

## Original Article

# Escopoletin treatment induces apoptosis and arrests cell cycle at G0/G1 phase in the oral squamous cancer cell lines

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**Abstract:** This study was aimed to investigate the antiproliferative effect of escopoletin on CAL 27 and CAL 33 oral squamous cancer cell lines. MTT assay and flow cytometry were used for the analysis of escopoletin effect on cell viability and apoptosis, respectively. Western blot analysis was used for the examination of cyclin and cyclin-dependent kinase expression after treatment of CAL 33 cells with escopoletin. The results showed a marked decrease in the viability of CAL 27 and CAL 33 cell lines after 48 h of escopoletin treatment. Treatment of CAL 33 cells with escopoletin led to the induction of apoptosis and arrest of cell cycle at G0/G1 phase. In the cells treated with escopoletin cyclin D1 and E expression was reduced and CDK1 expression was inactivated. The above findings suggest that escopoletin exhibits inhibitory effect on the oral squamous cancer through induction of apoptosis and arrest of cell cycle. Therefore escopoletin can be a promising candidate for the prevention of oral squamous cancer.

**Keywords:** Antiproliferative, squamous, escopoletin, apoptosis, cyclins

## Introduction

Oral squamous cell carcinoma (OSCC) has an incidence of around 263,000 yearly throughout the globe and constitutes the sixth most abundant cancer [1, 2]. In the United States and some countries of the Europe the number of patients with OSCC has been found to increase every year [3-5]. The treatments used currently for OSCC include radiotherapy, chemotherapy and surgery of the patients. Ineffectiveness of chemotherapy [6-8] in patients with advanced OSCC demands for the discovery new molecules with more efficient results and better cure rates [9-11]. In the patients diagnosed with OSCC, the rate of 5-year survival has been reported to be less than 60% based on the tumor stage [5].

Compounds isolated from the natural sources including crude drugs exhibit promising antioxidant activities. Flavonoids comprise an important class of compounds present in plants and possess potent effect on the health of humans [12]. Some of the promising activities of flavonoids and their analogs include anticancer,

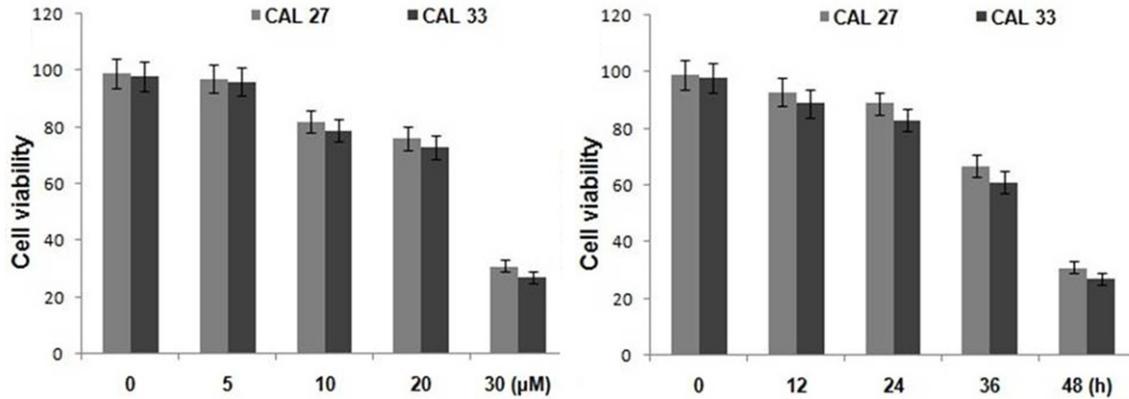
anti-inflammatory, antibacterial, antiviral, and anti-allergic [12, 13]. Flavonoids exhibit their activity through the mechanism involving antioxidant property. They are reported to play their vital roles by acting as the radical scavengers for oxygen radicals and other free radicals generated during the course of various diseases [14]. It has been reported that flavonoid consumption in humans is associated with the decrease in the risk of diseases like cancer and cardiovascular diseases. The present study was performed to investigate the inhibitory effect of escopoletin on the growth of OSCC cells along with the mechanism of action involved.

## Materials and methods

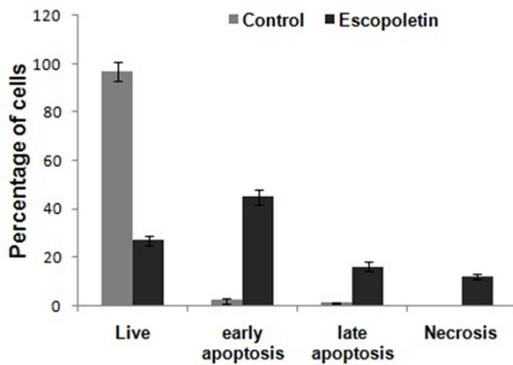
### Cell lines and culture

Human oral squamous cancer cell lines, CAL 27 and CAL 33 were obtained from American Type Culture Collection (Rockville, MD, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma Aldrich Co., St. Louis, MO, USA) supplemented with fetal bovine

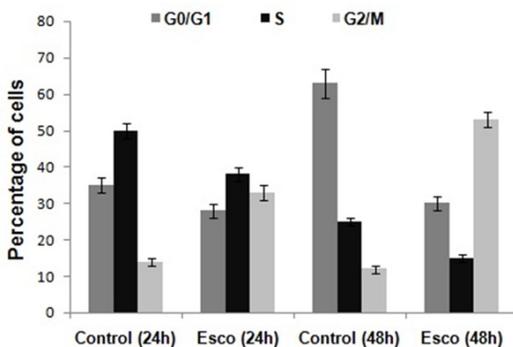
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**Figure 1.** Escopoletin inhibits survival of CAL 27 and CAL 33 cells in a concentration and time dependent manner. CAL 27 and CAL 33 cells were treated either with DMSO or escopoletin (5, 10, 20 and 30  $\mu\text{M}$ ) and incubated for 12, 24, 36 and 48 h and MTT assay was used to evaluate cell survival.



**Figure 2.** Effect of escopoletin on induction of apoptosis using annexin V (A)/propidium iodide (PI) staining. Incubation of CAL 33 cells with 30  $\mu\text{M}$  of escopoletin for 48 h was followed by FITC conjugated Annexin V and propidium iodide staining.



**Figure 3.** Escopoletin effects the distribution of cells in cell cycle cells in CAL 33 cells.

serum (FBS) and antibiotic solution in a humidified atmosphere containing 5%  $\text{CO}_2$  at 37°C.

### Drug

Escopoletin was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in DMSO (St. Louis, MO, USA) to prepare stock solution.

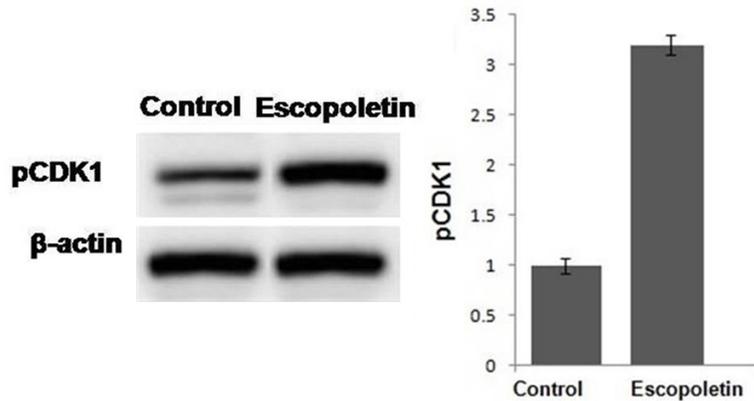
### MTT assay

For investigation of the effect of escopoletin on the vitality of CAL 27 and CAL 33 cell lines, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used. For this purpose the cells at a density of  $2 \times 10^5$  cells/ $\text{cm}^2$  were distributed onto 96-well plates and incubated with escopoletin. After 24 and 48 h, MTT was added to the cell cultures following removal of the medium. After incubation with MTT for 5 h DMSO was added to each well for dissolving the formazan crystals formed. The microplate reader (Bio-Rad, Hercules, CA, USA) was then used to measure the absorbance at 560 nm. The cells treated with only DMSO were used as the control for the estimation of the  $\text{IC}_{50}$  value of escopoletin.

### Flow cytometric analysis

Annexin V-FITC/propidium iodide Apoptosis Detection kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used to measure the rate of cell apoptosis induced by escopoletin treatment in CAL 27 and CAL cells. Fluorescence was measured using activated cell sorting Calibur instrument (Beckman Coulter Inc., Miami, FL, USA) and data were analyzed by CellQuest software (Becton-

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**Figure 4.** Escopoletin treatment enhances the phosphorylation of CDK1 (pCDK1) in CAL 33 cell cultures. The cells were cultured for 24 h with 30  $\mu$ M escopoletin or in absence of escopoletin as the control.

Dickinson, San Jose, CA, USA) according to the manual protocol.

### Western blot analysis

The cells treated with escopoletin after washing with PBS were lysed in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM ethylene glycol tetraacetic acid, 1 mM sodium orthovanadate, 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin and 1 mM NaF). After 20 min the cell lysates were centrifuged at 12,000 $\times$  g for 45 min at 4°C to isolate the supernatant. Bradford protein assay was used to determine the concentration of proteins in the supernatant. The proteins after separation on 12% SDS-PAGE were transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA) blocked with 5% non-fat milk. The membranes were incubated with primary antibodies for cyclin E, cyclin D1,  $\beta$ -actin, cyclin-dependent kinase 1 (CDK1), phospho-CDK1 (Cell Signaling Technology, Danvers, CA, USA) for overnight. The membrane after washing with PBS was probed with horseradish peroxidase-conjugated secondary antibody for 45 min. The electrochemiluminescence reagent (GE Healthcare, Amersham, UK) was then used to analyze the antibody bound complexes. Expression of  $\beta$ -actin was used as an internal control. The gel imaging analysis system (Kodak ID, Kodak, Rochester, NY, USA) was used for the analysis of band images and densitometric analysis for quantification of intensities of the immunoreactive bands.

### Analysis of cell cycle distribution

After escopoletin treatment, the cells were harvested, washed with PBS and suspended in cold 70% ethyl alcohol at 4°C overnight. The cells were then again washed with PBS incubated in PI (5  $\mu$ g/ml) and RNase (12.5  $\mu$ g/ml) for 48 h. For synchronization, the cells were maintained in serum-free medium for 36 and 48 h, respectively, followed by treatment with escopoletin in complete medium. FACSCantol flow cytometer

(BD Biosciences, Buccinasco, Italy) was used to analyze the content of DNA where as the ModFit LT (Verity House Software, Topsham, ME, USA) for the analysis of cell cycle distribution.

### Statistical analysis

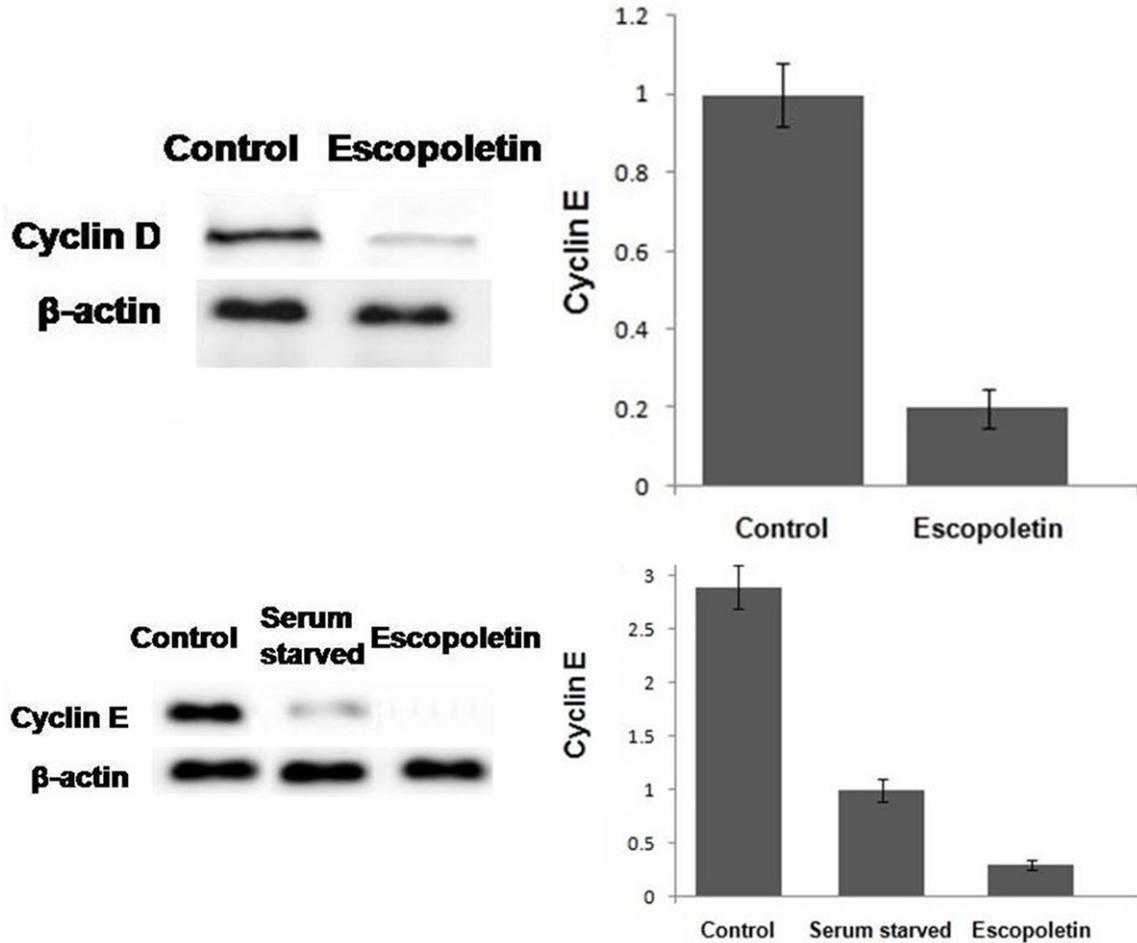
The SPSS 13.0 statistical software package (SPSS, Inc., Chicago, IL, USA) was used for the statistical analyses of the data. The data expressed are the mean  $\pm$  standard error. One-way analysis of variance with Fisher's Least Significant Difference test was employed for the analysis of the results.  $P < 0.05$  was taken as the statistically significant difference.

## Results

### Inhibition of oral squamous cancer cell proliferation by escopoletin

Treatment of CAL 27 and CAL 33 carcinoma cell lines with various concentrations of escopoletin induced inhibition of cell proliferation in a concentration and time dependent manner. Among various concentrations of escopoletin tested the cell viability was less than 50% at the concentration of 30  $\mu$ M (Figure 1A). Although the decrease in CAL 27 and CAL 33 cell viability started at 10  $\mu$ M concentration of escopoletin but was significant at 30  $\mu$ M compared to untreated cells.

Examination of the effect of time on the viability of CAL 27 and CAL 33 cells revealed that treatment with 30  $\mu$ M concentration of escopoletin



**Figure 5.** Escopoletin treatment decreases the expression of cyclin D1 and E expression in CAL 33 synchronized cells. The cells were cultured for 24 h in the presence of 30  $\mu\text{M}$  escopoletin or in its absence as control.

led to a significant decrease in cell viability at 48 h compared to 24 h. The viability of CAL 27 cells at 24 and 48 h was reduced by 33 and 69% respectively. Similarly, the viability of CAL 33 cells was decreased from 39 to 73%, respectively at 24 and 48 h (**Figure 1B**).

*Induction of apoptosis in oral squamous cancer cells by escopoletin*

The results from flow cytometric analysis revealed that exposure of CAL 33 cells to escopoletin at a concentration of 30  $\mu\text{M}$  led to a marked increase in the proportion of apoptotic cells compared to untreated (**Figure 2**). The percentage of live, early apoptotic, late apoptotic and the necrotic cells in the escopoletin treated cultures were 23.45, 45.37, 13.76 and 11.23% compared to 96.43, 1.45, 0.92 and 0% in untreated cultures, respectively.

*Effect of escopoletin on cell cycle alteration in CAL 33 cells*

Analysis of the cell cycle in escopoletin (30  $\mu\text{M}$ ) treated cells after 24 and 48 h showed a marked increase in the percentage of cells in G2/M phase in CAL 33 cells compared to untreated cells. The increase in the percentage of cells in G2/M phase was accompanied by subsequent decrease in the percentage cells in G0/G1 and S phase. Treatment of cells with escopoletin (30  $\mu\text{M}$ ) increased the percentage of cells in G2/M to  $53.6 \pm 3.0\%$  compared to  $7.5 \pm 1.2\%$  in untreated cells (**Figure 3**).

*Escopoletin inactivates CDK1 in SCC-25 cells*

Examination of the expression of cyclin B1 involved in G2/M transition revealed no significant changes in its expression in the escopoletin

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tin treated and untreated cells. Further, escopoletin treatment enhanced the phosphorylation of CDK1 markedly compared to untreated cells in CAL 33 cell cultures (Figure 4).

### *Escopoletin decreases cyclin D1 and E expression*

Cyclin D1 is involved in the progression through the G1 phase of cell cycle and its expression was examined in the synchronized cells after treatment with escopoletin (30  $\mu$ M) for 48 h. It was observed that escopoletin treatment induced a significant decrease in the expression of cyclin D1 (Figure 5). The expression of cyclin E, present abundantly in mid G1 phase was markedly decreased in the cells treated with escopoletin (30  $\mu$ M) compared to untreated cells.

### Discussion

Flavonoids like apigenin have shown promising results against various types of cancers [15-20]. Taking into account the efficient results shown by the flavonoid therapy, the current study was performed to investigate the effect of escopoletin, a flavonoid on the inhibition of OSCC. The results revealed that escopoletin treatment induced a marked decrease in the viability of oral squamous cancer cells. Earlier it was reported that flavonoids induce inhibition of proliferation in prostate carcinoma cells and SCC-15 OSCC [16, 21]. The results from the present study showed that escopoletin treatment also induced apoptosis in CAL 33 cells as well as altered the progression of cell cycle. The analysis of cell cycle distribution showed a marked increase in the percentage of cells in G2/M phase after escopoletin treatment in CAL 33 cells. It is well known that cell cycle is arrested in the G2/M in pancreatic and breast cancer cells after exposure to flavonoid [22, 17]. The main regulator of G2/M progression is the CDK1/cyclin B complex whose activity is required for cells to enter mitosis. Our results revealed that escopoletin induced phosphorylation of CDK1 in CAL 33 cells without any effect on the expression level of cyclin B.

The results of our study showed that escopoletin-induced a significant decrease in the expression of cyclin E in CAL 33 cells. The concentration of cyclin E is found to be markedly higher in oral carcinoma cells [24, 25]. These observa-

tions suggest that escopoletin treatment leads to a decrease in the expression of cyclin D1 and cyclin E along with inactivation of CDK1.

### Conclusion

Thus escopoletin acts as a potential agent for oral cancer prevention and treatment through the induction of cell cycle arrest and apoptosis in OSCC cells.

### Disclosure of conflict of interest

None.

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