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Evaluation of the effects of *Glycyrrhiza glabra* and *Syzygium aromaticum* extracts on gene expression of *Streptococcus mutans* in patients with dental caries

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Abstract. *Al-Amili ML, Al-Jobori KM. 2025. Evaluation of the effects of* Glycyrrhiza glabra *and* Syzygium aromaticum *extracts on gene expression of* Streptococcus mutans *in patients with dental caries. Biodiversitas 26: 418-423.* Dental caries, primarily associated with *Streptococcus mutans*, is among the most widespread diseases, particularly in developing regions such as Iraq. Licorice (*Glycyrrhiza glabra*) and clove (*Syzygium aromaticum*) are plants with significant economic value and antibacterial properties that potentially serve as alternatives to chemically synthesized antibiofilm agents. This study aimed to evaluate the antibacterial activity of *G. glabra* and *S. aromaticum* extracts against *S. mutans* and to compare their effects with those of antibiotics, mouthwashes, and toothpaste through gene expression analysis of *gtfB* and *gtfD* using RT-qPCR. In total, it did not include the methodology or provide details about the Sub-MIC because it was published separately as a second research study in another journal.100 specimens were collected from patients clinically diagnosed by dental physicians at the Hay Al-Hussein Specialized Center in Maysan City, Iraq. RNA was extracted from dentinal lesion specimens and reverse transcribed into complementary DNA (cDNA). Quantitative polymerase chain reaction (qPCR) was performed to analyze the expression of the *gtfB* and *gtfD* genes using the housekeeping gene 16S rRNA as an internal control. The analysis assessed the effects of licorice extract, clove extract, combined extracts, chlorhexidine mouthwash at sub-minimum inhibitory concentration (sub-MIC), and lacalut toothpaste on *S. mutans.* RT-qPCR results revealed that clove extract significantly reduced the expression of *gtfB* compared to other treatments, with fold changes of 0.178, 0.454, and 0.191. Licorice extract notably suppressed *gtfD* expression, with fold changes of 0.215, 0.390, and 0.003 for isolates 74, 80, and 46, respectively. These findings suggest that the plant extracts inhibited specific biofilm-related genes without necessarily reducing the overall bacterial growth. Therefore, these natural extracts can be developed as innovative natural anti-plaque agents.

Keywords: 16S rRNA, chlorhexidine, dental caries, *Glycyrrhiza glabra*, *Syzygium aromaticum*

INTRODUCTION

Dental caries is a common condition in Iraq as well as in other developed and developing nations and is the most serious threat to oral health and the most common oral infection disease (Alhelfi and Hobi 2023). Owing to a lack of knowledge about oral healthcare, the number of patients with dental caries has increased. *Streptococcus mutans*, a key member of the oral microbiota, is recognized as the primary causative agent of dental caries (Cornejo et al. 2013; Abd and Ali 2016). The genus *Streptococcus* includes at many as eight monophyletic groups, and the oral streptococci are mainly distributed in six of these groups: Mitis, Sanguinis, Anginosus, Mutans, Salivarius, and Downei, each of which is named with a representative species. Among these, the Mutans and Downei groups are known to contain many caries-promoting species, which are normally minor in oral microbiome under healthy condition but can grow to markedly higher proportions under disease condition (Whiley and Beighton 1998; Balakrishnan et al. 2000; Kilian et al. 2016).

Dental caries result from the accumulation of plaque on tooth surfaces. When *S. mutans* is present, its acid production significantly increases the risk of tooth demineral. (Marsh 2010; Mays et al. 2024) Tooth discoloration and toxicity have been recorded with commercial antiseptics, antibacterial agents, and antioxidants used in the prevention and treatment of plaque formation. Moreover, *S. mutans* has been shown to be resistant to antibiotics, including penicillin, amoxicillin, tetracycline, fluoroquinolones, and chloramphenicol, because these drugs can cause genetic alterations in their target or mutation, which lowers the affinity of the drug for its substrate (Li et al. 2022). As a result, natural product extracts must be investigated as a potential treatment and preventative measure for plaque development. Oral bacteria, oral environment, host, and time are the four factors that represent the currently recognized etiology of dental caries. An excess of carbohydrates in the diet leads to the colonization of microbes in the mouth, which are both acid- and acid-resistant. The dental biofilm that sticks to the enamel surface becomes bio-imbalanced, leading to tooth caries (Taghreed et al. 2021).

Plants generate a wide range of chemicals, including flavonoids, simple phenolics, terpenoids, and alkaloids (Lafta and Sadeq 2024) Consequently, given that natural compounds might have fewer adverse effects than synthetic antimicrobials, they may be useful for the prevention of dental caries (Chen et al. 2020). Antibacterial phytochemicals found in plants and other natural sources are highly effective at

preventing the growth of harmful bacteria, particularly streptococci. Recently, there has been a focus on finding natural compounds that help fight against *S. mutans* (Gabe et al. 2019). In order to lessen the issue, the development of novel treatment approaches for this oral condition may be helpful. Given the mounting evidence of *S. aromaticum* and *G. glabra* extracts antibacterial qualities, which are comparable to antibiotics' actions against oral pathogens; the hypothesis is that these extracts, alone or in combination, will exhibit more favorable activity than antibiotics when tested against facultatively resistant oral bacteria.

Streptococcus mutans possesses several virulence factors. *GTF*s are useful for absorbing more oral bacteria to create a dental biofilm. *gtf*B, *gtf*C, *Gtf*D, and SpaP are examples of virulence factors that may be linked to the target genes for PCR (Abdelkader et al. 2021; Abo Bakr et al. 2022; Rajabi et al. 2022). According to Xu et al. (2018), biofilms can cause bacteria to become more resistant to antibiotics, making it difficult for host inflammatory cells to phagocytose biofilm cells. One important virulence factor in the pathophysiology of dental caries in humans and animals is the capacity of *S. mutans* to synthesize extracellular glucans (Maria et al. 2010; Al-Qazzaz et al. 2014; Zhang et al. 2021). Glucan production can be affected by changes in the expression level of the *gtf* genes, which in turn impact bacterial adherence and biofilm formation (Wang et al. 2016). 16S rRNA sequencing has been used to examine the features of the oral microbiome in patients with and without caries. In studies on the human oral microbiome, taking advantage of the heterogeneity in the 16S rRNA gene sequence has been seen as a low-cost, high-throughput characterization strategy. Based on 16S rRNA gene sequencing, this method has been verified and shown to be accurate and practical for oral microbiome studies (Bertolo et al. 2024; Moorlag et al. 2024).

A dental caries treatment method that offers an alternative to synthetic chemicals is the use of natural plant components. *Glycyrrhiza glabra* and *Syzygium aromaticum*, are used as extracts to treat dental bacterial infections. The aim of this study was to analyze the antibacterial activity of the extracts of *G. glabra* and *S. aromaticum* against *S. mutans*, as well as to compare their effects with antibiotics, mouthwashes, and toothpaste by gene expression analysis of *gtf*B and *gtf*D using RT-PCR.

MATERIALS AND METHODS

Sample collection and bacterial growth conditions

One hundred specimens were obtained from patients with dental caries clinically diagnosed at the Hay Al Hussein Specialized Center at Mysan City, Iraq, between May 2023 and July 2023 by taking swabs from the mouth cavity of patients with different dental caries (pit, fissure, and dental roots). The specimens were sent to the laboratory and processed on the same day. To prepare for inoculation, the sample was vortexed for 15 s and then diluted one thousand times in an isotonic saline solution. One loop (1/1000 mL of sample) was inoculated on Mutans Sanguis Agar medium and incubated for 48 h under anaerobic conditions in an anaerobic environment (anaerobic container, and sterile transparent adhesive tape was used to seal the container cover).

Preparation of methanolic *Glycyrrhiza glabra* and *Syzygium aromaticum* extracts

Glycyrrhiza glabra *extract*

The roots of *G. glabra* were washed thoroughly with distilled water to remove dust. The roots were dried in a shaded place at room temperature. Dried roots were finely ground using an electric grinder and stored in an airtight container. A methanol solution of licorice root was prepared by mixing 500 g of root powder with 2500 mL of 95% methanol as the solvent in a Sechelt apparatus for 8h. The solvent was removed from the filtrate by passing it through a Whatman filter and evaporating under vacuum for five hours at 60°C in a rotary evaporator. The extract was then sealed in a container and maintained at 40°C (Malvania et al. 2019). To prepare a 1% stock solution, one gm of the dry substrate was mixed with 100 mL of 95% methanol.

Syzygium aromaticum *extract*

The cloves were washed thoroughly with distilled water to remove dust. The samples were then dried in a shaded place at room temperature. Dried cloves were finely ground using an electric grinder and stored in an airtight container. The powder (200 g) was mixed with 2000 mL of 95% methanol as a solvent in a Soxhlet apparatus for 8h. The solvent was removed from the filtrate by passing it through a Whatman filter and evaporating under vacuum using a rotary evaporator set at 40°C for 5 h. The extract was sealed in an airtight container and stored at 4°C until needed (Ishwarya et al. 2022). To prepare a 1% stock solution, one gm of the dry substrate was mixed with 100 mL of 95% methanol.

Determination of sub-minimum inhibitory concentration (Sub-MIC)

In a separate study, we determined the MIC and sub-MIC values for clove, licorice, a clove-licorice mixture, and a mouthwash (chlorhexidine) against *S. mutans*. Subsequently, we used sub-MIC concentrations to analyze the gene expression of *gtf*B and *gtf*D, with the 16S rRNA gene serving as a reference.

Preparation of stock solutions of 2% chlorhexidine

Aliquot of 0.4 mL of chlorhexidine was added to 9.6 mL of sterile distilled water to achieve a concentration of 100 µg/mL (Qaiyumi 2007).

Preparation of stock of toothpaste suspension

Toothpaste suspensions were produced by mixing toothpaste with 0.9% saline solution (NaCl) at a ratio of 1:2 by vortexing (Paqué et al. 2022)

RNA extraction and cDNA synthesis

Following the manufacturer's instructions, total RNA was extracted using the TransZol Up Plus RNA Kit (TransGen Biotech, China). Total RNA was extracted from *S. mutans* cultures of non-treated (control) and 24 h treated

S. mutans with Sub-MIC concentrations of clove was 3.125-6.25 µg/mL, 50 µg/mL of licorice, 12.5 µg/mL of combined clove and licorice, 6.25 µg/mL of mouthwash (chlorhexidine) and 1 µg/mL of toothpaste (lacalut) for the expression of *gtf*B gene. For *gtf*D gene, Sub-MIC concentrations of clove was 1.6-3.12 µg/mL, 1.6-6.25 µg/mL of licorice and combined clove and licorice, 0.8-3.1 µg/mL of mouthwash (chlorhexidine) and 1 µg/mL of toothpaste (lacalut). The following steps were followed using PCR amplification equipment manufactured by Pioneer, Korea, to reverse transcribe 1 µg of extracted RNA using the One-Step gDNA Removal and cDNA Synthesis SuperMix Kit from TransGen Biotech, China, in order to synthesize cDNA.

Real-time quantitative PCR and gene expression analysis

This step was performed to measure the changes in *gtfB* and *gtfD* gene expression in *S. mutans* when exposed to several extracts, including clove, licorice, a combination of the two, chlorhexidine mouthwash, and lacalut toothpaste. The *S. mutans* was not treated. Using a quantitative real-time qRT-PCR SYBR Green test, target gene expression was verified. The primer sequences for the *gtf*B and *gtf*D genes were created and synthesized by Alpha DNA (Canada) (Table 1), which were then lyophilized and kept at -20°C. The endogenous control gene 16S rRNA levels were amplified and used to normalize the *gtf*B and *gtf*D genes. The total reaction volume was (20 µL) containing 10 µL of Top Green qPCR Super Mix, 2 µL template cDNA, 2 µL forward and reverse primers and 6 µL nuclease free water. The RT-PCR system (QIAGEN Rotor gene Qs, Germany) was used for product amplification and detection under the following cycling conditions: initial denaturation at 95°C for 5 min, denaturation at 95°C for 15 s, 40 cycles of annealing at 58°C for 1 min and 60°C for 1 minute for 16S rRNA, and finally, 5 min of extension at 72°C.

Calculation of gene expression

The program StepOne ™ was used to estimate the threshold cycle (Cт) values and to compute the relative expression levels using the comparative Cт (ΔΔCт) approach. The amounts of *gtfB* and *gtfD* gene cDNA were normalized to the 16S rRNA gene cDNA quantities in the same sample. The change in gene expression levels in each test sample was determined by comparing these values with those of the untreated control.

RESULTS AND DISCUSSION

The effect of the methanolic extract of clove, licorice, and their combination on virulence gene expression was evaluated. Real-time PCR was used to quantify *gtf*B- and *gtf*D-specific mRNA expression in comparison with mouthwash (chlorhexidine) and toothpaste (lacalut). The 16S rRNA gene was used as a housekeeping gene. The Ct values of the 16S rRNA gene were similar in all treatments, and it was found that the expression of the 16s RNA gene was not affected by the studied treatments. Thus, the 16S rRNA gene can be successfully used as a reference gene under current conditions. Regarding *gtfB* and *gtfD*, the effect of methanolic extracts of clove, licorice, and their combination, chlorhexidine, and toothpaste exposure, there was variation in Ct (Figures 1 and 2).

The analysis showed that Variations in the *gtf* genes were observed in the mRNA transcripts of the *gftB* and *gtfD* genes among different treatments. Sub-MIC levels of methanolic extracts of clove, licorice, and their combination and chlorhexidine were able to suppress *gtfB* and *gtfD* gene expression in the strongest isolates of *S. mutans* (74, 80, and 46), and a similar effect was observed with toothpaste. The comparative effects of the studied treatments on *gtf* gene expression are presented in Tables 2 and 3.

The effect of clove extract resulted in a more significant reduction in *gtfB* compared to other treatments, with fold values of 0.178, 0.454, and 0.191, whereas licorice extract showed a decrease in *gtfD*, which was more remarkable than other treatments, with fold values of 0.215, 0.390, and 0.003 for isolates 74, 80, and 46, respectively. This indicated the high efficiency of the studied treatments in reducing the adhesive-promoting genes of *S. mutans*.

Table 1. Sequence of universal primer used in the study

Primers	Sequences	Products size (bp)	References
16S rRNA	F: CCTACGGGAGGCAGCAGTAG	101	Vahid-Dastjerdi et al. (2016)
16S rRNA	R: CAACAGAGCTTTACGATCCGAAA		
GtfB	F: AGCAATGCAGCCAATCTACAAAT	96	
GtfB	R: ACGAACTTTGCCGTTATTGTCA		
GtfD	F: ACAGCAGACAGCAGCCAAGA	94	
GtfD	R: ACTGGGTTTGCTGCGTTTG		

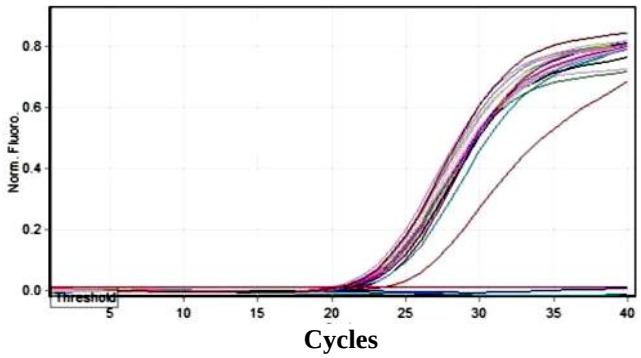

Figure 1. The amplification plots of *gtf*B gene before and after treatment showing cycles

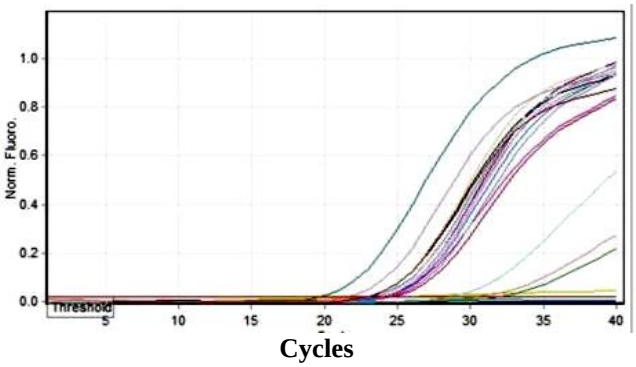

Figure 2. Amplification plots of the *gtf*D gene before and after treatment showing cycles

Table 2. Fold-change of *gtf*B gene expression according to the $2^{-\Delta\Delta Ct}$ method

Plant extracts	Bacterial isolates no.	Concentration	*gtf*B gene CT	16S rRNA gene CT	ΔCT	ΔΔCT	Folding
Clove (C)	74	6.25	23.59	16.24	7.35	2.49	0.178
	80	3.125	21.9	15.9	6.00	1.14	0.454
	46	3.125	22.25	15.00	7.25	2.39	0.191
	-	Control	21.21	16.35	4.86	0.00	1.000
	74	50	21.09	15.75	5.34	0.68	0.624
Licorice (L)	80	50	21.84	16.41	5.43	0.77	0.586
	46	50	21.26	15.89	5.37	0.71	0.611
	-	Control	21.21	16.55	4.66	0.00	1.000
C+L	74	12.5	21.01	15.76	5.25	0.0263	0.5173
	80	12.5	21.20	15.89	5.31	0.0252	0.4962
	46	12.5	21.34	15.8	5.54	0.0215	0.4231
	-	Control	21.21	16.91	4.30	0.0508	1.0000
Mouthwash	74	6.25	20.94	15.66	5.28	0.0257	0.7417
(Chlorhexidine)	80	6.25	21.00	15.56	5.44	0.0230	0.6638
	46	6.25	20.65	15.47	5.18	0.0276	0.7949
	-	Control	20.35	15.50	4.85	0.0347	1.0000
Toothpaste	74	1.0	21.10	15.53	5.57	1.96	0.253
(Lacalut)	80	1.0	20.90	16.16	4.74	1.13	0.457
	46	1.0	20.62	16.71	3.91	0.30	0.812
	-	Control	20.20	16.59	3.61	0.00	1.000

Table 3. Fold-change of *gtfD* gene expression according to the $2^{-\Delta\Delta Ct}$ method

Plant extracts	Bacterial isolates no.	Concentration	*gtfD* gene CT	16S rRNA gene CT	ΔCT	ΔΔCt	Folding
Clove (C)	74	3.12	24.75	15.9	8.85	0.53	0.693
	80	1.6	30.3	16.35	13.950	5.63	0.020
	46	1.6	24.02	15.00	9.020	0.70	0.616
	-	Control	24.56	16.24	8.320	0.00	1.000
Licorice(L)	74	1.6	24.20	15.75	8.450	2.22	0.215
	80	3.6	24.00	16.41	7.590	1.36	0.390
	46	6.25	30.52	15.89	14.630	8.40	0.003
	-	Control	22.78	16.55	6.230	0.00	1.000
C+L	74	1.6	32.00	15.76	16.240	8.89	0.002
	80	6.25	24.04	15.89	8.150	0.80	0.574
	46	3.25	30.3	16.91	13.390	6.04	0.015
	-	Control	23.15	15.8	7.350	0.00	1.000
Mouthwash (Chlorhexidine)	74	3.1	23.49	15.75	7.930	0.067	0.955
	80	1.6	28.57	16.41	13.100	0.584	0.667
	46	0.8	23.32	15.89	7.820	0.56	0.678
	-	Control	22.92	15.66	7.260	0.00	1.000
Toothpaste (Lacalut)	74	1.0	24.54	15.53	9.010	3.52	0.087
	80	1.0	23.96	16.59	7.370	1.88	0.272
	46	1.0	23.00	16.71	6.290	0.80	0.574
	-	Control	21.65	16.16	5.490	0.00	1.000

Discussion

The main cause of dental plaque formation is *S. mutans*, which uses *GTF*s to synthesize extracellular adherent glucans from dietary sucrose, which encourages oral bacteria to accumulate and colonize tooth surfaces. According to Rezaei et al. (2023) and Bushra et al. (2023), bacterial attachment is a crucial first stage in the development of mature biofilms, which eventually causes caries and erosion of the tooth enamel. Thus, substances that have the ability to block *S. mutans'* initial surface attachment of *S. mutans* can effectively stop the development of biofilms, the expression of virulence genes related to *S. mutans*, including *gbpC*, *spaP*, *gtfBCD*, and *ftf*, which encode surface adhesions and enzymes that Produce Extracellular Polysaccharides (EPS), as well as the maturation of dental plaque (Rudin et al. 2023). Proactive avoidance is simpler and more crucial than mechanical interventions. Several previous studies have provided evidence that some herbal extracts have inhibitory effects on biofilm formation by suppressing the *gtf* family genes in *S. mutans* (Zhang et al. 2021; Rudin et al. 2023; Wang et al. 2024).

Plant extracts influence *GTF*s expression in *S. mutans*. Real-time PCR assay results showed a significant reduction in the expression of *gtfB, gtfC,* and *gtfD* after treatment of *S. mutans* with the plant extracts. In the current study, a decrease in the expression of *gtfB* and *gtfD* was observed when *S. mutans* was cultured with sub-MIC concentrations of *S. aromaticum* and *G. glabra* extracts. The findings of this study showed that when *S. aromaticum* and *G. glabra* extracts, chlorhexidine, or toothpaste were applied to *S. mutans*, the expression of 16S rRNA remained unchanged. This implies that, in the investigation of expression under these circumstances, the 16S rRNA gene can be utilized as a reference gene.

Most of the bacterial strains tested for biofilm formation were not effectively inhibited by mouthwash solutions that did not contain cetylpyridinum chloride or chlorohexidine gluconate, such as Colgate. This may be due to the presence of potent ingredients such as chlorhexidine digluconate, which is related to the mechanism of action of chlorohexidine as it acts on many bacterial sites; lacalut active dental mouthwash exhibits the best effect when compared to other types of mouthwashes. According to Khan et al. (2020), the expression of *gtfB*, which is implicated in the development of biofilms, decreased by 0.6-fold when chlorhexidine was present. These findings are consistent with those of the current investigation of the *gtfB* and *gtfD* genes. Plant products, such as essential oils, can function as viable substitute antibacterial agents for oral hygiene because they can be just as effective as chlorhexidine (Khan et al. 2020). Lacalut toothpaste contains ingredients that are not all available in any other toothpaste, such as aqua,hydrogented staech hydrolysate, hydrated silica, poloxame188, sodium lauryl sulfate, aroma, hydroxyethylocellulose, propylene glycol, aluminum lactate, sodium myristyl sulfate, olaflur, sodium fluoride, sodium cetyl sulfate, chlorhexidine digluconate, and bisabolol. The presence of these compounds may reduce the expression of the *gtfB* and *gtfD* genes. Chaudhary et al. (2020) demonstrated that toothpaste is effective in preventing *S. mutans* from growing.

In conclusion, the use of natural herbs, such as clove licorice and many others, either as a single herb or in combination, is safe and beneficial in treating a variety of oral health issues. The results of the present study revealed that clove and licorice extracts altered the expression of the *gtfB* and *gtfD* genes, with a reduction in fold change. It was also observed that they inhibit specific genes linked to bacterial biofilm formation without necessarily reducing oral bacterial growth; thus, they may be utilized as innovative, natural antiplaque agents. Therefore, it would be a good substitute for current medicines that are typically considered while treating dental caries.

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