

Antimicrobial and Cytotoxic Effects of Hydroalcoholic Extract of *Ephedra major* Against Some Microbial Agents of the Oral Cavity

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ABSTRACT

Ephedra major is a valuable medicinal plant that has various therapeutic effects, including antimicrobial and anti-inflammatory activities. The aim of the present study was to investigate the antimicrobial effect of the hydroalcoholic extract of *Ephedra major* on oral cavity bacteria including *Streptococcus mutans*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus Salivarius* and *Candida albicans*. In this study, after preparing the hydroalcoholic extract of *Ephedra major*, the minimum inhibitory concentration (MIC) and minimum lethal concentration (MBC) were determined by microdilution in 96-well plates. Then, cytotoxicity was measured by the MTT method. The tests of determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) showed that the antimicrobial activity of the extract depends on the type of microorganism. The lowest MIC value for *Streptococcus salivarius* was 78 µg/mL and the highest resistance was for *Streptococcus mutans* with 2500 µg/mL. Also, strains of *Enterococcus faecalis*, *Staphylococcus epidermidis* and *Candida albicans* were inhibited at a concentration of 625 µg/mL. Based on the results of the cytotoxicity test, the extract had concentration-dependent cytotoxic effects. Compared to doxorubicin, it showed less toxicity on HaCaT cells. The results of this study showed that the hydroalcoholic extract of *Ephedra major* has significant antimicrobial effects against a range of microorganisms associated with oral infections and has less cytotoxicity than the chemical drug. Therefore, this plant can be suggested as a natural and safe option in the development of disinfectant products in the field of dentistry.

Keywords: *Ephedra major*, Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC), Cytotoxicity, HaCaT cells, Oral infections.

Mathematics Subject Classification : 62J12, 62G99

Journal of Economic Literature (JEL) Classification : Q12, D24

1. INTRODUCTION

The use of medicinal plants in the treatment of many diseases has a history of several thousand years. Traditional medicine and medicinal plants are among the sources in which antimicrobial drugs are sought (Organization 2022). In recent years, with the tendency of people to use drugs of plant origin due to the side effects and adverse effects of chemical drugs, the world's attention to this topic has increased, and with the new developments that have occurred in the pharmaceutical industry, due to drug resistance in bacterial and fungal strains, the need for new antimicrobial drugs with fewer side effects is felt more than ever. Recently, herbal products have been subjected to more detailed examination to discover their potential in preventing oral and dental diseases, especially diseases related to plaque and tooth decay. Plants and their compounds, including essential oils and extracts, have the potential to replace chemical drugs. Most of these herbal extracts and essential oils have antifungal, antiparasitic, antibacterial, antiviral, antioxidant, and cytotoxic properties (Bissett and Preshaw 2011).

Ephedra is one of the oldest medicinal plants in traditional Chinese medicine, belonging to the Ephedracea family of the order Ephedrales. Its medicinal species are used as stimulants and antiasthmatics for the treatment of colds, bronchial asthma, coughs, fever, influenza, headaches, edema, and allergies (Bissett and Preshaw 2011). The medicinal and therapeutic effects of these plants are related to alkaloids such as ephedrine, pseudoephedrine, norepinephrine, and methylephedrine, which are the most common compounds found in Ephedra. In addition, a wide range of alkaloid and non-alkaloid compounds have been identified in different Ephedra species (Jung and Shin 2008). The Ermak plant, with the scientific name *Ephedra major*, belongs to the family Ephedracea and is intermediate between the gymnosperms and angiosperms. ^[4] This plant is a single or double-stemmed, climbing plant that grows as a shrub with woody members about one meter or more in height (Kassebaum, Bernabé et al. 2014). This plant has several species, including *E. pachycada*, *E. strobiliacea*, *E. procera* and *E. geradiana*. Almost all species of this plant are adapted to the environmental and climatic conditions of Iran and grow in different regions of Iran, including South Khorasan. Ephedrales has significant antimicrobial properties. The alkaloid ephedrine and its isomeric derivatives are the most important components of the Ermak plant, and most of the medicinal uses of this plant are related to these compounds. This plant is recommended in traditional Chinese medicine for the treatment of diseases such as colds, flu, bronchitis, nasal congestion, hay fever, arthritis, hives, headaches, bone and joint pain, and lowering blood pressure (Peres, Nascimento et al. 2022). Research has shown that the extracts of this plant can be effective against a variety of microorganisms. The active compounds in the plant may act by disrupting the cell membrane of microorganisms. They act by inhibiting the synthesis of proteins and enzymes essential for growth (Peres, Nascimento et al. 2022). Aqueous and alcoholic extracts of the plant have a positive effect on various bacteria such as *Staphylococcus aureus* and *Escherichia coli*. These extracts can be used as a natural source to control bacterial infections. Extracts of this plant also have antifungal properties and can affect fungi such as *Candida albicans* (Casamassimo, Flaitz et al. 2018).

Although various species of *Ephedra* have been of medicinal interest for their active compounds, particularly ephedrine and pseudoephedrine, most pharmacological and antimicrobial studies have focused on well-known species such as *Ephedra sinica*. A review of the research literature shows that no study has been conducted to date on the antibacterial effects of *E. major* against bacteria associated with oral diseases. This is despite the increasing drug resistance and the discovery of natural antimicrobial agents, highlighting the need to evaluate less studied species.

Streptococcus mutans is one of the main causes of tooth decay. Studies have shown that some plant compounds can reduce the growth of this bacterium. *Streptococcus salivarius* is known as a probiotic and can be useful against oral infections (Husain 2018). *E. major* was chosen in this study not only because of its widespread growth and easy accessibility in Iran but also because of its scientific and economic importance. Although other species, including *E. sinica*, are known as the main source of ephedrine in the world, the chemical composition of *E. major* is also similar and contains significant amounts of active alkaloids; however, due to the lack of studies, its biological potential is not yet fully known. On the other hand, despite having natural resources of *E. major*, Iran is still an importer of ephedrine raw materials; therefore, identifying the medicinal and antimicrobial capacity of this native species can help develop domestic pharmaceutical products. The aim of the study was to investigate the antibacterial effect of *E. major* on a number of oral cavity bacteria (*Streptococcus mutans*, *Streptococcus salivarius*, *Staphylococcus aureus*, *Enterococcus faecalis* and *Staphylococcus epidermidis*).

2. GENERATION OF THE DATA

In this experimental study, bacterial strains *Streptococcus mutans* ATCC10682, *Streptococcus salivarius* PTCC1448, *Staphylococcus aureus* ATCC25923, *Enterococcus faecalis* ATCC29212, *Staphylococcus epidermidis* ATCC11294, and *Candida albicans* 10231 were obtained from the Iranian Center for Biological and Genetic Resources.

2.1. Preparation of hydroalcoholic extract of *E. major*

In order to extract the extract of *E. major*, 10 grams of dried plant powder was dissolved in 100 ml of 70% alcohol, then in order to optimize the extraction process of active compounds, it was placed at a controlled temperature of 45 to 50 ° C overnight. The use of this temperature was selected based on previous studies that showed that an average temperature between 45 and 50 ° C provides the best conditions for the extraction of biologically active substances. After completing this stage, the solution was subjected to the influence of ultrasound waves (sonicator) for one hour. After that, the solution was passed through special filters. In the next stage, the resulting extract was subjected to a centrifugation process to completely remove the suspended and fine particles. Using this technique, the remaining solid particles were completely precipitated and a clear and pure liquid was prepared for the next stages. Finally, the clear extract obtained was dried using an oven at 40°C. The final dried extract was stored at 4°C.

2.2. Minimum Inhibitory Concentration (MIC) of the hydroalcoholic extract of *E. major*

To perform the MIC test, Mueller Hinton Broth medium was used in 96-well microplates, as well as different concentrations of the hydroalcoholic extract and the broad-spectrum antibiotic chloramphenicol for bacteria and nystatin for fungi. 10 μ L of bacterial suspension prepared with half McFarland concentration were added to wells containing 100 μ L of Mueller Hinton Broth containing specific concentrations of the hydroalcoholic extract (dilutions in serial series of 1.2 mg/ml, the highest concentration of which was 20 mg/ml) and also the concentrations prepared from chloramphenicol. The plates were incubated at 37°C for 24 hours. Then, 10 μ L of TTC (2, 3, 5-triphenyltetrazolium chloride solution) with a specific initial concentration of 20 mg/ml was added to all wells of the microplate. After three hours of incubation, the 96-well plates were observed for the formation of a red color. The first well in which no microbial growth occurred and no red color was observed was considered as the MIC (Gilavand, Saki et al. 2020).

The extract concentration range for determining MIC was selected based on a pilot experiment. First, the extract was prepared at a concentration of 5000 μ g/mL, and then consecutive two-fold dilutions (5000, 2500, 1250, 625, 312.5, 156 μ g/mL, etc.) were performed according to the standard dilution instructions.

2.3. Minimum Bactericidal Concentration (MBC) of Hydroalcoholic Extract of *E. major*

To determine MBC, the determined MIC and higher concentrations were used separately, 10 μ L of the bacterial suspension was taken with a sampler and cultured in enriched Mueller Hinton Agar medium, and the hydroalcoholic extract with MIC and higher concentrations was added to the culture media, and the 96-well microplates were placed in a 37°C incubator for 24 hours. After this time, the culture media were observed and examined, and the lowest concentration in which nearly 99.9% of the bacteria did not grow was considered the minimum bactericidal concentration (Mirzaei, Somaghian et al. 2021).

2.4. The toxicity effect of hydroalcoholic extract of *E. major* on HaCaT cell line

HaCaT cells were maintained and passaged in high glucose DMEM culture medium supplemented with 10% FBS and 1% Pen/strep in a CO₂ incubator at 37°C and 5% CO₂; 0.05% trypsin–EDTA was used during passage and the cells were examined for their healthy condition and proper adhesion. For the cell test, 96-well flat-bottom plates were seeded at a recommended density of 1.0×10^4 cells per well to achieve adhesion and metabolic stabilization after 18–24 hours; then the culture medium was replaced with fresh medium and hydroalcoholic extract or reference drug was added as desired working dilutions. Each treatment group included at least three replicates. In this test, colchicine was used as a positive control. After 24 and 48 hours, cell viability was determined by MTT assay: MTT solution was added to each well and after appropriate incubation (usually 3–4 hours), the formazan formed was dissolved with the solubilizing solvent (DMSO or isopropanol) and the absorption was measured at a wavelength of 570 nm with a reference of 630–690 nm; the percentage of viability was calculated as the ratio of the OD of the sample to the OD of the negative control and the mean and standard deviation were reported for each group. The results were analyzed using GraphPad Prism 10 software.

2.5. Ethics approval

The present study was approved by Lorestan University of Medical Sciences with ethics code IR.LUMS.REC.1404.061.

2.6. Statistical analysis

After data collection, the information will be analyzed using SPSS software. For variables, mean and standard deviation will be calculated. To compare MIC and MBC between groups, considering the normality of the data, two-way analysis of variance (Two-way ANOVA) test was used.

3. RESULTS

3.1. Minimum Inhibitory Concentration (MIC) of the hydroalcoholic extract of *E. major*

According to the results (Table 1), the highest antibacterial effect of the hydroalcoholic extract of *E. major* was observed against *Streptococcus Salivarius* with an MIC of 78 µg/ mL. While the lowest antibacterial effect was reported for *Streptococcus mutans* with an MIC of 2500 µg/ mL.

Table 1. MIC levels of *E. major* hydroalcoholic extract against bacterial strains

<i>Bacteria</i>	MIC	
	Extract	Antibiotic
<i>Enterococcus faecalis</i>	625 µg/mL	19 µg/ mL
<i>Streptococcus mutans</i>	2500 µg/ mL	9.5 µg/ mL
<i>Staphylococcus aureus</i>	1250 µg/ mL	9.5 µg/ mL
<i>Staphylococcus epidermidis</i>	625 µg/ mL	39 µg/ mL
<i>Streptococcus Salivarius</i>	78 µg/ mL	17 µg/ mL
<i>Candida albicans</i>	625 µg/ mL	19 µg/ mL

3.2. Minimum Bactericidal Concentration (MBC) of the hydroalcoholic extract of *E. major*

The results of the MBC of the hydroalcoholic extract of *E. major* are shown in Table 2. The lowest MBC was for *Streptococcus salivarius* (625 µg/mL), which indicates the highest activity of the extract among the strains studied. In contrast, the highest MBC was reported for *Streptococcus mutans* (5000 µg/mL), which indicates the weaker effectiveness of the extract against this bacterium.

Table 2: MBC of the hydroalcoholic extract of *E. major*

<i>Bacteria name</i>	MBC	
	Extract	Antibiotic
<i>Enterococcus faecalis</i>	1250 µg/ mL	31 µg/ mL
<i>Streptococcus mutans</i>	5000 µg/ mL	15 µg/ mL
<i>Staphylococcus aureus</i>	1250 µg/ mL	31 µg/ mL
<i>Staphylococcus epidermidis</i>	1250 µg/ mL	39 µg/ mL
<i>Streptococcus Salivarius</i>	625 µg/ mL	19 µg/ mL
<i>Candida albicans</i>	1250 µg/ mL	19 µg/ mL

3.3. Effect of hydroalcoholic extract of *E. major* on HaCaT cells

The results of the effect of hydroalcoholic extract of *E. major* on HaCaT cells showed that this extract has a concentration-dependent cytotoxic activity (Figure 1). So that at high concentrations (500–1000 µg/ mL) the cell viability decreased significantly and reached values less than 50%. In contrast, at lower concentrations (15.62–62.5 µg/ mL) the effects of the extract were milder and the survival rate reached about 70 to 80%. This pattern indicates the existence of a direct relationship between the concentration of the extract and the level of cytotoxicity. Comparing the effect of the plant extract with Doxorubicin

showed that the aforementioned drug has a more intense cytotoxic activity than the extract at all concentrations. Especially at medium and high concentrations, cell viability in the presence of Doxorubicin decreased to less than 20%, while the plant extract-maintained survival in a higher range at the same concentrations.

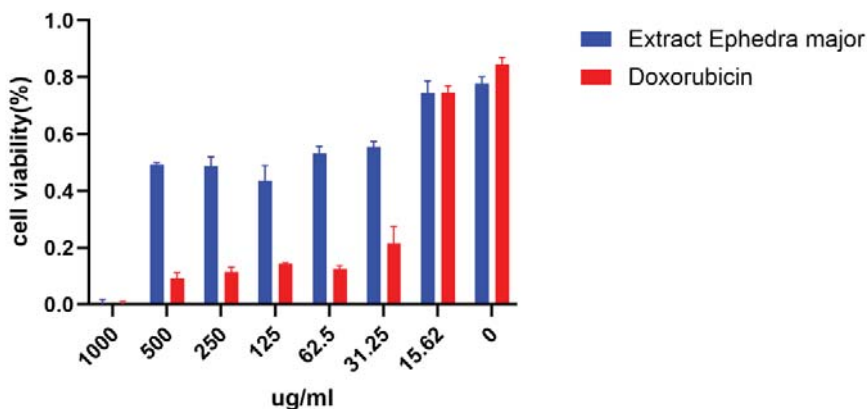


Figure 1. Cytotoxic effect of hydroalcoholic extract of *E. major* on HaCaT cells

Two-way analysis of variance was used to compare the estimated survival effect of different concentrations of *E. major* extract and Doxorubicin at different concentrations. The test results showed that the average percentage of cell viability in the two substances was significantly different from each other ($P < 0.001$). The effect of concentrations on cell viability was also significant ($P < 0.001$). In addition, the interaction effect of substance and concentration was also shown to be significant ($P < 0.001$) (Figure 2). At concentrations of 25.31 to 500, there was a large difference between the two groups, but at concentrations less than 25.31 and concentrations greater than 500, the difference between the two groups was greatly reduced.

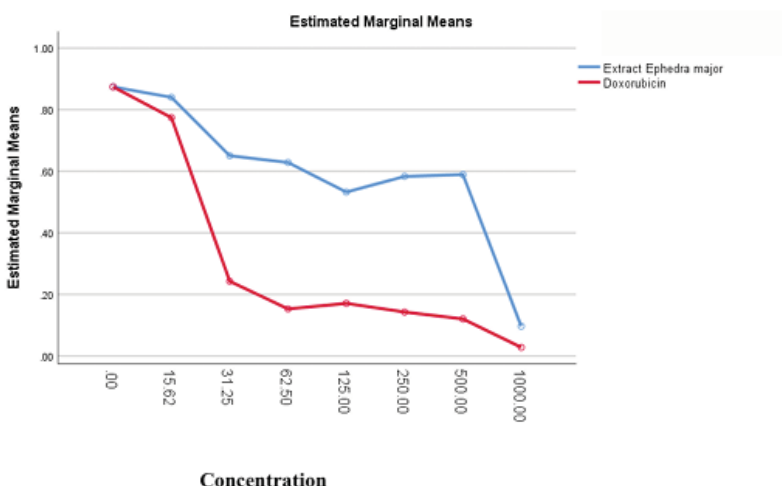


Figure 2. Survival effect of different concentrations of *E. major* extract and Doxorubicin at different concentrations

4. DISCUSSION AND CONCLUSION

The aim of this study was to investigate the effects of the hydroalcoholic extract of *E. major* on antimicrobial activity against various bacterial and fungal species, as well as to evaluate its cytotoxicity on the HaCaT cell line in comparison with the chemotherapy drug Doxorubicin. The findings showed that the plant extract, in addition to having significant antimicrobial activity, also exhibited concentration-dependent cytotoxic effects. The results of the MIC test showed that *E. major* extract has antimicrobial effects dependent on the type of microorganism. The lowest inhibitory concentration for *Streptococcus salivarius* was 78 µg/mL, and the highest resistance was observed in *Streptococcus mutans* at 2500 µg/mL. *Enterococcus faecalis*, *Staphylococcus epidermidis* and *Candida albicans* were inhibited at a concentration of 625 µg/mL, indicating moderate susceptibility of these microorganisms to the extract. MBC results also showed that the extract had a bactericidal effect on microorganisms at higher concentrations. The lowest and highest MBC was recorded against *Streptococcus salivarius* at 625 µg/mL and *Streptococcus mutans* at 5000 µg/mL. These findings were consistent with the MIC results and indicated that cellular structure and virulence factors play an important role in the susceptibility of microorganisms to the extract. Cytotoxicity assay showed that the hydroalcoholic extract of *E. major* has a concentration-dependent cytotoxic activity. At high concentrations (500–1000 µg/mL), cell viability decreased to less than 50%, while at lower concentrations (15.62–62.5 µg/mL) this effect was milder and survival remained around 70–80%. Comparison with Doxorubicin showed that the chemical drug had more severe cytotoxic effects and at all concentrations the cell viability was reduced to less than 20%. However, the lower toxicity of the plant extract could be an advantage for its use in alternative or complementary therapies.

In a study by Nasiri et al., in 2025, the antioxidant and anticancer properties of extracts and essential oil of *Ephedra major* were also confirmed. Nasiri et al., study showed anticancer activity of ethanolic extract and essential oil on A549 cells and IC₅₀ values were determined as 700 µg/mL and 280 µg/mL, respectively, at 48 hours. Also, flow cytometry showed an increase in primary and secondary apoptosis up to 22% and 29.3% for the extract and 14.8% and 25.4% for the essential oil (Nasiri-Varzeghani, Hosseini-Boldaji et al. 2025). In the present study, HaCaT cells were examined as a normal cell line and it was observed that at high concentrations (500 to 1000 µg/mL) the cell viability decreased to less than 50%, while at lower concentrations (15.62–5.62 µg/mL) the survival remained between 70–80%. Comparison with the drug Doxorubicin showed that this drug reduced cell survival to less than 20% at all concentrations and had more severe cytotoxic effects than the plant extract.

In the study by Fazeli-Nasab et al., (Fazeli-Nasab and Mousavi 2019) ethanolic and hydroalcoholic extracts of *E. sinica* were investigated against *Pseudomonas aeruginosa*. The results showed that the lowest MIC of ethanolic extract for standard and clinical strains was 12.5 and 25 µg/ml, respectively, and for hydroalcoholic extract in both strains was 25 µg/ml. Also, the lowest MBC of ethanolic extract was reported for standard and clinical strains was 25 and 50 µg/ml, respectively, and for hydroalcoholic extract in both strains was 25 µg/ml. These results indicate the strong antimicrobial effect of *E. sinica* extracts, especially at low concentrations. In the present study, the MIC results showed that the lowest inhibitory concentration of hydroalcoholic extract was 78 µg/ml for *Streptococcus salivarius* and the highest for *Streptococcus mutans* was 2500 µg/ml, while *Enterococcus faecalis*, *Staphylococcus*

epidermidis and *Candida albicans* were inhibited at a concentration of 625 µg/ml. MBC analysis also showed that the lowest lethal concentration for *Streptococcus salivarius* was 625 µg/ml and the highest for *Streptococcus mutans* was 5000 µg/ml.

In a study by Berreghioua et al., in 2025 on *E. alata* from the Bechar region of Algeria, the antibacterial activity of various extracts was investigated. The results showed that the methanol extract had the highest inhibitory effect on all bacterial strains, and the dichloromethane extract showed the lowest effect on *Escherichia coli*. A comparison of the results of this study with the present study shows that in Berreghioua et al., study on *E. alata*, the evaluation of antibacterial activity was reported qualitatively and MIC and MBC data were not provided. In contrast, the present study on *E. major* provided accurate quantitative MIC and MBC data against several bacterial and fungal species and also investigated the cytotoxicity of the extract (Berreghioua and Ziane 2025).

In summary, it can be concluded that *E. major* extract, with its antimicrobial properties and controlled cytotoxicity, has the potential for further investigation for use in pharmaceutical products or combination therapies, although further in vivo and molecular studies are necessary to determine its safety and precise efficacy.

The results of this study showed that the hydroalcoholic extract of *E. major* has significant antimicrobial effects and concentration-dependent cytotoxicity. The pattern of inhibition of the growth of microorganisms was not the same, and *S. salivarius* showed the highest sensitivity and *S. mutans* the highest resistance to the extract. Cytotoxicity study on the HaCaT cell line also showed that at lower concentrations, cell survival and viability were maintained at an acceptable level; while increasing the concentration caused a decrease in cell viability. Also, comparison with doxorubicin indicated that the studied herbal extract has much lower toxicity than the chemical drug. Despite the promising results, the findings of this study are limited to *in vitro* experiments and it is necessary to conduct additional studies to evaluate the molecular mechanism of action, evaluate toxicity in animal models, and ultimately clinical trials.

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