



Original Article

Anticancer and Antibacterial Effect of Eucalyptus Camaldulensis, in Vitro

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ABSTRACT

Background and objectives: The spread of infectious diseases and malignant diseases has been increasing in the recent years. The use of chemical drugs, in addition to the development of drug resistance, also cause serious side effects. We conducted the present study to examine the antibacterial, antiviral, and anti-cancer effects of *E. camaldulensis* as a herbal remedy.

Methods: We extracted *E. camaldulensis* using a hydroalcoholic solution. The antiviral effect of the plant was investigated at the time of the Herpes simplex virus entry and once the virus entered the cell. Moreover, we evaluated MIC and MBC of *E. camaldulensis* on *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Acinetobacter baumannii*, and *Corynebacterium glutamicum*. For the evaluation of cell cytotoxicity, HFF-2 (NCBI: C163) and A549 (ATCC: CCL81) cell lines were utilized.

Results: The results of the cytotoxicity test indicated that both cell lines were sensitive to the hydroalcoholic extracts of *E. camaldulensis*. The MIC for *A. baumannii*, *K. pneumoniae*, and *C. glutamicum* was 6.25 µg/ml, and the MIC for *S. aureus*, *S. pyogenes*, and *S. agalactiae* was 12.5 µg/ml. MBC was evaluated as 25 µg/ml for *S. aureus*, *S. pyogenes*, and *S. Agalactiae*. It was 12.5 µg/ml for *A. baumannii*, *K. pneumoniae*, and *S. Agalactiae*. IC50 value on entering the virus into the cell was 40 µg/ml, and following the absorption of the virus, the IC50 value was 80 µg/ml.

Conclusion: The results of this study demonstrated that *E. camaldulensis* is of antibacterial, antiviral, and anti-cancer potentials and could be used as a candidate for the preparation of a new drug.

Keywords: *E. camaldulensis*, Antibacterial, Antiviral, Anti-cancer, HSV-1

INTRODUCTION

Cancer is known to be a group of diseases involving abnormal cell growth with the potential to spread to other parts of the body (1). It is triggered by the uncontrolled division of cells, which are the effects of environmental factors and genetic disorders (2). Cancer is the second leading cause of death after heart disease (3). Lung cancer is the most prevalent cancer worldwide and is considered an epidemic (4). In 2002, over 1 million people suffered from the disease, accounting for 29% of all the cancer-related deaths. According to the World Health Organization's Cancer Research Unit, the incidence of cancer in developed countries are 2 times higher than that in developing countries (5).

Bacterial infections are believed to be one of the most common infectious agents in the nature, which severely threaten the life of all creatures, particularly humans. Bacteria can infect most human organs. Lung is one of the most important organs due to its high humidity, suitable temperature, and high oxygen content.

Klebsiella pneumoniae, *Staphylococcus aureus*, *Streptococcus pyrogens*, *Streptococcus agalactiae*, *Acinetobacter baumannii*, and *Corynebacterium glutamicum* are the most pathogenic bacterial infections.

K. pneumoniae is a normal flora for mouth, skin, and intestine, which is an important infection in the respiratory tract. This infection mainly occurs in patients with respiratory tract immune defects and also in patients with diabetes, malignancy, alcoholism, and liver diseases. *S. aureus* is gram-positive bacteria that are mainly found in the upper respiratory system and also in the skin; it is estimated that 20 to 30% of humans are infected with these bacteria. *S. pyrogens* is extracellular bacteria that are clinically important for humans and other hosts. *S. pyrogens* is responsible for 1-5% of the throat, vaginal, and rectal infections. *S. agalactiae* is a harmless commensal bacterium in humans, yet neonatal infections are severely dangerous and can cause chorioamnionitis in mothers during or after pregnancy. *A. baumannii* is an unprincipled bacterial pathogen mainly accompanying

hospital-acquired infections and is prevalent in immunosuppressed patients (6, 7).

Herpes simplex virus 1 and 2 are transmitted through oral secretions or sores on the skin. HSV-1 is transmitted through kissing or sharing mouth-related stuff, for example toothbrushes. Blister on genitals, rectum, areas and typically mouth are the common manifestations of HSV-1 (8). The treatments such as Famvir, Zovirax, and Valtrex are usually recommended for HSV-1 infections (9).

There are different treatments for cancer; surgery, chemotherapy, radiotherapy, hormone therapy, and immunotherapy for instance. In case of lung cancer, there are also different recommended treatments and systemic chemotherapy is one of them. However, the lack of selective toxicity often results in unbearable side effects (5, 10). That is due to the fact that current anti-viral and antibiotic drugs do not have therapeutic effect against infections. In addition, in the recent years, several cases of drug resistance have been reported by microorganisms. All these results confirm the need for further studies on new and non-toxic treatments (11).

E. camaldulensis is one of the most famous herbal remedies considered owing to its antimicrobial and anti-inflammatory effects (12). It has also shown an antimicrobial activity on a wide range of gram-negative bacteria, such as *Staphylococcus aureus*, *Shigella flexneri*, *Salmonella paratyphi*, *Escherichia coli*, and *Bacillus cereus*. *E. camaldulensis* is employed to treat several diseases, for instance influenza, tonsillitis, dysentery, and skin dysplasia. *E. camaldulensis* strengthens body's intimate immune system and attacks viruses, bacteria, and infections (13, 14).

To assess the effects of this drug, viability tests are usually used. To evaluate the toxicity and liver enzymes, such as ASL, ALT and ALP, cell culture is normally used (5, 15-17). The current study aimed to evaluate the effects of *E. camaldulensis* hydroalcoholic extract on the human lung cancer cell line and analyze its antibacterial and antiviral properties.

MATERIALS AND METHODS

The aerial parts of *E. camaldulensis* were collected from Tarbiat Modares University.

Following the identification of the herbarium, the plant was dried at room temperature and in shade by botanical experts at the Faculty of Biology Sciences, Tarbiat Modares University. The plant parts were crushed with a mill. An amount of 200 g of the processed powder was incubated in the dark in 1000ml of hydroalcoholic solution (water and ethanol (Merck, Germany)) at temperature of 25 °C. After 48 hours, the solution was passed through whatman and the resulting solution evaporated at 45 °C for 4 days to evaporate the solvents and the dilutions were prepared in PBS(18, 19).

The HFF-2 cell line (NCBI: C163) and the A 549 (ATCC: CCL81) human were purchased from Iran Pasteur institute and were cultured in DMEM (Dulbecco's Modified Eagle Medium) culture medium containing 10% Fetal Bovine Serum (FBS) in a sterile cell culture flask. After 2 to 3 days, their culture medium must be changed. To determine the total number of cells, we employed hemocytometer slide and staining technique with trypan blue solution (Sigma-Aldrich). Once the cultured cells were grown enough, they were carefully counted utilizing a hemocytometer slide and then incubated with the concentrations of 1, 10, 100 and 1000 µg/ml of the treated *E. camaldulensis* extract for a period of 24 hours. Subsequently, in each case, 10 MT of MTT and 90 µl of DMEM medium containing 10% FBS were incubated at 37 °C for 3-4 hours. After the incubation time, the supernatant was removed and 100 µL of DMSO was added. Finally, we measured the absorbance at 570 nm (20).

Minimum Inhibitory Concentration (MIC)

K. pneumonia ATCC: 1290, *Staphylococcus aureus* ATCC: 1337, *Streptococcus pyrogens* ATCC: 1447, *Streptococcus agalactiae* ATCC: 1864, *Acinetobacter baumannii* ATCC: 1855, *Corynebacterium glutamicum* ATCC: 1532 were purchased from Industrial Research Institute of Iran.

To determine the MIC of the hydroalcoholic extract, dilution series of 1.56, 3.12, 6.25, 12.5, 25, 50, 100, 200, 400 and 800 µg /ml were prepared in the Muller Hinton Broth medium. Afterwards, the bacterial

suspension with a concentration of 1.5×10^6 CFU/ml was added to the well of 96-well plate. Positive control wells (bacterial and culture medium without extracts) and negative controls (bacterial and culture medium) were also prepared. After 24 hours of incubation at 37 °C, the wells were examined for turbidity. According to the definition of the last well in which no cloudiness was created was equal to the MIC. All the stages of this work were done in quadruplicate(21).

Minimum Bactericidal Concentration (MBC) To determine the MBC, 100 µl of the third well before the separation of MIC concentration on the Muller Hinton Agar medium was cultured. The lowest concentration of the extract, with no growth, was reported as MBC(21).

Trypsin-EDTA solution was added to the single-layer cell culture medium, the culture medium was added and then was entered into the new flasks. The flasks were incubated with CO 25%, and humidified at 37°C. Following single layer formation, the cells were transferred to 96 cell culture microplates and then incubated for 24 h to allow cells to grow to 80% of the chuck floor. The virus was prepared in six dilutions. Subsequently, it was transferred to 96-wells plate. Initially, the culture medium was pulled out of the wells and the cell surface was washed three times with PBS. 50 µl of each dilution of the virus was poured into 4 wells. For 1 hour, viruses were exposed to cells at 37 °C to allow the adsorption. Afterwards, 150 µl of DMEM medium containing 5% FBS was added to each well. It was then transferred to an incubator with 5% CO₂ and humidity at 37 °C. For 7 days, the cells were microscopically monitored for observation of CytoPathic Effect (CPEs) and compared with the control cells. Ultimately, the virus titer was calculated using the RaamaKrishan formula (2016)(22).

Antiviral effects of *E. camaldulensis* on HSV-1 virus at the site of entry and connection

The cells were transferred to 96 wells plate, and the virus of 10^6 particles/ml was added to the well. Primarily, the culture medium was pulled out of the wells and the cell surface was washed three times with PBS.

50 µl of the virus suspension was poured into each well. *E. camaldulensis* extract was added to the well in different dilutions and each dilution was added to four wells (Drugs and viruses are added together). The rest of the wells were taken as the controls containing no extracts and viruses, cells with virus-free extract was consider as negative control. The viruses and extracts were exposed to cells at 37 ° C for 1 hour to allow the absorption. After one hour, the DMEM medium containing 5% FBS and antibiotic (Pen-Strep) was added to the flask. The flasks were then incubated with CO 25%, humidified at 37 °C. Following 24 hours, the cells were examined for CPE(22).

2.4.2. Antiviral Effects of *E. camaldulensis* on HSV-1 Virus on the Replication and Exit

At this stage, the cells were transferred to 96 well plates. Once more than 80% of the cells were single-layered, the number of 10⁶ viruses/ml was added to the wells. The culture medium was primarily pulled out of the wells and the cell surface was washed three times with PBS. 50 µL of the virus was poured into each well and the rest of the wells were as controls, containing the highest dilution cell, extracellular cell, cell-free virus, and virus-free cell line with acyclovir (at a concentration of 50 µL/ml). They were considered as effective drugs against the virus and positive control. The viruses were incubated at 37 ° C for 1 hour. After one hour, DMEM medium containing 5% FBS and antibiotic (Pen-Strep) was added to the flask. 50 µL of *E. camaldulensis* extract was added to concentrations of 1, 10, 100 and 1000 µg/ml. The flasks were incubated with CO 25%, humidified at 37 °C, and after 24 hours, the cells were examined for CPE(22). The statistical analysis was done using SPSS,

version 19 Software. We determined the variances between the treatments and the control groups utilizing one-way ANOVA, Paired sample t-test, and chi² square tests. $P < 0.05$ was considered as significant difference.

RESULTS

1. Cell cytotoxicity

The results of the cytotoxicity test indicated that both cell lines were sensitive to the hydroalcoholic extracts of *E. camaldulensis* ($P < 0.05$).

This increase is dependent on concentration and time, and with the increase in the concentration and treatment time in both cell lines, the cell viability decreases. Additionally, the results revealed the greater sensitivity of the cancer cells to the extract. The IC₅₀ value for HFF was 110.4 µg/ml and 34.85 µg/ml, and for A549 was 88.23 µg/ml and 22.63 µg/ml respectively after 24 and 48 hours(Fig. 1).

• Antibacterial effects:

E. camaldulensis in all the concentrations was effective against *A. baumannii*, *K. pneumonia*, *S. aureus*, *S. pyogenes*, *C. glutamicum*, and *S. agalactiae*. The MIC for *A. baumannii*, *K. pneumonia*, and *Corynebacterium glutamicum* was 6.12 µg/ml, and the MIC for *S. aureus*, *S. pyogenes*, and *S. agalactiae* was 12.5 µg/ml.

• Antiviral effects of the hydroalcoholic extract of *E. camaldulensis*

In an antiviral study, the effects of the drug on entering the virus into the cell and the effects of the drug on the virus inside the cell have been investigated. IC₅₀ value on entering the virus into the cell was 40 µg / ml and after absorption of the virus, the IC₅₀ value was 80 µg/ml (Fig. 2).

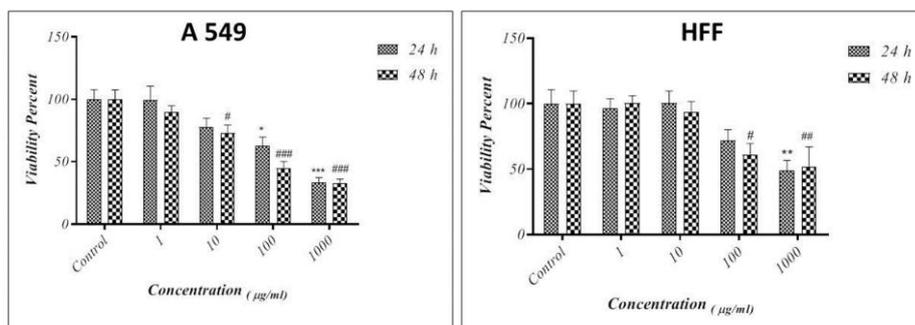


Fig1: Cytotoxicity of *E. camaldulensis* on the HFF and A549 cell lines after 24 and 48 hours. **Significant $P < 0.001$, *Significant $P < 0.05$

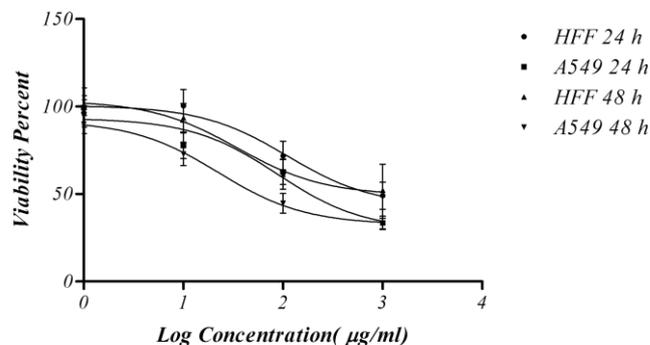


Fig. 2: IC50 value of *E. camaldulensis* against HFF and A549 cell lines.

Table 1: MIC and MBC of the hydroalcoholic extract of *E. camaldulensis* in different concentrations against *A. baumannii*, *K. pneumonia*, *S. aureus*, *S. pyogenes*, *C. glutamicum*, and *S. Agalactiae*

	MIC µg/ml	MBC µg/ml
<i>A. baumannii</i>	6.25	12.5
<i>K.pneumonia</i>	6.25	12.5
<i>S. aureus</i>	12.5	25
<i>S. pyogenes</i>	12.5	25
<i>C. glutamicum</i>	6.25	12.5
<i>S. Agalactiae</i>	12.5	25

MBC values for the hydroalcoholic extract of *E. camaldulensis* were evaluated as 25 µg/ml for *S. aureus*, *S. pyogenes*, and *S. Agalactiae* and were 12.5 µg/ml for *A. baumannii*, *K. pneumonia*, and *S. agalactiae*.

DISCUSSION

In the current study, the hydroalcoholic extract of *E. camaldulensis* in different concentrations indicated its antiviral and antibacterial effects. Furthermore, our results showed that HFF and A549 cancer cell lines were sensitive to *E. camaldulensis*. After 24 and 48 hours, A549 cell lines were more sensitive with an IC50 of respectively 88.23 and 22.63 µg/ml, and the IC50 for HFF cell lines were respectively 110.4 and 34.85 µg/ml.

In several researches, anti-cancer and anti-oxidant effects of *E. camaldulensis* have also been reported. In the current work, we evaluated cytotoxicity effects of *E. camaldulensis* on the HFF and A549 cell lines after 24 and 48 hours. The results revealed that *E. camaldulensis* hydroalcoholic extract in the concentration of the 100 µg/ml was the most effective up to 50%. Vuong et al. (2015) evaluated the anti-cancer effects of *Eucalyptus robusta* against breast, skin, pancreas, brain, ovary, colon, and lung cancer cell lines. According to their results, *E. robusta* leaf aqueous extract showed the best effects on pancreas cell lines (23). Madhulika et al. (2012) examined the anticancer effects of six different extracts from

the leaves of *E. citriodora* in an in-vitro study on colon, lung, prostate, ovary, cervix, neuroblastoma, and liver cancer cell lines. Their findings depicted that ethyl acetate extracts were the most effective extract and suppressed 29.79% of the growth of Ehrlich ascites carcinoma (24). Similarly, Döll-Boscardin et al. (2012) assessed the anti-cancer effects of young and adult leaves of *E. benthamii* against Jurkat, J774A.1, and HeLa cells lines and revealed that *E. benthamii* have cytotoxicity against the examined tumor cell lines, against Jurkat cell line in particular (25). In 2005, the antibacterial effects of *E. camaldulensis* aqueous extracts were studied against *Pseudomonas aeruginosa*. The results indicated that alcoholic and aqueous extracts can efficiently prevent *P. aeruginosa* growth. In 2012, Ismaili and colleagues studied the effect of *E. camaldulensis* on *Helicobacter pylori* bacteria and reported *E. camaldulensis* as one of the most effective antibacterial herbal medicines (26). Tameshkel et al. 2012 evaluated the anti-Giardial activity of *E. camaldulensis* against *G. lamblia* cysts in an in vitro assay. Based on their findings, *E. camaldulensis* is of anti-parasitic effects on top of antibacterial effects (27).

In the current study, the antibacterial effects of *E. camaldulensis* were assessed against *A. baumannii*, *K. pneumonia*, *S. aureus*, *S. pyogenes*, *C. glutamicum*, and *S. Agalactiae*; the MIC and MBC were evaluated. Our results implied that *S. aureus*, *S. pyogenes*, and *S. agalactiae* were the most resistant bacteria with MIC and MBC of 12.5 and 25 µg/ml, respectively. In this study, along with anti-cancer and anti-parasitic activity, antibacterial effects of *E. camaldulensis* were also studied and most of the pathogenic bacteria were assessed (28, 29). The essential oil of *E. globulus* and *E. camaldulensis* were used for the evaluation of their anti-bacterial effects against certain gram-positive and gram-negative bacteria. The results revealed a more efficient inhibitory effect on *S. aureus* compared to that on *E. coli* (30). Elaissi et al. (2011) examined the antibacterial effects of the essential oils of 20 *E. camaldulensis* species and the results exhibited that *S. aureus* and *P. aeruginosa* were respectively the most sensitive and the most resistant strains (31). The IC50 value of the HSV-1 was 40 µg/ml whereas it was 80 µg/ml after the absorption. Antiviral activities of the essential oil of *E. camaldulensis* were examined by Farouk et al. (2015) against *Rotavirus*, *Adenovirus*, *Coxsackieviruses*, and *Herpes Simplex Virus*. They reported that 90% of the reduction was seen in *Herpes Simplex Virus* load, and the reduction load of *Rotavirus* and *Coxsackievirus* was 50% and 53%, respectively. According to their results, no reduction was observed in the virus load in *Adenovirus* (32). Cermelli et al. (2008) evaluated the antiviral effects of *E. camaldulensis* essential oil on *Mumps virus* and *Adenovirus*. They reported that *E. camaldulensis* essential oil did not have any effects on *Adenovirus* whereas *Mumps virus* was sensitive (33).

In the present study, *E. camaldulensis* showed acceptable anti-cancer, anti-bacterial, and anti-viral effects. In fact, the effects of medicinal plants against cancer, parasite, and bacterial cells are directly related to the plant's components. For instance, the antimicrobial effects of phenol, pinene, and terpineol have been demonstrated in in vitro and in vitro studies. 1,8-cineole and α-terpineol are the main components of various

E. camaldulensis species, which play pivotal roles in bioactivity effects (34, 35)

CONCLUSION

The obtained results herein revealed that *E. camaldulensis* has an antibacterial, antiviral, and anti-cancer potentials and could be used as a candidate for the preparation of new drugs. Obviously, in the future studies, the plant components need to be investigated using decomposition techniques, and each composition should be examined separately to determine the active ingredient of the plant.

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