Dental Research Journal

Original Article

Antibacterial and antibiofilm activities of tribulus terrestris methanolic extract against *Streptococcus mutans*, *Streptococcus sobrinus*, and *Lactobacillus acidophilus*: An *in vitro* study

Ali Azarm[1], Fatemeh Ayoobi[2], Mohammad Zare-Bidaki[3], Mohammad Taheri[4], Ebrahim Rezazadeh Zarandi[3]

[1]Student Research Committee, Rafsanjan University of Medical Sciences, [2]Non-Communicable Diseases Research Center, Rafsanjan University of Medical Sciences, [3]Immunology of Infection of Diseases Research Center, Research Institute of Basic Medical Sciences, Rafsanjan University of Medical Sciences, Rafsanjan, [4]Department of Microbiology, School of Medicine, Hamadan University of Medical Sciences, Hamadan, Iran

ABSTRACT

Background: *Tribulus terrestris* (TT) extract has shown good antibacterial activity against some bacteria. However, there are limited data on its cariogenic properties. This *in vitro* study aimed to evaluate the antibacterial and antibiofilm activities of TT extract against *Streptococcus mutans* (*S. mutans*), *Streptococcus sobrinus* (*S. sorbinus*), and *Lactobacillus acidophilus* (*L. acidophilus*) as the important cariogenic bacteria.

Materials and Methods: This study was designed in an experimental model (*in vitro*). Phytochemical tests were carried out to detect herbal compounds in the TT extract. Agar well diffusion was performed to compare the extract (500–62.5 mg/mL) with different concentrations of chlorhexidine (2–0.25 mg/mL). The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the TT extract and chlorhexidine were also determined. The lowest concentration showing ≥50% inhibition of biofilm formation ($MBIC_{50}$) was determined using crystal violet assay. Further, the time-kill assay (Log of CFU/mL) was performed, and acid production (pH) was measured at 1 × MIC concentration in 2, 4, 8, 12, and 24 h. Data analysis conducted using SPSS software (v26, IBM) involved One-way analysis of variance, Tukey *post hoc* tests, and *t*-test to compare concentrations and groups. Significance level is set at 0.05.

Results: The TT extract mostly consisted of flavonoids. Its inhibition zones in the well diffusion test were statistically comparable with chlorhexidine in some concentrations ($P > 0.05$). The MIC of the TT extract was 15.625 mg/mL for all tested bacteria, whereas the MBC ranged from 31.25 to 62.5 mg/mL. Further, the $MBIC_{50}$ ranged from 7.8125 to 15.625 mg/mL for the extract. Time-kill assay showed that the bactericidal activity of the TT extract lasted for 8, 12, and 2 h for *S. mutans*, *S. sobrinus*, and *L. acidophilus*, respectively. The acid production decreased obviously after 8 h.

Conclusion: The TT extract showed good time-dependent antibacterial and antibiofilm activity, as well as acid production inhibition, against cariogenic bacteria in laboratory experiments.

Key Words: Dental caries, *Lactobacillus acidophilus*, plant extracts, *Streptococcus mutans*, *Streptococcus sobrinus*, *Tribulus terrestris*

Received: 10-Aug-2023
Revised: 27-Aug-2024
Accepted: 09-Sep-2024
Published: 21-Oct-2024

Address for correspondence:
Dr. Ebrahim Rezazadeh Zarandi,
Immunology of Infection of Diseases Research Center, Research Institute of Basic Medical Sciences, Rafsanjan University of Medical Sciences, Rafsanjan, Iran.
E-mail: erezazadehzarandi50@gmail.com

Access this article online

Website: www.drj.ir
www.drjjournal.net
www.ncbi.nlm.nih.gov/pmc/journals/1480

How to cite this article: Azarm A, Ayoobi F, Zare-Bidaki M, Taheri M, Zarandi ER. Antibacterial and antibiofilm activities of tribulus terrestris methanolic extract against *Streptococcus mutans*, *Streptococcus sobrinus*, and *Lactobacillus acidophilus*: An *in vitro* study. Dent Res J 2024;21:57.

INTRODUCTION

Dental caries (tooth decay) is known as the most common chronic and infectious disease of the mouth in the world.[1] The mouth provides a unique environment for bacteria to accumulate on tooth surfaces and form the oral biofilm. Cariogenic bacteria produce acid by fermenting carbohydrates, which destroys the surface of tooth enamel.[2,3] Among dental plaque bacteria, *Streptococcus* spp. plays an important role in the initial phase of dental caries, whereas *lactobacilli* are very active in the development of tooth decay.[4] Of the 300 species living in dental plaque, *Streptococcus mutans* (*S. mutans*) and *Lactobacillus acidophilus* (*L. acidophilus*) are the two leading causes of tooth decay.[5] Epidemiological and laboratory studies have shown that *Streptococcus sobrinus,* another cause of caries, can be even more cariogenic than *S. mutans*. Clinical studies have also revealed that the co-existence of both *S. mutans* and *S. sobrinus* in preschool and 15-year-old children can increase dental caries rates compared to *S. mutans* alone.[6-8]

To prevent dental caries, a mechanical and chemical approach should be considered to remove and control plaque. Various synthetic chemicals, such as chlorhexidine, are used to control plaque. Despite the sound antibacterial effects of such substances, a variety of side effects, such as discoloration of teeth and restorations, taste disturbance, and desquamation of the oral lining, can be seen in long-term use.[9] Therefore, the search for alternative substances in plants as a possible source of the desired compounds has been considered more than ever.[10,11]

Tribulus terrestris (TT) is an herb belonging to the genus *Tribulus* and *Zygophyllaceae family*. It is a perennial flowering plant commonly found worldwide but primarily in warm and tropical areas. TT can also grow in poor climates and soils unsuitable for other herbs.[12] It contains flavonoids, steroids, saponins, alkaloids, unsaturated fatty acids, vitamins, tannins, resins, potassium, nitrate, phosphorus, iron, sodium, calcium, sulfur, chlorine, aspartic acid, and 14-aspartic acid. Furthermore, it has antimicrobial, antioxidant, cyclooxygenase inhibition, free radical scavenging, lipid peroxidation inhibition, and inflammatory agent modulation properties. In traditional medicine, various diseases, such as hypotension, diabetes, renal, cardiovascular, gastrointestinal, sexual (in men), and hepatic disorders, have been treated. This plant is used in traditional medicine in China, India, Iraq, Bulgaria, South Africa, and Iran.[13] Some *in vitro* studies have shown good antibacterial efficacy of TT extracts against pathogenic and non-pathogenic microorganisms such as *Staphylococcus epidermidis*, *Entrococcus faecalis*, *Salmonella parathyphi*, *Shigella dysenteriae*, *Candida albicans*, and *Mycobacterium tuberculosis*.[14,15] However, only a few researchers have explained its antibacterial effects on cariogenic bacteria.[16,17]

This *in vitro* study aimed to assess the antibacterial and antibiofilm activities of TT methanolic extract against *S. mutans*, *S. sobrinus*, and *L. acidophilus* as three important etiologic elements of dental caries.

MATERIALS AND METHODS

This study was designed in an experimental model (*in vitro*), and it was ethically approved by the Research Ethics Committees of Rafsanjan University of Medical Sciences with an ethical code of (IR.RUMS.REC.1400.026).

Plant and bacterial strains

TT fruits were obtained from Pakan Bazr Isfahan Corporation (collected from the Chadegan region of Isfahan province). The standard bacterial strains were also purchased from the Iranian Research Organization for Science and Technology, including *S. mutans* (ATCC 35668), *S. sobrinus* (ATCC 27607), and *L. acidophilus* (ATCC 4356).

Preparation of *Tribulus terrestris* extract

Plant extraction was performed as described previously with some modifications.[18] Fruits of TT were washed with distilled water and dried in a dark place for 3 days at 20°C. They were completely crushed by an electrical grinder. Two hundred grams of the produced powder were soaked with 1 L of pure methanol (Merck, Germany) for 4 days. After filtering with Whatman paper No. 1, the solvent was removed using a rotary evaporator (Heidolphlaborota 4000, Germany). The dry remaining mass was dissolved in 10% dimethylsulfoxide (DMSO, Merk), resulting in a final concentration of 500 mg/mL. This stock solution was stored at 4°C for the following use.

Phytochemical analysis

Standard preliminary phytochemical tests were performed to identify specific compounds in the TT extract. Mayer's test, Foam test, Ammonia test, Braymer's test, Ferric chloride test, Libermann-Burchard's test, and Salkowski's test were

performed to detect alkaloids, saponins, flavonoids, tannins, phenols, steroids, and terpenoids, respectively.[19]

Antibacterial assays

Agar well diffusion

The inhibitory effect of the TT extract on *S. mutans*, *S. sobrinus*, and *L. acidophilus* was determined by the agar well diffusion assay.[20] Briefly, the fresh bacteria from a 0.5 McFarland suspension (1.5×10^7 CFU/mL) were subcultured on a Mueller–Hinton agar by a swab. The 8 mm wells were created on the mediums, and different concentrations (500, 250, 125, and 62.5 mg/mL) of the TT extract were injected into the wells. All cultures were incubated at 37°C for 24 h. The inhibition zones were measured with a ruler. Furthermore, a diluted series of chlorhexidine (Donya Behdasht, Tehran, Iran) with concentrations from 2 to 0.25 mg/ml was used for comparison. All test was carried out in triplicate for each bacterium and concentration (3 samples for the control versus 3 for the test group), and average zones were reported.

Determination of minimum inhibitory concentrations

The microdilution method was used for minimum inhibitory concentration (MIC) determination. Two-fold dilutions of the TT extract (0.49–250 mg/mL) and chlorhexidine (0.062–2 mg/mL) were prepared in a 96-well microplate. Bacterial suspensions with a concentration of 10^6 CFU/mL were prepared in BHI broth. 100 µl of this bacterial suspension was added to each well containing 100 µl of the TT extract. The microplate was incubated for 24 h at 37 °C. The concentration with no visible bacterial growth after 24 h of incubation was considered the MIC.[21] These experiments were performed in triplicate (three experiments for the TT extract group and three for the chlorhexidine group) for each bacterium.

Determination of minimal bactericidal concentration

After determining MIC values, 10 µL from each well of the microplate was spread on the BHI agar and incubated for 24 h in a microaerophilic condition. The lowest concentration of the TT extract, killing 99.99% of bacteria, was defined as minimum bactericidal concentration (MBC).[21] This procedure was also conducted in triplicate (three experiments for the TT extract group and three for the chlorhexidine group) for each bacterium.

Time-kill assay

A time-kill kinetic assay was conducted using broth microdilution. The following BHI mediums, including 10^6 CFU/mL bacteria plus TT extract (1 × MIC) and 10^6 CFU/mL bacteria plus PBS (negative control), were prepared and incubated at 37°C. After 2, 4, 8, 12, and 24 h of incubation, 100 µl of each bacterial solution was serially diluted with phosphate-buffered saline (PBS, pH: 7). Finally, 10 µl of these dilutions were cultured onto BHI agar for colony counting. Enumeration of CFU was performed after incubation of cultures at 37°C for 24 h in microaerophilic conditions. Time-kill curves were traced with the $\log_{10}$ CFU/mL over the 24 h. This test was performed in triplicate for each bacterium and group (control and test, with 18 time-kill assays in total).

Acid production

Acid production was measured using Matsumoto *et al.*'s methodology with some modifications.[22] In brief, the TT extract (6.45 mL of 500 mg/mL stock solution) was added to 200 mL of BHI broth (Difco, USA) containing 10^6 CFU/mL supplemented with 1% glucose, resulting in MIC (15.625 mg/mL) in each broth medium. 5 mL of the cultures were removed at 2, 4, 8, 12, and 24 h after incubation at 37°C, and the pH was measured using a pH meter (Model 220, Corning). A BHI broth without the TT extract was also used as the control. This experiment was performed once for each bacterium and group (control and test) for 24 h, with 6 acid production assays in total.

Biofilm formation assay

The effect of the TT extract and chlorhexidine on biofilm formation was evaluated by the microdilution method. Two-fold serial dilutions of the extract (ranging from 0.49 mg/mL to 250 mg/mL) and chlorhexidine (0.0625–2 mg/mL) were prepared. Bacterial suspensions were prepared in the same manner as in the MIC determination, 20 µL of bacterial suspensions were added in the wells of a 96-well microplate, leading to the final concentration of 1×10^6 CFU/mL. Following incubation at 37°C for 24 h, wells were washed with PBS and fixed with methanol for 15 min. 0.1% (w/v) crystal violet was used for staining in 5 min. Then, all wells were rinsed thoroughly with water, and the control wells became colorless. 200 µL of 95% ethanol was added to each well, shaken for 30 min, and finally, absorbance was measured at a wavelength of 590 nm by a microplate reader. The percentage of inhibition was calculated by the following formula: (1-[absorbance of the test/absorbance of the control]) ×100. The lowest concentration showing ≥50% inhibition of biofilm

formation was defined as the minimum biofilm inhibition concentration ($MBIC_{50}$). This assay was performed in triplicate (three experiments for the TT extract group and three for the chlorhexidine group) for each bacterium.

Statistical analysis

Data analysis was performed with SPSS software (version 26.0; SPSS Inc., Chicago, IL, USA) using one-way analysis of variance (ANOVA) and Tukey *post hoc* tests to compare different concentrations and groups. *T*-test analysis was also performed to compare the decrease of bacteria growth in the TT extract group with the control shown in the time-kill assay. The statistical significance level was set at 0.05.

RESULTS

Phytochemical analysis

The standard preliminary photochemical tests showed that the majority of the TT extract consisted of flavonoids, as demonstrated in Table 1. It included tannins, phenols, steroids, and terpenoid compounds. The TT extract was devoid of saponin compounds [Table 1].

Antibacterial assays

Agar well diffusion

One-way ANOVA showed a statistically significant difference between groups of *S. mutans* ($F_{[7,16]} = 451.95$, $P < 0.001$), *S. sobrinus* ($F_{[7,16]} = 347.54$, $P < 0.001$), and *L. acidophilus* ($F_{[7,16]} = 35.47$, $P < 0.001$). The Tukey *post hoc* test showed that inhibition zones of 125 and 250 mg/mL of the TT extract against *S. mutans* did not significantly differ from 0.5 and 0.25 mg/mL of chlorhexidine, respectively. The data showed no significant difference between 500 and 250 mg/mL of the TT extract with 0.5 mg/mL of chlorhexidine on *S. sobrinus*. Similar results were also found between 125 mg/mL of the TT extract and 0.25 mg/mL of chlorhexidine ($P > 0.05$).

The inhibition zone of 250 mg/mL of the TT extract against *L. acidophilus* was not significantly larger than 0.25 and 0.5 mg/mL of chlorhexidine, according to the Tukey *post hoc* test. Moreover, 500 mg/mL and 62.5 mg/mL of the extract had statistically similar zones to chlorhexidine with concentrations of 0.5 and 0.25 mg/mL, respectively ($P > 0.05$). The comparison results between all other concentrations of the TT extract and chlorhexidine were statistically significant, in which the agent showing a larger inhibition zone was statistically more effective ($P < 0.05$). The results of the agar well diffusion assay are presented in Table 2.

Table 1: Phytochemical analysis of *Tribulus terrestris*

Plant constituent	Methanolic extract
Alkaloids	+
Saponins	–
Flavonoids	+++
Tannins	++
Phenols	++
Steroids	++
Terpenoids	++

(–) means no detection of the secondary metabolite, and (+) to (+++) are positive for the presence of the desired metabolite

Minimum inhibitory concentration, minimum bactericidal concentration, and $MBIC_{50}$

MIC, MBC, and $MBIC_{50}$ values of the TT extract and chlorhexidine are reported in Table 3.

Time-kill assay

According to the *t*-test analysis of the time-kill assay, the TT extract had a significant ability to inhibit the growth of all three tested bacteria in all periods in comparison with control groups ($P < 0.05$) except for *L. acidophilus* in 24 h ($P = 0.371$). The bactericidal activity of the TT extract lasted 8 h, 12 h, and 2 h for *S. mutans*, *S. sobrinus*, and *L. acidophilus*, respectively, showing no visible viable colonies. All test bacteria showed visible growth after the mentioned period [Figure 1]. *L. acidophilus* was the most resistant bacteria, whereas *S. sobrinus* was the most susceptible with longer growth inhibition. Details of Log CFU/mL of per bacteria strains are presented in Table 4.

Acid production

Acid production of all tested microorganisms did not decrease compared with negative control up to 4 h. After 8 h, a decrease in acid production rate was evident in the three bacteria [Figure 2]. Details of pH changes are provided in Table 5.

DISCUSSION

The TT extract has been widely used for the treatment of toothache, dental caries, and periodontal diseases in traditional Chinese medicine.[23,24] Three-time oral rinse with the TT extract has also been recommended for the healing of gingivitis in the traditional herbal

Table 2: Antimicrobial activities of the *Tribulus terrestris* extract and chlorhexidine against tested microorganisms in millimeters using the well diffusion method (mean±standard deviation) (*n*=3; for each concentration and bacteria)

Agent	Concentration (mg/mL)	*Streptococcus mutans* (*n*=24)	*Streptococcus sobrinus* (*n*=24)	*Lactobacillus acidophilus* (*n*=24)
Tribulus terrestris (*n*=36)	500	16.33±0.58	16.67±0.76	14.83±0.29
	250	14.16±0.58	15.33±0.58	12.50±0.00
	125	12.33±0.29	13.33±0.58	12.17±0.29
	62.5	10.33±0.29	11.00±0.00	9.83±0.29
Chlorhexidine (*n*=36)	2	23.00±0.00	24.00±0.00	21.00±0.00
	1	19.00±0.00	21.50±0.00	18.33±0.29
	0.5	14.17±0.29	16.17±0.29	13.67±0.29
	0.25	12.00±0.00	13.00±0.00	10.83±0.29

Table 3: Minimum inhibitory concentration, minimum bactericidal concentration, and minimum biofilm inhibition concentration 50 values of the *Tribulus terrestris* extract and chlorhexidine against tested microorganisms (*n*=3; for each test and bacteria)

Bacteria strains	*Tribulus terrestris* extract (mg/mL) (*n*=27)			Chlorhexidine (mg/mL) (*n*=27)		
	MIC	MBC	$MBIC_{50}$	MIC	MBC	$MBIC_{50}$
Streptococcus mutans (*n*=18)	15.625	31.25	15.625	0.0625	0.125	0.0625
Streptococcus sobrinus (*n*=18)	15.625	31.25	7.8125	0.0625	0.125	0.0625
Lactobacillus acidophilus (*n*=18)	15.625	62.50	15.625	0.0625	0.25	0.0625

MIC: Minimum inhibitory concentration; MBC: Minimum bactericidal concentration; $MBIC_{50}$: Minimum biofilm inhibition concentration 50

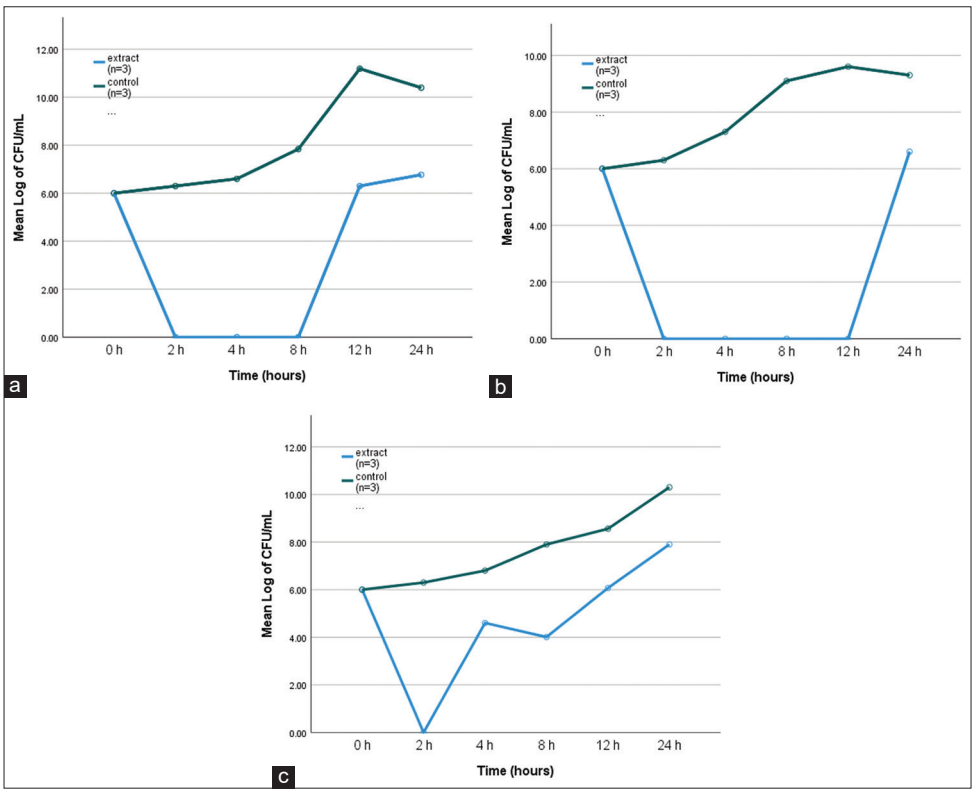

Figure 1: Time-kill assay of bacteria strains during 24-h exposure to *Tribulus terrestris* extract (1 × minimum inhibitory concentration) compared to negative control; (a) *Streptococcus mutans,* (b) *Streptococcus sobrinus,* (c) *Lactobacillus acidophilus* (*n* = 3 for each group).

medicine of Mexico.[25] Another *in vitro* study has also strengthened the theory of this extract's usefulness in oral and dental health.[17]

In the present study, the results of phytochemical screening of the methanolic extract of the native TT in the Chadegan region of Isfahan demonstrated that

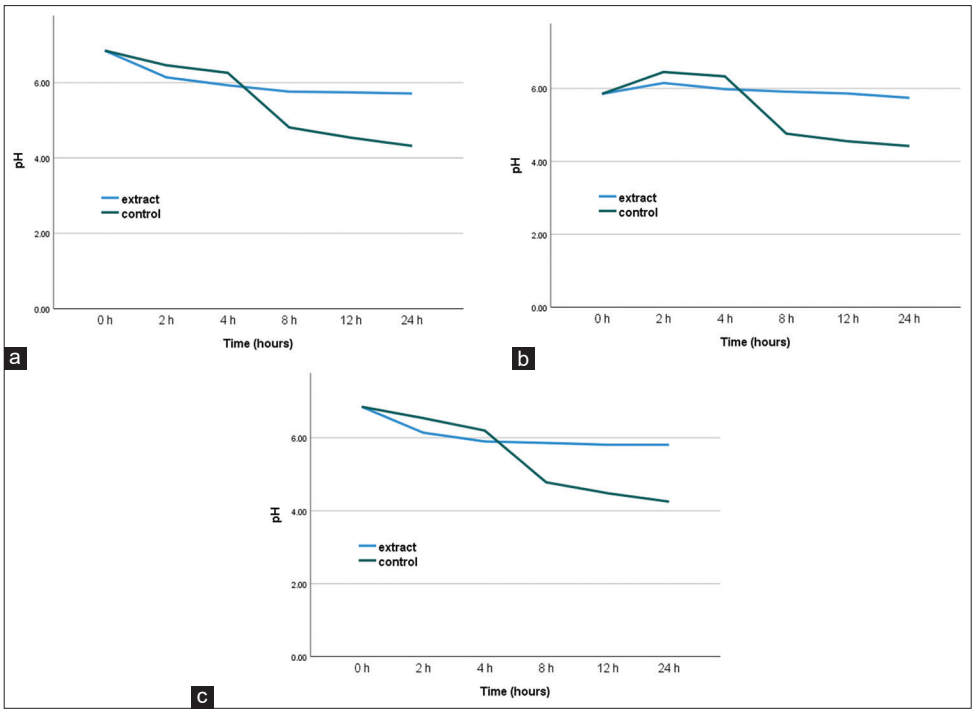

Figure 2: pH changes during 24-h exposure of bacteria strains to *Tribulus terrestris* extract (1 × minimum inhibitory concentration) compared to negative control; (a) *Streptococcus mutans,* (b) *Streptococcus sobrinus,* (c) *Lactobacillus acidophilus* (*n* = 1; for each bacterium and group).

the extract contained flavonoids (the main compound), alkaloids, tannins, phenols, steroids, and terpenoids. Oh *et al*. showed that the ethanolic TT extract had a wide range of constituents, including phenols, glycosides, peptides, flavonoids, steroids, terpenoids, and organic acids. No alkaloids were detected, whereas steroids and terpenoids were the maximum secondary metabolites.[16] *T. terrestris,* which grows in Punjab in Pakistan, had no tannins, whereas other compounds, such as terpenoids, phlorotannins, anthraquinone, and phenolics, were present as the majority of flavonoids.[18] It has been revealed that different constituents of the TT extract are linked to different factors, such as plant origin, climate conditions, and geographical region. Moreover, the extraction method and duration can also affect the final composition of the extract.[26]

The TT extract could significantly inhibit three bacteria in comparison with previous studies. In Soleimanpour *et al*., well diffusion tests showed that 50 and 100 mg/mL of the TT extract led to zones of 21.8 ± 0.3 and 22.2 ± 0.2 mm against *S. mutans,* respectively. Zones of 18 ± 0.0 and 20.4 ± 0.5 mm were also reported against *S. sanguis* for 50 and 100 mg/mL, respectively.[17] Moreover, the methanolic extract of TT fruits showed good antibacterial activity against *Staphylococcus aureus, Klebsiella pneumonia,* and *Acinetobacter baumannii*.[18] Inhibition zones of chlorhexidine against *S. mutans* and *S. sanguis* were 18 ± 0.0 and 14.2 ± 0.2 for 0.5 mg/mL and 15 ± 0.0 and 13.7 ± 0.99 mm for 0.25 mg/mL, respectively.[17] Different bacteria species, extraction methods, and plant origins can cause various inhibition zones.[26] To our knowledge, limited studies have compared the TT extract with chlorhexidine, especially against *S. sobrinus* and *L. acidophilus*. The present study suggested that the antibacterial activity of the TT extract could not significantly differ from chlorhexidine in some concentrations. Furthermore, this could be the high concentration of the TT extract or its richness in various medicinal compounds according to the phytochemical screening results.

MIC and MBC of the TT extract have been studied previously. MIC and MBC of the aqueous extract of TT against *S. mutans* and *S. sanguis* were higher than the reported concentration for methanolic extract in the present research. On the other hand, the MBC value reported by Kiani *et al*. was lower than that in our study; however, it was higher than the MBC value reported by Soleimanpour *et al*. against *S. mutans*.[17,27] The TT methanolic extract seems more effective than the aqueous extract, suggesting a higher

Table 4: Log CFU/mL of bacteria strains during 24-h exposure to *Tribulus terrestris* extract (1× minimum inhibitory concentration) in comparison with negative control in triplicate

Time	0 h	2 h	4 h	8 h	12 h	24 h
Streptococcus mutans						
Extract 1	6	NA	NA	NA	6.35	6.83
Extract 2	6	NA	NA	NA	6.28	6.75
Extract 3	6	NA	NA	NA	6.27	6.73
Mean±SE	6±0	NA	NA	NA	6.3±0.020	6.77±0.025
Control 1	6	6.5	6.58	7.83	11.18	10.42
Control 2	6	5.8	6.51	7.79	11.16	10.35
Control 3	6	6.6	6.71	7.9	11.23	10.43
Mean±SE	6±0	6.3±0.205	6.6±0.049	7.84±0.026	11.19±0.017	10.4±0.020
Streptococcus sobrinus						
Extract 1	6	NA	NA	NA	NA	6.45
Extract 2	6	NA	NA	NA	NA	6.64
Extract 3	6	NA	NA	NA	NA	6.71
Mean±SE	6±0	NA	NA	NA	NA	6.6±0.063
Control 1	6	6.34	7.32	9.2	9.73	9.24
Control 2	6	6.32	7.34	9.14	9.64	9.35
Control 3	6	6.24	7.24	8.96	9.43	9.31
Mean±SE	6±0	6.3±0.025	7.3±0.025	9.1±0.059	9.6±0.073	9.3±0.026
Lactobacillus acidophilus						
Extract 1	6	NA	4.76	4.13	6.21	8.14
Extract 2	6	NA	4.38	3.93	5.85	7.68
Extract 3	6	NA	4.66	3.97	6.15	7.88
Mean±SE	6±0	NA	4.6±0.093	4.01±0.050	6.07±0.091	7.9±0.109
Control 1	6	6.29	6.82	7.88	8.71	10.37
Control 2	6	6.06	6.75	7.7	8.44	10.27
Control 3	6	6.55	6.83	8.12	8.53	10.26
Mean±SE	6±0	6.3±0.116	6.8±0.0205	7.9±0.0993	8.56±0.065	10.3±0.027

NA: Not available; SE: Standard error

Table 5: pH changes during 24-h exposure of bacteria strains to *Tribulus terrestris* extract (1× minimum inhibitory concentration) in comparison with negative control

Time	0 h	2 h	4 h	8 h	12 h	24 h
Streptococcus mutans						
Extract	6.85	6.14	5.93	5.76	5.74	5.71
Control	6.85	6.46	6.26	4.81	4.54	4.32
Streptococcus sobrinus						
Extract	6.85	6.15	5.98	5.91	5.86	5.74
Control	6.85	6.45	6.33	4.76	4.55	4.42
Lactobacillus acidophilus						
Extract	6.85	6.14	5.9	5.86	5.81	5.81
Control	6.85	6.54	6.2	4.78	4.48	4.25

n=1; for each bacterium and group

concentration of antibacterial substances released from the methanolic extract.

According to our results, biofilm formation can be significantly inhibited in exposure to the TT methanolic extract. The ethanolic extract of TT fruits can inhibit the adhesion of *S. mutans* to saliva-coated hydroxyapatite and the synthesis of water-insoluble glucan in a dose-dependent manner (0.1–0.5 mg/mL).[16] The cell surface hydrophobicity of *S. mutans* has an important role in biofilm formation. It has been shown that other strains, such as *S. sanguis* and *S. salivarius*, are not able to adhere to hydroxyapatite due to the lack of this property.[28,29] Further studies are needed to find whether the TT extract can reduce the cell surface hydrophobicity. Studies have demonstrated that the existence of strongly attached and insoluble glucans within a biofilm enhances its mechanical stability

by binding bacterial cells together and the apatite surface. These polymers are crucial for maintaining the three-dimensional structure of the biofilm, in addition to interacting with the proteins related to the specific Gbps genes expressed by *S. mutans* and other oral microorganisms. As a result, they play a role in regulating the development of cariogenic biofilm.[30-32] This may explain the biofilm formation reduction of other *Streptococci* spp., whereas the main mechanism for *lactobacilli* remains unclear.

The time-kill assay results of the present research showed the bactericidal effect of the TT extract to be time-dependent for *S. mutans*, *S. sobrinus,* and *L. acidophilus*. Tolerance and resistance to herbal drugs have also been reported for *Streptococci* and *Lactobacilli* spp.[33] This can be caused by nutrient compounds in the TT extract or the creation and growth of resistant strains through time. This new data are hard to discuss due to the absence of a similar study.

Based on the present study, the TT extract can reduce acid production after 8 h compared to the control. These results are in line with Oh *et al*.'s findings after 24 h, suggesting that methanolic extract can inhibit the acid production of *S. mutans*.[16] The other two bacteria examined in our study have been investigated for the first time. Oh *et al*. suggested that the anticariogenic properties of the TT extract against *S. mutans,* including reducing acid production, could be related to active compounds of TT.[16]

The main components responsible for the antibacterial and antibiofilm effects of the TT extract are unclear. Some studies have mentioned alkaloids,[34] whereas some have mentioned saponins as the main compound.[35,36] Flavonoids of the TT extract have also been proven to have antibacterial activity against different species, such as *Streptococci*.[37,38] The TT extract of the present study also showed high amounts of flavonoids, which can explain the antibacterial and antibiofilm effects in line with the previous studies.[37,38]

CONCLUSION

The TT methanolic extract seems to have good time-dependent antibacterial activity against cariogenic bacteria. It could inhibit the growth, biofilm formation, and acid production of *S. mutans*, *S. sobrinus*, and *L. acidophilus*. However, *in vivo* studies are needed to find out whether the TT extract or its compounds are a possible candidate for use in oral health products.

Financial support and sponsorship
Nil.

Conflicts of interest
The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or non-financial in this article.

REFERENCES

1. Peres MA, Macpherson LM, Weyant RJ, Daly B, Venturelli R, Mathur MR, *et al.* Oral diseases: A global public health challenge. Lancet 2019;394:249-60.
2. Lippert F. The effects of lesion baseline characteristics and different Sr: Ca ratios in plaque fluid-like solutions on caries lesion de- and remineralization. Arch Oral Biol 2012;57:1299-306.
3. Qiu W, Zhou Y, Li Z, Huang T, Xiao Y, Cheng L, *et al.* Application of antibiotics/antimicrobial agents on dental caries. Biomed Res Int 2020;2020:565 8212.
4. Venkataraghavan K, Shah S, Choudhary P, Trivedi K. Effectiveness of a new generation anti-cavity mouthwash on *Streptococcus mutans* and *Lactobacillus acidophilus* count: A microbiological study. World J Dent 2014;5:152-5.
5. Viuda-Martos M, Ruiz-Navajas Y, Fernández-López J, Pérez-Alvarez JA. Functional properties of honey, propolis, and royal jelly. J Food Sci 2008;73:R117-24.
6. Conrads G, de Soet JJ, Song L, Henne K, Sztajer H, Wagner-Döbler I, *et al.* Comparing the cariogenic species *Streptococcus sobrinus* and *S. mutans* on whole genome level. J Oral Microbiol 2014;6:26189.
7. Okada M, Soda Y, Hayashi F, Doi T, Suzuki J, Miura K, *et al.* Longitudinal study of dental caries incidence associated with *Streptococcus mutans* and *Streptococcus sobrinus* in pre-school children. J Med Microbiol 2005;54:661-5.
8. Sánchez-Acedo M, Montiel-Company JM, Dasí-Fernández F, Almerich-Silla JM. *Streptococcus mutans* and *Streptococcus sobrinus* detection by polymerase chain reaction and their relation to dental caries in 12 and 15 year-old schoolchildren in Valencia (Spain). Med Oral Patol Oral Cir Bucal 2013;18:e839-45.
9. Supranoto SC, Slot DE, Addy M, Van der Weijden GA. The effect of chlorhexidine dentifrice or gel versus chlorhexidine mouthwash on plaque, gingivitis, bleeding and tooth discoloration: A systematic review. Int J Dent Hyg 2015;13:83-92.
10. Karygianni L, Al-Ahmad A, Argyropoulou A, Hellwig E, Anderson AC, Skaltsounis AL. Natural antimicrobials and oral microorganisms: A systematic review on herbal interventions for the eradication of multispecies oral biofilms. Front Microbiol 2015;6:1529.
11. Teanpaisan R, Kawsud P, Pahumunto N, Puripattanavong J. Screening for antibacterial and antibiofilm activity in Thai medicinal plant extracts against oral microorganisms. J Tradit Complement Med 2017;7:172-7.

12. Hanif MA, Yousaf S, Rehman R, Hanif A, Nadeem R. Chapter 42—Puncture Vine. Medicinal Plants of South Asia: Elsevier; 2020. p. 571-85.
13. Naseri L, Khazaei M. A review on therapeutic effects of *Tribulus terrestris*. J Med Plants Stud 2019;18:1-22.
14. Al-Bayati FA, Al-Mola HF. Antibacterial and antifungal activities of different parts of *Tribulus terrestris* L. growing in Iraq. J Zhejiang Univ Sci B 2008;9:154-9.
15. Arulmozhi P, Vijayakumar S, Kumar T. Phytochemical analysis and antimicrobial activity of some medicinal plants against selected pathogenic microorganisms. Microb Pathog 2018;123:219-26.
16. Oh HK, Park SJ, Moon HD, Jun SH, Choi NY, You YO. *Tribulus terrestris* inhibits caries-inducing properties of *Streptococcus mutans*. J Med Plants Res 2011;5:6061-6.
17. Soleimanpour S, Sedighinia FS, Safipour Afshar A, Zarif R, Ghazvini K. Antibacterial activity of *Tribulus terrestris* and its synergistic effect with *Capsella bursa-pastoris* and *Glycyrrhiza glabra* against oral pathogens: An *in-vitro* study. Avicenna J Phytomed 2015;5:210-7.
18. Naz R, Ayub H, Nawaz S, Islam ZU, Yasmin T, Bano A, *et al.* Antimicrobial activity, toxicity and anti-inflammatory potential of methanolic extracts of four ethnomedicinal plant species from Punjab, Pakistan. BMC Complement Altern Med 2017;17:302.
19. Shaikh JR, Patil M. Qualitative tests for preliminary phytochemical screening: An overview. Int J Chem Stud 2020;8:603-8.
20. Suriyakala G, Sathiyaraj S, Gandhi AD, Vadakkan K, Rao UM, Babujanarthanam R. *Plumeria pudica* Jacq. flower extract-mediated silver nanoparticles: Characterization and evaluation of biomedical applications. Inorg Chem Commun 2021;126:108470.
21. Xu YT, Wu Q, Chen YM, Smales RJ, Shi SY, Wang MT. Antimicrobial effects of a bioactive glass combined with fluoride or triclosan on *Streptococcus mutans* biofilm. Arch Oral Biol 2015;60:1059-65.
22. Matsumoto M, Minami T, Sasaki H, Sobue S, Hamada S, Ooshima T. Inhibitory effects of oolong tea extract on caries-inducing properties of mutans streptococci. Caries Res 1999;33:441-5.
23. Kim R, Jeon S, Choi Y. EuiBangRuChui (6). Seoul: Yeokang Publications; 1991.
24. Lee S. Dongeui Bogam. Seoul, Korea: Yeokang Publication; 1986.
25. Gauthaman K, Adaikan PG, Prasad RN. Aphrodisiac properties of *Tribulus Terrestris* extract (Protodioscin) in normal and castrated rats. Life Sci 2002;71:1385-96.
26. Ștefănescu R, Tero-Vescan A, Negroiu A, Aurică E, Vari CE. A comprehensive review of the phytochemical, pharmacological, and toxicological properties of *Tribulus terrestris* L. Biomolecules 2020;10:752.
27. Kiani Z, Tabas PM, Moghadam MG, Zare-Bidaki M. Evaluation of *in vitro* antimicrobial effects of aqueous extract of *Tribulus terrestris* against oral bacteria. Med Lab J 2021;15:7-12.
28. Westergren G, Olsson J. Hydrophobicity and adherence of oral streptococci after repeated subculture *in vitro*. Infect Immun 1983;40:432-5.
29. Yamanaka-Okada A, Sato E, Kouchi T, Kimizuka R, Kato T, Okuda K. Inhibitory effect of cranberry polyphenol on cariogenic bacteria. Bull Tokyo Dent Coll 2008;49:107-12.
30. Koo H, Xiao J, Klein MI, Jeon JG. Exopolysaccharides produced by *Streptococcus mutans* glucosyltransferases modulate the establishment of microcolonies within multispecies biofilms. J Bacteriol 2010;192:3024-32.
31. Lynch DJ, Fountain TL, Mazurkiewicz JE, Banas JA. Glucan-binding proteins are essential for shaping *Streptococcus mutans* biofilm architecture. FEMS Microbiol Lett 2007;268:158-65.
32. Xiao J, Koo H. Structural organization and dynamics of exopolysaccharide matrix and microcolonies formation by *Streptococcus mutans* in biofilms. J Appl Microbiol 2010;108:2103-13.
33. Vadhana P, Singh BR, Bharadwaj M, Singh SV. Emergence of herbal antimicrobial drug resistance in clinical bacterial isolates. Pharm Anal Acta 2015;6:434.
34. Jindal A, Kumar P, Gautam K. Evaluation of antibiotic potential of alkaloids of *Tribulus terrestris* L. against some pathogenic microorganisms. Int J Green Pharm IJGP 2013;7:102-5.
35. Bangham AD, Horne RW, Glauert AM, Dingle JT, Lucy JA. Action of saponin on biological cell membranes. Nature 1962;196:952-5.
36. Böttger S, Hofmann K, Melzig MF. Saponins can perturb biologic membranes and reduce the surface tension of aqueous solutions: A correlation? Bioorg Med Chem 2012;20:2822-8.
37. Tian C, Chang Y, Zhang Z, Wang H, Xiao S, Cui C, *et al.* Extraction technology, component analysis, antioxidant, antibacterial, analgesic and anti-inflammatory activities of flavonoids fraction from *Tribulus terrestris* L. leaves. Heliyon 2019;5:e02234.
38. Tian C, Zhang Z, Wang H, Guo Y, Zhao J, Liu M. Extraction technology, component analysis, and *in vitro* antioxidant and antibacterial activities of total flavonoids and fatty acids from *Tribulus terrestris* L. fruits. Biomed Chromatogr 2019;33:e4474.