

ORIGINAL ARTICLE

Anticancer action of lactucopicrin in SKMEL-5 human skin cancer cells is mediated via apoptosis induction, G2/M cell cycle arrest and downregulation of m-TOR/PI3K/AKT signalling pathway

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Summary

Purpose: Skin cancer is one of the cancers responsible for significant morbidity and mortality across the globe. The treatment options for skin cancer are limited and associated with significant toxicity. Therefore, researches have been directed towards exploring molecules that could prove beneficial in the treatment of this disease. Lactucopicrin is an important sesquiterpene lactone with important pharmacological potential.

Methods: In the present study the anticancer effects of lactucopicrin against human skin SKMEL-5 cancer cell line were investigated. Antiproliferative effects were examined by CCK-8 assay. Apoptosis was detected by DAPI and annexin V/propidium iodide (PI) staining. Cell cycle analysis was carried out by flow cytometry. Protein expression was determined by western blotting.

Results: The results indicated that lactucopicrin exerts significant anticancer effects on the SKMEL-5 cells with an IC_{50} of 7.5 μ M. Its anticancer effects were due to induction of apoptosis. Lactucopicrin could upregulate the expression of Bax which was associated with concomitant downregulation of Bcl-2 expression. Additionally, lactucopicrin induced G2/M cell cycle arrest in SKMEL-5 cells in a dose-dependent manner and also inhibited the m-TOR/PI3K/AKT signalling pathway.

Conclusion: These results indicate that lactucopicrin shows potent anticancer action in the tested skin cancer cells and may prove a prospective lead molecule for the treatment of skin cancer.

Key words: apoptosis, cell cycle arrest, lactucopicrin, skin cancer

Introduction

Skin cancer is the one of the most prevalent types of cancer across the globe. In United States alone around 90,000 deaths due to skin cancer are recorded every year [1]. It is believed that the increased incidence of skin cancer is mainly due to the regular exposure to UV radiation and other environmental carcinogens. Therefore, limited exposure to these carcinogens may help to prevent the development of skin cancers [2]. Additionally, skin cancer chemoprevention is a beneficial model for the prevention of skin cancer genesis. Generally,

skin cancer induced by chemicals and UV radiation in murine models consists of three important stages which are distinct from each other. These stages include, initiation, promotion and progression [2-4]. Currently, the treatment options for skin cancer are too limited and associated with severe side effects that adversely affect the patient quality of life [5]. Therefore, there is a need to identify molecules that could help inhibit the growth of cancer cells and prevent their spread to other parts of the body. Over the last 30 years

natural products have been considered as a very significant source of cancer chemotherapy. In reality, natural products are expected to offer many of the lead chemical scaffolds that could act as templates for the synthesis of novel compounds with enhanced anticancer activities and limited toxicity to the normal cells [6].

Sesquiterpene lactones (SLs) are among the plant secondary metabolites that have been reported to exhibit tremendous anticancer potential. They have been shown to inhibit the growth of diverse cancer types, such as breast, skin, colon and many other types of cancer [7]. Lactucopicrin is also an important SL normally found in many species of the *Asteraceae* family [8] (Figure 1) and has been shown to be responsible for the anticancer activity of several plant extracts. However, the anticancer properties of lactucopicrin have not been generally explored, especially against skin cancer.

The main objective of this study was to investigate the anticancer potential of lactucopicrin against skin SKMEL-5 cell cancer line along with the demonstration of the mechanism of action by studying its effects on apoptosis, Bax and Bcl-2 expressions and the effects on the cell cycle and m-TOR/PI3/AKT signaling pathway.

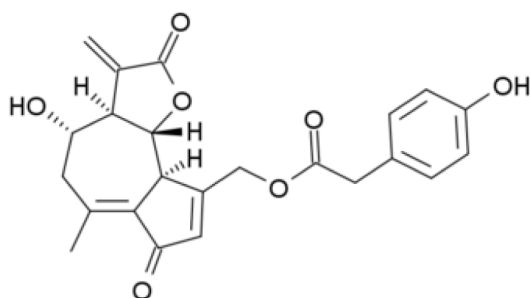


Figure 1. Chemical structure of lactucopicrin.

Methods

Chemicals, reagents and cell cultures

All chemicals (including lactucopicrin) and reagents were procured from Sigma-Aldrich, USA. The SKMEL-5 cell line was obtained from Type Culture Collection of Chinese Academy of Sciences, Shanghai, China. The cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 100U/mL penicillin and 100µg/mL streptomycin and maintained in a humidified atmosphere containing 5% CO₂.

Antiproliferative assay

The antiproliferative effect of lactucopicrin on the proliferation of human skin SKMEL-5 cells was investigated by CCK8 assay. In brief, 5×10^3 cells were seeded in a 96-well plate and then kept at 37°C for incubation in a humidified, 5% CO₂ atmosphere. After an overnight incubation the cells were treated with different concen-

trations of lactucopicrin (0, 7.5, 15, and 30 µM) for 24 hrs. Thereafter, 10 µL of CCK8 was added into each well and again incubated at 37°C for 1 hr. The optical density (OD) at OD₄₅₀ nm was determined by microplate spectrophotometer (BioRad, Segrate, Italy). The cell viability was assessed as percentage of the control.

Apoptosis assay

SKMEL-5 skin cancer cells were seeded at a density of 2×10^5 cells/well in 96-well plates, treated with varying concentrations of lactucopicrin (0, 7.5, 15, and 30 µM) and incubated for 24 hrs. DAPI staining was carried by incubating the cells with DAPI. The cells were then washed with phosphate buffered saline (PBS) and fixed in formaldehyde (10%). The DAPI-stained cells were then examined by fluorescence microscope. For annexin V/PI assay a similar procedure as for DAPI was followed except for the cells stained with annexin V/PI and investigated by flow cytometry.

Cell cycle analysis

For estimating the distribution of SKMEL-5 skin cancer cells in different phases of the cell cycle, the lactucopicrin-treated cells (0, 7.5, 15, and 30 µM) were harvested and washed twice with PBS. Thereafter, the cells were fixed with ethanol (70%) for about an hour and then washed again by PBS. The cells were finally resuspended in solution of PI (50µl/ml) and RNase1 (250µg/ml). This was followed by incubation for a period of 30 min at room temperature and fluorescence-activated cell sorting was done using cater-plus cytometer using 10,000 cells/group.

Western blotting

The lactucopicrin treated SKMEL-5 cells were lysed in RIPA buffer and protein extracts were collected. Equal protein extracts from each group were run on SDS PAGE and then transferred to a polyvinylidene fluoride membrane. This was followed by blocking with 5% non-fat milk and incubating at 25°C for 1 hr. Thereafter, the membranes were incubated with primary antibodies (antibodies against Bax, Bcl-2, PI3K, m-TOR) at 4°C overnight. This was followed by washing in washing buffer and incubation for 1 hr with the suitable secondary antibody (horseradish peroxidase-conjugated goat anti-mouse). The protein bands of interest were visualised by ECL Advanced Western Blot Detection Kit (GE Healthcare, Little Chalfont, UK).

Statistics

Data were expressed as the mean \pm SD of three experiments. Statistical analysis was carried out using Student's t test by GraphPad prism 7 software. The values were considered significant at $p < 0.01$.

Results

Anticancer effect of lactucopicrin SKMEL-5 cells

The anticancer effect of lactucopicrin on human SKMEL-5 skin cancer cells was examined

by CCK8 assay at 0, 7.5, 15 and 30 μM concentrations of lactucopicrin. The results showed that lactucopicrin inhibited the proliferation of these cells. The antiproliferative effects of lactucopicrin were concentration-dependent and increased with increase of its concentration (Figure 2).

Effect of lactucopicrin on apoptosis of SKMEL-5 cells

To examine whether the antiproliferative effects of lactucopicrin on human SKMEL-5 skin cancer cells were due to induction of apoptosis, we carried out DAPI staining. The results revealed that lactucopicrin induced apoptosis in SKMEL-5 human skin cancer cells as evidenced from the development of apoptotic crops and cell blebbing (Figure 3). Moreover, the apoptosis inducing potential of lactucopicrin was found to be concentration-dependent and the percentage of apoptotic cells significantly increased ($p < 0.01$) with increased concentration of lactucopicrin. Furthermore, annexin V/PI staining revealed that the apoptotic cell population increased from 1.75% in control to about 38% at 30 μM concentration of lactucopicrin (Figure 4).

Lactucopicrin alters Bcl-2/Bax ratio

To further confirm the apoptotic cell death of SKMEL-5 skin cancer cells, we determined the expression of Bax and Bcl-2 in lactucopicrin-treated cells. The results indicated that lactucopicrin treatment upregulated the expression of Bax in a dose-dependent manner which was associated with downregulation of Bcl-2 expression (Figure 5). This caused decrease in the Bcl-2/Bax ratio, ultimately favoring apoptosis.

Lactucopicrin triggers cell cycle arrest in of SKMEL-5 cells

Cell cycle is considered as one of the mechanisms by which antiproliferative agents exert

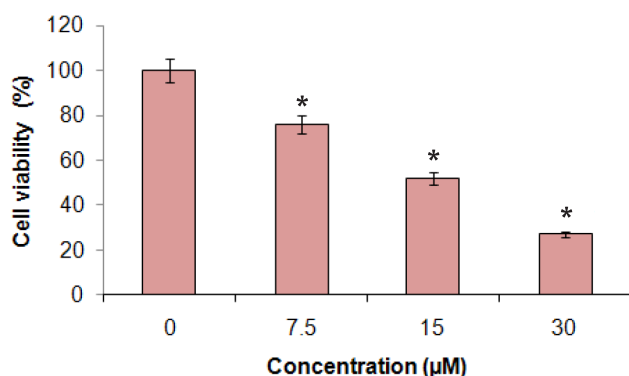


Figure 2. Effect of lactucopicrin on the cell viability of human SKMEL-5 human skin cancer cells at indicated doses. The results are the mean \pm SD of three experiments. The values were considered significant at $*p < 0.01$.

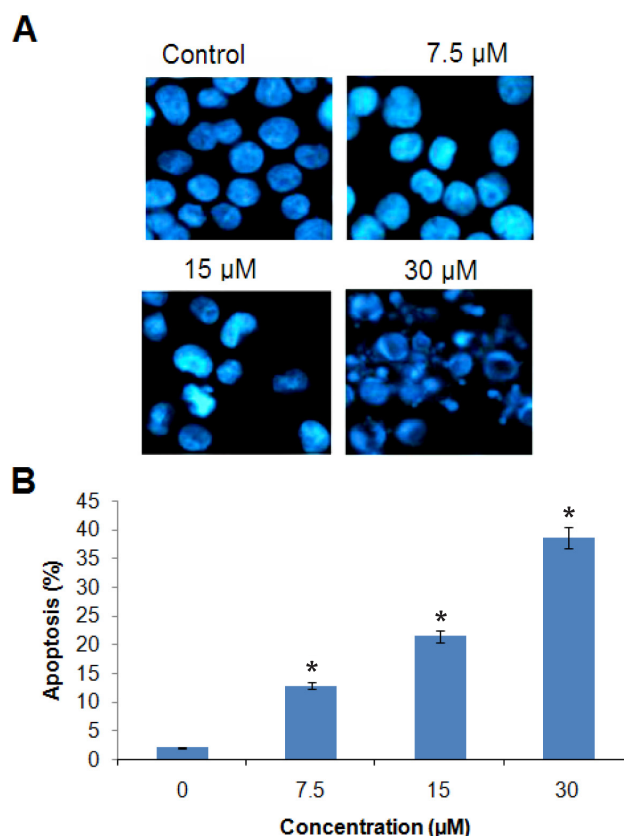


Figure 3. Lactucopicrin-induced apoptosis in human SKMEL-5 human skin cancer cells. (A) DAPI staining at indicated concentrations. (B) estimation of the apoptotic cell populations. The results are the mean \pm SD of three experiments. $*p < 0.01$.

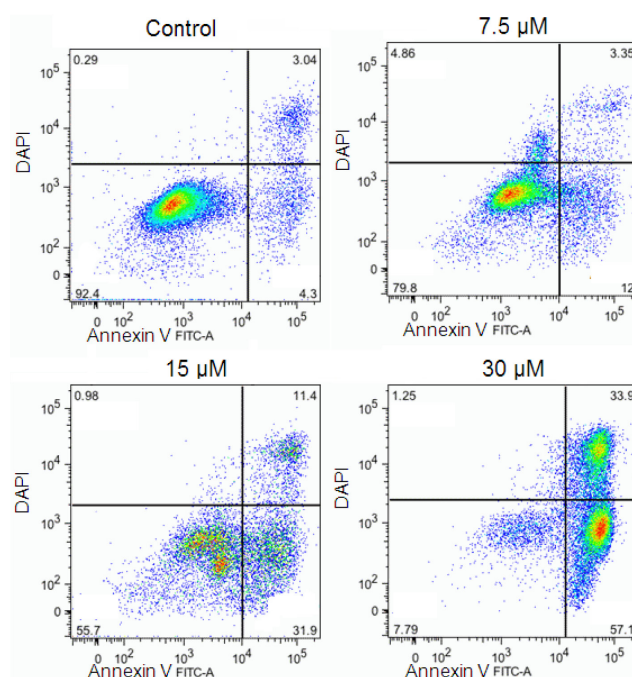


Figure 4. Estimation of apoptotic cell population by annexin V/PI staining at indicated doses of lactucopicrin. Experiments were carried out in triplicate. The results indicate that with increasing doses of the compound, the population of both early and late apoptotic cells increased significantly.

their effects. Therefore, we investigated whether lactucopicrin induces cell cycle arrest in human SKMEL-5 skin cancer cells. Interestingly, it was observed that lactucopicrin caused significant increase in the G2 cell populations of these cells, ultimately leading to G2/M cell cycle arrest. These effects of lactucopicrin were also concentration-dependent (Figure 6).

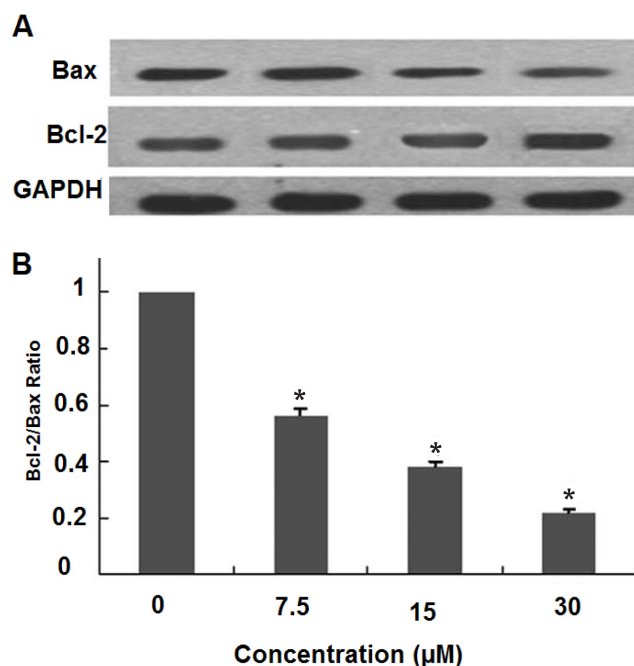


Figure 5. Effect of lactucopicrin on the expression of Bax and Bcl-2 expression in SKMEL-5 human skin cancer cells at indicated doses by (A) western blotting and (B) quantification of Bcl-2/Bax ratio. The results are the mean \pm SD of three experiments. * $p < 0.01$.

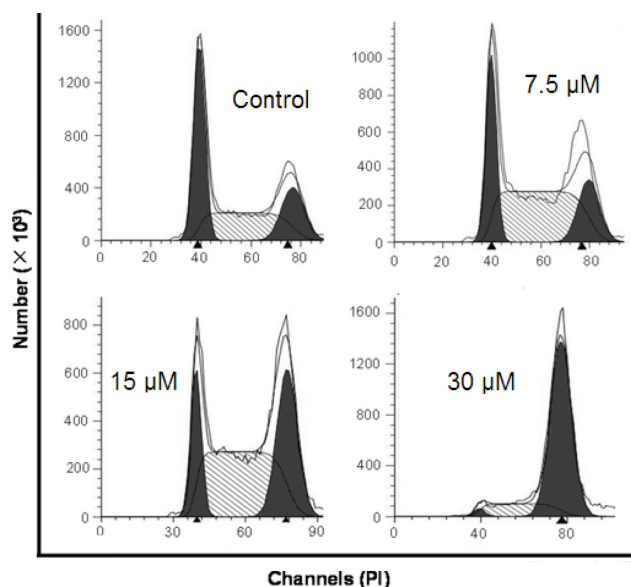


Figure 6. Effect of lactucopicrin on cell cycle phase distribution of SKMEL-5 human skin cancer cells at indicated doses. The results are the mean \pm SD of three experiments. With increasing doses of the compound, the fraction of cells in G2/M cell cycle increased considerably.

Lactucopicrin suppresses PI3K/AKT/mTOR pathway in SKMEL-5 cells

The PI3K/AKT/mTOR signaling pathway plays very important role in the progression of different cancers and also acquisition of chemoresistance properties. Hence, we investigated the status of activated PI3K, Akt and mTOR signaling in untreated or lactucopicrin-treated SKMEL-5 cells (Figure 6). It was observed that lactucopicrin treatment resulted in a dose-dependent decrease in the levels of p-PI3K, p-Akt and p-mTOR, while the levels of total PI3K, Akt and m-TOR remained unaltered (Figure 7).

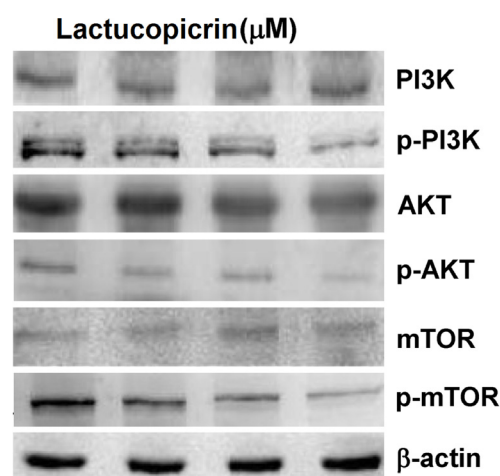


Figure 7. Effect of lactucopicrin on the PI3K/AKT/mTOR protein expression in SKMEL-5 human skin cancer cells at indicated doses by western blotting. Lactucopicrin treatment resulted in a dose-dependent decrease in the levels of p-PI3K, p-AKT and p-mTOR.

Discussion

Skin cancer is one of the prevalent types of cancer and accounting for about 90,000 deaths in United States alone [1]. The existing treatments for skin cancer are limited and the treatment regimes have a lot of side effects [2]. Therefore, there is a pressing need to develop novel and efficient treatment strategies for the treatment of skin cancer. Against this background, we investigated the anticancer activity of SL lactucopicrin against skin cancer and investigated the probable underlying mechanisms for its anticancer activity. Our results indicated that lactucopicrin exerted significant anticancer activity against human SKMEL-5 skin cancer cells with an IC_{50} of 7.5 μ M. Our results are also supported by a previous study wherein SLs have been reported to exhibit anticancer activity against a range of cancer cell types [9]. This anticancer activity of SLs has been attributed to the reaction of an α , β -unsaturated lactone moiety with thiols, such as those in the cysteine residues

of proteins. Over the years, the anticancer activities of SLs have drawn a lot of attention. Extensive research has been carried out to identify the underlying mechanisms of their anticancer activities. However, it has been reported that the anticancer activities of SLs are controlled by the α,β -unsaturated carbonyl functions [9]. Since it has been previously reported that several SLs induce apoptosis in the cancer cells [10], we investigated whether lactucopicrin could induce apoptosis in SKMEL-5 skin cancer cells. Interestingly, our results showed that lactucopicrin induced apoptosis in these cells and the apoptotic cell populations increased in a dose-dependent manner. Moreover, the apoptosis of SKMEL-5 cells was associated with upregulation of Bax and downregulation of the BCL-2 expression, confirming that lactucopicrin induces apoptosis through the mitochondrial pathway.

Analysis of the cell cycle revealed that the G2 cell populations increased with increase in the dose of lactucopicrin, leading to G2/M cell cycle arrest and these results are also in concordance with previous studies wherein SLs have been re-

ported to induce G2/M cell cycle arrest in cancer cells [11,12].

The m-TOR/PI3K/AKT signalling pathway has been reported to be activated in several cancer types and is involved in the progression and tumorigenesis of many cancer types [13]. Therefore, this pathway is considered as an important target for the treatment with anticancer agents. In our study we observed that lactucopicrin could downregulate the expression of several proteins of this pathway.

Conclusion

Based on our results we conclude that lactucopicrin exerts anticancer effects on skin cancer cells, and the anticancer effects are due to induction of apoptosis, cell cycle arrest and inhibition of the m-TOR/PI3K/AKT signalling pathway. Therefore, we propose that lactucopicrin could prove an important lead molecule for the treatment of skin cancer.

Conflict of interests

The authors declare no conflict of interests.

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