and cancer cell migration and invasion.

Methods

Cell culture and cell proliferation assay

The Cell Bank of the Chinese Academy of Sciences was kind enough to provide us the human bladder cancer cell line T24. The cells were suspended in Dulbecco's modified Eagle's medium (DMEM), supplemented

with 10% fetal bovine serum (FBS) antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin), and 2 mM glutamine. The viability of T24 cells was assessed by WST-1 assay. In brief, T24 cells were cultured in 96-well plates at the density of 1×10^5 cells /well and treated with 0, 2.5, 5, 10, 20, 40, 80, 160 and 320 µM concentrations of limonene for 24 h at 37°C. Subsequently, the T24 cells were incubated with WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) at 37°C for 2 h. The absorbance was then measured at 450 nm with a microplate reader to determine the cell viability

Studying apoptosis using DAPI and annexin V/PI staining using fluorescence microscopy

Fluorescence microscopy was used to study the apoptotic effects induced by limonene in T24 bladder cancer cells using DAPI staining. The apoptotic effects were also evaluated by annexin V/ propidium iodid (PI) staining using flow cytometry. In brief, T24 cells were placed in 6-well plates (1×10^6 cells/well) for 24 h. The cells were then treated with 0, 9, 18 and 36 µM concentration of limonene for 24 h at 37°C. Around 25 µl cell cultures were put onto a glass slide and stained with DAPI solution. The slides were then cover-slipped and examined with a fluorescent microscope (Nikon Instruments Inc., NY, USA). For annexin V/PI analysis, T24 human bladder cells were put on 6-well plates for 12 h and then treated with 0, 9, 18, and 36 µM dose of limonene for 12 h. The apoptotic percentage of cells was evaluated following the guidelines provided by the Bio-Vision annexin V-FITC apoptosis detection kit (BioVision, CA, USA). The results were evaluated using a flow cytometer.

Flow cytometric analysis of the cell cycle

The effects of limonene on the cell cycle progression were analysed by flow cytometry using Annexin V/PI staining. The cultured human bladder T24 cells were originally treated with 0, 9, 18 and 36 µM concentration of limonene for 24 hours at 37°C. The cells were then washed with phosphate buffered saline (PBS). Subsequently, the T24 bladder cancer cells were stained with PI and the distribution of the cells in various cell cycle phases was measured by FACS flow cytometer (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ, USA).

Wound-healing and transwell assays for cell migration and invasion evaluation

The limonene-treated cells (0, 9, 18 and 36 µM) were cultured till 90% confluence. Subsequently, the DMEM medium was removed and the cell culture was washed with PBS twice. After that, a wound was scratched with a sterile pipette tip and a picture was taken. The plates were then incubated for about 12 h at 37°C and then photographed using an inverted microscope.

For cell invasion assay, transwell chambers with Matrigel were used to check whether limonene affects bladder cancer cell invasion. Nearly 300 µl cell culture were placed onto the upper chambers and plain medium was placed in the bottom wells. Following 24-h incuba-

tion, the cells were removed from the upper chamber and the cells that invaded through the chambers were fixed with methyl alcohol and stained with crystal violet. Inverted microscope was used to count the number of invaded cells (200x magnification) (Nikon Instruments Inc., NY, USA).

Western blot assay

The apoptotic effects induced by limonene were further examined by western blot assay. The T24 human bladder cells were washed with ice-cold PBS and then suspended in a lysis buffer at 4°C. Afterwards, Bradford assay was used for assessing the protein content of each cell extract. About, 60 µg of protein was loaded from each sample and separated by SDS-PAGE before being shifted to polyvinylidene fluoride membrane. The membranes were then subjected to TBS treatment and exposed to primary antibodies at 4°C. The cells were treated with appropriate secondary antibodies and the proteins of interest were visualised by enhanced chemiluminescence reagent. Finally the signal was detected by Odyssey Infrared Imaging System. Actin and GAPDH were used as control for normalisation.

Statistics

The results are presented as mean \pm standard deviation values from three independent experiments. Differences between the groups were examined by Student's *t*-test using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Limonene induced significant cell cytotoxicity in T24 human bladder cancer cells

The cytotoxic effects induced by limonene (Figure 1A) in T24 bladder cancer cells were examined by WST-1 cell proliferation assay at increasing doses of limonene (0, 2.5, 5, 10, 20, 40, 80, 160 and 320 µM). and the results indicated that limonene triggered dose-dependent cytotoxic effects in these cells. In order to evaluate its potency, IC_{50} value was calculated and was found to be 9 µM, indicating that only 9 µM dose of limonene can cause 50% cell cytotoxicity in T24 human bladder cancer cells (Figure 1B).

Limonene caused apoptosis in T24 bladder cancer cells

T24 human bladder cancer cells were treated with 0, 9, 18 and 36 µM doses of limonene, stained with DAPI and analysed by fluorescence microscopy in order to confirm whether limonene has a role to play in inducing apoptosis in these cells. The results clearly indicated that limonene caused a significant nuclear fragmentation, chromatin condensation and splitting of the nucleus, all representative of the apoptotic cascade (Figure 2). Annexin V/PI staining was also carried out and the apoptotic T24 bladder cancer cell

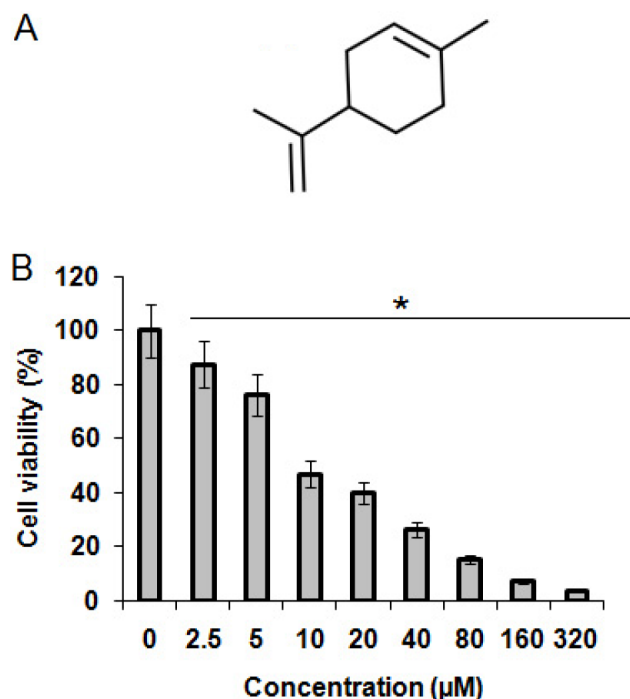


Figure 1. A: Chemical structure of limonene. **B:** WTS-1 assay showing cytotoxic effects of limonene on the viability of T24 human bladder cancer cells. The experiments were performed in triplicate and shown as mean \pm SD (* $p < 0.05$).

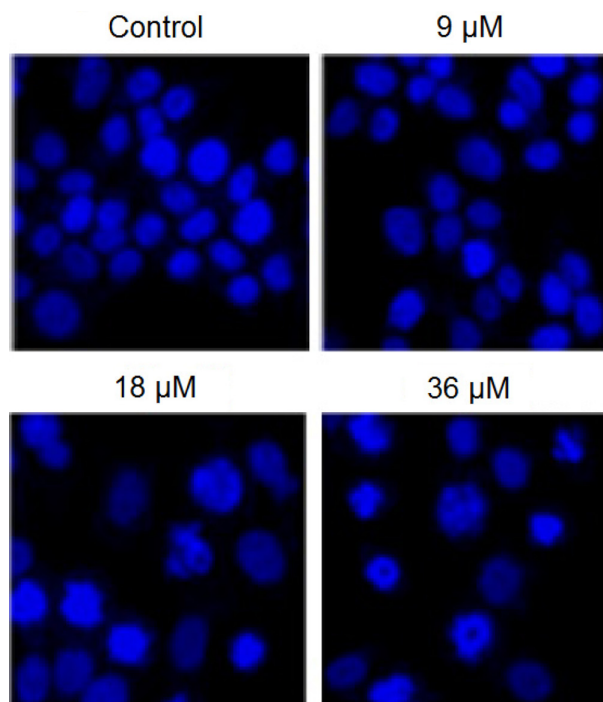


Figure 2. Apoptotic studies using DAPI (4',6-diamidino-2-phenylindole) staining, showing that limonene induces apoptosis in T24 human bladder cancer cells. The experiments were performed in triplicate.

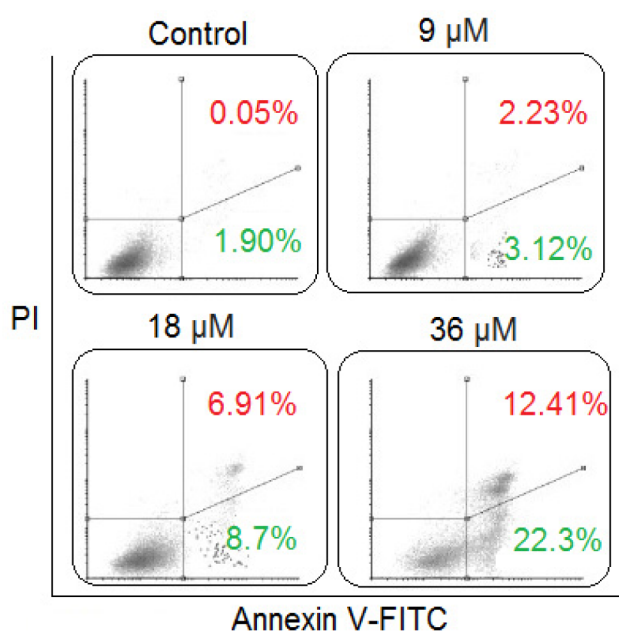


Figure 3. Annexin V/PI assay using flow cytometry showing that limonene increases the T24 human bladder apoptotic cells with increasing concentrations. The experiments were performed in triplicate.

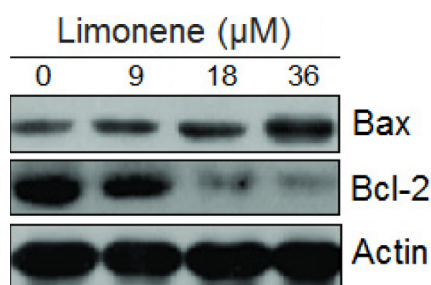


Figure 4. Effect of tested doses of limonene on the expression of apoptosis-related proteins (Bcl-2 and Bax) by western blot analysis. The Figure shows that Bax expression increased and Bcl-2 decreased upon limonene treatment. The experiments were performed in triplicate.

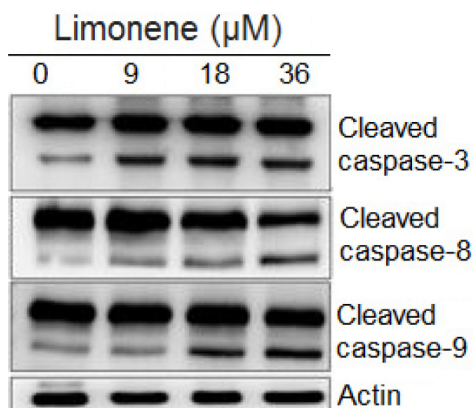


Figure 5. Effect of limonene on the expression levels of various caspases by western blot assay. The Figure shows that limonene activated the cleavage of caspase-3, 8 and 9 in T4 cells. The experiments were performed in triplicate.

percentage was measured at 0, 9, 18 and 36 μM concentrations of limonene. The apoptotic cell percentage was 1.95, 5.35, 15.61 and 34.71% at the limonene concentrations of 0, 9, 18 and 36 μM (Figure 3). Furthermore, the apoptotic effects of limonene were also confirmed by Western blot analysis and the results showed increase in the expression of Bax and caspase-3 and decrease of Bcl-2 expression. Both these effects were found to be concentration-dependent (Figure 4). Moreover, limonene augmented the expression of caspase-3 and 9 and also stimulated their cleavage (Figure 5). The above bioassays clearly confirmed the fact that limonene inhibits the growth of T24 human bladder cells by triggering cell apoptosis.

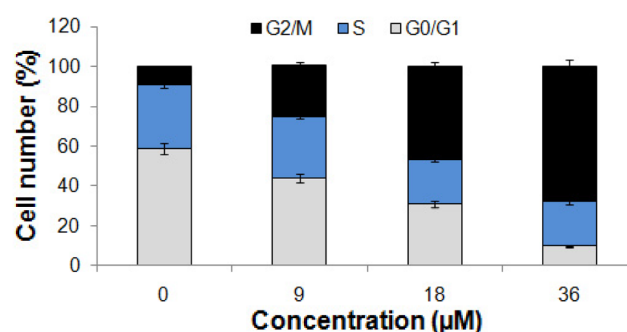


Figure 6. Limonene induced G2/M cell cycle arrest of the T24 human bladder cancer cells as shown by flow cytometry. The experiments were performed in triplicate and shown as mean ± SD (*p<0.05).

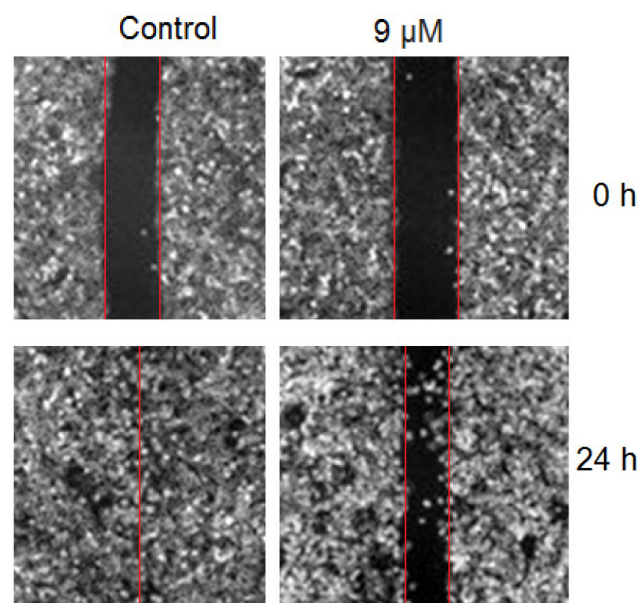


Figure 7. Wound healing assay showing that limonene induced dose-dependent inhibition of migration in T24 human bladder cancer cells. The experiments were performed in triplicate.

Limonene triggered G2/M cell cycle arrest in T24 human bladder cancer cells

The growth inhibitory effects of limonene were further seen to be facilitated through hindering cell cycle phase distribution. The results obtained from flow cytometric measurements indicated that limonene led to G2/M cell cycle arrest in a concentration-dependent manner. With increase in the limonene concentration, the T24 cells in G2/M phase were also seen to upsurge and at 36 μM dose, 73% of the cells were seen to lie in G2/M phase of the cell cycle (Figure 6).

Limonene induced suppression of bladder cancer cell migration and invasion

The fact whether limonene caused suppression of cancer cell migration and invasion was examined by wound healing and transwell assays, respectively. These assays indicated that limonene not only suppressed cell migration but it inhibited cell invasion also, showing its potential that it might be useful against in stopping bladder cancer metastasis. It was seen that limonene at IC_{50} dosage showed potent inhibitory effects on cancer cell migration and invasion (Figure 7 and 8). The cell migration inhibition effects of limonene were particularly significant as clearly evidenced from wound width at increasing dosage of limonene.

Discussion

Plant-derived secondary metabolites constitute an important source of drugs for treating hu-

man illnesses. The majority of the clinically approved drugs for cancer treatment are either pure natural products or their semisynthetic/synthetic derivatives. Molecules derived from plants exert their anticancer actions through a wide array of molecular mechanisms including cell cycle arrest, inducing apoptosis or programmed cell death, autophagy, necrosis, ROS generation, mitochondrial membrane potential collapse, and inhibition of cell migration and invasion. In addition, it has been reported that plant-derived molecules and their synthetic derivatives also target specific biochemical signalling pathways including PI3K/AKT, JNK/p38, Raf/MEK/ERK signalling pathways. Various published reports show that plant molecules are comparatively safer and display minimal or even no adverse effects [11-13]. There is a significant and promising amount of published studies showing the *in vitro* anticancer activity of a wide variety of plant molecules against a wide-spectrum of cancer cell lines [14]. Limonene is a plant monoterpene mainly found in citrus fruits. It has been shown that limonene induced antitumor effects in human breast and pancreatic cancer cells [15]. Limonene has also been reported to induced cytotoxicity in K562 (leukemia), MCF-7 (breast cancer), PC-12, HT-29 (colon cancer), A-549 (lung cancer) and HepG2 liver cancer cell lines [16-18]. Some authors have shown that the anticancer activity of limonene was mediated via a caspase-dependent apoptosis in leukemia cells. Limonene was also shown to induce apoptosis in HL-60 leukemia cells by activating caspase-8 [19,20]. Some workers have reported chemopreventive effects of limonene in aflatoxin B1-induced liver carcinogenesis and it was shown that the molecule suppressed aflatoxin-DNA adduct formation in hepatocytes.

Taking cue from these published reports, we focussed our attention on limonene and investigated its effects on the growth inhibition of human bladder cancer cells A-724). We also investigated its effects on apoptosis induction, cell cycle, cell migration and invasion. Our results revealed that the molecule triggered dose-dependent cytotoxic effects in these cancer cells and its IC_{50} value was calculated to be 9 μM . Further, it was mandatory to check how growth inhibitory effects of limonene were exerted, so we focussed on whether limonene induced apoptosis. For this, fluorescence microscopy and flow cytometric measurements were carried out and it was seen that limonene induced significant apoptotic effects in T24 human bladder cancer cells. It caused nuclear fragmentation and chromatin condensation which are apoptotic features. The apoptotic cell percentage was 1.95, 5.35, 15.61 and 34.71% at limonene concentrations of 0, 9, 18 and

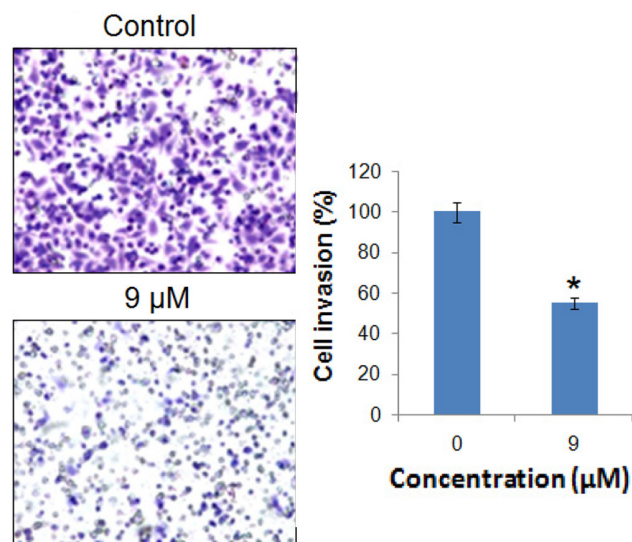


Figure 8. Transwell assay showing that limonene induced dose-dependent inhibition of invasion in T24 human bladder cells. The experiments were performed in triplicate and shown as mean \pm SD (* $p < 0.05$).

36 μ M. Further, the apoptotic effects of limonene were also confirmed by Western blot analysis and the results showed increase in the expression of Bax and caspase-3 and decrease of Bcl-2 expression. Limonene also caused G2/M phase cell cycle arrest in addition to causing inhibition of cancer cell migration and invasion.

Conclusion

Taking all the results into consideration, it is

concluded that limonene inhibits bladder cancer cell growth by inducing programmed cell death, cell cycle arrest and suppression of cell migration and invasion. As such limonene might act as a potent anticancer agent against bladder cancer provided further studies are carried out.

Conflict of interests

The authors declare no conflict of interests.

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