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ORIGINAL ARTICLE

Lupeol triterpene exhibits potent antitumor effects in A427 human lung carcinoma cells via mitochondrial mediated apoptosis, ROS generation, loss of mitochondrial membrane potential and downregulation of m-TOR/PI3K/AKT signalling pathway

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Summary

Purpose: *Lung cancer is one among the most commonly diagnosed malignancies in developed countries with highest rates of morbidity and mortality. The treatment options for lung cancer are limited and have side effects. Hence, researches are being directed at the exploration and evaluation of new molecules showing anticancer activity. In this study we investigated the anticancer effects of lupeol on the lung cancer A427 cancer cells and normal MRC-5 cells.*

Methods: *Growth inhibitory effects were checked by MTT assay. Apoptosis was detected by DAPI and annexin V/PI staining. Reactive oxygen species (ROS) and mitochondrial membrane potential (MMP) were estimated by flow cytometry. Protein expression analysis was performed by western blotting.*

Results: *The results showed that lupeol inhibited the growth of A427 lung cancer cells as observed by MTT and colony formation assay. The antiproliferative effects were due to induction of apoptosis as indicated by DAPI and annexin V/PI staining. This was further confirmed by Bax and Bcl-2 expression. Moreover, lupeol triggered generation of ROS and decrease of MMP. Lupeol could also inhibit the mTOR/PI3K/AKT signalling pathway. Taken together, lupeol could prove beneficial in the treatment of lung cancer.*

Conclusions: *In brief, the present study indicated that lupeol induced potent and selective cytotoxic effects and these effects were mediated via apoptosis, ROS generation, loss of MMP and inhibition of mTOR/PI3K/AKT signalling pathway.*

Key words: *apoptosis, flow cytometry, lung cancer, lupeol, ROS*

Introduction

Plants are versatile chemical factories that exhibit the capacity to synthesize a vast array of chemicals of with diverse structures [1]. Among these chemicals, the secondary metabolites that are used by the plants to combat stress are of utmost importance to humans as drugs [2]. Plants and their secondary metabolites have been used as a source of medicines since ages and still a large part of world's population depends on plants for their primary health care needs. Among plant secondary metabolites, triterpenes have been reported to be of immense pharmacological potential due to their bioactivities such as anticancer, antimicrobial and others [3]. Lupeol is one such triterpene that is isolated from several plant species and has been shown to have a number of bioactivities [4].

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In this study we evaluated the anticancer activity of lupeol against the lung cancer cell line A-427. Lung cancer is one among the most commonly diagnosed malignancies in developed countries with highest rates of morbidity and mortality [5]. There are no clear symptoms for lung cancer during the early disease stage and it is therefore detected at an advanced stage [6]. Only around 20% of the patients with lung cancer are diagnosed in early stage [7]. Studies are being performed to understand the mechanisms involved in lung cancer genesis and the therapeutic potential of various factors [8]. The prognosis of this disease is dismal [9] and the currently used treatment options consist of radical resection, use of chemotherapeutic agents and radiotherapy. However, in most of the cases lung cancer recurrence is observed after successful completion of the treatment [10]. Thus the discovery of novel molecules which can prevent cancer recurrence or inhibit the tumor progression is highly needed. Over the past few decades many anticancer agents have been found but the development of resistance and the toxicity to the currently used drugs demands the discovery of new chemotherapeutics [11].

In this study we report that lupeol exhibits antiproliferative effects on lung cancer cells. The anticancer effects are due to the induction of apoptosis mediated by accretion of ROS, reduction of the MMP and inhibition of mTOR/PI3K/AKT signalling pathway. Given these results, lupeol could prove to be an important molecule for the treatment of lung cancer.

Methods

Cell lines and culture conditions

The human lung cancer cell lines A427 and normal lung cell line MRC-5 were procured from the Shanghai Xiang Biotechnology Co., Ltd. (Shanghai, China). Maintenance of the cells was performed in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). The cells were incubated in an incubator containing 95% air and 5% CO_2 at 37°C. Ten mM stock solution of 3-tetrazolylmethyl-3-hydroxy-oxindole hybrid was prepared in dimethyl sulfoxide (DMSO) and dilution was performed in DMEM medium to the required concentration. MTT and DMSO were procured from Sigma-Aldrich (St. Louis, MO, USA).

MTT assay

A427 cancer cells were cultured in the 96-well plates containing DMEM medium at a density of 2×10^6 cells per well. The cells were incubated for 48 hrs with the conditioned medium containing 3-tetrazolylmethyl-3-hydroxy-oxindole hybrid at 37°C. After incubation the cells were treated with 5 mg/ml solution of MTT for 4 hrs. Extraction of the cells with acidic isopropyl alcohol was followed by absorbance measurement at 570-nm wavelength (A570) using an enzyme-labelling instrument (ELx800 Absorbance Microplate Reader type, Bio-Tek Instruments, Inc., Winooski, VT, USA). Colony formation assay was carried out as described previously [12].

Apoptosis assay, ROS and MMP determination

The lung cancer A427 cancer cells were seeded in 6-well plates (2×10^5 cells per well) and treated with lupeol at the concentrations of 0, 6, 12 and 24 µM. The cells were then DAPI-stained to detect the apoptosis by fluorescence microscopy as previously reported [13]. For the estimation of the percentage of the apoptotic cells a FITC-Annexin V/PI Apoptosis detection kit was employed as per the instructions of the manufacturer. ROS and MMP levels were determined as described previously [14].

Western blot analysis

The lung cancer cells treated with lupeol were collected and treated with lysis buffer [Tris-HCl, sodium-dodecyl sulfate (SDS), mercaptoethanol and glycerol]. The extracts were boiled for 10 min in the presence of loading buffer followed by separation of cell extracts using 15% SDS-PAGE gel. The samples were then put onto polyvinylidene fluoride membranes and blocked using 5% skimmed milk powder. Membrane incubation with primary antibodies was performed overnight at 4°C. The membranes were incubated with horseradish peroxidase-linked secondary biotinylated antibodies at 1:1,000 dilution for 2 hrs. Washing of the membranes with PBS was followed by visualization of the immunoreactive bands using the ECL-PLUS / Kit according to the manufacturer's instructions. The immune complexes development was carried out using an ECL detection kit according to the manual protocol (ECL GST western blotting detection kit, Pierce Biotechnology, Inc., Waltham, MA, USA). The bands were analyzed using GelGDoc2000 imaging system (Bio-Rad Laboratories GmbH, Munich, Germany).

Statistics

The experiments were performed thrice and presented as mean ± SD. One way ANOVA followed by Tukey's t-test using GraphPad7 software were used for statistical analysis. A p value <0.01 was considered significant.

Results

Lupeol exhibits antiproliferative effects on lung cancer cells

Lupeol belongs to triterpene class of natural products (Figure 1). The growth inhibitory effects of lupeol were evaluated in A427 lung cancer lung cancer cell line and one normal MRC-5 cell line. It was observed that lupeol could inhibit the growth

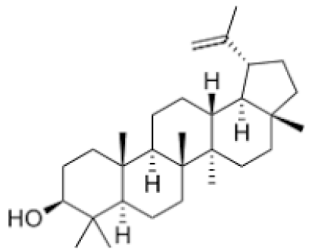

Figure 1. Chemical structure of lupeol.

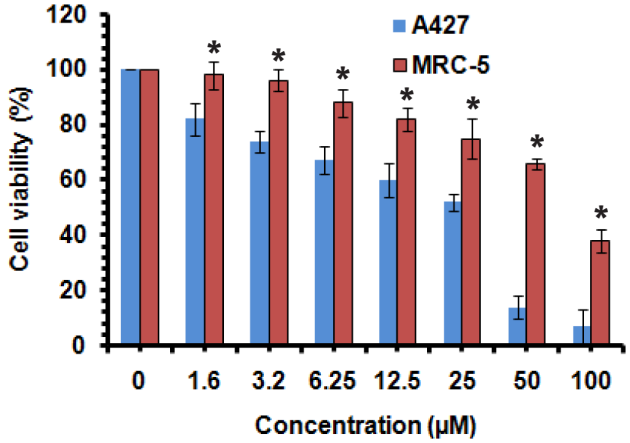

Figure 2. Antiproliferative activity of lupeol on lung cancer (A427) and normal cell (MRC-5) lines as depicted by MTT assay. The results are mean of three biological experiments and expressed as mean ± SD (*p<0.01). The results indicate that with increasing concentration of lupeol, the cell viability decreased significantly.

of A427 cancer cells in a dose-dependent manner with an IC_{50} of 25 μM. However, the anticancer effects against the normal MRC-5 cell line were comparatively less with an IC_{50} of 75 μM (Figure 2). The antiproliferative effects of lupeol were further confirmed by the results of the colony formation assay where it was observed that lupeol could inhibit the colony formation ability of the A427 cells in a dose-dependent manner (Figure 3).

Lupeol triggers apoptosis in lung cancer cells

To assess the underlying anticancer mechanism of lupeol, we first investigated whether lupeol triggers apoptosis in A427 lung cancer cells. The results revealed that lupeol induced apoptosis in a concentration-dependent manner (Figure 4). Moreover, annexin V/PI staining showed that the percentage of the apoptotic cells increased from 0.46% in the control to 32.12% at 50 μM concentration (Figure 5). We further checked the expression of Bax and Bcl-2 and observed that the expression of Bax increased on lupeol treatment while bcl-2 decreased, further confirming the apoptotic cell death (Figure 6).

Lupeol enhances ROS production in A427 lung cancer cells

After treatment with varying concentrations of lupeol, we estimated the ROS levels in A427

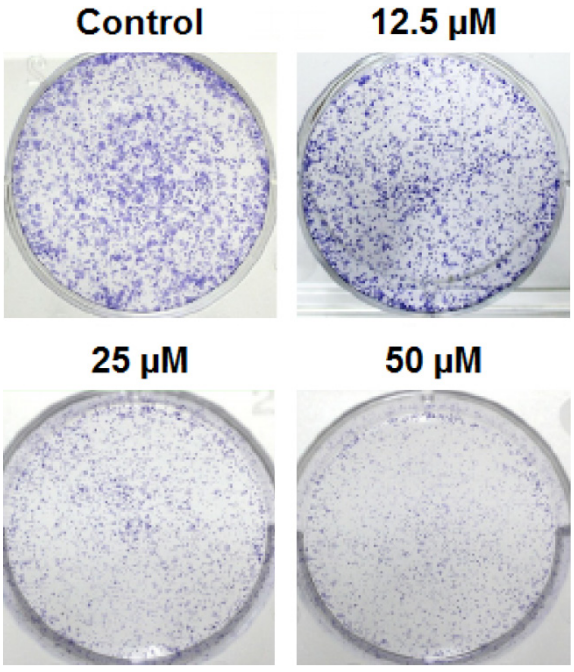

Figure 3. Inhibition of colony formation of A427 cancer cells by lupeol at various concentrations. The experiments were carried out in triplicate. The number of cancer cell colonies decreased as the dose of lupeol increased from 12.5 to 50 μM.

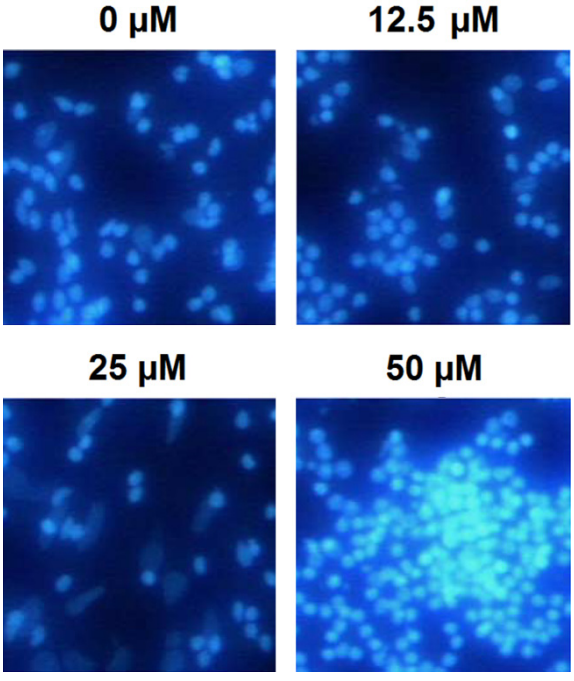

Figure 4. Induction of apoptosis by lupeol in A427 cancer cells at indicated concentrations as determined by DAPI staining. The results are mean of three biological experiments. Higher doses of lupeol induced chromatin condensation, evidenced by bright fluorescence.

lung cancer cells. The results showed that lupeol could significantly enhance the production of ROS in the A427 cancer cells. The ROS levels increased to 192% at 50 μM concentration as compared to the control (Figure 7).

Lupeol reduces the MPP in 427 lung cancer cells

The results showed that upon increase in the concentration of lupeol the MMP levels decreased significantly. This effect of lupeol was found to be concentration-dependent and at 50 μM concentration of lupeol the MMP level decreased to 43% (Figure 8).

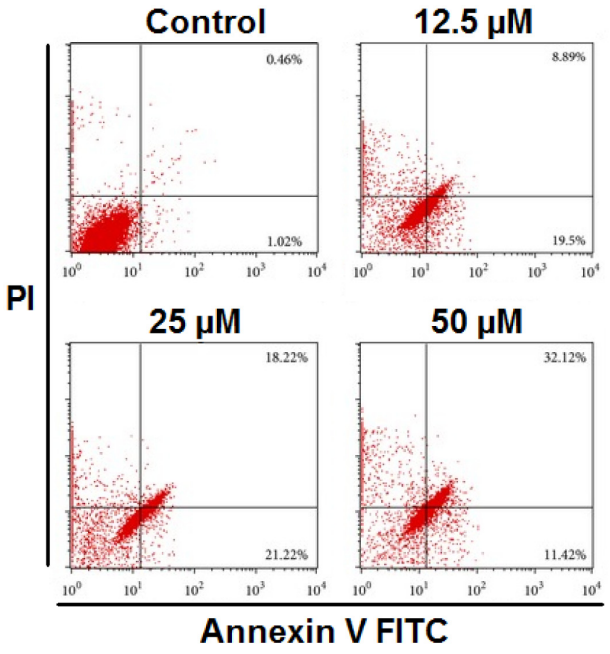

Figure 5. Estimation of A427 apoptotic cell populations by annexin V/PI staining at indicated concentrations of lupeol. The experiments were carried out in triplicate. The fraction of cells with both early and late apoptosis increased with increasing dose of lupeol.

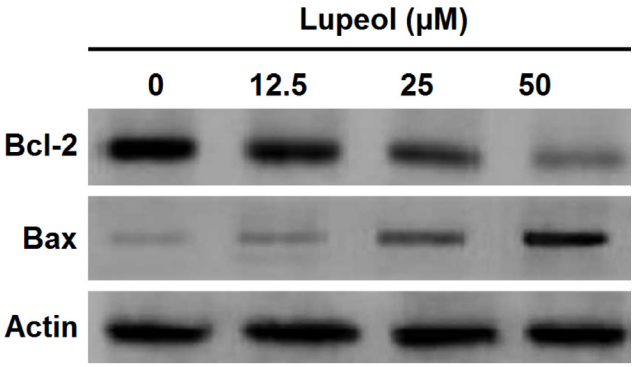

Figure 6. Effect of lupeol on the expression of Bax and Bcl-2 (western blot). The experiments were carried out in triplicate. The expression of Bax increased with lupeol treatment, while Bcl-2 decreased, further confirming the apoptotic cell death.

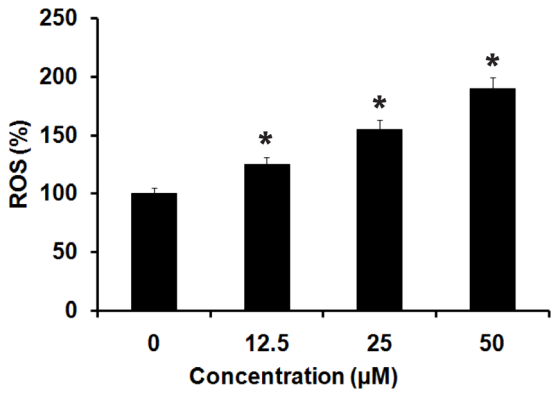

Figure 7. Determination of ROS levels in A427 cancer cells at indicated concentrations of lupeol by flow cytometry. The results are mean of three biological experiments and expressed as mean ± SD (*p <0.01). Lupeol treatment led to a dose-dependent increase in ROS production.

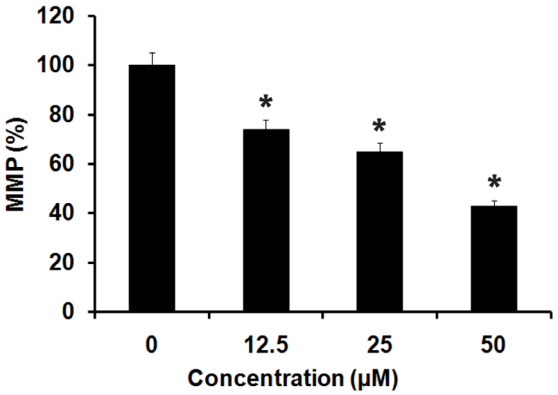

Figure 8. Determination of mitochondrial membrane potential (MMP) levels in A427 cancer cells at indicated concentrations of lupeol by flow cytometry. The results are mean of three biological experiments and expressed as mean ± SD (*p <0.01). Lupeol treatment led to a dose-dependent decrease in MMP.

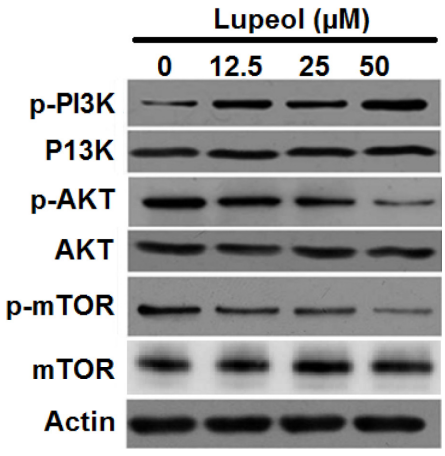

Figure 9. Inhibition of mTOR/PI3K/AKT signalling pathway by lupeol at indicated concentrations as shown by the western blotting. The experiments were carried out in triplicate and revealed that the expression of mTOR, PI3K and AKT remained unaltered. However, the expression of p-mTOR, p-PI3K and p-AKT decreased significantly in a concentration-dependent manner.

Lupeol inhibits mTOR/PI3K/AKT signalling pathway in A427 cancer cells

Evaluation of the effect of lupeol at 0, 12.5, 25 and 50 μM concentrations on the mTOR/PI3K/AKT signalling pathway revealed that the expression of mTOR, PI3K and AKT remained unaltered. However, the expression of p-mTOR, p-PI3K and p-AKT decreased significantly and in a concentration-dependent manner (Figure 9).

Discussion

Despite the developments made in the field of science and technology, lung cancer still remains the most common type of solid malignancy across the world. Lung cancer is mostly diagnosed at advanced stages and as such is often difficult to treat [5]. Moreover, the available chemotherapeutic agents are less efficient, creating also considerable side effects [15]. Therefore, screening of novel molecules for their anticancer activity against the lung cancer cells is the need of the hour. In this study we observed that lupeol exerted growth inhibitory effects on the A427 lung cancer cells in a concentration-dependent manner. Moreover, these antiproliferative effects were further confirmed by the colony formation assay. Our results are in agreement with previous studies wherein lupeol has been reported to exert antiproliferative effects on cancer cells [16]. To further unravel the reason behind the growth inhibitory effects of lupeol on the A427 cancer, we examined whether lupeol produces apoptosis in the A427 lung cancer cells by DAPI and annexin V/PI staining. It was observed that lupeol could initiate apoptosis in these cells and the percentage of apoptotic cell populations increased with increasing concentration of lupeol. Apoptosis is considered one of the important mechanisms to prevent the development of diseases such as cancer and the organism eliminates the harmful cells via this process [17]. As such, anticancer drugs such as cisplatin that triggers apoptosis of cancer cells are considered important for anticancer chemotherapy. Additionally it is reported that apoptosis prevents the development of drug resistance in cancer cells [18]. Bax and Bcl-2 are important indicators for apoptosis [19] and in the present study we observed that lupeol could enhance the expression level of Bax and decrease the expression of Bcl-2 at the same time, further confirming the apoptotic cell death.

It has been previously reported that several anticancer agents initiate apoptosis by generating high ROS levels in the cancer cells by reducing MMP [20]. In this study we observed that lupeol also triggered accretion of ROS and reduction in the MMP of the lung cancer A427 cancer cells. It has been reported that mTOR/PI3K/AKT signalling pathway is one of the pathways that is dysregulated in cancer cells and as such is considered to be important for development of anticancer drugs [21]. In the present study we examined the effects of lupeol on mTOR/PI3K/AKT signalling pathway and observed that lupeol could inhibit this pathway in lung cancer A427 cancer cells.

Conclusion

The results of the present study revealed that lupeol inhibited the growth of A427 lung cancer cells by triggering apoptosis, accretion of ROS and reduction of MMP. Furthermore, lupeol could also inhibit the mTOR/PI3K/AKT signalling cascade and may therefore prove to be an important lead molecule for the treatment of lung cancer, deserving *in vivo* evaluation.

Conflict of interests

The authors declare no conflict of interests.

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