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Evaluation of in Vitro Antimicrobial Effects of Aqueous Extract of *Tribulus terrestris* Against Oral Bacteria

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ABSTRACT

Background and objectives: Medicinal plants have long been considered as one of the most important pillars of traditional medicine. Existing challenges in the treatment of diseases, particularly infectious diseases, are major drivers for herbal medicine studies. *Tribulus terrestris* has been widely used in traditional medicine to treat various diseases. This study aimed to investigate in vitro antibacterial effect of the aqueous extract of *T. terrestris* on several oral bacteria.

Methods: In this experimental study, after preparing the aqueous extract of *T. terrestris*, minimum inhibitory and bactericidal concentrations (MIC and MBC) of the extract were determined against standard strains of *Streptococcus mutans*, *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Streptococcus pyogenes* using the broth microdilution method. The experiments were repeated three times and the results were analyzed with SPSS 22 using the one-way analysis of variance (ANOVA) and LSD statistical tests with the significance level set at 0.05.

Results: The aqueous extract of *T. terrestris* had the highest inhibitory effect on *S. pyogenes* and *S. mutans*, and the difference between the MIC and MBC values was significant ($P < 0.05$). However, no such effect was observed against *S. aureus* and *K. pneumonia* at concentrations below 50 mg/ml when compared to ampicillin and chlorhexidine.

Conclusion: The aqueous extract of *T. terrestris* has significant antibacterial effects against *S. pyogenes* and *S. mutans*. Therefore, it can be incorporated into topical formulations such as toothpaste and mouthwash products after further in vivo and toxicity experiments.

Keywords: Tribulus, Anti-Bacterial Agents, bacteria.

INTRODUCTION

Nowadays, emergence of drug-resistant infectious diseases due to the inappropriate use of antibiotics is an important public health challenge. Alternative methods such as the use of bacteriocins, medicinal plants essential oils, antibodies, phage therapy, quorum-sensing inhibitors, and nanotherapy have been suggested to tackle this problem (1, 2). Medicinal plants have long been considered as one of the most important pillars of traditional medicine around the world. It is estimated that almost 80% of the world's population depends on herbal products for healthcare needs, especially in developing countries (3). Medicinal plants have several bioactive compounds such as flavonoids, terpenes and terpinoids, which have shown antibacterial, anti-inflammatory, anticancer and antiviral effects (4-7). The mechanisms through which medicinal plants kill bacteria are different than antibiotics. This is a clinically significant advantage in the treatment of resistant infections (8, 9). *Tribulus terrestris*, commonly known as puncture vine, is a medicinal plant belonging to the *Zygophyllaceae* family. It is one of the native plants of the southeastern region of Iran that grows in tropical and arid regions. The plant contains bioactive compound such as triterpene glycosides (saponins), flavonoids, alkaloids and tannins that have been shown to have diuretic, aphrodisiac, anti-urolithic, immunomodulatory, hypolipidemic, cardioprotective, antidepressant, anxiolytic, hepatoprotective, anti-inflammatory, analgesic, antispasmodic, anticancer and antibacterial effects (10-12).

As the most prevalent chronic infection of oral cavity, periodontitis and dental caries have serious health and economic burdens. They are caused by deregulation of oral microbiota and subsequently not only affects oral health, but also correlates with some systemic diseases, such as diabetes, cancer and atherosclerosis, which indicates that the prevention and treatment of dental caries are important to attenuate this global health risk (13-15). *Streptococcus mutans* is a common resident microflora in dental plaque and also known as the main cause of dental caries. *Staphylococcus aureus* is a transient microflora of nasal mucosa but sometimes leads to purulent infections, systemic infections as well as nosocomial infections. *Klebsiella pneumoniae* is a transient normal microflora of the nasopharynx that may cause a type of pulmonary pneumonia. *Streptococcus pyogenes* is another transient normal flora of the upper respiratory tract and the main causative agent of purulent sore throat (16-18). Due to the presence of active compounds in *T. terrestris* and their potential antibacterial effects, in this study, we evaluated the in vitro effects of aqueous extract of the plant against the above mentioned oral bacteria to find scientific evidence for the production of a cheap and effective topical formulation of *T. terrestris*.

MATERIALS AND METHODS

The fruit of *T. terrestris* was purchased from a traditional market in Birjand, Iran, and authenticated by an expert botanist (Department of Botany, University of Birjand, Iran). It was washed, shade-dried and then powdered. To obtain an aqueous extract, 100 g of the powder were added to 1000 ml of boiling distilled water and boiled for 30 minutes. The solution was filtered through a filter paper (Whatman paper No. 41) and lyophilized using a vacuum freeze dryer (Dena Vacuum Industry, model FD-5005-BT, Iran). Then, the lyophilized extract was dissolved in autoclaved distilled water and passed through a 0.45 µm filter (19).

Four standard strains of different bacterial species including *S. aureus* (ATCC 29213), *K. pneumoniae* (ATCC 700603), *S. mutans* (ATCC 35668) and *S. pyogenes* (ATCC 10403) were purchased from the Pasteur Institute of Iran (Tehran, Iran). After preparation of bacterial suspensions, these suspensions were placed in cryotube and kept at -70 °C until use. To regenerate each strain of bacteria, we took a bullet impregnated with the desired bacteria from each cryotube and placed it in a test tube containing 3 ml of nutrient broth medium (Merck Co., Germany). Then, the tubes were incubated at 37 °C for 24 hours. After regenerating the bacterial strains, they were cultured on nutrient agar and blood agar and then isolated as pure colonies. These colonies were used to prepare microbial suspensions with a concentration of 0.5 McFarland (1.5 X 10^8 cfu/ml). To grow *S. mutans* and *S. pyogenes*, they were incubated in candle jars.

We used the latest edition of the Clinical and Laboratory *Standards* Institute(CLSI) guidelines to determine minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of the extract on the studied bacteria (20). To prepare the required concentrations of the extract, we dissolved 100 mg of the extract in 1 ml of sterile distilled water. The resulting solution was sterilized using a 0.45 µm filter paper and stored in a sterile, dark glass container at 3 °C until use. The MIC values were determined using the broth microdilution method (21).

Different dilutions were prepared from the *T. terrestris* extract. From each dilution, 100 µl were added to wells of a sterile 96-well microplate (Microteb Co., Iran) containing 100 µl of bacterial suspension in Mueller Hinton broth (Merck Co., Germany) with a concentration equal to half the McFarland standard. A well containing only medium and another containing only the extract were considered as negative control. Wells containing the bacterial suspension and the medium were considered as positive controls. The experiments were performed with chlorhexidine and ampicillin to compare data.

After inoculating the bacteria, the microplate was shaken for 30 seconds to make the mixture completely uniform.

In the last step, the microplate was incubated at 37 °C for 24 hours. After incubation, turbidity of the wells was observed visually. The lowest concentration of extract that inhibited the growth of bacteria was determined as the MIC.

The experiment was performed in triplicate. To determine the MBC of the extract, 10 µl were taken from turbid wells (MIC concentration and above) in sterile condition and inoculated on Mueller Hinton agar (Merck Co., Germany). Streptococci were inoculated on blood agar (Merck Co., Germany). After 24 hours of incubation at 37 °C, the lowest dilution in which no colony appeared was determined as the MBC (22). All experiments were repeated three times.

Data were expressed as mean ± standard deviation (SD). Statistical analyses were performed by SPSS Statistics 22.0 (IBM SPSS Statistics for Windows, IBM Corp, USA). Data were compared using the one-way analysis of variance (ANOVA) and LSD tests. P-values < 0.05 were considered statistically significant.

RESULTS

The aqueous extract of *T. terrestris* showed antibacterial effects against the selected bacteria. As shown in table 1, *S. pyogenes* was most sensitive to the extract with MIC and MBC values of 8.33 ± 2.89 and 16.67 ± 5.77, respectively. MIC and MBC values against *S. mutans* were 43.33 ± 5.77 and 46.67 ± 5.77, respectively. Bacterial growth was not observed in the wells containing the extract and culture medium as well as the culture medium alone (negative control). However, growth was observed in all wells containing the culture medium and the bacterial suspension (Positive control), which confirms the accuracy of the experiments.

Table 1- The MIC and MBC of the aqueous extract of *T. terrestris*, chlorhexidine and ampicillin on some oral pathogenic bacteria

	T. terrestris		**Chlorhexidine**		**Ampicillin**	
Microorganism	**MIC** ($\frac{mg}{ml}$)	**MBC** ($\frac{mg}{ml}$)	**MIC** ($\frac{\mu I}{ml}$)	**MBC** ($\frac{\mu B}{ml}$)	**MIC** ($\frac{\mu I}{ml}$)	**MBC** ($\frac{\mu B}{ml}$)
S. pyogenes	**8.33 ± 2.89**	**16.67 ± 5.77**	**500**	**500**	**0.25**	**0.25**
S. mutans	**43.33 ± 5.77**	**46.67 ± 5.77**	**500**	**500**	**3**	**3**
S. aureus	**>50**	**>50**	**8**	**8**	**32**	**32**
K. pneumoniae	**>50**	**>50**	**8**	**16**	**64**	**64**

DISCUSSION

In this study, we examined the antibacterial effect of the aqueous extract of *T. terrestris* against several normal microflora and pathogenic bacteria of the oral cavity. Based on the findings, the aqueous extract of this plant had significant antibacterial effects on standard strains of the tested bacteria. The results indicated that the extract was more effective against *S. pyogenes* (MIC=8.33 mg/ml) and *S. mutans* (MIC=43.33 mg/ml). Al-Bayati et al. investigated the effects of aqueous, ethanolic and chloroform extracts of different parts of the *Iraqis T. terrestris* on some gram-positive and gram-negative bacteria. They reported that the aqueous extract of the plant had significant antibacterial effects on most of the bacteria tested, such as *S. aureus* (MIC=2.5 mg/ml) and *K. pneumoniae* (2.5 mg/ml). The lowest MIC values were observed for the ethanolic extract of fruit, leaf and root of the plant (23). The difference between the MIC value in our study and that in the Al-Bayati et al. may be due to the different geographical locations of the plant, as well as the different strains of bacteria in the two studies. Soleimanpour et al. reported the antibacterial effects of ethanolic extract of *T. terrestris* against some bacteria, including of *S. mutans* (MIC and MBC=25 mg/ml) and *S. aureus* (MIC and MBC=50 mg/ml). They also reported the MIC and MBC values of chlorhexidine to be 0.0625 mg/ml and 0.125 mg/ml, respectively (24). The inconsistency between the results of their study and ours could be due to the use of different types of extracts.

Hakemi-Vala et al. examined the effects of the total aqueous and dimethyl sulfoxide extracts and phytophenol fraction (Benzoxazine derivative) of *T. terrestris* on some bacteria. The total extract showed a good antibacterial effect against *P. aeruginosa* (MIC=125 mg/ml), *E. coli* (MIC=62.5 mg/ml) and *B. subtilis* (MIC=500 mg/ml) but had no effect against *S. aureus*, *S. epidermidis*, *P. aeruginosa*, *K. pneumonia*, and *Candida albicans*. They also showed that the antibacterial effect was not due to the benzoxazine derivatives of the plant (25) .Antibacterial effects of *T. terrestris* may be due to bioactive compounds such as frostanol, spirostanol, saponins, tigogenin, etc. By separating saponins from *T. terrestris* and performing antibacterial assays, Mohammed *et al.* showed that *saponins* can inhibit the growth of *S. aureus* and *K. pneumonia* by the disk diffusion method. They suggested that these compounds alter the surface tension of the bacterial outer membrane, causing changes in the membrane and killing the microorganism. In addition, due to their free carbonyl group, flavonoids can react with proteins on the surface of the bacterial membrane and kill the microorganism. The biological content of *T. terrestris* may vary in different geographical regions (10, 26, 27).

With the rapid emergence of antimicrobial-resistant microorganisms, there is an urgent need to implement some strategies including antibiotic stewardship programs and find alternatives for antibiotics. Indeed, medicinal plants have bioactive compounds with different mechanisms of action that have great potential to provide effective, biocompatible and economical solutions to accelerate the development of antimicrobial drugs. Therefore, more research is needed to provide a comprehensive knowledge of medicinal plants.

CONCLUSION

This study showed that *T. terrestris* has significant antibacterial effects against *S. pyogenes* and *S. mutans*. Therefore, it can be incorporated into topical formulations such as toothpaste and mouthwash products after further *in vivo* and toxicity experiments.

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ETHICS APPROVAL

This study was approved by the ethics committee of Birjand University of Medical Sciences, Iran (IR.BUMS.REC.1400.056)

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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