

Down-Regulation of Glycosyl Transferase Genes in *Streptococcus Mutans* by *Punica Granatum* L. Flower and *Rhus Coriaria* L. Fruit Water Extracts

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Abstract

In our previous studies, we showed the inhibitory effects of *Punica granatum* L. flower and *Rhus coriaria* L. fruit water extracts on dental plaque accumulation by several bacteria, especially *Streptococcus mutans* (*S. mutans*), on orthodontic wire by *in-vitro* assays. In this study, the anti-cariogenic properties of the extracts were evaluated by assessing their effects on expression of glycosyltransferase (*gtf*) genes, which are responsible for initial biofilm formation by *S. mutans*.

In this study, the effect of herbal extracts on expression of *gtfB*, *C* (encoding enzymes that produce water-insoluble glucans) and *D* (encoding enzymes that produce water-soluble glucans) genes in *S. mutans* growing in planktonic state was evaluated quantitatively by real-time polymerase chain reaction (PCR) method.

The minimum biofilm inhibitory concentration (MBIC) of understudied herbal water extracts significantly suppressed *gtfB*, *C* and *D* gene expression by $85.3 \pm 7.5\%$, $33.3 \pm 6.4\%$ and $25 \pm 14\%$, respectively for *Punica granatum* L. extract and $73.4 \pm 7.3\%$, $93.8 \pm 2.7\%$ and $59.3 \pm 9.8\%$, respectively for *Rhus coriaria* L. extract compared to the non-treated control group ($P < 0.05$). Also, the real-time PCR showed that the inhibitory effect of *Rhus coriaria* L. extract on *gtfC* and *D* was significantly greater (10.8 and 1.8 fold, respectively) than that of *Punica granatum* L. extract.

These findings suggest that *Punica granatum* L. and especially *Rhus coriaria* L. maybe used as novel, natural antiplaque agents since they inhibit specific genes associated with bacterial biofilm formation without necessarily affecting the growth of oral bacteria.

Keywords: *Rhus coriaria* L.; *Punica granatum* L.; *Streptococcus mutans*; Glycosyltransferase; Biofilm.

Introduction

Dental caries is among the most prevalent and costly oral infectious diseases worldwide (1).

The etiology of dental caries is associated with bacterial metabolism of carbohydrates leading to plaque acidification and demineralization of dental hard tissues. Classic bacterial virulence factors contributing to the initiation and progression of dental caries consist of three components, *i.e.* stable biofilm formation, efficient acid

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production, and sophisticated environmental stress adaptation (2-5) *Streptococcus mutans*; one of the primary etiologic agents of dental caries (6, 7) that produces glycosyl transferases (GTFs), which synthesize intracellular and extracellular polysaccharides. The extracellular polysaccharides, especially the water-insoluble glucans, facilitate the initial adherence of *S. mutans* to tooth surfaces and the formation of mature dental plaque biofilm (8-10). Deletion of *gtfB* and *gtfC* genes in *S. mutans* resulted in diminished biofilm formation with minimal accumulation of bacteria and polysaccharides *in-vitro* (10). This suggested that suppression of *gtf* genes may represent an alternative approach for disrupting biofilm formation.

Fixed orthodontic treatment with the use of orthodontic wires provides new surfaces susceptible to plaque accumulation, resulting in an increase in oral microbial count, especially *S. mutans* (11, 12). Therefore, prevention of bacterial adhesion to orthodontic wires and inhibition of biofilm formation by the use of novel natural anti-plaque agents is an important priority for dental scientists.

Punica granatum L. (Punicaceae), known as pomegranate (Golnar-e-Farsi), is a small tree that is native in the region from Iran to northern India. This medical herb has important antibacterial, antifungal, and antiviral activity (13).

Rhus Coriaria L. (Anacardiaceae), known as sumac (also spelled sumach), grows wild in Iran and Afghanistan and is traditionally used in foods. Antibacterial and antioxidant effects of *Rhus coriaria* L. extract have been confirmed in several studies (14, 15).

In our previous study, viable cell counting and scanning electron microscopic (SEM) analysis were performed *in-vitro* to assess the growth of microorganisms and to investigate the effect of *Punica granatum* L. and *Rhus coriaria* L. water extracts on microbial attachment to orthodontic wires. These herbal extracts significantly decreased biofilm formation on orthodontic wires (16, 17).

In the current study, quantitative real-time PCR was carried out to assess the effect of *Punica granatum* L. flower and *Rhus coriaria* L. fruit water extracts on the transcriptional

expression of *S. mutans* glycosyltransferase (*gtfB*, *C* and *D*) genes.

Experimental

Bacterial growth and extract treatment

For this study, *S. mutans* ATCC 35668 was purchased from the Industrial Fungi and Bacteria Collection Center (Tehran, Iran). Bacterial suspension was prepared using a pure culture of this bacterial strain, grown in brain heart infusion (BHI) broth (Merck, Germany) medium. The inoculated medium was incubated at 37°C. After this time, broth culture was inoculated in 8.5 % saline solution to obtain an optical turbidity of 1 McFarland standard. In this study, the water extracts of *Punica granatum* L. flowers and *Rhus coriaria* L. fruit, prepared in our previous studies, were used (16, 17). The stock of extracts was prepared with a concentration of 4.68 mg/mL and dissolved in deionized water. Prepared solutions were filter sterilized through a 0.2 µm pore-size polycarbonate filter. Then, extract solutions in a final MBIC (0.39 mg/mL for *Rhus coriaria* L. and 6.125 mg/mL for *Punica granatum* L.) were added to BHI broth medium in sterile test tubes. These tubes were then inoculated with the bacterial suspension prepared equivalent to 1 McFarland. The tubes were incubated overnight.

RNA extraction and cDNA synthesis

Total RNA was extracted from planktonic cultures of non-treated (control) and 24 h treated *S. mutans* with MBIC concentrations of *Rhus coriaria* L. (0.39 mg/mL) and *Punica granatum* L. (6.125 mg/mL) (Table 1) (16, 17). Before extraction, bacterial cultures (three cultures for each group; n = 3) were mixed with 2 volumes of RNA protect solution (Qiagen, USA) in order to stabilize bacterial RNA. After 5 min of incubation at room temperature, bacterial cells were collected by centrifugation (5000 g, 10 min). The cells were disrupted with re-suspension of the pellets in TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0) containing 1 mg/mL lysozyme (Sigma-Aldrich, Germany) and incubation at 37°C for 20 min. Then, the bacterial total RNA was isolated and purified using High

Table 1. Effect of *Punica granatum* L. flower and *Rhus coriaria* L. fruit water extracts on the formation (MBIC 50) of *S. mutans* biofilm on orthodontic wire (values were acquired in our previous studies)(16, 17).

Test agent	MBC ¹ (mg/mL)	MIC ² (mg/mL)	MBIC ³ (mg/mL)
<i>Punica granatum</i> L.	100	50	6.125
<i>Rhus coriaria</i> L.	6.125	1.56	0.39

¹Minimum bactericidal concentration, the lowest concentration of the agent that killed bacteria.

²Minimum inhibitory concentration, the lowest concentration of the agent that inhibited bacterial growth.

³Minimum biofilm inhibitory concentration, the lowest concentration of the agent that inhibited biofilm formation.

Pure RNA Isolation Kit (Roche, Germany) according to the manufacturer's instructions. The extraction procedure contained on-column digestion step with RNase-free DNase I in order to eliminate the residual contaminating DNA. The RNA concentration was determined spectrophotometrically using the NanoDrop instrument (mySPEC, Austria). The quality and integrity of the RNAs were determined by measuring OD260/280 ratio and agarose gel electrophoresis of RNA respectively.

In order to synthesize cDNA, 1 µg of isolated RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) according to the manufacturer's instructions in the presence of random primers on a PCR amplification machine (PeQlab, USA). For additional control for residual genomic DNA contamination, the cDNA synthesis reaction was carried out with each purified total RNA sample in the absence of reverse transcriptase enzyme and served as negative control.

Real-time quantitative PCR and gene expression analysis

In order to quantify the changes in *gtfB*, *C* and *D* mRNA gene expression in *S. mutans* exposed to the two understudy extracts in comparison with the control (non-treated *S. mutans*), real-time PCR was performed. All primers (*gtf* genes and housekeeping genes) for real-time PCR were controlled with NCBI Primer Blast software and obtained commercially from Takapuzist Company (Bioneer, Korea) (Table 2).

The resulting cDNAs and negative controls (no-RT) were amplified using the SYBR®Green PCR Master Mix (Applied Biosystems, USA). The reaction mixture (20 µL) contained 10 µL of Master Mix, 200 ng of template cDNA (2µL) and 200 nM of forward and reverse primers placed in MicroAmp® Optical tube (Applied Biosystems, USA). Three repeats of the same reaction tubes were used for each test sample and three reaction tubes without cDNA template were used as negative controls (NTC) for each primer pair in order to check for DNA contamination.

Table 2. Specific primers used for real-time PCR.

Gene	Sequence (5'→3')	Product Size (bp)
<i>gtfB</i>	Forward: AGCAATGCAGCCAATCTACAAAT	96
	Reverse: ACGAACTTTGCCGTTATTGTCA	
<i>gtfC</i>	Forward: GGTTTAACGTCAAAATTAGCTGTATTAGC	91
	Reverse: CTCAACCAACCGCCACTGTT	
<i>gtfD</i>	Forward: ACAGCAGACAGCAGCCAAGA	94
	Reverse: ACTGGGTTTGCTGCGTTT	
<i>16sRNA</i>	Forward: CCTACGGGAGGCAGCAGTAG	101
	Reverse: CAACAGAGCTTTACGATCCGAAA	
<i>recA</i>	Forward: GCGTGCCTTGAAGTTTTATTCTTC	75
	Reverse: TGTTCCCGGTTCTTAAATT	

Amplification and detection of specific products were performed on the ABI StepOne™ detection system (Applied Biosystems, USA) with the following thermal cycling conditions: initial denaturation at 95°C for 10 min (holding stage), denaturation at 95°C for 15 seconds, followed by 40 cycles of annealing and extension at 60°C for 1 min (cycling stage). All primer pairs were checked for primer dimer by adding a melt curve stage to the end of protocol.

Threshold cycle (C_T) values were determined, and relative expression levels were calculated by StepOne™ software version 2.1 (Applied Biosystems, USA) according to the comparative C_T (ΔΔC_T) method. The quantities of cDNA for *gtfB*, *gtfC*, and *gtfD* genes were normalized to that of cDNA synthesized from *recA* gene in the same sample. These values were then compared to those obtained from the non-treated control to determine the change in *gtf* gene expression level in each test sample.

Statistical analysis

All real-time PCR assays for three target genes and two target samples were performed in triplicate at the same time and reproduced at least three separate experiments. The differences between the experimental group and the non-treated control group were analyzed by GraphPad Prism (version 6.01, USA). The data from fluorescent intensities were assessed by one-way analysis of variance (ANOVA) in the Tukey-Kramer post deviation test for all pairs. A P value < 0.05 was considered statistically significant.

Results

In order to determine the effect of *Rhus coriaria* L. fruit (sumac) and *Punica granatum* L. (pomegranate) flower (Golnar) water extracts on the virulence factors associated with attachment and biofilm formation by *S. mutans*, we performed real-time PCR to quantify the abundance of *gtfB*, *gtfC* and *gtfD* specific mRNA expression.

Interestingly, we found that expression of 16sRNA gene (internal control) decreased by understudy water extracts (data not shown). According to these findings, *recA* was selected

as internal control for real-time PCR reactions. Melt curves revealed the absence of non-specific products in all amplification reactions.

The analysis revealed that the *gtf* genes were more abundantly expressed in non-treated cultures. Significant differences were observed in *gtfB*, *gtfC* and *gtfD* mRNA transcripts among different treatment groups. The mRNA expression of *gtfB*, *C*, and *D* was significantly down-regulated when *S. mutans* was cultured with sub-MIC (MIBC) concentrations of extracts.

Water extracts of *Punica granatum* L. flower and *Rhus coriaria* L. fruit at MBIC (sub-MIC) level (6.125 and 0.39 mg/mL, respectively) significantly inhibited the *gtfB*, *C* and *D* gene expression by 85.3 ± 7.5%, 33.3 ± 6.4% and 25 ± 14% respectively for *Punica granatum* L. extract (Figure 1-A, p < 2.7% ± 93.8, 7.3% ± 73.4, (0.05, and 59.3 ± 9.8% respectively for *Rhus coriaria* L. extract (Figure 1-B, P < 0.05) compared to the non-treated control.

Figure 2 shows the comparison effects of two studied extracts on *gtf* gene expression. The effect of *Rhus coriaria* L. extract in MBIC concentration on down-regulation of *gtfC* and *gtfD* was more remarkable than that of *Punica granatum* L. extract in its MBIC concentration. The mRNA expression of *gtfC* and *gtfD* was significantly higher in *S. mutans* after treatment with *Punica granatum* extract, 10.8-fold, and 1.8-fold respectively compared to *Rhus coriaria* L. extract (P < 0.05).

Discussion

Dental plaque is a complex bacterial biofilm that develops naturally on the tooth's smooth surface. Many species of bacteria especially *Streptococcus mutans*, *fusobacterium*, and *actinobacteria* are involved in the formation of biofilm. *S. mutans*, the primary constituent of dental plaque, synthesizes extracellular adherent glucans from dietary sucrose via GTFs and thus, promotes the accumulation and colonization of oral bacteria on teeth surfaces. Bacterial attachment is an important initial step towards the subsequent formation of mature biofilm. Therefore, compounds with the potential of inhibiting the initial attachment of *S. mutans* to

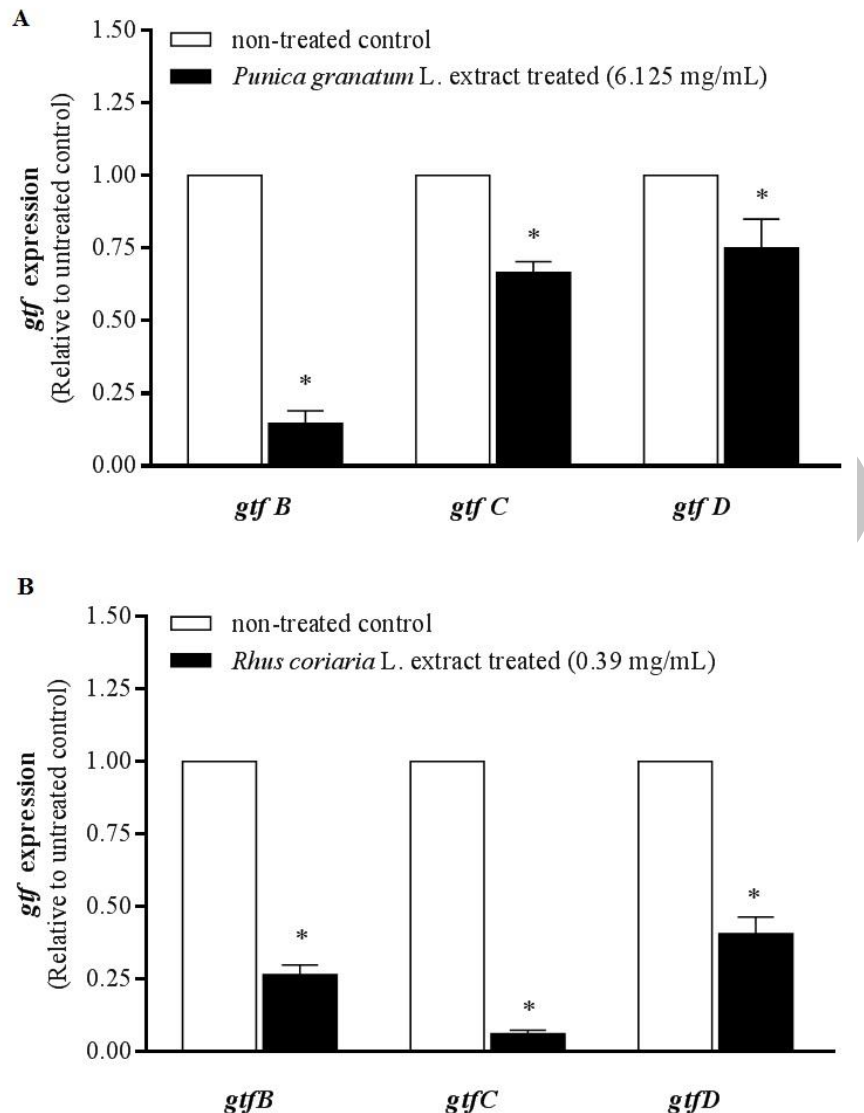


Figure 1. Real-time PCR analysis of *gtfB*, *gtfC*, and *gtfD* gene expression in *S. mutans* growing in presence of 6.125 mg/mL *Punica granatum* L. flower (A) and 0.39 mg/mL *Rhus coriaria* L. fruit (B) water extracts. The mRNA levels of each gene was normalized to that of *recA* (internal control) and then expression in treated cells was compared to that in non-treated control (value of 1). The results are presented as mean \pm SE of three independent experiments ($n = 3$, * $P < 0.05$).

the surface can efficiently prevent the formation of biofilm and maturation of dental plaque (18). Initial prevention is easier and more important than subsequent mechanical treatments (*e.g.* scaling and root planning) especially in patients with fixed orthodontic appliances.

In our previous studies, we reported that *Punica granatum* L. flower and *Rhus coriaria* L. fruit water extracts inhibited *S. mutans* biofilm formation *in-vitro* at MBICs of 6.125 mg/mL

and 0.39 mg/mL, respectively. (16, 17).

In the current study, we aimed to confirm the inhibitory effects of *Punica granatum* L. and *Rhus coriaria* L. extracts on dental biofilm formation by analyzing *gtf* gene expression in *S. mutans*.

Several studies have shown that some herbal extracts have inhibitory effects on biofilm formation by *gtf* family gene suppression. For example, one study showed that Tea catechin

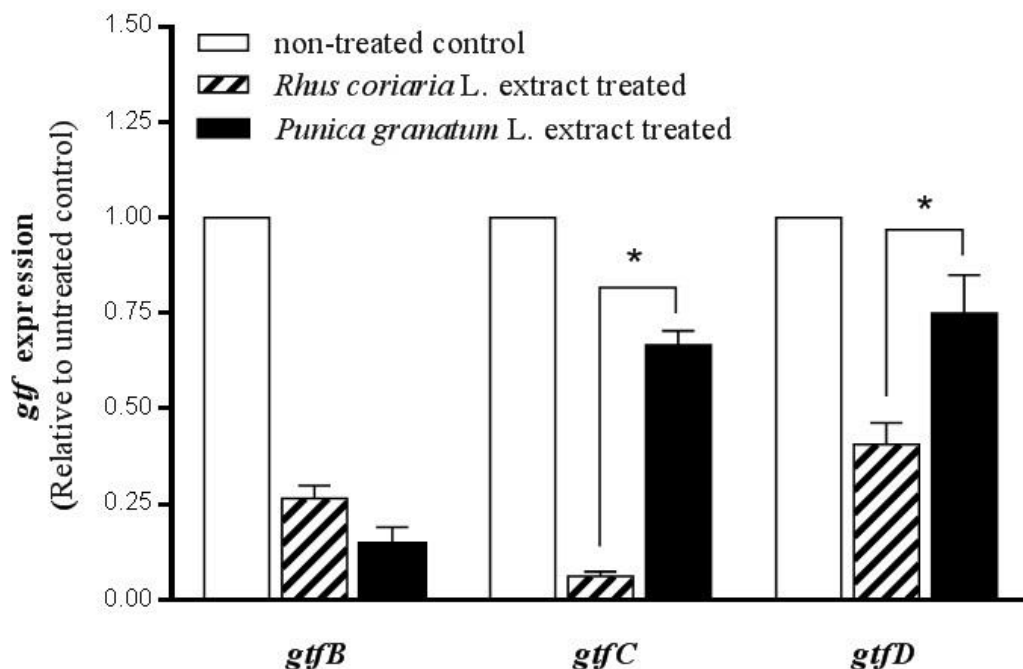


Figure 2. Comparison effects of *Punica granatum* L. flower and *Rhus coriaria* L. fruit water extracts on *gtfB*, *gtfC*, and *gtfD* gene expression. The planktonic cultures of *S. mutans* were treated with MBIC (sub-MIC) level of water extracts (6.125 mg/mL for *Punica granatum* L. and 0.39 mg/mL for *Rhus coriaria* L.). The expression rate in non-treated cultures (control) was considered to be 1. Stars (*) in-between the columns indicate significant differences (n-fold) between the groups ($P < 0.05$).

epigallocatechin gallate inhibited *S. mutans* biofilm formation by suppressing *gtfB*, *C* and *D* genes at sub-MIC levels (19).

Another study indicated that the phenolic fraction from the pomace of *Vitis coignetia* may play an important role in prevention of adherence of *S. mutans* and has a medicinal potential for reducing dental caries when used as a food additive (20).

Also, significant inhibition of GTFs activity by *Nidus vespae* extract revealed its potential as a promising natural product for prevention of dental caries (21).

In the current study, real time PCR assay revealed significant decrease in *gtfB*, *gtfC* and *gtfD* expression following *Punica granatum* L. flower and *Rhus coriaria* L. fruit water extract treatments of *S. mutans*. Based on our findings, both extracts at sub-lethal concentrations are capable of decreasing biofilm formation by *S. mutans* via down-regulating all three *gtf* genes without necessarily suppressing the growth of oral bacteria. These suppressing effects were more remarkable for *Rhus coriaria* L. extract in

comparison with *Punica granatum* L. extract.

In conclusion, *Punica granatum* L. flower and *Rhus coriaria* L. fruit water extracts (especially *Rhus coriaria* L.) are promising natural antiplaque agents and may be used as food additives or in the formulation of dental care products (e.g. mouth washes and toothpastes).

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