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ORIGINAL ARTICLE

Linalool monoterpene exerts potent antitumor effects in OECM 1 human oral cancer cells by inducing sub-G1 cell cycle arrest, loss of mitochondrial membrane potential and inhibition of PI3K/AKT biochemical pathway

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Summary

Purpose: *Oral cancer is one of the prevalent types of cancer and has been reported to responsible for significant mortality and morbidity. Since treatment options for oral cancer are limited, there is need to explore novel molecules for treatment of oral cancer. In the current study we evaluated the anticancer activity of a plant derived monoterpene, Linalool against oral cancer cell line, OECM-1.*

Methods: *Cell viability was determined by MTT assay. Apoptosis was detected by DAPI and annexin V/PI staining. Cell cycle analysis was carried out by flow cytometry. Cell migration was assessed by wound healing assay and the expression of the proteins was determined by western blotting.*

Results: *The results showed that Linalool inhibited the viability of oral cancer OECM-1 cells in a concentration-dependent manner. The IC_{50} of Linalool against OECM-1 oral cancer cells was 10 μM as compared to its IC_{50} of 65 μM against non-cancer FR-2 cells. The anticancer effects were due to the induction of the apoptosis and sub-G1 cell cycle arrest. The results of annexin V/PI further revealed that the apoptotic cell populations increased from 2.6% in the control to 61.3% at 20 μM concentrations. It was observed that Linalool decreased the expression of p-PI3K and p-AKT in a concentration-dependent manner. However, the expression of PI3K and AKT remained almost unaltered.*

Conclusions: *Taken together it was shown that Linalool monoterpene exerted significant anticancer effects in OECM-1 human oral cancer cells via inducing cell cycle arrest, loss of mitochondrial membrane potential (MMP) and suppressing PI3K/AKT signalling pathway.*

Key words: *apoptosis, cell cycle, cell migration, cell viability, Linalool, oral cancer*

Introduction

Chemotherapy is one of the main treatment strategies for advanced cancers. Several plant derived products have been reported to offer considerable protection against a diversity of cancers [1,2]. In the recent years there has been increased attention towards understanding the basis of the anticancer properties of these plant products so as to provide an effective and viable treatment strategy for the treatment of cancer patients. OECM-1 is the prevalent type of head and neck cancer [3]. It has been found to exhibit poor prognosis and is often associated with recurrent lymph node metastasis. The metastasis and invasion to distant parts of the body is the principal cause of death in oral squamous cell carcinoma and other types of cancers as well [4]. Plant-derived natural products have been considered as prospective anticancer agents and are therefore being frequently screened for possible anticancer activity. Among plant-derived natural products, monoterpenes have been reported to

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exhibit tremendous pharmacological potential [5]. They have been reported to exhibit, antimicrobial, anticancer, anti-inflammatory and several other activities [5,6]. These monoterpenes are basically components of our diet, and hence considered safe [6,7]. They are prevalent in the essential oils of several plants and mainly function as chemo-attractants and repellents at physiological level and are also responsible for the distinct fragrance of the plants. Chemically, monoterpenes are 10-carbon isoperenoids that are derived from the mevalonate pathway in plants. Among the monoterpenes, carvone is one important monoterpene [8]. In the present study we evaluated the anticancer effects of Linalool against oral cancer cell line OECM-1 and normal oral cell line CMMT.

Methods

Chemicals, reagents and culture conditions

The chemicals and reagents used in the present study were procured from Sigma-Aldrich Co. Primary and secondary antibodies were purchased from Santa Cruz Biotechnology Inc. Human oral cancer OECM-1 and non-cancer FR-2 cell lines were purchased 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), 100 U/ml penicillin and 100 µg/ml streptomycin and maintained in a humidified atmosphere containing 5% CO_2.

MTT assay for cell viability

For assessment of the cell viability, the OECM-1 and FR-2 cells were cultured in a 96-well plates at a density of 5×10^3 cells/well. The cells were incubated overnight and then the RPMI-1640 medium was removed and replaced with a fresh medium with Linalool separately at different concentrations (0-100 µM) for 24 hrs. Thereafter, 0.5 mg/ml MTT solution was added for the last 4 hrs of incubation and finally the absorbance was measured at 570 nm using spectrophotometer.

Analysis of apoptosis

Oral cancer OECM-1 cells were seeded at the density of 2×10^5 cells/well in 6-well plates. The cells were then administered with 0, 5, 10 and 20 µM of Linalool and incubated for 24 hrs. DAPI staining was carried by incubating the cells in 6-well plates with DAPI. The cells were then washed with phosphate buffered saline (PBS), fixed in formalin (10%) and then again washed with PBS. The DAPI stained cells were then examined by fluorescence microscope. For estimation of apoptotic cell populations, similar procedure was carried out except for the cells were stained with annexin V/PI staining and analysed by flow cytometer.

Analysis of cell cycle

To estimate the number of cells in different phases of the cell cycle, the Linalool treated OECM-1 oral cancer cells were harvested and washed with PBS. Thereafter the cells were fixed with ethanol (70%) for about an hr and then washed again by PBS. The cells were finally resuspended in solution of PI (50 µl/ml) and RNase1 (250 µg/ml). This was followed by incubation for 30 min at room temperature and final assessment under a fluorescence-activated cell sorting cater-plus cytometer using 10,000 cells/group.

Determination of mitochondrial membrane potential

OECM-1 cells were cultured in 6 well plates with 2×10^5 cells per well, incubated for 24 hrs and administered 0, 5, 10 and 20 µM Linalool for 24 hrs at 37°C in 5% CO_2 and 95% air. Next, the cells from each of the samples were harvested and PBS was used to wash the cells twice. This was followed by suspension of the cells in 500 µl of $DiOC_6$ (1 µmol/l) for determination MMP levels at 37°C in the dark for 35 min. Each sample was then investigated by a flow cytometer.

Western blotting analysis

Total protein from untreated and Linalool-treated OECM-1 oral cancer cells was isolated in RIPA lysis buffer. Equal volumes of the proteins from each sample were run on SDS PAGE. This was followed by transference to a polyvinylidene fluoride membrane. Afterwards, blocking was done with 5% non-fat milk followed by incubation at room temperature for 1 hr. The membranes were then subjected to treatment with either specific primary antibody at 4°C for 20 hrs. Thereafter, washing in washing buffer was carried out and then the membranes were incubated with secondary antibody for 1 hr. The protein bands were then visualised by an ECL Advanced Western Blot Detection Kit.

Results

Linalool decreased the viability of OECM-1 oral cancer cells

The effects of Linalool (Figure 1) on cell viability were evaluated by MTT assay. The OECM-1 cells were treated with Linalool at varied concentrations

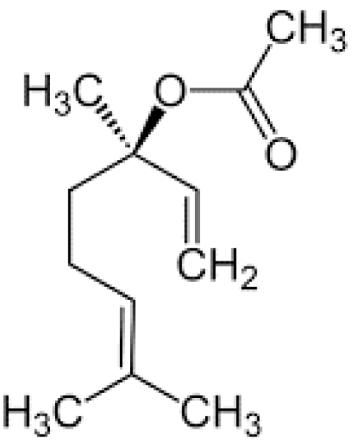

Figure 1. Chemical structure of Linalool.

(0, 5, 10 and 20 µM) and the results revealed that Linalool exhibited considerable antiproliferative effects on the OECM-1 cells and the antiproliferative effects were found to be concentration-dependent (Figure 2). It was observed that the IC_{50} of Linalool against OECM-1 oral cancer cells was 10 µM as compared to IC_{50} of 65 µM against non-cancer FR-2 cells. These results clearly showed that Linalool selectively exerted anticancer effects on oral cancer cells.

Linalool induced apoptosis in OECM-1 oral cancer cells

It was previously reported that Linalool could induce apoptosis in cancer cells [7], therefore in

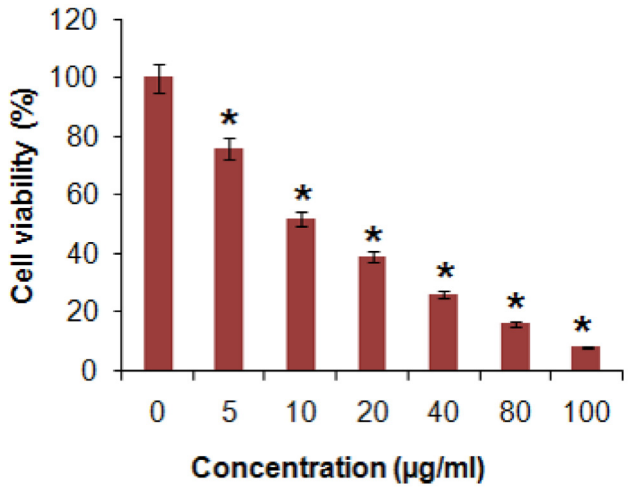

Figure 2. Effect of indicated concentrations of Linalool on the cell viability of OECM-1 and FR-2 cancer cells. The Figure depicts that Linalool reduces the OECM-1 cells in a concentration-dependent manner. The experiments were carried out in three biological replicates and expressed as mean ± SD (*p <0.01).

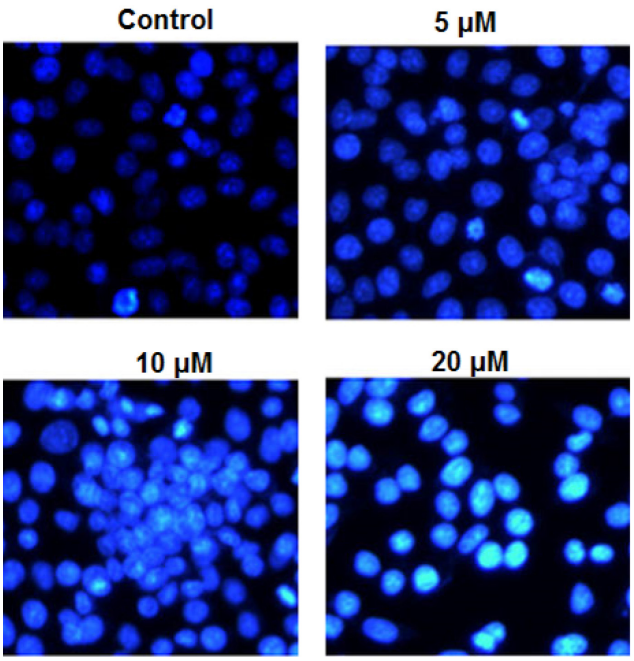

Figure 3. Linalool induces apoptosis in OECM-1 cancer cells as indicated by DAPI staining. The experiments were carried out in three biological replicates. The Figure depicts that Linalool induces apoptosis in a concentration-dependent manner.

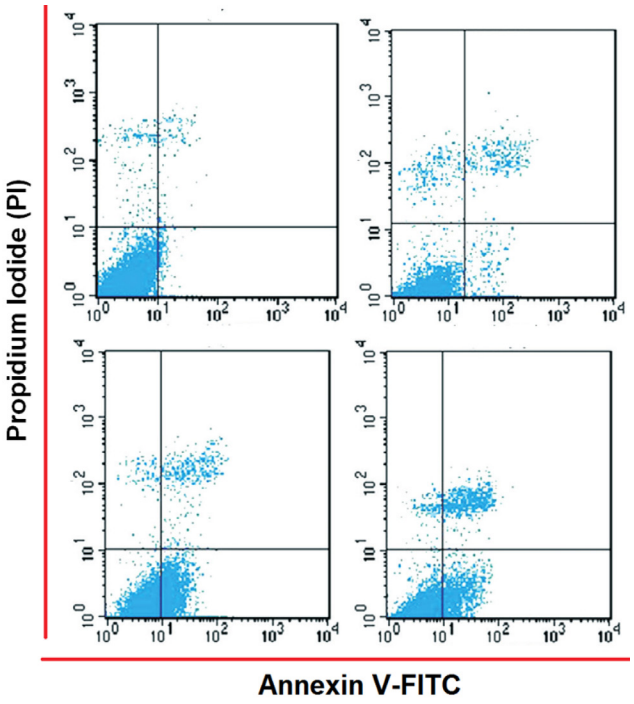

Figure 4. Estimation of apoptotic cell populations of OECM-1 oral cancer cells at indicated concentrations of Linalool by annexin V/PI staining. The experiments were carried out in three biological replicates. The Figure depicts that the percentage of the apoptotic OECM-1 cells increases in a concentration-dependent manner.

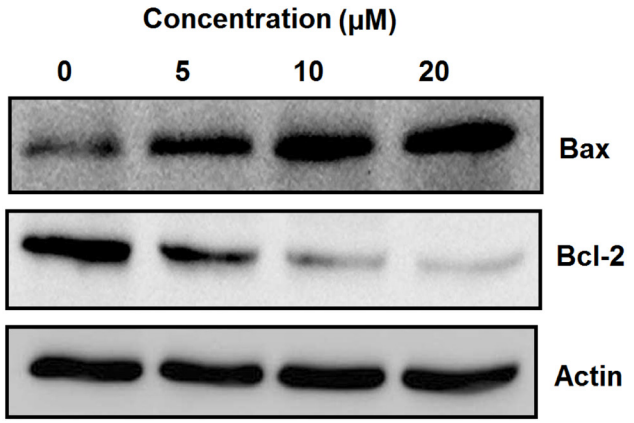

Figure 5. Effect of indicated concentrations of Linalool on the expression of Bax and Bcl2 of OECM-1 oral cancer cells as indicated by western blotting. The Figure depicts that the expression of Bax increases and that of Bcl-2 decreases in a concentration-dependent manner. The experiments were performed in triplicate.

this study it was investigated by DAPI staining if Linalool could also trigger apoptosis in OECM-1 oral cancer cells. The OECM-1 oral cancer cells were first treated with Linalool at different concentrations, and then subjected to DAPI staining and finally observed under fluorescence microscope. It was observed that Linalool induced apoptosis in OECM-1 cells as evidenced from the increased number of cells with white colour nuclei (Figure 3). The results of annexin V/PI staining further revealed that the apoptotic cell populations increased from 2.6% in the control to 61.3% at 20 μM concentrations (Figure 4). To further confirm the apoptosis at molecular level, we determined the expression of Bax and Bcl-2 proteins. The results showed that Linalool treatment increased the expression of Bax and decreased the expression of Bcl-2 in a concentration-dependent manner (Figure 5). We further investigated MMP and observed that Linalool decreased MMP in a concentration-dependent manner (Figure 6).

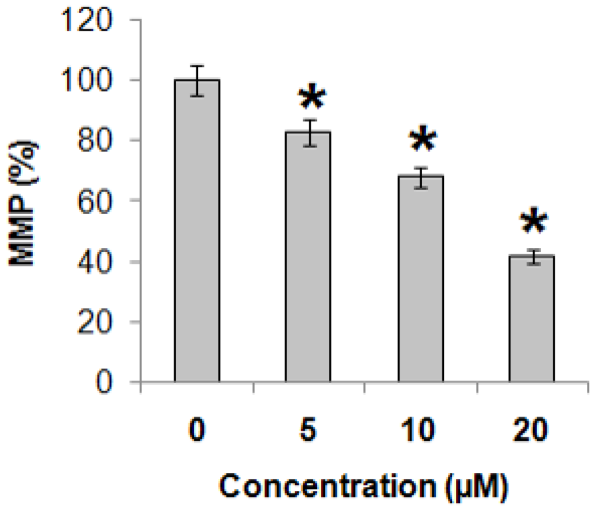

Figure 6. Effect of Linalool on the mitochondrial membrane potential (MMP) of OECM-1 oral cancer cells. The Figure depicts that Linalool causes decrease of MMP levels in OECM-1 cells in a concentration-dependent manner. The experiments were performed in triplicate and the results are expressed as mean±SD (*p<0.01).

Linalool caused sub-G1 cell cycle arrest in OECM-1 oral cancer cells

The distribution of OECM-1 cells in the different cell cycle phases after treatment with Linalool at varied concentrations was determined by flow cytometry. The results showed that Linalool led to accumulation of OECM-1 cells in the sub-G1 phase of the cell cycle and ultimately prompting sub-G1 cell cycle arrest (Figure 7).

Linalool inhibited PI3K/AKT signalling pathway

PI3K/AKT signalling pathway has been found to be involved the progression and tumorigenesis of different types of cancers [8]. In the present study, the effect of Linalool was also investigated on PI3K/AKT signalling pathway. It was observed that Linalool decreased the expression of p-PI3K and p-AKT in a concentration-dependent manner. However, the expression of PI3K and AKT remained almost unaltered (Figure 8).

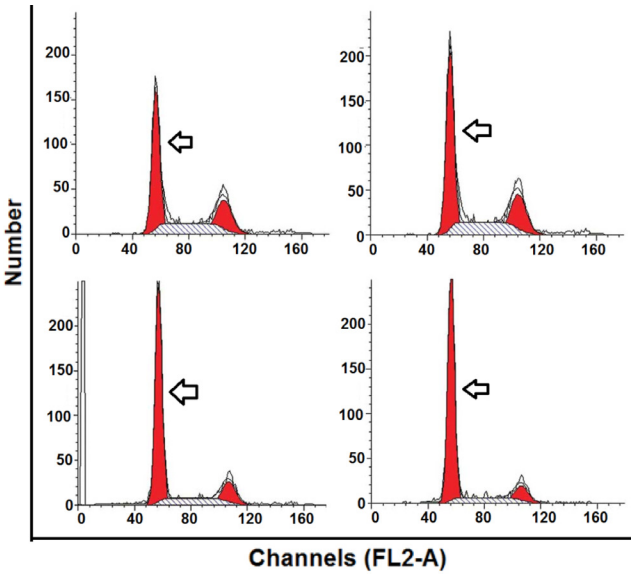

Figure 7. Linalool induces sub-G1 arrest in Linalool-treated OECM-1 cells. The experiments were carried out in three biological replicates. The Figure depicts that Linalool causes arrest of OECM-1 cells at the sub-G1 phase of the cell cycle in a concentration-dependent manner.

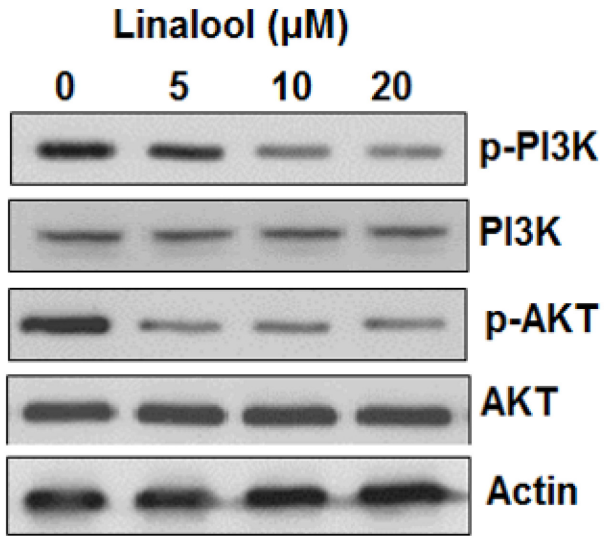

Figure 8. Effect of indicated concentrations of Linalool on the PI3K/AKT signalling pathway as indicated by western blotting. The Figure depicts that Linalool blocks the PI3K/AKT pathway in OECM-1 cells concentration-dependently. The experiments were performed in triplicate.

Discussion

OECM-1 is the prevalent type of Head & Neck cancer [9]. It has been found to exhibit poor prognosis and is often associated with recurrent lymph node metastasis [10,11]. Therefore, there is an urgent need to look for effective and novel therapeutic targets to curb the growing incidence of oral cancer. Over the years, plant-derived secondary metabolites have attained considerable attention as bioactive molecules. They have been shown to have anticancer activities against a range of cancer types [12]. In this connection, the present study was carried out to investigate the anticancer effects of Linalool against oral cancer OECM-1 cancer cells. The results showed that Linalool exhibits considerable anticancer activity with an IC_{50} of 10 µM against OECM-1 oral cancer cells as compared to IC_{50} of 65 µM against normal oral cells. To further unveil the reasons behind the anticancer effects of Linalool we carried out DAPI staining and observed that Linalool exerted anticancer effects via induction of apoptosis. Moreover, the apoptotic effects of Linalool were concentration-dependent and the apoptotic cell populations increased with increase in the concentration of Linalool as evidenced from the annexin V/PI staining. Apoptosis involves a form of cell death by which programmed series of actions lead to destruction of cells without release of any harmful chemicals. It is an important mechanism by which several of the chemotherapeutic drugs exert their anti-proliferative actions [13]. The results of the present study are well supported by previous studies where Linalool has been reported to trigger apoptosis in glioma cells [7]. To understand if the Linalool-induced apoptosis follows the mitochondrial pathway, we estimated the expression of Bax and Bcl-2 proteins. The results of the western blotting revealed that the expression of Bax was increased and that of Bcl-2 was suppressed in response to Linalool treatment. Another important mechanism that has been reported to contribute to the anticancer effects of many well-known drugs is cell cycle arrest [14]. Some anticancer drugs halt the progression of the cells from the one phase of the cell cycle to another by targeting specific proteins leading to accumulation cancer cells at a particular phase. Arrest of the cell cycle prevents the cancer cell to develop into tumours and to spread to other parts of the body [15]. Consistent with this, we observed that Linalool caused sub-G1 cell cycle arrest of OECM-1 cells in a concentration-dependent manner. Anticancer agents that inhibit the migration of cancer cells have been reported to be of importance as they may efficiently inhibit the metastasis of the cancer cells [16]. In the present study we also observed that Linalool could efficiently inhibit the migration of OECM-1 cancer cells. It was earlier reported that many anticancer molecules target PI3K/AKT signalling pathway in cancer cells [8]. Therefore we investigated the effect of Linalool on the expression of p-AKT, AKT, p-p-PI3K and PI3K and observed that Linalool decreased the expression of p-PI3K and p-AKT, indicating that the anticancer effects of Linalool may in part due to inhibition of PI3K/AKT signalling pathway.

Conclusion

Taken together it is concluded that Linalool exhibits significant anticancer activity against oral cancer cells. The anticancer activity can be attributed to the ability to trigger apoptosis, cell cycle arrest and inhibition of PI3K/AKT signalling pathway. Our results suggest that Linalool may prove a promising lead molecule for the development of new therapy for oral cancer and deserve further research efforts.

Conflict of interests

The authors declare no conflict of interests.

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