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Original Article

# Comparison of antibacterial effect of methanolic and hydro-alcoholic ziziphus spina-christi extract with 2.5% sodium hypochlorite on enterococcus faecalis: an in vitro study

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## Abstract

**Introduction:** Root treatment will not be successful, without a proper root canal irrigation with less disadvantages and antibacterial effect. The aim of this study was to compare antimicrobial effect of cedar extract and 2.5%NaOCl on E. faecalis.

**Materials & Methods:** In disk diffusion test, a standard suspension of E. faecalis (ATCC 29212) was cultured on plate and different concentrations (0.05, 0.15, 0.25, 0.35, 0.45 g/ml) of methanolic or hydro-alcoholic extracts, 2.5% NaOCl and physiologic serum (as negative control) were infused on paper disks. The inhibition zone measured after 48 h. In microdilution test, serial dilution of methanolic and hydro-alcoholic extracts, 2.5% NaOCl and physiologic serum in 1:2 proportion was performed in Brain Heart Infusion (BHI) culture medium. Then, standard suspension of E. faecalis was added to each well of micro plate. Data were analyzed using ANOVA.

**Results:** Hydro-alcoholic and methanolic extracts had antibacterial effect on *E.* faecalis. Inhibition zone of 2.5% NaOCl was significantly higher than that of other extracts ($p<0.001$). In microdilution test, *E.* faecalis bacterium was sensitive to both hydro-alcoholic and methanolic extracts but it was more sensitive to 2.5% NaOCl.

**Conclusion:** Totally, 2.5% NaOCl had the highest antibacterial effect on E.faecalis followed by hydro-alcoholic and methanolic extracts. NaOCl is an effective irrigant in root treatment until the studies like this can find a good alternative for it.

**Keywords:** Enterococcus faecalis, Methanol, Sodium hypochlorite, Ziziphus

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# مقایسه اثر ضد باکتری عصاره متانولی وهیدروالکلی گیاه سدربر انتروکوکوس فکالیس با هیپوکلریت سدیم ۲/۵ ٪ درصد: (مطالعه آزمایشگاهی)

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## چکیده

**مقدمه:** بدون یک شوینده مناسب کانال با عوارض کمتر و اثر ضدمیکروبی، درمان ریشه موفق نخواهد بود. هدف این مطالعه مقایسه اثر ضد میکروبی عصاره سدر و هیپوکلریت سدیم ۲/۵ ٪ روی انتروکوکوس فکالیس است.

**مواد و روش ها:** در روش انتشار دیسک، یک سوسپانسیون استاندارد از باکتری انتروکوکوس فکالیس (ATCC 29212) روی محیط کشت داده شد و غلظت های مختلف عصاره های متانولی و هیدروالکلی سدر (۰/۰۵، ۰/۱۵، ۰/۲۵، ۰/۳۵، ۰/۴۵ g/ml) ، هیپوکلریت سدیم ۲/۵ ٪ و سرم فیزیولوژی، روی دیسک های کاغذی ریخته شدند. بعد از ۴۸ ساعت هاله عدم رشد باکتری اندازه گیری شد. در روش میکرودایلوشن، رقیق سازی سریال عصاره های متانولی و هیدروالکلی، هیپوکلریت سدیم ۲/۵ ٪ و سرم فیزیولوژی در نسبت ۱:۲ در محیط BHI انجام شد و سپس سوسپانسیون استاندارد باکتری به خانه های میکروپلیت اضافه گردید. آنالیزهای آماری با استفاده از آزمون ANOVA انجام شد.

**یافته ها:** عصاره های متانولی و هیدروالکلی اثر ضدمیکروبی روی باکتری انتروکوکوس فکالیس داشتند. قطر هاله عدم رشد هیپوکلریت سدیم ۲/۵ ٪ در مقایسه با عصاره ها به طور معناداری بزرگتر بود($p<0.001$). در تست میکرودایلوشن، انتروکوکوس فکالیس به هر دو عصاره متانولی و هیدروالکلی حساس بود اما نسبت به هیپوکلریت سدیم ۲/۵ ٪ بیشتر حساس بود.

**نتیجه گیری:** به این ترتیب هیپوکلریت سدیم ۲/۵ ٪ ، عصاره های هیدروالکلی و متانولی بیشترین اثر ضد میکروبی را نشان دادند. هیپوکلریت سدیم یک شستشودهنده موثر در درمان ریشه است تا زمانی که مطالعاتی اینچنینی بتوانند جایگزین مناسبی برای آن پیدا کنند.


**واژگان کلیدی:** انتروکوکوس فکالیس ، متانول، هیپوکلریت سدیم، Ziziphus

## Introduction

Bacteria are the most important factor in creating pulp and periapical disease. When trauma and caries cause pathologic changes in pulp of tooth, microorganisms get into pulp system and invade root canal. Because of bacteria competition for reaching to low amount of food and oxygen in root canal, specific number of bacteria species in endodontic infections will persist. E. faecalis is a facultative anaerobe Gram-positive bacterium which is one of the bacteria in constant periapical lesions. It can attack dentinal tubules and cope with hard condition of root canal, so it is a resistant microorganism. [1]

Effective cleaning and complete disinfection of root canal system are needed to achieve long-term success of root canal therapy. [2] Root canal cleaning contains two parts: instrumentation and irrigation processes. Instrumentation removes pulp remains, scrapes dentin from root walls and prepares them for aggression from canal. Irrigators remove debris of filing process from root canal and kill microorganisms of root canal. [3] NaOCl is used as a proper irrigator in endodontics therapy that is effective against many bacteria and can solve necrotic pulp tissues. Toxicity for periapical tissues, inability in eliminating smear layer, change in physical structure of canal dentin and unfavorable smell and taste are some of disadvantages of NaOCl. [4] Considering that without a proper root canal irrigation, root treatment will not be successful and since the root canal disinfection at the best condition cannot eliminate all microorganisms, the use of an irrigator, which has an antibacterial effect, and can close dentin tubules and neutralize bacteria with this way, is needed. [2]

Cedar with scientific name, Ziziphus spina-christi, and English name, Lote tree or Christ's Thom is a plant of jujube family with 10 meter height, which grows self-growingly in Saudi Arabia, North Africa and Iran (in Khuzestan, Fars, Hormozgan provinces and Khark Island). Cedar is an evergreen plant with prickly branches, oval and taper leaves. Its fruit is sweet, sour, spherical, and aromatic, and has two-centimeter diameter. Its leaves powder is known as cedar and used



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for hair irrigation and reinforcement as well as hair loss prevention. Another use of its leaf is disinfecting the wound. As mentioned before, water-alcohol extract of its leaf prevents the growth of some fungi. [5] Researches have shown that cedar leaf extract can have bactericidal effect against Gram-positive bacteria such as Bacillus subtilis, Streptococcus pyogenes, and E. faecalis as well as Gram-negative bacteria such as Acinetobacter baumannii, Haemophilus influenzae, Salmonella enteritidis, and Escherichia coli. [6] Since that cedar is a native plant of Iran and its south provinces, and its different pharmaceutical and antibacterial effects have been proved, the aim of this study was to compare antimicrobial effect of cedar and sodium hypochlorite 2.5% on E. faecalis bacterium.

## Materials & Methods

**1- Cedar leaf collection and extract preparation:** The leaves of Z. spina-christi used in this study were collected in July 2017 from Ahvaz University of Medical Sciences in Khuzestan province of Iran. The leaves were dried at the shadow, room temperature for 48 h and crushed into powder. First, 90 gr of dried leaves were soaked in 800 ml of methanol and another 90 gr were soaked in 800 ml of ethanol 70% and allowed to stand in shaker (Labnet-America) for 72 h. Then, the solutions were filtered with No.42What man filter paper. After that, solvents evaporated in oven (Shimi Fann-Iran) with 46°*C* for about 72 h. Finally, precipitates were scratched into powder using sterilized scalpel. [7]

**2- 2.5% sodium hypochlorite:** Solution of 5.25% sodium hypochlorite (chloraxid 5.25%- cerkamed) was diluted 1:2 with physiologic serum.

**3-E. faecalis strain preparation:** Standard strain of E. faecalis bacterium (ATCC 29212) was obtained from Iranian Research Organization for Science and Technology (Tehran-Iran).

**4- Disk diffusion test:** To evaluate antibacterial activity of Z. spina-christi leaves methanolic and hydro-alcoholic extracts and NaOCl, disk diffusion test was performed. First, standard strain of *E.* faecalis was inoculated in Tryptic soy broth (TSB) (Merck, Darmstadt, Germany) medium and incubated in 37 °C for 22 hours. Then, according to McFarland turbidometry, a suspension with $10^8$ CFU/ml final cell concentration was prepared from this culture. This microbial suspension was cultured on Mueller Hinton agar plate (Merck, Darmstadt, Germany). Seven 6-mm diameter sterile paper disks (Padtanteb-Iran) were put on Mueller Hinton agar plate, and immediately 40 µl of each prepared concentration of methanolic or hydro-alcoholic extracts (0.05, 0.15, 0.25, 0.35, 0.45 g/ml), 20 µl of 2.5% NaOCl and 20 µl of physiologic serum (as negative control) were infused on two other paper disks. Plates were put in laboratory temperature for one hour till extracts were being absorbed to disks, then plates were incubated for 48 hours in 37 °C. [8] The inhibition zone around each disk was measured. This test was repeated three times and performed based on CLSI 2014 standard.

**5- Microdilution test:** To determine MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration), culture of *E.* faecalis bacterium was diluted in sterile physiologic serum that a $10^6$ CFU/ml inoculum was gained with reference to 0.5 Mc Farland. In each well of microplate, serial dilution of methanolic and hydro-alcoholic extracts, 2.5% NaOCl and physiologic serum in 1:2 proportion was performed in Brain Heart Infusion (BHI) culture medium (Merck, Darmstadt, Germany) and 10 µl of standard suspension of *E.* faecalis was added to each well. After that, micro-plates were incubated in 37 °C. After 24 h, because of extract turbidity, MIC determining was not possible; therefore, we cultured 10 µl of each well at blood agar plates (Merck, Darmstadt, Germany) and incubated them in 37°C for 24 h. The lowest concentration of extract with less and no colony growth was considered as the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), respectively. [9] This test was performed based on CLSI 2014 standard.

**6- Statistical analyses:** Data were analyzed using ANOVA. In case of significance, Scheffe's multiple comparison was used for two-by-two groups' comparison. $P<0.05$ was statistically considered significant.

## Results

The results showed that hydro-alcoholic and methanolic extracts have antibacterial effect on E. faecalis bacterium and have better activity than physiologic serum as negative control (inhibition zone=0mm). The results of disc diffusion test indicated that inhibitory action of different concentrations of hydro-alcoholic and methanolic extracts against E.

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faecalis bacterium was increased when used in higher concentration (figure-1).

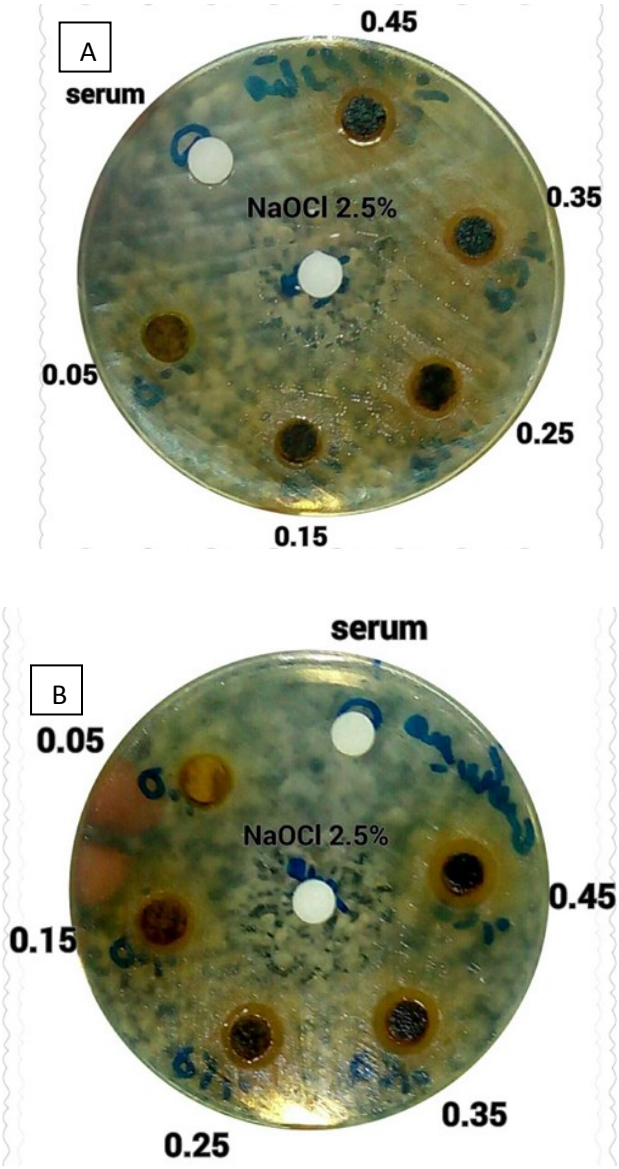



**Figure1. (a) Inhibition zone of methanolic extract and (b) Inhibition zone of hydro-alcoholic extract**

Hydro-alcoholic extract has intermediate antibacterial activity on E. faecalis and it was more effective than methanolic extract (table-1). The inhibition zone of 2.5% NaOCl was significantly higher than other extracts ($p<0.001$). Only one concentration (0.35g/ml) of methanolic extract was statistically comparable to hydro-alcoholic extract and 2.5% NaOCl. Mean inhibition zone of methanolic extract (0.35g/ml) was not significant ($p<0.174$) compared to hydro-alcoholic extract (0.35g/ml), but it was significant in comparison with 2.5%NaOCl ($p<0.001$). Mean inhibition zone of other concentrations (0.05, 0.15, 0.25 and 0.45 g/ml) of methanolic extract was 0, 0, 8 and 9 mm, respectively. The results of microdilution test demonstrated that E. faecalis bacterium was sensitive to both hydro-alcoholic and methanolic extracts but it was more sensitive to 2.5% NaOCl (table-2).

**Table1.The comparison of mean inhibition zone (mm)**

| | Mean | SD | P-value* |
|---|---|---|---|
| **NaOCl 2.5%** | 16.5 | 1.871 | |
| **Methanolic (0.05)** | 0 | 0.000 | - |
| **(0.15)** | 0 | 0.000 | - |
| **(0.25)** | 8 | 0.000 | P <0.001 |
| **(0.35)** | 8.33 | 0.577 | P <0.001 |
| **(0.45)** | 9 | 0.000 | P <0.001 |
| **Hydro-alcoholic (0.05)** | 8.33 | 0.577 | P <0.001 |
| **(0.15)** | 10 | 0.000 | P <0.001 |
| **(0.25)** | 10.67 | 0.577 | P <0.001 |
| **(0.35)** | 10.67 | 0.577 | P <0.001 |
| **(0.45)** | 11 | 1.000 | P <0.001 |

**Table2. Minimum inhibitory and bactericidal concentrations of cedar methanolic and hydro-alcoholic extracts and 2.5% NaOCl on E. faecalis**

| | MIC | MBC |
|---|---|---|
| Cedar methanolic extract | 62.5mg/ml | 62.5mg/ml |
| Cedar hydro-alcoholic extract | 62.5mg/ml | 62.5mg/ml |
| 2.5% NaOCl | 0.625% | 0.625% |

## Discussion

This study showed that methanolic and hydro-alcoholic extracts of Z. Spina-christi have antibacterial activity against E. faecalis in in vitro. Ramezanali et al.'s study in 2016 represented that green tea has acceptable antibacterial effect on the biofilm of E. faecalis [10], which is consistent with the study of Hudson et al. who found that cedar leaf (Thuja pelicata) oil, can kill E. faecalis. [6] The hydro-alcoholic extract than methanolic extract showed better inhibitory action, meaning it could be used more widely. According to Motamedi et al. in 2009, the ethanolic extract of Z. spina-christi illustrated slightly better killing action than methanolic extract against some Gram-positive and-negative bacteria, which are compatible with our results.[8] However, these both extracts showed lower potency compared to 2.5% NaOCl in both disc diffusion and microdilution tests. To

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the best our knowledge, there is no study to comparatively evaluate the antibacterial activity of Z. spina-christi and 2.5% NaOCl against E. faecalis. Another study stated that the mean inhibition zone in descending order was found as sodium hypochlorite > Propolis > AI > Triphala > C. longa = MC > ethanol. [11] Recently, scientists from divergent fields are investigating plants for their antimicrobial usefulness. [12] The components of plants with antibacterial activity include tannins, saponins, phenolic compounds, essential oils and flavonoids. [13]

Bacteria are the most important factor in creating pulp and periapical disease. One of the bacteria in constant periapical lesions is E. faecalis. [1] The standard irrigator (2.5% NaOCl) used as positive control compared to the extracts showed higher antibacterial activity on E. faecalis. This is not surprising because it is well refined industrial product so there is no doubt its activity will be more than crude herbal extracts. If the extracts used in the present work were refined, more and better activity could be observed. [14] The major advantages of using herbal extracts alternatives are easy availability, low toxicity, increased shelf life and cost effectiveness. [15] This was a preliminary study that evaluated antibacterial activity of Z. spina-christi extract against E. faecalis in vitro; thus, further animal experimental studies are needed to determine its safety, toxicity, biocompatibility and efficacy. Considering disadvantages of 2.5% NaOCl which are mentioned previously and physiological benefits of this herbal extract, further studies using improved extraction technique and comparing common irrigants on other bacteria species can be carried out to consider it as an alternative for NaOCl at least in clinical situations like an open apex or in young permanent teeth, in which NaOCl causes toxicity.

## Conclusion

As a result, 2.5% NaOCl had the highest antibacterial effect on E.faecalis followed by hydro-alcoholic and methanolic extracts. Therefore, NaOCl is an effective irrigator in root treatment until the studies like this can find a good alternative for it.

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**Conflict of interest:** The authors state that they have no conflict of interest.

## Authors' contributions

The study was designed by Akam Saeidi and Mahmoodreza Hamidi. The study data was collected by Zahra Nikouee Rad. Analysis and interpretation of data drafting of the manuscript, and critical revision of the manuscript for important intellectual content were performed by Soraya Khafri ,Akam Saeidi, Mahmoodreza Hamidi, Abolfazl davoodabadi and elham mahmoodi. Study supervision was conducted by Akam Saeidi and Mahmoodreza Hamidi and Abolfazl Davood abadi

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