Original Article
Food Science and Technology

ISSN 0101-2061 (Print)
ISSN 1678-457X (Online)

DOI: https://doi.org/10.1590/fst.38322

The antibiofilm and antibacterial effects of medicinal plant extracts on isolated sulfate-reducing bacteria from orthodontic appliances

Salmeh KALBASSI[1]*, Mahsa YARAHMADI[2], Hasan MOHAMMADIFARD[3], Fatemeh AHMADI[4]

Abstract

Sulfate-Reducing Bacteria (SRB) are a heterogeneous group of oral microflorae that cause dental caries, destructive periodontal infections, and biofilms on fixed orthodontic appliances. Plant extracts as a source of antibacterial components may have acceptable activity against SRB strains and their biofilm development. Our study aimed at determining the antibiofilm, antimicrobial efficacy, and cytotoxicity of Iran's endemic plants against pathogenic SRB. Plantago ovata, Terminalia chebula, Eugenia caryophyllata, and Aloe vera extracts were prepared according to standard methods. Patients and orthodontic appliances samples were collected, and SRB strains were isolated using standard tests and 16S rRNA analysis. The extracts' antimicrobial, antibiofilm activity, and cytotoxicity were determined using dilution broth micro, crystal violet staining, and MTT method, respectively. Biofilm involved gene expression of strains determined using Real-time PCR. Three SRB isolates harboring target genes were identified using 16S rDNA that showed a more than 90% of similarity to Desulfomicrobium orale spp. All investigated extracts showed good activity against SRB isolates. T. chebula, E. caryophyllata, and A. vera showed promising antibiofilm activity, and their subMIC concentrations downregulated biofilm-forming related genes in all isolates. T. Chebula had the lowest cytotoxicity, followed by E. caryophyllata and A. vera. T. Chebula also showed the best average selectivity index (SI) of 17.9, followed by E. caryophyllata (12.52). Extracts investigated in the present study had good antibacterial and antibiofilm activity against isolated sulfate-reducing bacteria isolated from patients' orthodontic appliances. Most of these extracts may be potential candidates for the development of antibacterial drugs.

Keywords: sulfate-reducing bacteria; medicinal plants; antibiofilm.

Practical Application: Control of SRB induced oral and dental biofilm and infections.

1 Introduction

The most important microorganisms involved in the dental biofilm formation process are SRB, which uses sulfate and produces hydrogen sulfide (H_2S) gas. In addition, these bacteria use iron in the environment and produce black SFe sediment (Amado et al., 2020). The SRB bacteria get their energy from reducing sulfate in humans and animals' gastrointestinal tracts (mouth and intestines). These microorganisms are found everywhere in the environment and are scattered in the water, air, and soil (Dula et al., 2021). Numerous studies have shown that bacteria in the oral environment infect the tissue around the tooth and cause inflammation. The resulting infection and inflammation lead to dental biofilm and orthodontic–periodontic problems. Chronic periodontitis is considered the most frequent form of periodontitis, which leads to inflammation in the supporting tissues of the teeth and their progressive loss of adhesion and gingival bone resorption. Bacteria that stay on the oral surfaces for a long time due to poor oral hygiene led to the development of a layer called dental plaque, which becomes a hard layer called calculus over time. These formations gradually spread to the subgingival space, which is difficult to clear through conventional hygienic methods and leads to periodontal disease (Kushkevych et al., 2020; Cwalina et al., 2017).

Periodontal pathogens are usually a small fraction of the total of 600 known bacterial species that can colonize the dental surfaces and the underside of the gums and oral mucosa. SRB bacteria play a significant role in the onset of persistent inflammation such as gum disease and dental plaque (Naginyte et al., 2019). Most periodontal pathogens are anaerobic, and their number in the infection medium depends on the biofilm environment created and the periodontal pocket (Winning et al., 2015; Cruz et al., 2022). A biofilm is a complex microbial community enclosed within a polysaccharide or protein matrix caused by gram-positive and gram-negative strains (Jakubovics et al., 2021). Bacterial resistance to antimicrobials in the biofilm phase is a major global issue today. In addition, studies have shown that biofilm formation on fixed orthodontic appliances, which happens due to etching, priming, and applying brackets to the tooth surface, can cause enamel demineralization and gingival tissue inflammation (Ghane et al., 2022; Jeon et al., 2020).

Received 15 Apr., 2022
Accepted 02 July, 2022
[1] *Independent researcher, Tehran, Tehran province, Iran*
[2] *Department of Clinical Pharmacy, Faculty of Pharmacy, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran*
[3] *Department of Pathobiology, Science and Research Branch, Islamic Azad University, Tehran, Iran*
[4] *Department of Microbiology, North Tehran Branch, Islamic Azad University, Tehran, Iran*
**Corresponding author: Kalbassi_salmeh@yahoo.com*

Investigates have shown that SRB-produced biofilms are resistant to many agents (Crispim et al., 2018; Shastry & Rekha, 2021), such as antibiotics and disinfectants, and their removal requires a new approach (Shin & Kim, 2022). Many genes are involved in the function of SRB strains. The enzyme adenosine 5-phosphosulfate reductase (APS), encoded by the aprAB gene, is a flavoprotein with two alpha and beta subunits that convert APS to sulfite or bisulfite and adenosine monophosphate. Another important enzyme in SRBs is the sulfite reductase enzyme, which has three subunits encoded by dsvABC genes. Another important enzyme in the function of SRB bacteria is ATP sulfurylase, which is encoded by the sat gene and plays a key role in the uptake of sulfate activated by APS sulfurylase (Tripathi et al., 2021; Li et al., 2021).

Over the last few years, with the propensity of people to use verdant medicines because of the adverse effects and antibiotic resistance caused by chemical drugs, and the emphasis of the World Health Organization on the replacing of chemicals with natural ingredients has led researchers to use these rich reservoirs of useful compounds in the pharmaceutical industry and the production of antimicrobials with the least side effects (Alwazeer et al., 2020; El-Shafei et al., 2020; Bulut et al., 2021; Yonjalli et al., 2020; Son et al., 2022). Studies have shown that these herbs, such as P. ovata, T. chebula, E. caryophyllata, and A. vera, were used to treat inflammation of the oral mucosa (Jokar et al., 2016; Karami et al., 2017; Lu et al., 2019). In addition, there is no investigation about SRB strains causing biofilm on fixed orthodontic appliances. Considering the above and confirming the antibacterial effects of various plant extracts, it seems that these plants can combat different types of dental caries and can be used as an active ingredient to prevent caries caused by SRB strains. Therefore, our study aimed to investigate the effect of different plant extracts on the biofilm formation and cytotoxicity of SRB strains isolated from orthodontic appliances of patients.

2 Material and methods

2.1 Sampling

The current study was performed on samples taken for diagnostic studies in Dr. Kalbassi dentistry clinic in Tehran and Isfahan provinces of Iran. Samples from orthodontic appliances including brackets, molar bonds, arch wires, and auxiliaries were collected by two orthodontists and kept in 4 ˚C until further examinations.

2.2 Isolation of bacteria

Two blood agar plates were used under aerobic and anaerobic conditions to isolate the bacteria. Then, common microbiological tests were used to accurately identify microbial isolates, such as Gram staining, API kit sugar tests, and biochemical tests. Finally, the bacteria were cultured in an SRB-specific culture medium to isolate pure strains. Postgate E culture medium (Merk, Germany) containing agar, 2.0 g; Sodium sulfate, 1.0 g; potassium dihydrogen phosphate, 0.5 g; ammonium chloride, 1.0 g; calcium chloride, 1.0 g; magnesium chloride hexahydrate, 1.83 g; ascorbic acid, 0.1 g; yeast extract, 1.0 g; sodium thioglycolate, 0.013 g; sodium citrate, 6.38 g; sodium lactate 1.75 mL; NaCl 3.5%, resazurin, 2.0 mL 0.025% P/V, ferrous sulfate heptahydrate, 0.5 g per liter was used for culture and isolation of SRB strains. A reducing solution (pH = 6.5) was also used to transfer optional anaerobic bacteria. Immediately after collecting the samples, each sample was subjected to a tube containing the reduction solution and shaken for 10 s. One mL of the solution was cultured in Postgate E medium for 28 days at 30 °C. The samples in reducing solution were then transferred to Nutrient Broth (NB) culture medium and cultured for 48 hours. Following confirmation and isolation of the colonies, they were subjected to molecular studies. Samples cultured in Postgate E were examined for 28 days. Changing the medium color to black due to the formation of iron sulfide was considered SRB bacteria. Also, the growth of anaerobic bacteria that did not reduce sulfate produced a clear white or pink color in the culture medium.

2.3 Multiplex PCR

DNA extraction from bacteria was performed by QIAmp DNA Mini Kit (QIAGEN) and following its instructions. The concentration of oligonucleotides was determined by the nanodrop device determining the OD at 260 and 280 nm (Eppendorf BioPhotometer D30, Germany). DNA purity was calculated by determining the OD ratio at 260 to 280 nm (1.5 to 2), indicating that the DNA-containing solution was properly purified. The conserved sequence of target genes and 16S rRNA of SRB bacteria was determined at the NCBI website. Primers were designed using Gene runner software (Table 1). Multiplex PCR amplified target sequences under the following conditions in 35 cycles. Preliminary denaturation at 95 °C for five min, denaturation at 95 °C for 60 s, annealing of primers at 55 °C for 60 s, polymerization at 71 °C for 3 min and elongation at 72 °C for 2 min employing a thermocycler (Bio-Rad, USA). DNA fragments were separated by electrophoresis on 2% agarose gel, and the resulting fragments were sequenced. Isolated strains were identified by 16S rRNA sequence analysis using a genetic analyzer (Bioneer, South Korea). BLASTN program of NCBI website used for search homologous nucleotide sequence encoding 16S rRNA gene.

2.4 Extraction of plants

Authenticated P. ovata (seeds), A. vera (leaf), T. chebula (fruit), and E. caryophyllata (flower bud) were prepared from the Tehran university botanical garden and washed several times. The herbs were identified by five experts in the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Tehran University of medical sciences, Tehran, Iran. Herbs were dried in a dark place for several days and then ground by a shredder. Extraction of 200 g of powdered material with 500 mL of water was performed by reflux condenser for 8 h at boiling temperature by distillation method. The hydroalcoholic extract of T. chebula was prepared according to Bag et al. (2013) study, the hydroalcoholic extract of P. ovata was carefully prepared by the method used in the study of Karami et al. (2017), and the hydroalcoholic extract of e. caryophyllata was prepared according to the Zam et al. (2020) study.

2.5 Gas Chromatography-Mass Spectrometry (GC-MS) analysis

Major phytochemical constituents of the hydroalcoholic extracts of the plants were determined using GC-MS analysis.

Table 1. Primers used in this study.

Primer	Sequence (5-3)	Product size
16S RNA	CCGTAGATATCTGGAGGAACATCAG ACATCTAGCATCCATCGTTTACAGC	1500 bp
BFR	CCGCGAAAGTCATCTGAGAAGTGC CATCGGCAAGTTCTCCGTAGTC	360 bp
AprB	TTCGTGCGTATGCCCCGATCGCAACC ATGCCAACCTTCGTGCGTCCCGATCG	411 bp
dsvA	CAAGTTCTCCGTAGAGTTCTCCGTAG GTTCTCCGTAGAGTTCTCCGTTCTCCG	510 bp
dsvB	AGTTCTCACATCAAGTTCTAGTTC TTCTCAAGCAAGTAAGTTTCTCCT	272 bp
dsvC	ACAGTTCTCATTCTAGTCAAGTTC GCGTATGCCCCGACAGTTCTC	455 bp
sat	TCACAGTTACAGTTCCACAGTTCTCTC TCACAACAGTGTTCTCCACTCAGTTCT	560 bp

Perkin-Elmer Clarus 680 system (USA) equipped with a fused silica column, packed with Elite-5MS) capillary column (HP-5 MS capillary column (30 m × 0.25 mm, 0.25 mm) was used for analysis. In this study, helium gas was used as the carrier (flow-rate of 1 mL/min) and an electron ionization energy method was adopted with high ionization energy of 70 eV (electron Volts) with 0.2 s of scan time and fragments ranging from 40 to 600 m/z. The injector temperature was maintained at 250 °C (constant) and injection quantity of one µL was used. The column oven temperature was set at 52 °C for 3.5 min, raised at 10 °C per min up to 280 °C, and final temperature was increased to 300 °C for 10 min. The contents of phytochemicals present in the test samples were identified based on comparison of their retention time (min), peak area, peak height and mass spectral patterns with those spectral databases of authentic compounds stored in the National Institute of Standards and Technology (NIST) library.

2.6 Determination of MIC of plant extracts

The minimum inhibitory concentration (MIC) of four herbal extracts against SRB strains was determined by the dilution broth micro method in 96 sterile plates. For this purpose, 90 µL of Müller-Hinton Broth culture medium (containing 2% sucrose for oral bacteria) containing 1.3 × 106 CFU/mL of the SRB bacteria were poured into each 96 microplate wells. Extracts were serially diluted in two folds to obtain the concentrations from 512 to 0.98 µg/mL and added to each well, and plates were incubated for 24 h at 37 °C. Then, the last well that showed turbidity was considered as MIC. Subminimum inhibitory concentrations of the extracts were used for gene expression studies. All antimicrobial tests were performed in triplicate.

2.7 Biofilm formation activity of SRB strains

The biofilm formation strength of the strains was evaluated according to the Famuyide et al. (2019) method. Briefly, fresh colonies of SRB isolates harboring target genes were inoculated to Luria-Bertani broth (LB) medium. The cultures were incubated overnight at 37 °C with shaking at 180 rpm. Next, the number of cells in each culture was quantified and optical density adjusted to 0.5 McFarland turbidity standard. Then, 150 µL of each bacterial suspension was subjected to eight wells of a 96-well flat-bottom microtiter plate containing Brain Heart Infusion Broth (BHI). Incubation was carried out at 37 °C for 24 h. Cultures were then aspirated, and the wells were washed three times with Phosphate Buffer Saline (PBS). Next, the plates were air-dried overnight and stained with 0.1% crystal violet. After staining for 30 min, the culture wells were washed three times with deionized water to remove the unbound crystal violet. The optical density of the wells was measured at 560 nm using an ELISA auto reader (Berthold Technologies). Sterile BHI broth was served as a negative control. All assays were done in triplicates.

2.8 Antibiofilm activity of the extracts

96-well plates were employed to assess biofilm formation under extracts stress. Overnight cultures of SRB strains harboring target genes were adjusted to an 0.5 McFarland standard in LB medium and co-cultured by subMIC concentration of extracts as treatment and without agents as a control for 24 h at 37 °C with slight shaking. Bacterial growth was detected by measuring absorbance at OD560 by Wensar Double Beam UV-VIS Spectrophotometer, LMSP-UV1900 (Wensar, India). Formed biofilms were stained with 100 µL of crystal violet for a half-hour at 25 °C. Then, plates were emptied and dried. Dried crystal violet was extracted with ethanol (95%, v/v), and total biofilm formation was then analyzed at OD570. The assessment was performed as triplicates, and results were performed as the averages with standard deviations. Extracts' ability to destroy preformed biofilms was investigated as previously described (Famuyide et al., 2019), and the biofilm biomass was examined using crystal violet staining.

2.9 Real-time PCR

Finally, the effect of plant subMIC concentrations on isolated SRB strains harboring target genes was investigated. Total RNA of SRB bacteria was extracted using an RNA isolation kit (QIAGEN) and stored at -80 °C. Then, cDNA was synthesized using a Thermoscript reverse transcriptase kit (Invitrogen), and real-time PCR was performed using a real-time PCR kit (QIAGEN) following factory instructions.

Real-time PCR was performed with thermal cyclers (Bio-Rad T100) using SYBR Green PCR Master MIX (Applied Biosystems). The relative rate of gene expression was determined using 16S rRNA as a housekeeping reference. Finally, the specificity of the PCR reaction was determined by melting curve analysis in the final stage of PCR. Real-time PCR data were analyzed using the software in ABI device by ΔΔCT-method, and the final number obtained from different iterations was statistically analyzed using Microsoft Office Excel software.

2.10 Cytotoxicity

In this study, the in vitro cellular toxicity of the extracts to Vero cells obtained from the collection of the Iranian Biological Resource Center (IBRC), Tehran, Iran, was determined using the 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide (MTT) method. Cells were grown in RPMI 1640 medium containing 5% fetal calf serum and 0.1% gentamicin in a 5% CO2 incubator. The cell suspension was plated at a density of 6 × 104 cells into each 96-well cell culture plate well and incubated for 24 h at 37 °C in a 5% CO2 incubator. Then, different concentrations of the extracts (0.025 to 1000 µg/mL) were added to wells and incubated for 48 h. Doxorubicin and dimethyl sulfoxide (DMSO) were used as a positive and negative control, respectively. Next, wells were rinsed with PBS, and fresh RPMI 1640 medium (200 µL) was dispensed to each well. Then 30 µL of MTT dissolved in PBS and added to wells, and plates were incubated for 5 h at 37 °C in a 5% CO2 incubator. Next, the medium aspirated and 100 µL of DMSO was added to each well and carefully pipetted. The produced formazan was assessed spectrophotometrically at 570 nm wavelength, and the cell viability percentage and selectivity index (SI = LD50/MIC) were calculated as previously described (Ser et al., 2015). Each extract concentration was examined in quadruplicate.

3 Results and discussion

Despite the availability of appropriate oral care and improving oral hygiene, the cases of infectious diseases of the oral cavity have been growing in recent decades. In this context, the oral cavity issues due to SRB strains had been increased. In addition, because of the irreversible side effects of chemical drugs, including tooth staining, hypersensitivity reactions, toxicity, as well as the resistance of bacterial strains to these treatments, which has become a global problem, researchers have turned to the use of plants in the pharmaceutical industry and the production of antimicrobials with minimal side effects. As there are rare studies about SRB strains biofilms causing oral and dental issues, we evaluated the antibiofilm, antimicrobial effects, and cytotoxicity of plant extracts against biofilm-forming SRB species.

3.1 Bacterial isolation

To conduct this study, samples of 30 patients with orthodontic appliances were examined. Generally, 30 samples were taken from orthodontic instruments, materials, and patients' fixed appliances. During different stages of isolation and culture, 18 SRB bacteria were isolated from samples and cultured in general-purpose media and specific SRB culture medium. Then the Gram-negative, non-spore-forming, rod-shaped and motile strains that blackened Postgate E medium as described in the Bergey's Manual of Systematics of Archaea and Bacteria were identified subjected to 16S rRNA analysis.

3.2 Multiplex PCR and sequencing

The complete 16S rRNA gene sequences showed that 18 sulfated reducing bacteria were isolated from samples, of which 3 harbored all target genes. The phylogenetic and genomic analyses indicated that strains are closely related to Desulfomicrobium orale strain NY677, Desulfomicrobium orale strain NY682 and Desulfomicrobium orale strain NY683 as they formed a distinct subclade at more than 90% bootstrap value (Figure 1). Heggendorn et al. (2014) examined patients and reported the presence of SRB strains in 66.67% of the samples and linked the periodontal diseases to these bacteria (Heggendorn et al., 2014). Here, we found SRB strains in 60% of the samples. The significant elevation of the SRB strains had been reported to be associated with periodontal diseases (Gopalakrishnan et al., 2019). Still, different species were found in both studies that could be attributed to the study population.

3.3 GC-MS analysis

The utilization of GC-MS was useful for the identification of the bioactive compounds in extracted plants. Table 2 listed the important bioactive compounds present in the hydroalcoholic extracts of the plants.

3.4 Antibacterial activity

The antibacterial activity of the extracts against isolated strains was detailed in Table 2. The MIC values for the extracts ranged between 0.11-96.7 µg/mL against strain 1, 57.2-219.22 µg/mL for strain 2, and 40.2-75.5 µg/mL for strain 3. These results indicated that the extracts were active against all SRB strains. Antibacterial activity of the aloe vera (Jain et al., 2016; Sánchez et al., 2020), E. caryophyllata (Azizan et al., 2019; Zainol et al., 2017), and T. chebula (Mishra et al., 2019; Patel et al., 2017) extracts against both gram-positive and gram-negative multi-drug resistant oral pathogens have been reported previously. In addition, some studies reported antibacterial properties of P. ovata (Hammami et al., 2020; Patel et al., 2018), but there was no survey about the effects of these extracts on SRB strains. This result appears that our study is the first study reporting the antibacterial and antibiofilm effects of these medicinal plants extracts against pathogenic SRB strains. Pyrogallol and eugenol as major components of T. Chebula fruit and E. caryophyllata, respectively showed antibacterial activities (Jokar et al., 2016; Azizan et al., 2019; Zainol et al., 2017).

3.5 Antibiofilm activity

Bacterial biofilm is a universal threat to human oral health because of resistance to treatment and the capability to exacerbate nosocomial conditions. Henceforth, the search for novel effective agents to challenge this issue is a priority. In this study biofilm, formation screening showed that three isolated

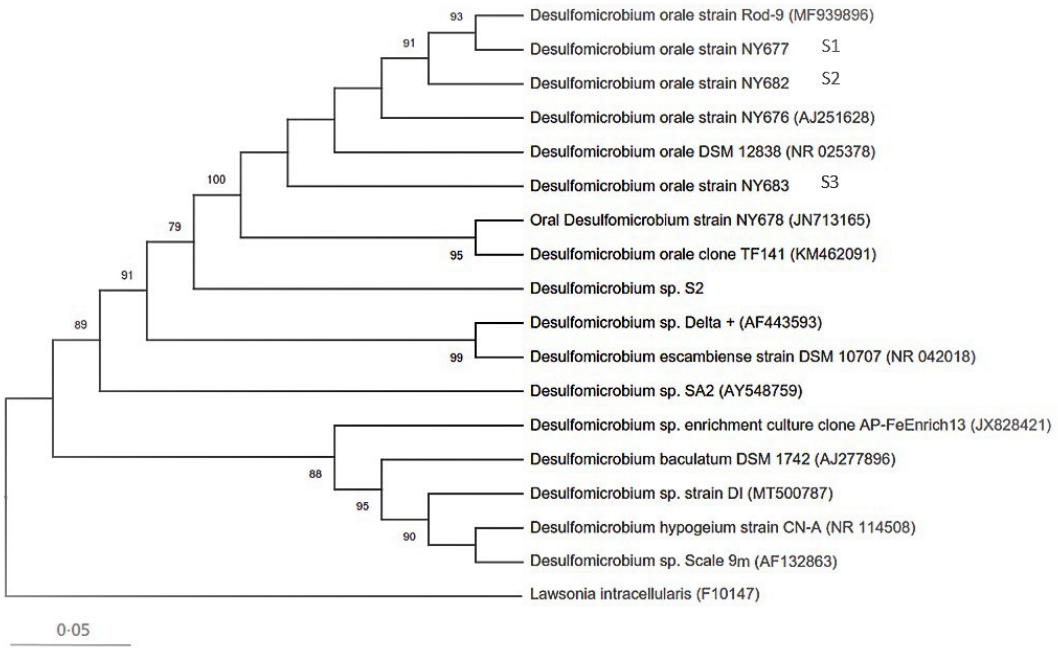

Figure 1. Maximum likelihood tree of 16S rRNA sequences using the Neighbor-Joining method showing the phylogenetic positions of isolated sulfate-reducing strains (S1, S2, and S3) to other members of the SRB family. Bootstrap values greater than 75% are shown.

Table 2. Major components identified in hydroalcoholic extracts of the plants by GC-MS analysis.

No.	Aloe vera	T. chebula	E. caryophyllata	P. ovata
1	β-sitosterol	Pyrogallol	Eugenol	Oleic acid
2	Oleic acid	9-Octadecene	Phenol,2-methoxy-4-(2-propenyl)	Palmitic acid
3	Phytosol	9-Eicosene	1,2,3-Benzenetriol	Octadecanoic acid
4	9-Octadecenoic acid, (2-phenyl-1,3dioxolan-4-yl)methyle ester.cis-	Phenol	Caryophyllene	Docosane
5	Hexadecanoic acid	10-Nonadecanone	Asarone	Tetracosane
6	Lupeol	Cyclooctacosane	2′,3′,4’Trimethoxyacetophenone	Pentacosane
7	n-Hexadecanoic acid	Hexadecanoic acid	1,4,7-Cycloundecatriene,1,5,9,9-tetramethyl-z,z,z	Tetradecane
8	Tocopherol	1-Tricosene	Caryophyllene oxide	Thymol
9	Nonadecane, 2-methyl	Heptafluorobutyric acid	Phenol,2-methoxy-4-(methoxymethyl)	Glycerylpalmitate
10	Octadecane, 2-methyl-	Triacontanoic acid	Farnesol,acetate	1-Tetracosanol

strains were capable of producing strong biofilms. Examination of the effect of subMIC extracts on these strains showed that A. vera, T. chebula, and E. caryophyllata had an anti-biofilm effect on two strains producing strong biofilm ($p < 0.05$). In contrast, P. ovata had an anti-biofilm impact on one of the strains. Strain 1 showed the highest sensitivity, and strain 3 had the highest resistance to extracts (Figure 2). The data showed that the three extracts had an inhibitory effect on all strains within 24 h after biofilm formation (Figures 3-4). After 48 h of biofilm formation, P. ovata extract did not show anti-biofilm activity. At the same time, the other three extracts, including aloe vera, T. chebula, and E. caryophyllata prevented the formation of biofilm (> 50%) ($p < 0.05$). The ability of antibacterial agents to destroy or interfere with the formation of biofilms is attributed to their active components that reduce the colonization of bacterial cells in epithelial mucosa and dental surfaces. In our study, except for P. ovata that just affected strain 1, other extracts inhibited the formation of all strain's biofilms. In addition, A. vera, T. chebula, and E. caryophyllata prevented attachment of all strains by over 65%, revealing a promising antibiofilm property. Previous studies exhibited that these extracts' major components, such as myrobalan and eugenol, exert their action by increasing the permeability of the cell membrane, causing the export of ATP and K+ molecules (Jokar et al., 2016). It has also been reported that these components subMIC concentrations inhibited P. gingivalis, S. aureus, S. agalactiae, and Enterococcus faecalis biofilm formation (Adil et al., 2014; Sarabhai et al., 2013). In this study, subMIC concentrations of the extracts inhibited SRB strains' biofilm formation, and 24-48 h treatment removed preformed biofilm. The outstanding function of the plant extracts to hinder

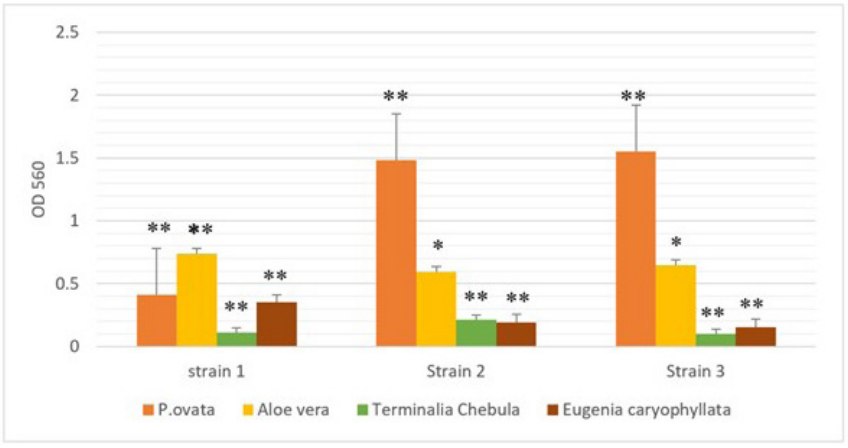

Figure 2. Antibiofilm effect of subMIC concentrations of four herbal extracts against strong biofilm-producing strains of sulfate-reducing bacteria. The untreated biofilm Odr = 1.6. Data are presented as Mean ± SD, *= $p < 0.05$, and **$p < 0.00$ versus control.

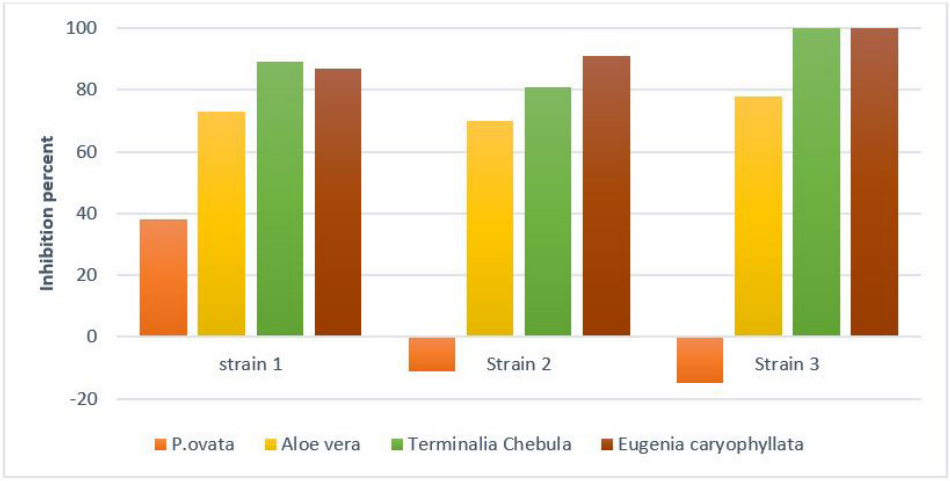

Figure 3. Effect of herbal extracts of four plants against formed biofilm by sulfate-reducing bacteria. The results ≤ 50% showed low activity, and > 50% showed high activity against the SRB strains. Negative values represent the development of formed biofilm.

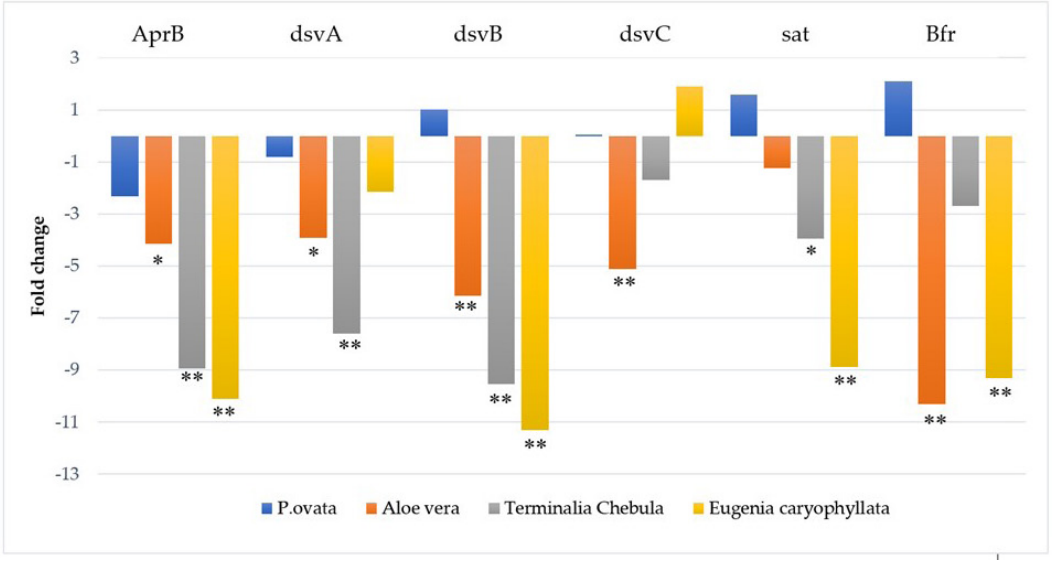

Figure 4. Bar graph presents the fold change in target genes with quantitative real time-PCR. Data are presented as Mean ± SD, * = $p < 0.05$, and **$p < 0.00$ versus control.

Table 3. The MIC (μg/mL), selectivity index, and in vitro cytotoxicity (LC50) of the four plant extracts against Vero cells.

Extract	Cytotoxicity (μg/mL)	Strain 1		Strain 2		Strain 3		Average SI
		MIC	SI	MIC	SI	MIC	SI	
Planta ovata	113 ± 0.08	0.11	10.27	219.22	1.94	40.02	3.54	5.71
Aloe vera	74 ± 0.03	96.7	13.07	163.02	22.03	60.01	8.11	14.4
Terminalia Chebula	39 ± 0.05	76.5	19.64	57.2	14.68	75.5	19.38	17.9
Eugenia caryophyllata	44 ± 0.001	61.5	13.99	44.8	10.2	58.9	13.39	12.52

the early stage of biofilm establishment of the SRB isolates may be attributed to interference with electrostatic interaction, van der Waals, and Brownian forces that mediates the deposition and adherence of bacteria to surfaces (Roy et al., 2018). Some studies showed that plants extracts inhibit the availability of nutrients for bacterial cell growth and cell adhesion. Following our findings, Aires et al. (2021) evaluated the bacteriostatic and bactericidal abilities of e. caryophyllata against oral bacteria in planktonic and biofilm states. They revealed promising antibiofilm activity of the plant (Aires et al., 2021). In another study, Aloe vera extract had acceptable antibiofilm activity against various drug resistant microorganisms (Galleh et al., 2021; Cherian et al., 2019; Cardoso et al., 2021). Another important of SRB strains is that in addition to producing mono-specie biofilm, they are also involved in the production of multispecies biofilms and major oral pathogens such as S. mutans and Porphyromonas gingivalis. Therefore, the nonspecific mechanism of action of the extracts may affect these strong multispecies biofilms as their component's antibacterial properties against drug-resistant oral pathogens have been demonstrated (Zhang et al., 2017; Miladi et al., 2017). Rare studies are examining biofilm inhibition on orthodontic appliances. In a study investigating the effect of Padma-hepaten herbal combination on the biofilm development of S. mutans on orthodontic surfaces, they revealed the antibiofilm effects of that extracts. Our findings prove the use of these extracts as antibiofilm agents. With increased antibiotic resistance, the use of natural medicinal plants to prevent biofilm formation and development without cytotoxicity against human cells may pave the way to an innovative therapeutic concept.

3.6 Gene expression analysis

The results of studying the expression of genes involved in biofilm formation in isolated SRB strains are detailed in Figure 4. They indicate that the fold change for these genes was significantly reduced due to treatment with plant extracts. These data suggest that these gene expressions were downregulated in the treated group compared to the untreated group ($p < 0.05$, CI = 95%). The suppression of these genes demonstrated that except P. ovata extract, all other extracts induced downregulation of these virulence factors, which interferes with bacterial colonization and the initial stage of biofilm formation. There was no investigation about the effects of antibiofilm agents on SRB strains' biofilm-related genes expression profile. Still, there is evidence about the influences of these extracts on virulence genes expression on other species (Lu et al., 2019; Zhang et al., 2017). The present study results indicate that A. vera, T. chebula, and E. caryophyllata decrease the formation and development of biofilm, and downregulating biofilm formation genes expression is one of the mechanisms of action of these agents. As a result, these extracts appear to be an ideal candidate to manage and prevent oral diseases. In line with our finding's studies revealed downregulation of the virulence genes of oral pathogens following treatment with eugenol, myrobalan, and acemannan as major components of E. caryophyllata, T. chebula, and A. vera, respectively (Adil et al., 2014; Farkash et al., 2020; Salah et al., 2017).

3.7 Cytotoxicity

The selectivity index and cytotoxic activity of the extracts against Vero lineage are detailed in Table 3. The hydroalcoholic extract of T. Chebula had the lowest cytotoxicity, followed by Eugenia caryophyllata and Aloe vera. P. ovata extract had LC50 > 100 μg/mL. In this investigation, the safety margin of the extracts measured using SI value and T. Chebula showed the best average SI value of 17.9 against isolated strains, followed by E. caryophyllata (12.52) and A. vera (14.4). It must be pointed out that P. ovata showed a relatively low SI value of 5.71. Although herbal extracts are considered safe, it is essential to evaluate their cytotoxicity scientifically. Previous reports showed that plant extract with LD50 ≤ 20 μg/mL must be considered highly cytotoxic agents (Kuete et al., 2011). In addition, it must be pointed out that in vitro evaluation of the agent's cytotoxicity is not adequate because various factors are involved. All the plant extract evaluated had low cytotoxicity against Vero cells with LD50 ≤ 20 μg/mL in the present study. The selectivity index of an antimicrobial agent is a significant factor as a SI > 1 reveals that the extract is more toxic to the strains than to normal animal cells. Plant extracts with SI value are safer for host cells and more potent to be developed as a safe, natural agent. Our results demonstrated that all extracts evaluated in this study have the potential to be developed into effective and safe natural antimicrobial agents to control biofilm in orthodontic appliances and periodontic infections.

4 Conclusion

There was rare information about the sulfate-reducing bacteria biofilms as an oral pathogen and antibiofilm activity of investigated plants against these strains. The present study revealed that E. caryophyllata, T. chebula, and A. vera had promising activity against biofilm formation and the development of isolated strains. All extracts showed good antimicrobial activity and relatively low cytotoxicity, making them nonnegligible candidates for isolation of functional components and possible development into natural products that can be considered templates for new antibiofilm and antibacterial agents. These extracts showed good activity against SRB strains that may stimulate further biological studies on underestimated medicinal plants, especially those native to Iran.

Conflict of interest

The authors declare no competing interests.

References

Adil, M., Singh, K., Verma, P. K., & Khan, A. U. (2014). Eugenol-induced suppression of biofilm-forming genes in Streptococcus mutans: an approach to inhibit biofilms. *Journal of Global Antimicrobial Resistance*, 2(4), 286-292. http://dx.doi.org/10.1016/j.jgar.2014.05.006. PMid:27873689.

Aires, A., Barreto, A. S., & Semedo-Lemsaddek, T. (2021). Antimicrobial effects of essential oils on oral microbiota biofilms: the toothbrush in vitro model. *Antibiotics*, 10(1), 21. http://dx.doi.org/10.3390/antibiotics10010021. PMid:33383788.

Alwazeer, D., Bulut, M., & Tunçtürk, Y. (2020). Fortification of milk with plant extracts modifies the acidification and reducing capacities of yoghurt bacteria. *International Journal of Dairy Technology*, 73(1), 117-125. http://dx.doi.org/10.1111/1471-0307.12643.

Amado, P. P. P., Kawamoto, D., Albuquerque-Souza, E., Franco, D. C., Saraiva, L., Casarin, R. C. V., Horliana, A. C. R. T., & Mayer, M. P. A. (2020). Oral and fecal microbiome in molar-incisor pattern periodontitis. *Frontiers in Cellular and Infection Microbiology*, 10, 583761. http://dx.doi.org/10.3389/fcimb.2020.583761. PMid:33117737.

Azizan, N., Mohd-Said, S., Mazlan, M. K. F., Chelvan, K. T., Hanafiah, R. M., & Zainal-Abidin, Z. (2019). In-vitro inhibitory effect of Cinnamomum zeylanicum and Eugenia caryophyllata oils on multispecies anaerobic oral biofilm. *Journal of International Dental and Medical Research*, 12(2), 411-417.

Bag, A., Bhattacharyya, S. K., & Pal, N. K. (2013). Antibacterial potential of hydroalcoholic extracts of Triphala components against multidrug-resistant uropathogenic bacteria–a preliminary report. *Indian Journal of Experimental Biology*, 51(9), 709-714. PMid:24377130.

Bulut, M., Tunçtürk, Y., & Alwazeer, D. (2021). Effect of fortification of set-type yoghurt with different plant extracts on its physicochemical, rheological, textural and sensory properties during storage. *International Journal of Dairy Technology*, 74(4), 723-736. http://dx.doi.org/10.1111/1471-0307.12803.

Cardoso, V. F. S., Roppa, R. H. A., Antunes, C., Moraes, A. N. S., Santi, L., & Konrath, E. L. (2021). Efficacy of medicinal plant extracts as dental and periodontal antibiofilm agents: a systematic review of randomized clinical trials. *Journal of Ethnopharmacology*, 281, 114541. http://dx.doi.org/10.1016/j.jep.2021.114541. PMid:34416298.

Cherian, T., Ali, K., Fatima, S., Saquib, Q., Ansari, S. M., Alwathnani, H. A., Al-Khedhairy, A. A., Al-Shaeri, M., & Musarrat, J. (2019). Myristica fragrans bio-active ester functionalized ZnO nanoparticles exhibit antibacterial and antibiofilm activities in clinical isolates. *Journal of Microbiological Methods*, 166, 105716. http://dx.doi.org/10.1016/j.mimet.2019.105716. PMid:31499093.

Crispim, J. S., Dias, R. S., Vidigal, P. M. P., Sousa, M. P., Silva, C. C., Santana, M. F., & Paula, S. O. (2018). Screening and characterization of prophages in Desulfovibrio genomes. *Scientific Reports*, 8(1), 9273. http://dx.doi.org/10.1038/s41598-018-27423-z. PMid:29915307.

Cruz, M. F., Magno, M. B., Jural, L. A., Pimentel, T. C., Ferreira, D. M. T. P., Esmerino, E. A., Ramos, G. L. P. A., Gomila, J. V., Silva, M. C., Cruz, A. G., Fidalgo, T. K. S., & Maia, L. C. (2022). Probiotics and dairy products in dentistry: a bibliometric and critical review of randomized clinical trials. *Food Research International*, 157, 111228. http://dx.doi.org/10.1016/j.foodres.2022.111228. PMid:35761544.

Cwalina, B., Dec, W., Michalska, J. K., Jaworska-Kik, M., & Student, S. (2017). Initial stage of the biofilm formation on the NiTi and Ti6Al4V surface by the sulphur-oxidizing bacteria and sulphate-reducing bacteria. *Journal of Materials Science: Materials in Medicine*, 28(11), 173. http://dx.doi.org/10.1007/s10856-017-5988-2. PMid:28956213.

Dula, S., Ajayeoba, T. A., & Ijabadeniyi, O. A. (2021). Bacterial biofilm formation on stainless steel in the food processing environment and its health implications. *Folia Microbiologica*, 66(3), 293-302. http://dx.doi.org/10.1007/s12223-021-00864-2. PMid:33768506.

El-Shafei, S. M., Sakr, S. S., & Abou-Soliman, N. H. (2020). The impact of supplementing goats' milk with quinoa extract on some properties of yoghurt. *International Journal of Dairy Technology*, 73(1), 126-133. http://dx.doi.org/10.1111/1471-0307.12628.

Famuyide, I. M., Aro, A. O., Fasina, F. O., Eloff, J. N., & McGaw, L. J. (2019). Antibacterial and antibiofilm activity of acetone leaf extracts of nine under-investigated south African *Eugenia* and *Syzygium* (Myrtaceae) species and their selectivity indices. *BMC Complementary and Alternative Medicine*, 19(1), 141. http://dx.doi.org/10.1186/s12906-019-2547-z. PMid:31221162.

Farkash, Y., Feldman, M., Ginsburg, I., Shalish, M., & Steinberg, D. (2020). The effect of Padma-hepaten herbal combination on the caries-inducing properties of Streptococcus mutans on orthodontic surfaces. *Journal of Herbal Medicine*, 20, 100321. http://dx.doi.org/10.1016/j.hermed.2019.100321.

Galleh, P. R., Nwosisi, C., & Mohammed, F. A. (2021). Phytochemical and antibiofilm activity of Aloe barbadensismiller (Aloe vera) on Candida albicans isolated from urinary catheter. *Journal of Pharmaceutical Research International*, 33(7), 93-103.

Ghane, M., Babaeekhou, L., & Shams, M. (2022). Antimicrobial activity of Rhus Coriaria L. and Salvia Urmiensis bunge against some food-borne pathogens and identification of active components using molecular networking and docking analyses. *Food Science and Technology*, 42, e08221. http://dx.doi.org/10.1590/fst.08221.

Gopalakrishnan, U., Felicita, A., Mahendra, L., Premkumar, S., & Madasamy, R. (2019). Prevalence of sulfate reducing bacteria in oral cavity: a narrative review. *African Journal of Clinical and Experimental Microbiology*, 20(2), 82-86. http://dx.doi.org/10.4314/ajcem.v20i2.1.

Hammami, S., Debbabi, H., Jlassi, I., Joshi, R. K., & Mokni, R. (2020). Chemical composition and antimicrobial activity of essential oil from the aerial parts of Plantago afra L.(Plantaginaceae) growing wild in Tunisia. *South African Journal of Botany*, 132, 410-414. http://dx.doi.org/10.1016/j.sajb.2020.05.012.

Heggendorn, F. L., Gonçalves, L. S., Dias, E. P., Heggendorn, C., & Lutterbach, M. T. (2014). Detection of sulphate-reducing bacteria and others cultivable facultative bacteria in dental tissues. *Acta Stomatologica Croatica*, 48(2), 116-122. http://dx.doi.org/10.15644/asc48/2.116. PMid:27688355.

Jain, S., Rathod, N., Nagi, R., Sur, J., Laheji, A., Gupta, N., Agrawal, P., & Prasad, S. (2016). Antibacterial effect of Aloe vera gel against oral pathogens: an in-vitro study. *Journal of Clinical and Diagnostic Research*, 10(11), ZC41-ZC44. http://dx.doi.org/10.7860/JCDR/2016/21450.8890. PMid:28050502.

Jakubovics, N. S., Goodman, S. D., Mashburn-Warren, L., Stafford, G. P., & Cieplik, F. (2021). The dental plaque biofilm matrix. *Periodontology 2000*, 86(1), 32-56. http://dx.doi.org/10.1111/prd.12361. PMid:33690911.

Jeon, D.-M., An, J.-S., Lim, B.-S., & Ahn, S.-J. (2020). Orthodontic bonding procedures significantly influence biofilm composition. *Progress in Orthodontics*, 21(1), 14. http://dx.doi.org/10.1186/s40510-020-00314-8. PMid:32476070.

Jokar, A., Masoomi, F., Sadeghpour, O., Nassiri-Toosi, M., & Hamedi, S. (2016). Potential therapeutic applications for Terminalia chebula in Iranian traditional medicine. *Journal of Traditional Chinese Medicine*,

36(2), 250-254. http://dx.doi.org/10.1016/S0254-6272(16)30035-8. PMid:27400482.

Karami, L., Ghahtan, N., & Habibi, H. (2017). Antibacterial effect of plantago ovata and lallemantia iberica seed extracts against some bacteria. *Research in Molecular Medicine*, 5(3), 32-36. http://dx.doi.org/10.29252/rmm.5.3.32.

Kuete, V., Krusche, B., Youns, M., Voukeng, I., Fankam, A. G., Tankeo, S., Lacmata, S., & Efferth, T. (2011). Cytotoxicity of some Cameroonian spices and selected medicinal plant extracts. *Journal of Ethnopharmacology*, 134(3), 803-812. http://dx.doi.org/10.1016/j.jep.2011.01.035. PMid:21291988.

Kushkevych, I., Coufalová, M., Vítězová, M., & Rittmann, S. K.-M. (2020). Sulfate-reducing bacteria of the oral cavity and their relation with periodontitis—recent advances. *Journal of Clinical Medicine*, 9(8), 2347. http://dx.doi.org/10.3390/jcm9082347. PMid:32717883.

Li, E., Wu, J., & Zhang, D. (2021). Exogenous autoinducer-2 inhibits biofilm development of Desulfovibrio sp. Huiquan2017. *World Journal of Microbiology & Biotechnology*, 37(7), 124. http://dx.doi.org/10.1007/s11274-021-03071-w. PMid:34170406.

Lu, L., Hu, W., Tian, Z., Yuan, D., Yi, G., Zhou, Y., Cheng, Q., Zhu, J., & Li, M. (2019). Developing natural products as potential anti-biofilm agents. *Chinese Medicine*, 14(1), 11. http://dx.doi.org/10.1186/s13020-019-0232-2. PMid:30936939.

Miladi, H., Zmantar, T., Kouidhi, B., Qurashi, Y. M. A., Bakhrouf, A., Chaabouni, Y., Mahdouani, K., & Chaieb, K. (2017). Synergistic effect of eugenol, carvacrol, thymol, p-cymene and γ-terpinene on inhibition of drug resistance and biofilm formation of oral bacteria. *Microbial Pathogenesis*, 112, 156-163. http://dx.doi.org/10.1016/j.micpath.2017.09.057. PMid:28964825.

Mishra, P., Marwah, N., Agarwal, N., Chaturvedi, Y., & Suohu, T. (2019). Comparison of Punica granatum, Terminalia chebula, and Vitis vinifera seed extracts used as mouthrinse on salivary Streptococcus mutans levels in children. *The Journal of Contemporary Dental Practice*, 20(8), 920-927. http://dx.doi.org/10.5005/jp-journals-10024-2617. PMid:31797848.

Naginyte, M., Do, T., Meade, J., Devine, D. A., & Marsh, P. D. (2019). Enrichment of periodontal pathogens from the biofilms of healthy adults. *Scientific Reports*, 9(1), 5491. http://dx.doi.org/10.1038/s41598-019-41882-y. PMid:30940882.

Patel, H., Padalia, H., Kaneria, M. J., Vaghasiya, Y., & Chanda, S. (2017). Anti-oxidant and anti-bacterial properties of extracts: Terminalia chebula and terminalia bellerica. In: M. Meghwal, M. R. Goyal & M. J. Kaneria (Eds.), *Food technology: applied research and production techniques* (pp. 213-231). New York: Apple Academic Press. http://dx.doi.org/10.1201/9781315365657-9.

Patel, M. K., Tanna, B., Mishra, A., & Jha, B. (2018). Physicochemical characterization, antioxidant and anti-proliferative activities of a polysaccharide extracted from psyllium (P. ovata) leaves. *International Journal of Biological Macromolecules*, 118(Pt A), 976-987. http://dx.doi.org/10.1016/j.ijbiomac.2018.06.139. PMid:29964112.

Roy, R., Tiwari, M., Donelli, G., & Tiwari, V. (2018). Strategies for combating bacterial biofilms: a focus on anti-biofilm agents and their mechanisms of action. *Virulence*, 9(1), 522-554. http://dx.doi.org/10.1080/21505594.2017.1313372. PMid:28362216.

Salah, F., Ghoul, Y., Mahdhi, A., Majdoub, H., Jarroux, N., & Sakli, F. (2017). Effect of the deacetylation degree on the antibacterial and antibiofilm activity of acemannan from Aloe vera. *Industrial Crops and Products*, 103, 13-18. http://dx.doi.org/10.1016/j.indcrop.2017.03.031.

Sánchez, M., González-Burgos, E., Iglesias, I., & Gómez-Serranillos, M. P. (2020). Pharmacological update properties of Aloe vera and its major active constituents. *Molecules*, 25(6), 1324. http://dx.doi.org/10.3390/molecules25061324. PMid:32183224.

Sarabhai, S., Sharma, P., & Capalash, N. (2013). Ellagic acid derivatives from Terminalia chebula Retz. downregulate the expression of quorum sensing genes to attenuate Pseudomonas aeruginosa PAO1 virulence. *PLoS One*, 8(1), e53441. http://dx.doi.org/10.1371/journal.pone.0053441. PMid:23320085.

Ser, H.-L., Mutalib, N.-S., Yin, W.-F., Chan, K.-G., Goh, B.-H., & Lee, L.-H. (2015). Evaluation of antioxidative and cytotoxic activities of Streptomyces pluripotens MUSC 137 isolated from mangrove soil in Malaysia. *Frontiers in Microbiology*, 6, 1398. http://dx.doi.org/10.3389/fmicb.2015.01398. PMid:26733951.

Shastry, R. P., & Rekha, P. (2021). Bacterial cross talk with gut microbiome and its implications: a short review. *Folia Microbiologica*, 66(1), 15-24. http://dx.doi.org/10.1007/s12223-020-00821-5. PMid:32949007.

Shin, G. H., & Kim, W. (2022). Antibacterial and antiseptic activities of various spices to the mala food ingredients. *Food Science and Technology*, 42, e115821. http://dx.doi.org/10.1590/fst.115821.

Son, N. H., Tuan, N. T., & Tran, T. M. (2022). Investigation of chemical composition and evaluation of antioxidant, antibacterial and antifungal activities of ethanol extract from Bidens pilosa L. *Food Science and Technology*, 42, e22722. http://dx.doi.org/10.1590/fst.22722.

Tripathi, A. K., Thakur, P., Saxena, P., Rauniyar, S., Gopalakrishnan, V., Singh, R. N., Gadhamshetty, V., Gnimpieba, E. Z., Jasthi, B. K., & Sani, R. K. (2021). Gene sets and mechanisms of sulfate-reducing bacteria biofilm formation and quorum sensing with impact on corrosion. *Frontiers in Microbiology*, 12, 754140. http://dx.doi.org/10.3389/fmicb.2021.754140. PMid:34777309.

Winning, L., Patterson, C. C., Cullen, K. M., Stevenson, K. A., Lundy, F. T., Kee, F., & Linden, G. J. (2015). The association between subgingival periodontal pathogens and systemic inflammation. *Journal of Clinical Periodontology*, 42(9), 799-806. http://dx.doi.org/10.1111/jcpe.12450. PMid:26309048.

Yonjalli, R. V., Aghjehgheshlagh, F. M., Mahdavi, A., Navidshad, B., & Staji, H. (2020). The effects of tannin extract and linseed oil on yield, physicochemical characteristics and fatty acid profile of ewe milk. *International Journal of Dairy Technology*, 73(4), 656-666. http://dx.doi.org/10.1111/1471-0307.12716.

Zainol, S., Said, S., Abidin, Z., Azizan, N., Majid, F. A. A., & Jantan, I. (2017). Synergistic benefit of Eugenia caryophyllata L. and Cinnamomum zeylanicum Blume essential oils against oral pathogenic bacteria. *Chemical Engineering Transactions*, 56, 1429-1434.

Zam, W., Ali, A., & Ibrahim, W. (2020). Antimicrobial activity of Myrtus communis L., Cinnamomum verum and Eugenia caryophyllata alcoholic mixtures. *Current Nutrition and Food Science*, 16(2), 207-212. http://dx.doi.org/10.2174/1573401315666190103111000.

Zhang, Y., Wang, Y., Zhu, X., Cao, P., Wei, S., & Lu, Y. (2017). Antibacterial and antibiofilm activities of eugenol from essential oil of Syzygium aromaticum (L.) Merr. & LM Perry (clove) leaf against periodontal pathogen Porphyromonas gingivalis. *Microbial Pathogenesis*, 113, 396-402. http://dx.doi.org/10.1016/j.micpath.2017.10.054. PMid:29101062.