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Antibacterial Activity of *Trachyspermum ammi* Essential Oil Against *Streptococcus mutans* Isolated from Human Dental Plaques

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ABSTRACT

Introduction: Mutans Streptococci are significant pathogens isolated from dental plaques. Due to the increasing frequency of antimicrobial resistance among pathogens causing dental caries, more studies have focused on using natural agents against them. This study aimed to evaluate the antibacterial effects of *Trachyspermum ammi* essential oil against *Streptococcus mutans* isolated from dental plaques. **Methods:** Twenty human dental plaque samples were collected, and eight *S. mutans* isolates were detected using biochemical and molecular tests. The antibiotic susceptibility patterns of the isolates were determined by the disc diffusion method. Also, *T. ammi* essential oil antibacterial activity was investigated using the disc diffusion method and determining minimum inhibitory concentration (MIC50) and minimum bactericidal concentration (MBC) values. **Results:** Phenotypic and genotypic characterization identified *S. mutans* in 40% of the samples. The antibiotic susceptibility assay revealed the highest resistance patterns against cefotaxime (100%), ceftriaxone (100%), and penicillin (87.5%). The *T. ammi* essential oil demonstrated 20 µg/ml MIC and 80µg/ml MBC against *S. mutans*. **Conclusion:** The present study revealed a potent antibacterial activity for *T. ammi* essential oil against *S. mutans* isolates.

INTRODUCTION

Dental caries is a significant public health problem worldwide [1]. Among oral Streptococci, the mutans group, *Streptococcus mutans*, and *Streptococcus sobrinus* are the most frequent bacterial pathogens isolated from dental plaque. These species are the primary etiologic agents of dental caries [2]. According to epidemiological reports, *S. mutans* is more prevalent than *S. sobrinus* in dental plaques, while *S. sobrinus* show more association with dental carries incidence. A significant virulence factor of *S. mutans* is its capacity to metabolize and process different sugar carbohydrates and use them to produce biofilms on the tooth enamel surface [3]. These biofilms usually contain surface-associated microbial cells enclosed in a 3D extracellular polymeric substance matrix. The matrix provides a stable and complex chemical microenvironment essential for biofilm formation and defense against the host protein interactions, enhancing bacteria tolerance to antimicrobial agents [4]. The carbohydrate metabolism and biofilm development process reduces pH in the oral cavity, resulting in the demineralization of hydroxyapatite, enamel dissolution, and carious lesions [5]. Accurate detection of oral Streptococci has been a significant parameter as it directly affects treatment strategies. During the last decades, various methods were developed for detecting *S. mutans* and *S. sobrinus*, including culture, microscopy, biochemical tests, enzyme-linked immunosorbent assays, polymerase chain reaction, and restriction fragment length polymorphism (PCR-RFLP), PCR-based species-specific primers, and PCR-based 16S rRNA gene [6].

As a precautionary measure, dentists usually prescribe antibiotics before beginning treatment to prevent systemic infections after cavity filling or tooth extraction [7]. The antibiotics kill the pathogenic bacteria but cause some adverse effects, such as the overgrowth of antibiotic-resistant bacteria. So, evaluating the antibiotic susceptibility of *S. mutans* species is critical [8]. The side

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effects of chemical and synthetic antimicrobial agents and widespread antibiotic resistance among many bacteria have driven attention to green solutions like medicinal plants that could selectively inhibit pathogens while leaving the commensal microorganisms intact [8]. *Trachyspermum ammi* is one of the members of the Apiaceae plants family. It is native to Egypt but grows in Iraq, Iran, Afghanistan, Pakistan, and India [9]. *T. ammi* seeds contain brown oil named *T. ammi* oil which contains fiber, minerals, vitamins, and antioxidants. However, the main component of this oil is thymol [10], a significant antibacterial among the herbal essence components [11].

This study investigated pathogenic bacteria involved in dental caries and evaluated the chemical composition and antibacterial activity of *T. ammi* essential oil on these bacteria.

MATERIAL AND METHODS

Isolation of Streptococci from dental plaques. Twenty plaque samples were collected from people referred to the Faculty of Dentistry, Tabriz University of Medical Sciences, Tabriz, Iran. All patients filled informed consent form, and all ethical standards have been respected in this study (approval reference No.: 2309894, Tabriz University of Medical Sciences). Dental plaque samples were collected from the labial surfaces of the upper primary central incisors using sterile toothpicks. A small visible amount of plaque on the tip of the toothpick was transferred to a sterile test tube. The plaque was washed with sterile PBS twice and centrifuged, and the supernatant was spread onto Mitis- Salivarius (MS) agar plates (Sigma Aldrich, USA) with (0.2U ml^{-1} bacitracin and 15% (W/V) sucrose (MSB plates) for two days at 37 ºC [12].

Bacterial identification. Pure cultures of each isolate on the MSB agar plate were identified by Gram staining, catalase test, and biochemical reactions, including mannitol, inulin, salicin, and raffinose fermentation tests [2].

Molecular differentiation of *S. mutans* and *S. sobrinus*. According to the manufacturer's instructions, DNA from isolates was extracted using a DNA extraction kit (CinnaGen, Iran). A PCR assay was used to target the *gtfB* gene from *S. mutans* and the *gtfI* gene from *S. sobrinus* (Table 1). Sequences of genes used were obtained from the GenBank database (acc. Nos. M17361 for *gtfB* and D90213 for *gtfI*). Twenty-five µl reactions comprised a ready-to-use master mix (Reddy, Sinaclone, Iran), and *S mutans* ATCC 25175 and *S. sobrinus* ATCC 33478 were included in the assays as positive controls. The amplicons were resolved by electrophoresis in 1.5% agarose gels [13].

Antimicrobial sensitivity testing. Once single *S. mutans* was isolated and identified from each sample, the standard Kirby- Bauer disk diffusion test was performed according to CLSI to determine the antibiotic susceptibility patterns of the isolates. Antibiotic disks included Cefotaxime (30µg), Erythromycin (15 µg), Penicillin (10 unit), Chloramphenicol (30 µg), Tetracycline (30 µg), Ceftriaxone (30 µg), Vancomycin (30 µg), and Azithromycin (15 µg) **(**Padtanteb, Iran)**.** [14, 15].

Plant sample. *T. ammi* seeds were purchased from the spicery store in Tabriz, East Azerbaijan province, Iran.

Extracting *T. ammi* essential oil. The essential oil was extracted by hydrodistillation of 100 g of dried seeds using Clevenger-type apparatus for three hours. As a collector solvent, 10 ml of diethyl ether was used. After evaporating the solvent, the essential oil was dried using anhydrous sodium sulfate and stored at -20 ºC [16].

GC-MS analysis. *T. ammi* essential oil ingredients were determined by gas chromatography-mass spectrometry (GC-MS). (Agilent 6890 gas chromatography equipped with Agilent 5973 mas selective detector, USA). The chromatograph had HP-5MS capillary column (30×0.2mm ID × 0.2µm film thickness). Data were acquired under the following conditions: Initial 70 ºC holding for 2 min, then increased to 220 ºC at a rate of 4 ºC. The injector temperature was 290 ºC, helium was used as the carrier gas, the split ratio was 0.8 ml-1 per min, and the final temperature was 300 ºC (holding for 2 min) [17].

Antibacterial activity screening. The sensitivity of the isolated bacteria to *T. ammi* essential oil was investigated using a broth microdilution assay. For minimal inhibitory concentration (MIC) determination, a stock solution of essential oil was prepared in dimethyl sulfoxide (DMSO; oil to DMSO ratio1:3). Then, serial two-fold dilutions of *T. ammi* essential oil in concentrations ranging from 80 to 0.036 µg/ml were added to the tubes containing 1 ml sterile Mueller Hinton broth tubes [18]. The bacterial strains were cultured for 12 h in nutrient broth media. Then the turbidity of suspensions was adjusted to 0.5 Mc-Farland standards. The 96-well plates were used in this study. Amounts of 95 µl of nutrient broth and 5 µl of microbial suspension of 10^9 CFU/mL were added to the plate, followed by 100 µl of the essential oil with the above concentrations. The last well of the plate contained 195 µl of nutrient broth medium and 5 µl of bacterial suspension with no essential oil as the negative control. Contents of each well were mixed on a plate shaker at 300 rpm for 20 s, then incubated anaerobically for 24 h at 37 ºC. Microbial growth was confirmed by absorbance at 600 nm by applying a microplate reader (Biotech Instrument, Highland Park, Vermont, USA) [19].

For minimal bactericidal concentration (MBC) determination, 10 µl of each negative well sample was cultivated anaerobically on a nutrient agar plate at 37 ºC for 24 h. The MIC and MBC were defined as the lowest concentration of the *T. ammi* essential oil, which inhibited the growth of Streptococcus isolates [16].

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Disc diffusion test. The Petri plates with 15 0 mm sterile Mueller Hinton agar were prepared. After culturing the bacteria in BHI broth for 24 h, the turbidity of bacterial suspensions was adjusted to 0.5 McFarland standard and then cultured on the Mueller Hinton agar plates. Sterile discs were saturated with 10 µl of different antimicrobials and serial concentrations of sterile essential oil ranging from 10-80µg/ml and placed on the plates, followed by incubation anaerobically at 37 ºC for 18-20 h. The inhibition zones were measured in millimeters and compared with each other [13, 20].

Statistical analysis. Descriptive statistics, such as the frequency of *S. mutans* isolates from dental plaques, and the main ingredients of *T. ammi* essence, were performed using SPSS 15.0. The mean values ± standard deviations were calculated. The data were analyzed by the one-way ANOVA method. Analysis of variance (ANOVA) was performed based on mean values to determine the significant difference between different concentrations of essential oil at $P \leq 0.05$.

RESULTS

Isolation and biochemical characterization of *S. mutans*. Among the 20 human dental plaque samples, 16 grew on MSB agar. According to the Gram staining, catalase test, and biochemical reactions, eight of these isolates were catalase-negative and Gram-positive cocci which could ferment mannitol, inulin, salicin, and raffinose. These characteristics belong to the *S. mutans.* However, *S. sobrinus* is a Gram-positive and catalase-negative coccus that could ferment mannitol and inulin but not raffinose and salicin. Hence, none of the isolates were *S. sobrinus*, while *S. mutans* was isolated from 40% of human dental plaques.

Molecular detection of isolates. The identity of the eight *S. mutans* isolates was confirmed via PCR amplification of 517-bp fragments of the *gtfB* gene. None of the isolates yielded a 712-bp fragment belonging to the *S. sobrinus gtfI* gene (Fig. 1).

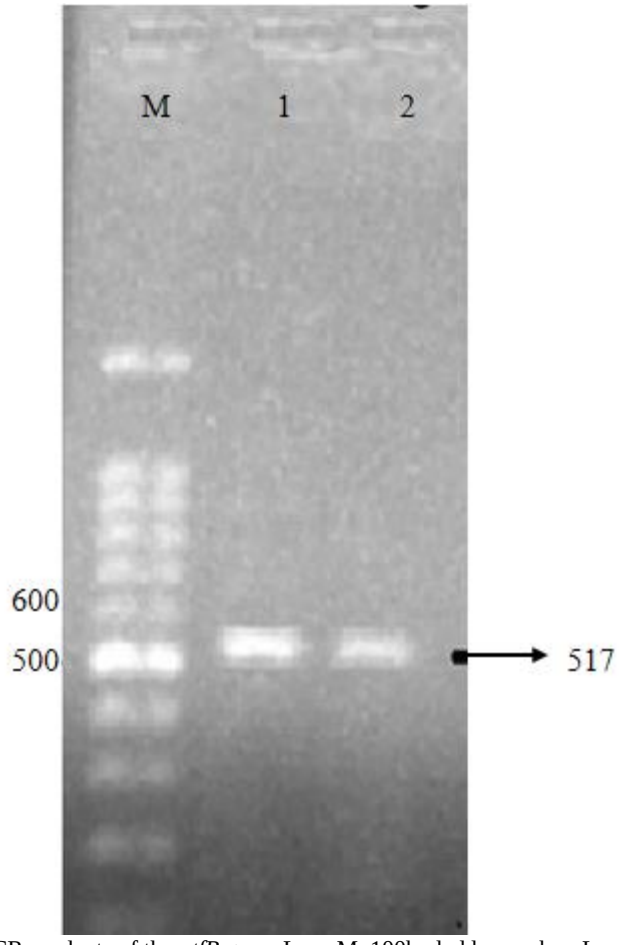

Fig. 1. Gel electrophoresis of the PCR products of the *gtfB* gene, Lane M, 100bp ladder marker; Lane 1, positive control; lane 2, the 517-bp fragment *of gtfB* gene

Antibiotic susceptibility patterns of the isolates. The isolates showed the highest resistance to cefotaxime (100%), ceftriaxone (100%), and penicillin (87.5%), and the highest susceptibility to tetracycline (0%) and erythromycin (12.5%) (Table2)

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Table 1. Primers and amplification conditions utilized for molecular detection of *S. mutans* and *S. sobrinus.*

Genes	Sequences	PCR conditions	Amplicon size bp	References
gtfB	F5′- ACTACACTTTCGGGTGGCTTGG-3´ R 5′- CAGTATAAGCGCCAGTTTCATC-3´	95 °C 4 min; 30 cycles (95 °C 30 s; 59 °C 30 s; 72 °C 1 min), 72 °C 5 min.	517	13
gtfI	F 5′- GATAACTACCTGACAGCTGACT-3´ R 5′- AAGCTGCCTTAAGGTAATCACT-3´		712	13

Table 2. The antibiotic susceptibility pattern of *S. mutant* isolates originated from human dental plaque

Antibiotics	Number of tested isolates	Percentage of resistant strains
Ceftriaxone (CRO)	8	100%
Cefotaxime (CTX)	8	100%
Penicillin (P)	8	87.5%
Azithromycin (AZM)	8	62.5%
Chloramphenicol (C)	8	25%
Vancomycin (V)	8	25%
Erythromycin (E)	8	12.5%
Tetracycline (TE)	8	0%

GC-MS analysis. Thirty-one compounds were detected in the oil, representing 94.48% of the entire oil. The main ingredients were phenol (42.26%), benzene methyl (23.11%), γ-terpinene (19.69%), and thymol (7.75%).

Antibacterial activity. The MIC and MBC values of the *T. ammi* essence and eight different antimicrobials for *Streptococcus* isolates are summarized in table 3. The *T. ammi* essence was more effective in inhibiting the growth of selected isolates than all antibiotics tested except

for Erythromycin and Tetracycline. Among different antimicrobials tested, Ceftriaxone, Cefotaxime, and Penicillin showed no bactericidal properties against selected isolates. However, the MBC value of *T. ammi* was like Azithromycin, Chloramphenicol, and Vancomycin. Moreover, Erythromycin and Tetracycline were more successful than *T. ammi* oil in killing bacterial isolates (Table 3).

Additionally, the mean±SD of zone inhibition for different concentrations of *T. ammi* oil and different antimicrobials has been shown in table 3. The inhibition zones of all concentrations of *T. ammi* oil were larger than Ceftriaxone, Cefotaxime, Penicillin, Azithromycin, and Chloramphenicol. However, they were all lower than the Tetracycline inhibition zone (Table 4).

Table 3. The effect of *T. ammi* essential oil and different antimicrobials on the *S. mutans* isolates

No of isolates	MIC (µg/ml)	MBC (µg/ml)
T. ammi	20	80
Ceftriaxone	80	-
Cefotaxime	60	-
Penicillin	60	-
Azitromycin	40	80
Chloramphenicol	40	80
Vancomycin	40	80
Erythromycin	20	60
Tetracycline	10	40

Table 4. Mean±SD of zone inhibition for *T. ammi* oil and different antimicrobials

Antimicrobial	Inhibition zone (mm)
T.ammi (80 µg/ml)	78.3
T.ammi(40 µg/ml)	67.0
T.ammi(20 µg/ml)	47.6
T.ammi(10 µg/ml)	42.4
Ceftriaxone	17.5
Cefotaxime	18.5
Penicillin	18.2
Azithromycin	15.7
Chloramphenicol	34.1
Vancomycin	43.7
Erythromycin	55.2
Tetracycline	88.1

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DISCUSSION

This study identified *S. mutans* isolated from human dental plaque using biochemical and molecular methods and investigated their antimicrobial resistance patterns. Also, the antibacterial property of *T. ammi* essential oil was assayed against bacterial isolates, and the values of MIC and MBC of essence were determined. Also, the inhibition zone for 4 *T. ammi* essential oil concentrations was determined. A similar study examined the prevalence of *S. mutans* and *S. sobrinus* from dental caries and, based on Gram staining and biochemical assays, identified 41 *S. mutans* and *S. sobrinus* isolates [18]. In another study, phenotypic differentiation between *S. mutans* and *S. sobrinus* was made based on culture on modified SB-20 medium and biochemical assays, with no significant differences between the two methods [21]. The differentiation among mutans group species is difficult, necessitating new methods like PCR. This molecular method is easy, rapid, and reliable for identifying these species. The species *S. mutans* and *S. sobrinus* were detected in dental plaques from preschool children in Brazil using the PCR method; this assay showed a good ability for differentiation between these two species, with results similar to ours [2]. Also, broad-range PCR followed by sequencing was used to identify *S. mutans* from heart valve and dental plaque specimens of people with infective endocarditis [12]. Furthermore, the colonization of *S. mutans* in the dental plaque was detected using standard PCR and quantitative real-time PCR tests. [22]. Medicinal plants have some bioactive compounds that could be precursors for synthesizing therapeutic drugs. They are famous for their low toxicity, pharmacological activities, and economic viability [23]. Essential oils are significant for their antimicrobial properties, including the ones against *S. mutans.* These are secondary metabolites formed by aromatic plants. Their antibacterial activity is due to their complex chemical components, such as terpenes, aromatic, and aliphatic constituents.

Also, investigating the antibacterial activity of twenty herbal essential oils against *S. mutans* showed that *A. gratissima*, *A. triphylla*, *B. dracunculifolia*, *L. sidoides*, *M. glomerata*, *S. guianenses*, *S. aromaticum,* and *C. sativum* had good antibacterial activity and might be used as new antibacterials [19]. The results of our study indicated that *T. ammi* essential oil showed similar antibacterial activity against *S. mutans* compared to the essences mentioned above. *T. ammi* essential oil has components like thymol, p-cymene, and gamma-terpinene, showing some antibacterial activities. The main components of the Iranian and African *T. ammi* essential oil are carvacrol gamma-terpinene and p-cymene, while the major component of south Indian plant oil is thymol. A previous analytical study reported that the essential oil from *T. ammi* mainly contains thymol (37.2%), p-Cymene (32.3%), and gamma-terpinene (27.3%) [24]. Another study detected 14 components, with thymol, γ-terpinene, and ρ-cymene comprising 94%-96% of the essential oil [25]. Here the GC-MS analysis results demonstrated that the major components of *T. ammi* essential oil were phenol, benzene methyl and gamma-terpinene, and thymol. Antimicrobial activities are primarily attributable to the presence of phenolic compounds such as thymol and hydrocarbons like γ-terpinene. These compounds, as the major components of the essential oil in the present study, indicate their prominent role in inhibiting *S. mutans* growth. Different chemical profiles for *T. ammi* oil may be due to differences in the growth stages of plants and the duration of the daylight that could affect the composition and extraction contents of the final essential oil. Such a physiological difference changes the chemical structure of essential oil components and affects their exact mode of action and antibacterial properties. Generally, the most potent antimicrobial activity of *T. ammi* oil is related to the high percentage of phenolic compounds such as carvacrol, eugenol, and thymol. The antimicrobial activity of phenolic compounds disturbs the cytoplasmic membrane, electron flow, active transport, proton motive force, and coagulation of cell contents. Other essential oil components also act differently on cell proteins embedded in the cytoplasmic membrane [26].

Similar studies screened the antibacterial properties of essential oils such as *T. ammi* against bacteria. One study evaluated *T. ammi* essential oil for controlling the *Listeria* monocytogenes infection in fish [27], and another investigated the inhibitory effect of this oil on *E. coli, Klebsiella,* and *Staphylococcus* bacteria. Based on their findings, *T. ammi* essential oil had antibacterial effects against human pathogens, similar to our results [2]. In 2017, the antibacterial effects of *T. ammi* essential oil against mutans Streptococci isolated from dog dental plaques were evaluated. This study indicated *S. mutans* and *S. sobrinus* in 40% and 10% of plaques. Also, MIC50 and MBC values of the essential oil against *S. mutans* isolates were 20 µg/ml and 80 µg/ml, respectively, and the mean ± SD of zone inhibition was 5 ± 31 and 2 ± 10 for 80 µg/ml and 20 µg/ml concentrations of the essential oil, respectively. The result of this study indicated an increase in the inhibition zone with increased concentrations of *T. ammi* essential oil [13]. Another study assessed the antibacterial activity of *T. ammi* oil against the oral microbes using an agar well diffusion assay with three concentrations, 40 µl/ml, 80 µl/ml, and 100 µl/ml. In this study, *T. ammi* oil showed dose-dependent antibacterial activity against *Enterococcus faecalis* and *S. mutans* [28].

We used *T. ammi* essential oil in the present study to control bacteria in dental plaques. According to our findings, *T. ammi* essential oil may be of therapeutic value in treating *S. mutans* isolates from human dental plaque.

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CONFLICT OF INTEREST

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The authors declare that there are no conflicts of interest associated with this manuscript.

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