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Evaluation of the Antimicrobial Activity of *Tribulus terrestris, Allium sativum, Salvia officinalis,* and *Allium hirtifolium* Boiss Against *Enterococcus faecalis*

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Abstract

Background: *Enterococcus faecalis* is a Gram-positive, commensal bacterium which can cause life-threatening infections in humans. *E. faecalis* has been frequently found in root canal-treated teeth and is resistant to many commonly used antimicrobial agents. Nowadays modern medicine recognizes herbalism as a form of alternative medicine. *Tribulus terrestris, Allium sativum, Salvia officinalis* and *Allium hirtifolium* Boiss are commonly found in Iran and used as antimicrobial agents in folklore medicine.

Objectives: In this study, antimicrobial activities of aqueous extracts of some plants were examined in vitro against *E. faecalis*.

Materials and Methods: Antibacterial activities of the extracts of *T. terrestris, A. sativum, S. officinalis* and *A. hirtifolium* Boiss were examined using disc and well diffusion methods, and the19 minimum inhibitory concentration (MIC) of aqueous extracts were determined against *E. faecalis* using agar and broth dilution methods.

Results: The obtained results showed that the extract of *A. hirtifolium* Boiss inhibited the growth of *E. faecalis* (MIC of 10 mg/mL). Other plants had no effect on the target bacterium.

Conclusion: According to the best effect of *A. hirtifolium* extract on *E. faecalis* and stability of this extract in thermal condition, we may purify this extract and use it for treatment of infections.

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Background

About 500 cultivable and non-cultivable bacterial species live in the human oral cavity, among which, the majority are considered to be commensals and opportunistic pathogens.

The role of human oral pathogens is pivotal for development of periodontitis and dental caries, the most prevalent infectious diseases in the world. In addition, a number of systemic diseases like infectious endocarditis, respiratory infections, cardiovascular diseases, and brain abscess are stated to cause the presence of oral pathogens. Several oral bacterial species resistant to antibiotics such as penicillins, macrolides and metronidazole, have been reported worldwide.[1] *Enterococcus faecalis* is a well-known example of the resistant microorganisms in the pathogenesis of some medical and dental pathoses. In medical literature, *E. faecalis* is known to be resistant to antibiotics.[2] In endodontic literature, it is well established that *E. faecalis* is resistant against certain intra-canal medicaments. Accordingly, this microorganism is considered to be a key element to evaluate the antimicrobial efficacy of various endodontic medicaments or root canal irrigants.[3-7] This microorganism is most frequently (32%-77%) isolated from persistent periapical lesions following endodontic treatment. Regarding the presence of *E. faecalis* in persistent endodontic diseases, its eradication from the root canal system can ensure the success of endodontic (re)treatment.[6]

According to the increasing rate of antibiotic resistance

as well as the side effects of synthetic drugs, researchers have focused more on herbal medicine to deal with odontogenic infections.[4,5]

Among the aforementioned herbals, *Tribulus terrestris*, *Allium sativum*, *Salvia officinalis* and *Allium hirtifolium* Boiss are commonly found in Iran. Garlic (*A. sativum*) has been extensively advised as an antimicrobial agent in medical folklore. It was shown that this herbal is capable of inhibiting many Gram-negative and Gram-positive pathogens including some vancomycin-resistant enterococci and methicillin-resistant *Staphylococcus aureus*. In addition, it has been demonstrated that garlic has strong antifungal properties against pathogens, such as *Candida*, *Aspergillus* and *Cryptococcus*. Likewise, it exhibits antimicrobial activity against many parasites including *Leptomonas, Leishmania, Trypanosoma, Trichomonas,* and *Entamoeba*, and was shown to be effective in the treatment of giardiasis.[8-14]

Tribulus terrestris L. *(Zygophllaceae)* is an annual plant of warm regions in Europe, America, Asia, Africa, and Australia. *T. terrestris* is used in medical folklore as anti-hypertensive, diuretic, tonic, aphrodisiac, analgesic, astringent, stomachic, lithontriptic, and urinary anti-infective agent. Its main constituents are demonstrated to be saponins, diosgenins, alkaloids, and amides.[15]

The antimicrobial activity of *S. officinalis* was recognized to be attributable to the presence of 1,8-cineole, thujone and camphor in high concentrations.[16]

Shallot (*Allium ascalonicum* L.) is frequently used in many Asian diets and in traditional medicine since ancient times and is considered to be one of the most important *Allium* species.

Contrary to its widespread consumption, there are few reports concerning the biological effects of shallot in the literature in comparison to other *Allium* species such as garlic and onion.

Shallot is well known for its hypocholesterolemic and antimicrobial effects.[17] Recently, it has been shown that shallot can exhibit antioxidant effects in scavenging free radicals.[18] These effects can be attributed to high contents of flavone, sulfur-containing compounds, and polyphenolic derivatives in the bulb of shallot. On the other hand, it has been shown that even some properties of shallot are superior to numerous onion varieties as well as some garlic preparations.[18]

Objective

Because of lack of pertinent data, the aim of this study was to evaluate the effect of Iranian species of the aforementioned plants on E. *faecalis*. In addition, historically the herbal extracts were boiled before administration.[19] Temperature also had variable results, for instance, extract of *Mangifera indica* increased its antibacterial effect,[19] whereas heating the garlic extract eliminated its antimicrobial effect against certain bacterial species.[20] Therefore, at the second stage the effect of temperature was assessed on the antibacterial properties of the herbal extracts.

Methods and Materials

Preparation of Plant Extracts

The bulbs of *A. hirtifolium* Boiss and *A. sativum*, the aerial part of *S. officinalis*, and the fruit of *T. terrestris* was obtained from the Iranian eastern city of Mashhad and was confirmed by Kerman University of Medical Sciences, Department of Pharmacognosy. Five hundred grams of each plant were grounded and suspended in 500 mL of distilled water for 24 hours. The mixture was then filtered through a Whatman Grade 1 filter paper. Some of the extracts were freshly used and some were dried in an oven at 50°C and stored in a cool room at 4°C.

In order to evaluate the antimicrobial effect, 160 mg of each dried extract was dissolved in 2 mL sterilized distilled water before being tested for antimicrobial efficacy. The dried extract was autoclaved at 121°C for 15 minutes and evaluated for antibacterial efficacy as heated extracts of *T. terrestris*, *A. sativum*, and *A. hirtifolium* Boiss.

Fresh *A. sativum* extract was taken from 500 g garlic bulb using a fruit juicer and filtered by a Whatman Grade 1 filter paper. In order to prepare fresh *A. sativum* extract, 500 g of garlic bulb was placed in a fruit juicer and the resultant juice was filtered with a Whatman Grade 1 filter paper. The filtered extract was used as the fresh *A. sativum* extract. An aliquot of the extract was heated at 121°C for 15 minutes using the aforementioned pressure autoclave for further evaluations as heated *A. sativum* extract.[21]

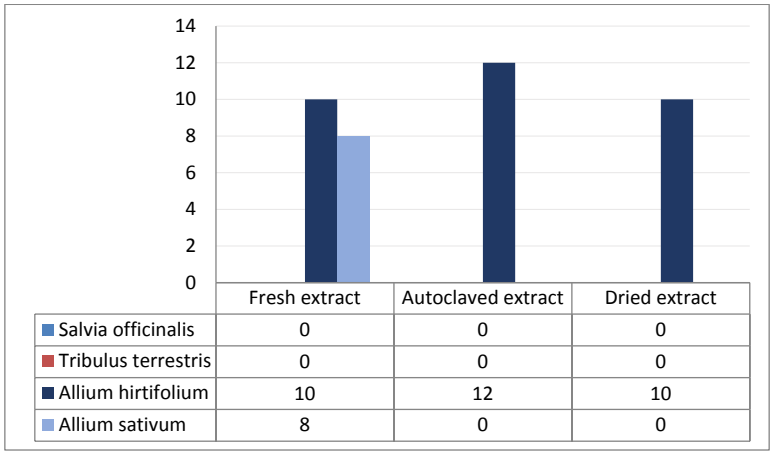
Microorganism and its Maintenance

Antibacterial assessment of aqueous extracts of the abovementioned plants were made by agar disc diffusion, using *E. faecalis* (PTCC 1394) obtained from the Persian Type Culture Collection. The bacterium was stored in trypticase soy broth containing 25% (v/v) glycerol (Merck, Germany) and refreshed on Mueller- Hinton agar (MHA) media (Hi media, India) before use.

Determination of Minimal Inhibitory Concentration

The freshly grown culture of *E. faecalis* was prepared in sterile saline and adjusted to a density of 10^6 cell mL^{-1}.

The plate of culture media (MHA) was inoculated by dipping a sterile cotton swab into the cell suspension and streaking it across the surface of the agar in three directions, and dried at ambient temperature for 15 minutes. Then, 8 sterile discs, 6 mm in diameter, were kept on the agar surface in a line. The aqueous extract of *A. hirtifolium* Boiss was serially diluted in water; and 10 µL of each dilution was separately used to impregnate the disc. The plate was incubated for 24 hours at 37°C. The minimal inhibitory concentration (MIC) values were read as the antimicrobial concentration at the point where dense colonial growth intersected the disc. The test was performed in quadruplicate for the tested bacterium.[22]



	Fresh extract	Autoclaved extract	Dried extract
Salvia officinalis	0	0	0
Tribulus terrestris	0	0	0
Allium hirtifolium	10	12	10
Allium sativum	8	0	0

Figure 1. The Antibacterial Effects of Different Plant Extracts Against *Enterococcus faecalis*.

Results

The obtained results showed that the extract of *A. hirtifolium* Boiss inhibited the growth of *E. faecalis* (MIC 10 mg/mL). There was no significant difference between autoclaved, dried and fresh extracts. The antimicrobial activity of *A. sativum* fresh extract was more effective against target bacterium (MIC = 8 mg/mL), however the autoclaved and dried extract lost its activity in those conditions. Other tested plants had no effect on the target bacterium (Figure 1).

Discussion

In contrast to other studies on plant extracts, the aqueous extract of *A. hirtifolium* Boiss showed an acceptable antimicrobial activity against *E. faecalis* even after being autoclaved. This was in accordance with the study results of Ghahremani-majd and colleagues.[23] They studied the antibacterial and antioxidant activities of *A. hirtifolium* Boiss extract, though they did not investigate the effect of heat on such properties. Allicin, diallyl disulphide and diallyl trisulphide are considered the major active components of *Allium* species and these substances are unstable and changeable to other products. There are components in these plants, such as polyphenols which are thermostable.[24-26] Contrary to *A. sativum*, the antimicrobial effect of *A. hirtifolium* Boiss extract on *E. faecalis* was maintained after heat treatment. This effect can be attributed to the pivotal role of polar compounds of *A. hirtifolium* Boiss extract such as polyphenols. This can be considered an area of interest for future investigations. Despite few number of similar studies on *A. hirtifolium* Boiss, a relevant investigation by Mohammadi-motlagh et al showed that heating could not alter the angiogenic effect of *A. hirtifolium* Boiss extract.[27]

Amin et al[28] demonstrated that *A. hirtifolium* Boiss extract maintained its antibacterial efficacy following pH changes from 4 to 8. It has been confirmed by several studies in which calcium hydroxide was ineffective when used as an intracanal medicament in elimination of *E. faecalis*.[2,6] This is mainly attributed to passive diffusion proton pump in *E. faecalis* cell membrane. Additionally, presence of dentin, as an agent for reducing calcium hydroxide alkalinity, eliminates its antimicrobial efficacy.[2,6] Maintenance of antibacterial efficacy of *A. hirtifolium* Boiss extract in pH values of 4 to 8 may overcome the defensive mechanism of *E. faecalis*. In previous investigations,[20,28-31] the effect of *A. hirtifolium* Boiss extract against bacterial species (such as *S. aureus, Listeria monocytogenes, Bacillus cereus, Serratia marcescens, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi,* and *Proteus mirabilis*) as well as fungal species (such as *Microsporum gypseum, Aureobasidium pullulans, Trichophyton mentagrophyte, Trichophyton rubrum, Fusarium oxysporum, Saccharomyces cerevisiae, Aspergillus niger, Aspergillus flavus, Aspergillus fumigatus,* and *Candida albicans*) has been studied; although, the results of these studies are obtained in vitro and cannot be easily extrapolated to in vivo conditions. These primary data can show that this extract can be used as intracanal medicament for further investigations. Surprisingly, Amin et al showed that *A. hirtifolium* Boiss extract maintained its antibacterial effect in variable temperatures, pH values, and in the presence of different enzymes and detergents. Its antimicrobial effect was constant from 1 to 6 months at 4°C.[28]

Contrary to the current study and other similar articles,[32] some authors stated that all parts of *T. terrestris* were effective against *E. faecalis*.[33,34] This result can be related to different methods in obtaining plant extracts, microbial species, and climate conditions in which plants have been cultivated.[16]

Several investigations have evaluated the antibacterial effect of *S. officinalis* with promising results.[16,35-38] Horiuchi et al demonstrated a synergistic effect of *S. officinalis* extract with aminoglycosides in elimination of certain infections.[39,40] On the contrary, some studies did indicate no antibacterial activity against *E. coli* and *P. aeruginosa*.[41] In general, according to the available literature, the effect of *S. officinalis* extract against *E. faecalis* is controversial.[16,33,42,43] Several Iranian papers have shown that *S. officinalis* extract possesses antimicrobial efficacy[44,45] without pointing to its specific

effect on *E. faecalis*. The current investigation revealed that the Iranian species of *S. officinalis* were not effective on *E. faecalis* which was congruent with the study by de Oliveira et al.[43]

In this study it was concluded that the fresh extract of *A. sativum* had an inhibitory activity against the growth of *E. faecalis* (MIC = 8 mg/mL). This finding was in accordance with the studies by Ruddock et al and Ruiz et al who worked on the effect of *A. sativum* extract on *E. coli*, *S. typhimurium*, *Neisseria gonorrhoeae*, *S. aureus*, and *E. faecalis*.[46,47] Our study revealed that the autoclave temperature eliminated the antibacterial effect of *A. sativum* extract. However, this effect was not considered in the studies by Ruddock et al and Ruiz et al. This can be related to the heat-induced destruction of allicin, the thermolabile antibacterial compound in garlic.[48-50] Correspondingly, Daka showed that autoclaving garlic extract reduced its antibacterial effect against *S. aureus*.[24] Additionally, Ali et al[51] stated that heat could diminish anti-platelet effect of *A. sativum*. The MIC of *A. sativum* extract against *E. faecalis* (8 mg/mL) was lower than those seen in the studies by Bakri and Douglas[52] (71.4 mg/mL) and Srinivasan et al[32] (33 mg/mL). According to Ruddock et al, the garlic natural health products with more allicin content had stronger antibacterial effects.[46] Therefore, it can be stated that allicin content in Iranian garlic species is higher than that in English and Indian species used in the aforementioned studies.[43,46,52] In other words, it can be related to the geographical and climate conditions in which these plants are cultivated.

Conclusion

It can be concluded that different types of *A. hirtifolium* Boiss extract had stronger, more prominent and thermally stable antimicrobial activity against *E. faecalis* in comparison to other extracts.

Authors' Contributions

All authors passed four criteria for authorship contribution based on recommendations of the International Committee of Medical Journal Editor.

Ethical Approval

The Ethics Committee of Ahvaz Jundishapur University of Medical Sciences approved the study.

Conflict of Interest Disclosures

The authors declare that they have no conflict of interests.

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