



# The Cytotoxicity and Anticancer Effects of Propolis against the Oral Squamous Cell Carcinoma: *In Vitro* Study

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## Abstract

**BACKGROUND:** Propolis has a wide range of therapeutic properties, including anti-cancer properties.

**METHOD AND MATERIALS:** Two types of propolis were collected from two Iranian Provinces (Ardabil (Khalkhal) and Gilan (Qaleh Rudkhan)). The study was performed using ethanolic extract of propolis on Human dermal fibroblast cells and oral squamous cell carcinoma (OSCC) cell lines. The cell viability was evaluated by MTT assay. Cancer cell lines were assessed for the expression of matrix metalloproteinases (mmp) 2 and 9, caspase 3 and 9, BCL2-associated X protein (*bax*), and B-cell lymphoma 2 (*bcl-2*).

**RESULTS:** Increased concentrations of two types of propolis reduced cell viability but did not cause significant cytotoxicity. Real-time polymerase chain reaction indicated that the Khalkhal propolis sample produced more effects as the level of mRNA gene expression of *bax* in the Khalkhal propolis sample was increased in concentration dependent manner, and mRNA levels of *bcl-2*, *mmp-2*, and *mmp-9* were reduced. The activities of caspase 3 and 9 were increased in concentration dependent manner.

**CONCLUSION:** Two types of propolis had cytotoxic effects on OSCC cell line, but the Khalkhal propolis was more effective than Gilan propolis.

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## Introduction

The most common type of cancer in southern Asia is the squamous cell cancer of the head and neck squamous cell cancer (HNSCC). This group of malignancies is considered the most prevalent cancer worldwide, and more than 550,000 new cases are diagnosed annually [1]. There are currently three main methods of treating HNSCC, namely, surgery, chemotherapy, and radiation therapy. Similar to other tumors, proliferating, invading, and metastasizing are key determining factors. An extreme microenvironment allows cancer cells to evade apoptosis [2].

The matrix metalloproteinase (mmp) is involved in invasion and metastasis of HNSCC. Basement membrane destruction and extracellular matrix degradation by *mmp-2* and *mmp-9* lead to

tumor invasion [2]. As a result of developmental and genetic processes, cancer arises when cells proliferate excessively and evade apoptosis [3]. Therefore, they may invade and metastasize into adjacent tissues and organs [4].

Even though billions of dollars are spent on cancer research annually, there is still much to be learned about its development. There are different advancements in cancer treatment methods, but no desired strategies exist for curing these cancers. Since the side effects of drugs have become evident, people have resorted to alternative therapies, such as herbs and natural medicines. Natural compounds have been considered potential resources for discovering new drugs [5], [6], [7].

Propolis is a complex resinous substance created by mixing the saliva of honeybees and the natural materials they consume [8]. Propolis

has been used for a long time due to various pharmacological and biological activities, including antibacterial [9], [10], [11], antifungal [9], anti-inflammatory [12], [13], [14], and antiviral [9], [15], [16], anti-tumoral [17], [18], [19], [20], [21], [22], [23], [24] effects. The traditional use of propolis in medicine has been found to have a wide range of uses. There are over 300 compounds in propolis, which originate from different geographical regions. These compounds include flavonoids, terpenes, phenolic acids, cinnamic acids, and caffeine.

There has been intensive research demonstrating that the proteins in propolis can be toxic against numerous types of cancer cells, such as DU145 prostate cancer cells, PC-3 human prostate cancer cells, HeLa cervical cancer cells, HT-29 colorectal cancer cells, MCF-7 breast cancer cells, Caco-2 colorectal cancer cells, and B16F1 melanoma cells [1]. Researchers have discovered that the phenolic compounds in propolis induce apoptosis [20], [25], [26], mitochondrial stress [27], and prevent cancer growth [20], [27], [28]. In this study, the various aspects of *in vitro* anticancer effects of two Iranian propolis were assessed by evaluating cell viability and apoptosis pathways on oral cancer cells.

## Materials and Methods

### Cell lines and materials

Based on this research, The Human dermal fibroblast (HDF, NCBI Code, C645) cells were obtained from Pasteur Institute of Iran and oral squamous cell carcinoma (OSCC) cell lines were obtained from the Iranian Biological Resource Center (Tehran, Iran). Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich. Phosphate-buffered saline (PBS), Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin, trypsin, Fetal bovine serum (FBS), and Beta-glycerol were obtained from Gibco (New York, U.S.A.). The MTT Kit was purchased from Bioidea, Tehran, Iran, TRIZOL reagent (Invitrogen, USA) Caspase-3 and -9 assay kit (Abcam, Cambridge, MA, USA).

### Propolis sampling and extraction

#### Propolis sampling

The raw propolis was collected from Khalkhal (Ardabil province) and Qaleh Rudkhan (Gilan Province) Qaleh Rudkhan in 2020.

### Propolis extract preparation

At first, the samples were frozen at  $-20^{\circ}\text{C}$  then were grounded in the cooled grinder. Approximately

10 g of raw propolis were extracted through diluting with a 10:1 volume of 70% ethanol solution in sealed tubes at room temperature for 3 days in the dark condition. After freezing the suspensions at  $-20^{\circ}\text{C}$  for 24 h to eliminate wax and less soluble compounds, the samples were filtered using Whatman filter papers (No.1). Three freezing-filtration cycles were performed. The final filtrates were called ethanolic extract of propolis as they represented the balsam (Tincture) of propolis. In the next step, under reduced pressure at  $64^{\circ}\text{C}$ , rotary evaporators (IRE 3001) (MedZist company, Iran) were used to evaporate the solutions to near-dryness, and then they were freeze-dried.

### Cell culture

The OSCC cell line was grown in monolayer method RPMI-1640 medium (Gibco, Germany) containing 10% FBS (Gibco, Germany), supplemented with an antibiotic solution of penicillin/streptomycin 1% (100 mg/L streptomycin and 100 U/mL penicillin, Gibco, Germany) ( $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$ , 72 h). HDF were cultured at  $37^{\circ}\text{C}$  in DMEM with 10% FBS and 1% penicillin/streptomycin antibiotics. A DMEM containing 0.1% FBS was added to the cultures after confluence. A culture of HDF cells were washed twice with PBS and then dissociated with 0.25% Trypsin-EDTA at  $37^{\circ}\text{C}$  for 5 min. By adding 15% FBS to DMEM, the enzyme was neutralized. A Neubauer chamber was used to count HDF cells using microscopy. The HDF cells were ready to be used for the next step after 3–4 passages.

### Cell viability assay (MTT assay)

MTT assay kit was used for cell toxicity. In first step,  $1 \times 10^4$  cells/mL were cultured in 96-well plates (with two types of propolis at 3.9, 7.8, 15.6, 31.2, 62.5, 125, 250, and 500  $\mu\text{g}/\text{ml}$  for 24, 48, and 72 h). Untreated cells of OSCC and HDF were considered as control groups in MTT test. Then, MTT solution was added to the cells ( $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$ , 4 h). Finally, the DMSO (100  $\mu\text{L}$ ) was replaced with the culture medium, and absorbance was determined (570 nm, the ELISA reader (BMG Labtech, Germany). For further assays and according MTT results, we continued our research work using the propolis that had maximum cytotoxicity on cancer cells and/or the highest survival rate on normal cells.

### Real-time polymerase chain reaction (RT-PCR)

The expression level of genes BCL2-associated X protein (*mmp-2*, *mmp-9*, *bcl-2*, and *bax*) (Table 1) [29], [30] was measured by RT-PCR using special oligonucleotide primers. After that, the mRNA of both untreated cells of OSCC (control group) and treated cells ( $5 \times 10^5$  cells/mL) with 31.2, 62.5, and

**Table 1: RT-PCR primer sequences**

Gene	Forward	Reverse
mmp-2	5'-CTCATCGCAGATGCCTGGAA-3'	5'-TTCAGGTAATAGGCACCCCTTGAAGA-3'
mmp-9	5'-ACGCACGACGCTTCCAGTA-3'	5'-CCACCTGGTTCAACTCACTCC-3'
bcl-2	5'-TGTGTGGAGAGCGTCAACC-3'	5'-TGGATCCAGGTGTGCAGGT-3'
bax	5'-GATGCGTCCACCAAGAAGC-3'	5'-AAGTCCAATGTCCAGCCAT-3'
GAPDH	5'-ATCACTGCCACCCAGAAGA-3'	5'-ATGAGGTCCACCACCTGT-3'

RT-PCR: Real-Time polymerase chain reaction.

125 µg/ml propolis for 72 h. Then, they were applied by implementing a cDNA Synthesis Kit (Revert Aid™ first stranded) for cDNA synthesis. The endogenous control gene used in the experiment was Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The PCR reaction mixture included 400 ng of template DNA, 12.5 µl of Master Mix (SYBR Green), forward and reverse primer (each 0.25 µM), and 12 µl of nuclease-free water. After denaturation for 5 min (95°C), RT-PCR was cycled 40 times, between 15 s (95°C), 30 s (Annealing temperature), and 30 s (72°C).

### Evaluation of caspase 3 and 9 activations

An assay kit of caspase-3 and -9 colorimetric measurements was applied on untreated cells of OSCC (control group) and treated cells ( $5 \times 10^5$  cells/mL) with 31.2, 62.5, and 125 µg/ml propolis for 72 h to measure the activity of caspase 3 and 9 as described in manufacturer instructions. As a final step, we measured the absorbance at 405 nm (ELISA Reader (BMG Labtech, Germany)) to determine the caspase activity in the sample.

### Statistical analysis

The statistical analysis was done by SPSS software, Ver 26.0 (I.B.M. Corp. Released 2021. Armonk, NY), GraphPad Prism 9 (GraphPad Software, La Jolla California, USA). Routinely, an ANOVA test was performed on distributed data. In this study,  $p < 0.05$  was considered statistically significant.

## Results

### Cell viability of propolis on HDF

The cell viability of Gilan and Khalkhal propolis was measured on fibroblast cells (normal cells) by MTT assay. As shown in Figure 1a, the percentage of cell viability was related to the extract concentrations. The results showed that the highest cytotoxicity and the lowest cell viability were at 500 and 250 µg/ml ( $p < 0.001$ ). In addition, the cell viability was higher than 99% at concentrations of  $\leq 0.156$  µg/ml. Cell viability at three concentrations of 125, 62.5, and 31.2 µg/ml ( $p < 0.001$  and  $p < 0.05$ ) was detected at more than 70% in 24 h of treatment. This cell survival rate was

increased by more than 80% after 48 h (Figure 1b). These three concentrations indicated lower cytotoxicity compared to the 24 h duration. Figure 1c shows 72 h of treatment, the rate of cell viability was notably increased with cells adaptation during this time.

### Cell viability of propolis on oral cancer cell line

The cytotoxicity of propolis extracts against OSCC cells was significantly high in 24 h (500 and 250 µg/ml) ( $p < 0.001$ ) and led the survival rate of cancer cells to  $< 10$  and 20%, respectively. The survival rate of cancer cells was approximately between 50 and 85% in three concentrations of 125, 62.5, and 31.2 µg/ml, and the cytotoxicity of Khalkhal extract was higher than Gilan extract (Figure 2a). The extract  $\leq 15.6$  µg/ml had little effect on the cytotoxicity of cancer cells. However, by increasing the concentration and extending the time to 48 and 72 h, the cytotoxicity of the extracts was increased. The survival rate of cancer cells in 48 h was lower than 24 h (Figure 2b). In addition, this rate dropped after 72 h (Figure 2c). The concentrations of 500 and 250 µg/ml had the highest level of cytotoxicity at 3 times intervals (24, 48, and 72 h), and the cytotoxicity of the three concentrations (125, 62.5, and 31.2 µg/ml) ( $p < 0.001$  and  $p < 0.05$ ) was measured approximately between 50 and 85%. According to the obtained results, Khalkhal extract was selected as the desirable one with maximum cytotoxicity on cancer cells.

### Expression level of genes that regulate apoptosis (bcl-2 and bax) and metastasis genes (mmp-2 and mmp-9)

Using RT-PCR, we measured the level of gene expression in oral cancer cells treated with propolis for the gene expression of mmp-2, mmp-9, bcl-2, and bax. A study showed that if the concentration of propolis was increased, there would be an increase in the amount of bax (proapoptotic mRNA) expressed. This is consistent with the fact that bax was a dose-dependent factor, with the highest level of expression being detected at 125 µg/ml ( $p < 0.001$ ). As a contrast, a higher concentration of propolis decreased mRNA expression levels of bcl-2 (antiapoptotic mRNA) in response to a higher concentration of propolis. As shown in Figure 3, the lowest expression was observed at 125 µg/ml ( $p < 0.001$ ). The level of expression of the bcl-2 gene occurred at concentrations of 125, 62.5, and 31.2 µg/ml, respectively, and was around 0.85, 0.79, and 0.75 fold of that of bcl-2. As shown in Figure 3, mmp-2 and mmp-9 expression levels were reduced while concentrations increased, indicating that the expression levels were dose-dependent in response to the concentration increase. According to the results of these experiments, the expression levels of these genes were reduced from 31.2 µg/ml

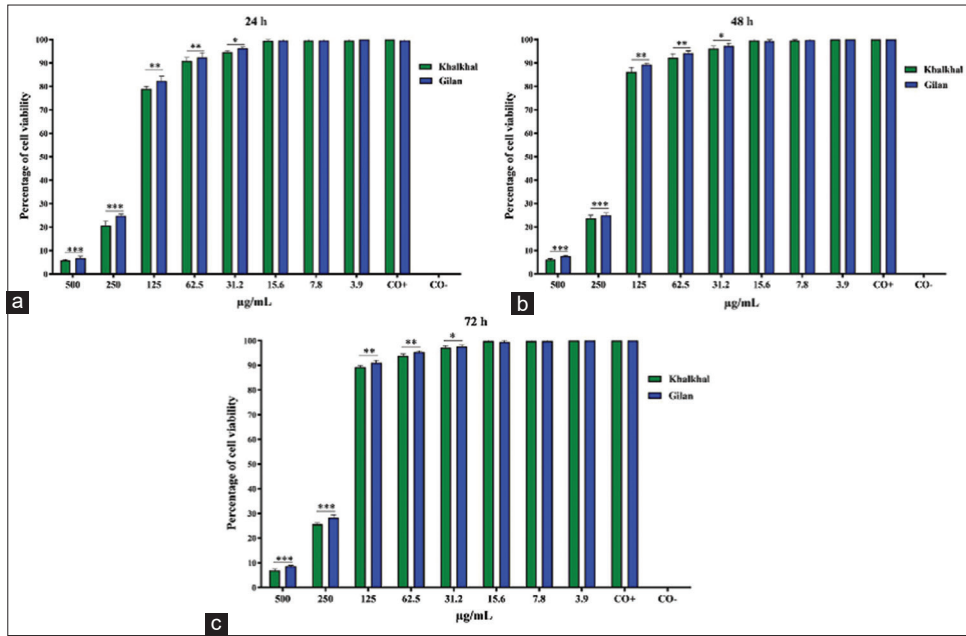


Figure 1: The cytotoxic effects of propolis against fibroblast cell lines After a: 24 h b: 48 h c: 72 h of incubation with different amounts of propolis (3.9–500 µg/ml). Data represent the mean ± S.E.M. of three independent experiments (\*p < 0.05 and \*\*p < 0.01 \*\*\*p < 0.001 demonstrate the groups with significant statistical differences)

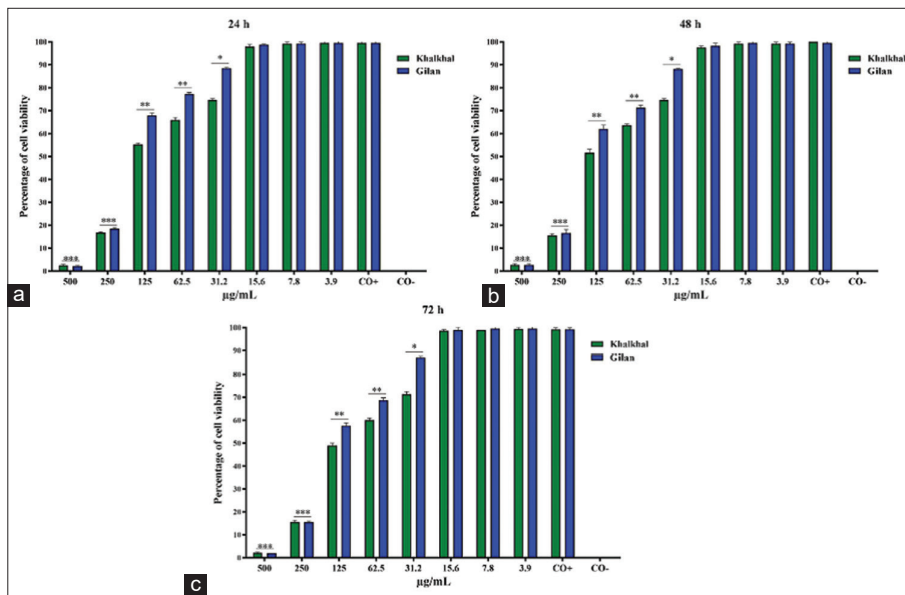


Figure 2: The cytotoxic effects of propolis against oral cancer cell line (SCC) after a:24 h b:48 h c: 72 h of incubation with different amounts of propolis (3.9–500 µg/ml). Data represent the mean ± S.E.M. of three independent experiments (\*p < 0.05 and \*\*p < 0.01 \*\*\*p < 0.001 demonstrate the groups with significant statistical differences)

(p < 0.05) to 62.5 µg/ml (p < 0.01) compared to the control, and the lowest expression level was found at 125 µg/ml (p < 0.01).

**Caspase 3 and 9 activities**

The activities of caspase 3 and 9 were measured in OSCC to determine the apoptosis. As regards Figure 4a, the caspase -3 activity was remarkably increased after the treatment at concentrations of 62.5 and 125 µg/ml (p < 0.01) compared to the control group. In addition, caspase 9 was similar to caspase 3 in these two concentrations (Figure 4b). The results indicated

that the activities of caspase 3 and 9 were increased by increasing the concentration of the extract. The highest level of caspase activity was at 125 µg/ml (p < 0.01), and the lowest level of caspase 3 and 9 activities was at the concentration of 31.2 µg/ml.

**Discussion**

Traditional medicine has been supplanted by the use of natural products to treat diseases rather

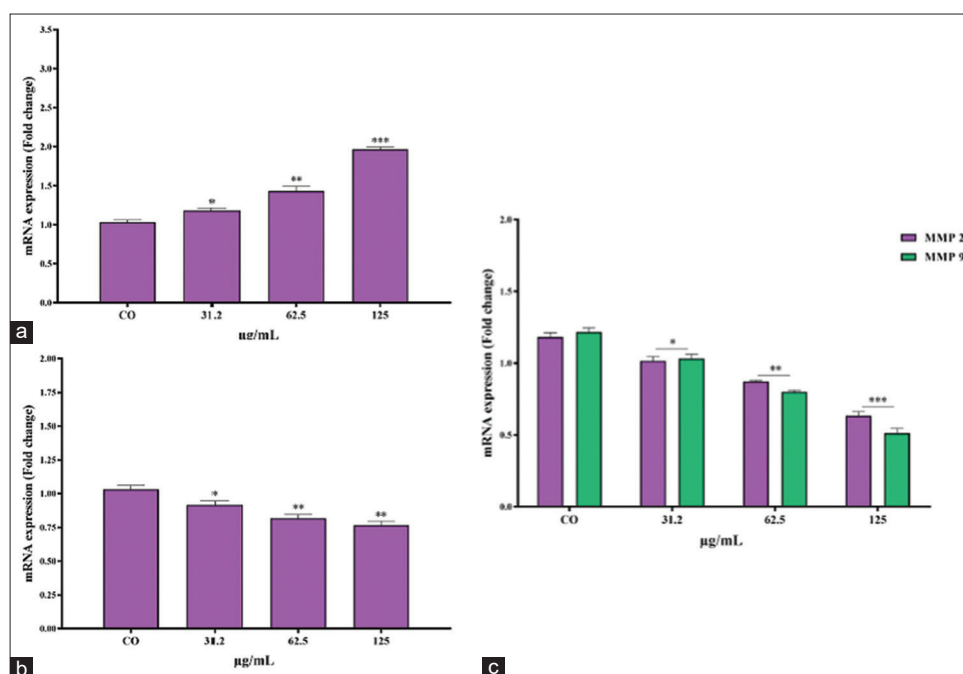


Figure 3: Relative mRNA levels of (a) *bax*, (b) *bcl-2*, (c) *mmp-2*, and *mmp-9*. Proapoptotic and antiapoptotic genes in treated SCC cells versus untreated cells are expressed as the mean  $\pm$  S.E.M. of at least three independent experiments (\* $p < 0.05$  and \*\* $p < 0.01$  \*\*\* $p < 0.001$  demonstrate the groups with significant statistical differences)

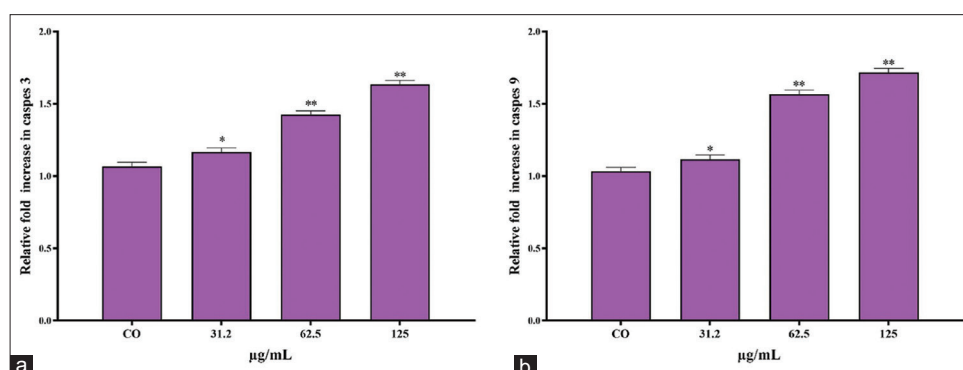


Figure 4: The propolis effects on caspase 3 and 9 activations in oral cancer cells. The relative fold caspase 3 (a) and 9 (b) activity were measured in SCC-treated cells with 31.2, 62.5, and 125  $\mu\text{g/ml}$  of propolis. Data represent the mean  $\pm$  S.E.M. of three independent experiments (\* $p < 0.05$  and \*\* $p < 0.01$  \*\*\* $p < 0.001$  demonstrate the groups with significant statistical differences)

than conventional treatments. Today's biomedical applications of natural products cover a broad spectrum of diseases that can be treated with them [31]. The ninth most prevalent type of malignancy is head and neck cancer worldwide, with a high mortality rate in developing countries [32], [33], [34], [35]. These tumors behave aggressively and can cause harmful diseases and form early local lymphoma and distant metastasis in the future [32], [36].

Propolis is a natural material that bees use to build and maintain their colonies [31]. There are a number of biological activities associated with the use of propolis, including anti-tumor properties, which make it a widely used bee product [20]. It has been found that several types of propolis have been shown to be effective in inhibiting breast cancer cell proliferation [37], lung cancer [38], and melanoma [39].

Jiang *et al.* in 2020 studied the anticancer properties of Chinese propolis in a similar manner to

the observations we made here that was altered by the expression of proteins involved in apoptosis [40]. It has also been reported that the ethanol extract of Chilean propolis has an antiproliferative activity against human mouth epidermoid carcinoma cells in another similar study was demonstrated by Russo *et al.* [41].

To assess the effect of propolis on proliferation and apoptotic processes *in vitro* in the first instance, a high-grade squamous cell carcinoma cell line, one of the most commonly used to model tumors, was used in this study to evaluate the efficacy of propolis on cell proliferation and apoptosis. Specifically, it has been found that Gilan and Khalkhal propolis both displayed antiproliferative activity against OSCC. This discovery was a result of measuring the cytotoxic activity of Iranian propolis *in vitro*. Human fibroblast cell lines were observed to have the lowest level of cytotoxic effects. Both propolis extracts showed great potential as anti-proliferative agents to inhibit the proliferation of oral cancer cells, according to the results

of the MTT test. In spite of the fact that Khalkhal propolis was found to inhibit oral cancer cells more effectively than Gilan, it was selected for further research based on its superior results.

The uncontrolled proliferation of cancerous cells is accompanied by a loss of resistance to apoptosis. Consequently, in the future, propolis ability to induce apoptosis may be considered as a potential characteristic for developing chemotherapeutics agent against cancer [42]. According to the results, the activities of caspases 3 and 9 increased in a concentration-dependent manner when Khalkhal propolis were used indicating that caspase pathways that control apoptosis are influenced by propolis.

Furthermore, the results of this study showed that by increasing the concentration of propolis the level of *bax* gene expression increased and the level of *bcl-2* gene expression decreased in OSCC cells, which are the genes that are responsible for the internal process of apoptosis, by a dose-dependent mechanism [43]. A high level of expression was observed in the *bax* gene when the concentration was 125 µg/ml. As a result of the *bax/bcl-2* pathway, several pharmaceutical agents can have a detrimental effect on antitumor proliferation and activity [44]. It was evident that *bcl-2* levels were decreasing and *bax* levels were increasing, indicating the dysregulation of two key molecules (*bcl-2* and *bax*) and the activation of caspases 3 and 9 in the cells [45].

As the concentration of propolis was increased, it was found that there was a decrease in the expression levels of *mmp-2* and *mmp-9* genes. We found that 62.5 µg/ml of *mmp-2* and 31.2 µg/ml of *mmp-9* gene expression were decreased, respectively. After treatment at concentrations of 125 and 62.5 µg/ml, the caspase 3 and 9 activities in oral cancer cells were significantly increased, when compared to the caspase 3 and 9 activities of normal cells.

## Conclusion

These results showed two types of propolis had cytotoxic effects on OSCC cell line, but the Khalkhal propolis was more effective than Gilan propolis and they can be considered as an appropriate candidate for preventing oral cancer cells.

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## Data Availability Statement

This published article includes all the data generated or analyzed during this study.

## Authors' contributions

All authors have done the same works.

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