Inhibition of Colorectal Cancer Cell Line CaCo-2 by Essential Oil of *Eucalyptus camaldulensis* Through Induction of Apoptosis

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Abstract- Treatment of colorectal cancer is one of the important challenges due to the increase of resistance to chemotherapeutic drugs. Isolated natural compounds from medicinal plants and other sources often are used as novel drugs for treatment of various human cancer. The aim of this study was to investigate the antioxidant and anticancer activity of *Eucalyptus camaldulensis* essential oil on colorectal cancer cell line Caco-2. The antioxidant activity of extracted *E. camaldulensis* essential oil (1000, 800, 400, 200, 100, 50, 25, 12.5, 6, and 3 µg/mL) was evaluated by free radicals inactivation method. Moreover, MTT assay was used to examine the cytotoxic effects of *E. camaldulensis* essential oil on the Caco-2 cell line. The mRNA expression of *BAX* and *BCL-2* genes was studied using quantitative Real-Time PCR method, in treated cancer cells compared to untreated cells. We indicated a significant, impressive antioxidant activity in 1000 µg/mL of *E. camaldulensis* essential oil, in a concentration-dependent manner. In addition, we found that this product exerted a cytotoxic effect on cancer cells when 100 µg/mL concentration was considered as half-maximal inhibitory concentration (IC_{50}). Also, the expression of *BAX* and *BCL-2* genes were significantly upregulated and downregulated, respectively, in the treated Caco-2 cells with *E. camaldulensis* essential oil. In conclusion, our study showed significant antioxidant and anticancer activity in *E. camaldulensis* essential oil in a concentration and time-dependent manner, which may be due to the reduction of free radicals and induction of apoptosis process in colorectal cancer cells.

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Keywords: Colorectal cancer; *Eucalyptus camaldulensis*; Apoptosis; Antioxidant

Introduction

Colorectal cancer is one of the most common cancers and causes of death in the world. The incidence of this cancer is much higher in developed countries such as European countries and the United States as compared with African and Asian countries (1). Nowadays, chemotherapy, followed by surgery, is the most important method for the treatment of patients with colorectal cancer. However, the efficacy of chemotherapy is limited due to the resistance of colorectal cancer cells to chemotherapeutic drugs (2). Therefore, the use of natural compounds and other therapies methods in the treatment of colorectal cancer have increased.

The recent studies suggested an appropriate anticancer potential of herbs and other nutritional supplements (3,4). The use of natural compounds, such as herbs in the treatment of various cancers, has been prevalent for several centuries. The evidence suggested more than 1400 plant genus, which has been used in the treatment of various cancers. Many studies on the medicinal plants, which used in cancer treatment in traditional medicine, have proven the presence of various antitumor compounds in these plants (5,6). One of the most common medicinal plants is Eucalyptus.

The *Eucalyptus camaldulensis* belongs to the Myrtaceae family and generated by fast-growing trees (7). There are many chemical compounds, such as

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acetate, ethylic alcohol, valeric aldehyde, a crystallization substance, etc. in the essential oil of most Eucalyptus species. Moreover, approximately 60-80% of the Eucalyptus essential oil consists of cineole (8,9). Eucalyptus species are highly used in traditional medicine due to its antioxidant, antibacterial, antivirus, and anticancer activities (10,1). However, the anticancer potential of *E. camaldulensis* and its mechanisms have not been investigated in colorectal cancer. Therefore, the aim of this study was to investigate the effect of *E. camaldulensis*on on the viability and proliferation of colorectal cancer cell line CaCo-2.

Materials and Methods

Preparation of essential oil

After collection of *E. camaldulensis* from Tabriz city, it was identified and approved by the Herbarium of the Islamic Azad University, Tabriz Branch. For isolation of the essential oil, the dried aerial parts of the plants (50 gr) were hydrodistilled in a Clevenger type apparatus for 3 hours. The oils were dried over anhydrous sodium sulfate. The obtained essential oil was lyophilized to powder form, and the stock solution was prepared by Dimethyl sulfoxide (DMSO).

Antioxidant DPPH assay

The antioxidant activity of *E. camaldulensis* was evaluated by free radicals inactivation produced by 2,2-diphenyl-1-picryl-hydrazyl (DPPH) and decolorization of dark purple solution. The DPPH methanolic solution (500 µM) and different concentrations of synthesized antioxidants of Butylated Hydroxytoluene (BHT) were prepared as reference antioxidants. The prepared concentrations of BHT (4 ml) and DPPH solution (1 ml) were combined, and the absorbance of the solution was measured after 30 minutes, at 517 nm by a spectrophotometer. Also, this experiment was performed with different concentrations of *E. camaldulensis*in different concentrations (1000, 800, 400, 200, 100, 50, 25, 12.5, 6 and 3 µg/mL). The following formula was used to evaluation of radical scavenging activity percentage (RSA%): RSA% = (Ac-As)/Ac × 100 (Ac = control absorption and As = sample absorption).

Cell culture

The colorectal cancer cell line CaCo-2 was purchased from the Pasteur Institute of Iran. The cancer cell culture was performed using Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (5,000 units/mL-5,000 mg/mL) and incubated at 37° C with 5% CO_2.

Cell viability assay

The Caco-2 cells were seeded in 96-well plates (1.5×10^4 cells/well) and incubated overnight. After this period, the CaCo-2 cells were treated with different concentrations of *E. camaldulensis* (400, 200, 100, 50, 25, and 12.5 µg/mL) for 24-96 hours. Then, treated cells were washed using phosphate-buffered saline (PBS), and 200 µL fresh medium contains 50 µL MTT solution (5 mg/mL reagent in PBS) was added and incubated for 4 hours. The culture medium was substituted with DMSO (50 µl) and incubated for 30 minutes. Finally, the optical densities (OD) of each well of plates were detected at 570 nm wavelength, and cell viability was evaluated.

Cell morphology assay

The Caco-2 cells were seeded in 96-well plates (1.5×10^4 cells/well) and incubated overnight. After this period, the CaCo-2 cells were treated with different concentrations of *E. camaldulensis* (400, 200, 100, 50, 25, and 12 µg/mL) for 24-96 hours. Finally, any visible alterations in the morphology of treated and control cells were monitored by inverted phase-contrast microscopy.

Gene expression assay

The RNA extraction was performed from treated and control cells using the TRIzol agent, as the manufacturer's instructions. The quantity and quality of extracted RNA samples were evaluated by Nanodrop instrument and electrophoresis on 1% agarose gel. The cDNA synthesis was performed using random hexamers. The expression levels of *BAX* and *BCL-2* genes was evaluated the Real-Time PCR method. The primer sequences were as following: *BAX* forward: 5′-CAGAGGATGATTGCTGACG-3′ and reverse: 5′-AAGGTAGAAGAGGGCAACCAC3'-3′; *BCL-2* forward: 5′-CAGAGATGTCCAGTCAGCTG-3′ and reverse: 5′-CTCAAAGAAGGCCACAATC-3′. The Real-Time PCR reaction was performed in a ten µl total volume: 5 µl Master Mix, 1 µg cDNA, and 0.5 µl from each forward and reverse primers (5 pmol). The cycles were as following: 1 cycle for initial denaturation in 94° C for 1 minute, 40 cycles for denaturation in 94° C for 10 seconds, 40 cycles for annealing in 59° C for 30 seconds, 40 cycles for extension in 72° C for 20 seconds. The *β-actin* gene was used as an endogenous control. The data analyzed by the comparative $2^{-\Delta\Delta Ct}$ threshold cycle.

Statistical analysis

The obtained data from three independent experiments are presented as mean±standard deviation (SD). Statistical analysis was performed by Student's *t*-test, Tukey (posthoc), and one-way analysis of variance (ANOVA) by the Graph Pad Prism software, and difference with $P<0.05$ was considered as significant.

Results

Antioxidant activity

Our study indicated that the antioxidant activity of *E. camaldulensis* essential oil was impressive and can inactivate produced free radicals. However, its antioxidant activity was less than BHT, in the same concentrations. The obtained results showed that the antioxidant activity of *E. camaldulensis* essential oil was in a concentration-dependent manner, and its highest antioxidant activity was at 1000 µg/mL concentration (Table 1).

Cell viability

This study showed that the anticancer activity of *E. camaldulensis* essential oil was appropriate and can inhibit the proliferation and growth of cancer cells. The obtained results showed that the anticancer activity of *E. camaldulensis* essential oil was in a time and concentration-dependent manner. We observed that the viability of treated Caco-2 cells was significantly decreased in high concentrations of *E. camaldulensis* essential oil. The proliferation and growth of CaCo-2 cells treated with *E. camaldulensis* essential oil (400 µg/mL at 96 hours) were decreased by 91.25%. Moreover, the inhibition concentration (IC_{50}) of *E. camaldulensis* essential oil on CaCo-2 cancer cells after 72 hours were 100 µg/mL (Figure 1).

Table 1. The free radicals inactivation by different concentrations of *E. camaldulensis* essential oil and BHT synthesized antioxidants

Sample	Concentrations (µg/mL)									
	3	**6**	**12.5**	**25**	**50**	**100**	**200**	**400**	**800**	**1000**
BHT (%)	15.9±02	23.4±0.4	32.6±0.6	44.5±0.7	56.5±0.3	70.1±0.1	81.3±0.5	86.3±0.2	93.2±0.5	97.3±0.1
***E. camaldulensis* (%)**	11.8±0.4	19.4±0.4	25.8±0.5	38.5±0.2	47.6±0.5	59.7±0.8	66.8±0.7	77.5±0.6	81.2±0.1	93.5±0.2

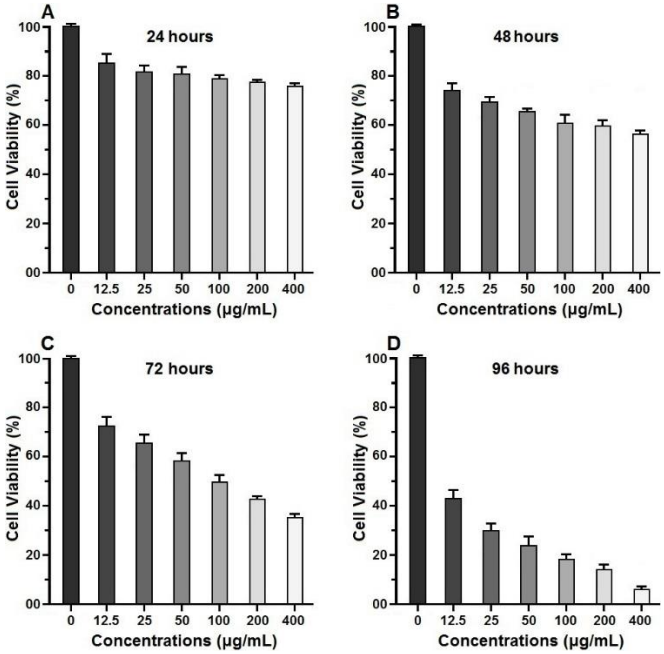

Figure 1. The inhibitory effects of *E. camaldulensis* essential oil on the viability of colorectal cancer cell line CaCo-2 at 24 (A), 48 (B), 72 (C), and 96 (D) hours

Cell morphology

We observed various morphological alterations in CaCo-2 cancer cells treated with *E. camaldulensis* essential oil, which are indicators of programmed cell death. The observed morphological alterations included cell shrinkage, fragmented nuclei, membrane damage,

and a decrease in cell size. The morphological alterations in CaCo-2 cancer cells treated with *E. camaldulensis* essential oil were in a time and concentration-dependent manner, and the highest morphological alterations were observed in 200 µg/mL concentration at 96 hours (Figure 2).

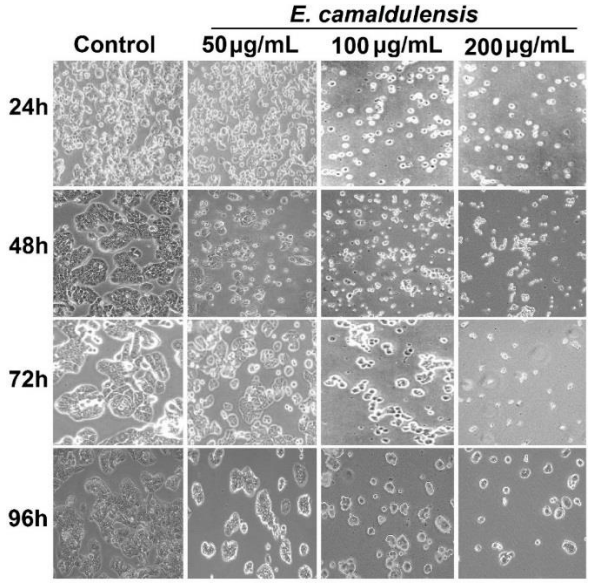

Figure 2. The effects of *E. camaldulensis* essential oil on morphological alteration of colorectal cancer cell line CaCo-2

Gene expression

Our results showed that the mRNA expression of the *BAX* gene was significantly increased (4.1 fold) in CaCo-2 cancer cells treated with *E. camaldulensis* essential oil in 100 µg/mL concentration at 72 hours (P<0.001). Moreover, the treatment of CaCo-2 cancer cells with *E. camaldulensis* essential oil in 200 µg/mL concentration at 72 hours cause to more increase (5.4 fold) in mRNA expression of *BAX* gene (P<0.0001) (Figure 3 A). On the other hands, the mRNA expression of *BCL-2* gene was significantly decreased 5.3 fold (P<0.001) and 8.2 fold (P<0.0001) in cancer cells treated with *E. camaldulensis* essential oil in 100 µg/mL and 200 µg/mL concentrations, respectively (Figure 3 B).

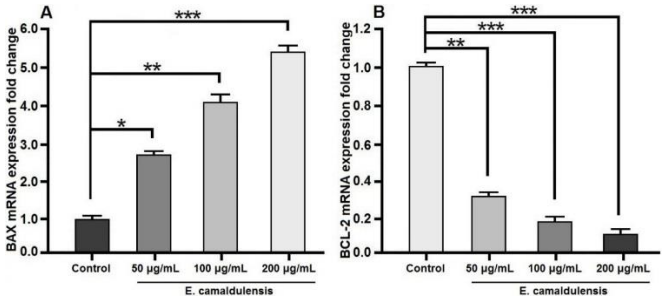

Figure 3. The effects of *E. camaldulensis* essential oil on mRNA expression of *BAX* (A) and *BCL-2* (B) genes (*P<0.01, **P<0.001, ***P<0.0001)

Discussion

More than 3500 years ago, natural resources such as plants had been used to treatment of various diseases (12). Modern pharmacy science has moved considerably toward medicinal herbs and treatment of many diseases, including cancer, using bio-active compounds over the past two decades (13). Uses of these medicinal plants in the treatment of cancer were increased due to their anticancer effects, fewer side effects, low costs, and especially its ability to target different signaling pathways (14). One of the most important medicinal plants was *E. camaldulensis*, which considered an antioxidant and antitumor herb (15). Therefore, in the present study, the

anticancer effects of *E. camaldulensis* essential oil on colorectal cancer cell line CaCo-2 have been investigated. In this study, we indicated that *E. camaldulensis* essential oil cause cancer cell death in a time and concentration-dependent manner. Moreover, many morphological alterations were observed in the colorectal cancer cells treated with *E. camaldulensis* essential oil, which can cause cell death. In several studies by Sidjiko *et al*., (2004), Takasaki *et al*., (2000), and Adebula *et al*., (1999) reported that Eucalyptus species could be used in the treatment of several diseases, such as dysentery, tonsillitis, influenza, various cancers, and skin diseases, which can be due to its anti-viral, anticancer, antioxidant, anti-inflammatory, and antifungal properties (16,17,18). In a study by Ashur (2008) suggested the anticancer and anti-proliferation effects of Eucalyptus essential oil on the breast and hepatocyte cancer cells (19). In another study by Bhagat *et al*., (2012) reported that the extraction of *E. citriodora* causes cancer cell death, such as colon, lung, prostate, ovary, cervix, neuroblastoma, and liver cancers (20). Moreover, Doll-Boscardin *et al*., (2012) reported an appropriate cytotoxic activity for Eucalyptus essential oil and terpenes on various cancer cell lines (21). Therefore, the anticancer activity of Eucalyptus can be due to the presence of several chemical compounds, such as terpene in its essential oil and extracts. The results of the mentioned studies were the same as our results, which showed an appropriate anticancer effect for Eucalyptus in time and concentration-dependent manner on different cancer cell lines, such as colorectal, breast, lung, prostate, ovary and cervix.

On the other hand, we showed several morphological alterations in colorectal cancer cells treated with *E. camaldulensis* essential oil, which was in time and concentration-dependent manner. The observed morphological alterations, such as cell shrinkage, fragmented nuclei, membrane damage, and decrease in cell size, can cause cell death. These presences of several chemical compounds such as acetate, ethylic alcohol, valeric aldehyde can show toxic effects and morphological alterations in cancer cells.

In the present study, we showed a high antioxidant activity in *E. camaldulensis* essential oil in 1000 µg/ml, which was in a concentration-dependent manner. Also, in a previous study by Mahdavi *et al*., (2017) reported a high antioxidant activity in *E. camaldulensis* ethanolic extraction (10). These antioxidant activities can be caused to anticancer activity in essential oil and extracts of Eucalyptus. Therefore, it can be mentioned that the anticancer activity of Eucalyptus essential oil in the present study is may be due to its antioxidant properties (15).

Our study indicated that the *E. camaldulensis* essential oil with targeted effects on mRNA expression of genes involved in the apoptosis process causes cancer cell death. The *BAX* and *BCL-2* genes are two of the most important genes involved in the apoptosis process. The *BAX* gene-encoded product induces apoptosis and causes to increases cell death. On the other hand, the *BCL-2* gene-encoded product inhibits the apoptosis and cause to cell survival (22). Therefore, many studies have been conducted to control the expression of *BAX* and *BCL-2* genes, as well as other genes involved in the apoptosis process to increase cell death in various cancer cells (23,24). A study by Sharifi *et al*., (2018) reported that Eucalyptus cause a significant increase of the *BAX* gene and a significant decrease of the *BCL-2* gene in lung cancer cell lines (25). Therefore, the results of the present study confirmed the results of the mentioned study and proved the anticancer and anti-proliferation function of Eucalyptus through apoptosis induction.

Generally, in the present study, we observed that *E. camaldulensis* essential oil has an acceptable cytotoxic effect on the colorectal cancer cell line CaCo-2 as a concentration and time-dependent manner. Also, we indicated that the anticancer activity of *E. camaldulensis* essential oil might be due to the reduction of free radicals and induction of the apoptosis process in the colorectal cancer cell line. However, further studies are required to more identification of the anticancer mechanisms of Eucalyptus for novel anticancer drug design, which may be used for the treatment of human malignancy, such as colorectal cancer.

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