Diketopiperazines, promising anti-adhesion metabolites of Glycomyces sediminimaris against dental pathogen Streptococcus mutans

Sheida Heidarian
Uppsala University
Faezeh Almasi
University of Tehran
Fatemeh Mohammadipanah (fmohammadipanah@ut.ac.ir)
University of Tehran

Research Article

Keywords: Dental plaque, Glycomyces sediminimaris, Natural anti-biofilm agents, Streptococcus mutans

Posted Date: April 5th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1455351/v2

Abstract

Oral biofilm formation by *Streptococcus mutans* as a critical and the predominant causative agent of several dental and physiologic disorders needs to be tackled. Investigating anti-biofilm compounds from marine *Actinobacteria* as a source of natural product for blocking the biofilm formation by *Streptococcus mutans* was conducted. Anti-adhesion activity of Actinobacterial extracts was investigated through crystal violet assay. Herein, the extract of *Glycomyces sediminimaris* UTMC 2460 (DSM 103727) prevented the adhesion potency of *S. mutans* up to 95.1% at 100 μg/mL. The Extracellular Polymeric Substance (EPS) production and cell hydrophobicity index of the strain was restrained up to 66% and 30%, respectively. The extract showed metabolism suppression up to 20% in *S. mutans* even in the biofilm state at the effective biofilm inhibition concentration (100 μg/mL). Metabolic profiling of *Glycomyces sediminimaris* revealed the presence of anti-biofilm diketopiperazines compounds as major components of the extract. The current finding postulate that the metabolic profile of marine *Glycomyces sediminimaris* includes biocompounds that can be evaluated in further studies as an anti-biofilm of *S.mutans* which has not been previously reported.

Introduction

Oral bacteria have evolved to form biofilms not only on dental hard tissue but also on a wide range of dental materials used in dentistry. The inherent resistant mechanisms in biofilm state make their disruption rather impossible when they transform to mature microbial communities called biofilm. This resistancy is related to the restricted penetration of anti-microbial agents, overexpression of multiple drug resistance pumps, reduced growth rate and metabolisms, changing the profile of outer membrane structures, etc (Rath et al., 2021; Sharma et al., 2019). Among bacterial species in dental plaque, *Streptococcus mutans* is considered the main etiologic agent of enamel caries and oral pathological states such as periodontal diseases, gingivitis and infective endocarditis (IE) on artificial structures (RadaicandKapila, 2021; Rosas-Piñón et al., 2012).

Unfortunately, simply brushing the teeth is not sufficient to eradicate the dental biofilm. Thus, approaches that will inhibit oral biofilm formation can greatly improve oral health. Specific anti-microbial design in the composition and application of new materials (e.g. bio-ceramic sealer, resin composite, implant coating) demonstrates an improvement of the anti-biofilm properties of dental materials compared to earlier generations. These materials affect biofilm growth by inhibiting the adhesion of bacteria or limiting their growth. Dental implants are also prone to spoilage by bacterial biofilm formations. Such biofilms are often highly resistant to current antibiotics and effective non-toxic implant coatings may help in long-term protection against biofilm formation (Eshed et al., 2013).

The impenetrable structure of mature biofilms (BeloinandGhigo, 2005) in comparison to the loose and reversible microbial attachment in the first steps of biofilm formation, justify the importance of inhibiting the bacterial attachment in the initial steps to hinder the formation of biofilm-associated infections

(Osherovich, 2012). Accordingly, any compound that decreases the affinity of microbial cell attachment should be a competent candidate as an anti-adhesion agent.

Regarding the diverse array of chemical structures and biocompatible properties, substances derived from nature have been considered as a prolific source of inspiration for numerous therapeutic agents. Microorganisms have shown an essential function in the development of the chemistry of natural products and have been considered a rich source of unique therapeutic molecules (Abdel-Razek et al., 2020). *Actinobacteria* are a group of potent microorganisms producing more than 40% of microbial bioactive compounds and about 70% of antibiotics, considering the never-ending source of bioactive compounds (Abdel-Razek et al., 2020; De SimeisandSerra, 2021).

Marine bacteria namely *Actinobacteria* are an exciting resource for the isolation of novel natural products, having peculiar characteristics that have not previously been reported in terrestrial ones, due to the extreme variations in ecological pressure (completion for space, nutrients, oxygen, light, etc) of marine habitats (WB Fenical, 2020). Enzyme inhibitory activities (Almasi et al., 2018), anti-cancer agents (Hozzein et al., 2021), anti-quorum sensing (NithyaandPandian, 2010; Romero et al., 2011), anti-fouling (Heidarian et al., 2018), and anti-biofilm agents (Bakkiyaraj et al., 2012; Golberg et al., 2013) are some of the reported pharmaceutical activities of marine *Actinobacteria*. Due to the intense competition and strong selective pressure in marine environments, it can be postulated that marine *Actinobacteria* are a source of anti-adhesion agents (Chen et al., 2016). Novel alkaloid compounds such as 2-bromo-N-methyltrypt amine, bufotenine and 1,3,7-trimethylisoguanine which were isolated from marine organisms are examples of natural products that exhibit anti-adhesion activity against biofilm-forming bacteria (Pénez et al., 2011).

Owing to the growing difficulty in treatment of resistance plaques, in the present study, we aimed at finding bioactive substances from marine *Actinobacteria* with anti-adhesion properties against the potent clinical biofilm-forming *Streptococcus mutans* ATCC 700610 as an initiator of the plaque formation. The bacteriostatic or bacteriocide effect of the actinobacterial metabolites was investigated to differentiate their effectiveness to suppress the primary colonization or eradication of biofilms.

Material And Methods

Bacterial cultivation and growth conditions

Streptococcus mutans ATCC 700610 as the reference strain and ten sediment isolated marine *Actinobacteria* collected from different depths of the Persian Gulf and the Oman sea, were obtained from the University of Tehran Microorganism Collection (UTMC). *S. mutans* was grown on Nutrient agar (Merck, Darmstadt, Germany) and brain heart infusion agar (Merck, Darmstadt, Germany) medium under microaerophilic condition in a candle jar. at 37°C. The selected marine *Actinobacteria* were grown on ISP2 medium (Malt extract 10 g L^{-1}, Yeast extract 4 g L^{-1}, Glucose 4

g L^{-1}, $CaCO_3$ 2 g L^{-1} and Agar 14 g L^{-1}), and preserved in the same one fifth strength medium using 30% glycerol as the protectant.

Fermentation and extraction of extracellular metabolites

The pre-culture was prepared by inoculating pieces (1×1 cm^2) of grown *Actinobacteria* on ISP2 agar cultures in 50 mL of ISP2 broth, and incubated at 28 °C with shaking at 160 rpm for 48 h. Then, 5% v/v of a pre-culture medium was inoculated in the fermentation medium and incubated at 28 °C, 180 rev min^{-1} for 7 days. The supernatant was collected using centrifugation at 4000 rpm for 10 min. Then, the biomass free supernatant was extracted twice using ethyl acetate (1:1 v/v) by vigorous shaking for 1 h. The separated organic phase was concentrated using a rotator vacuum evaporator (Heidolph, Germany) at 35 °C under reduced pressure and preserved at -20°C before conducting anti-adhesion assays.

Biofilm formation assessment of *S. mutans* by crystal violet assay

The effect of nutrient media in enhancing the *in vitro* biofilm formation of *Streptococcus mutans* ATCC 700610 was evaluated using nutrient, BHI and, Todd-Hewitt-2% yeast extract (THY) broth media (Merck, Darmstadt, Germany) supplemented with 1% glucose and sucrose. The intensity and the thickness of biofilms produced by *Streptococcus mutans* ATCC 700610 were measured in 96-well polystyrene microplates (Biofil, China) following a described method (Stepanović et al., 2000) with slight modifications. Briefly, fresh aliquots of the *S. mutans* with a cell density of 0.2 at 600 nm were inoculated above mentioned media and supplemented with saccharides; and incubated at 37 °C. The content of the wells were discarded after 72 h of incubation and weakly adherent cells were washed twice using sterile physiological saline. The resulting biofilms firstly were fixed using 250 µL methanol for 15 min, then were emptied and left to air dry. Staining was performed by adding 250 µL of crystal violet (0.05%, v/v) for 10 min. The stain was withdrawn and the wells were washed twice with sterile distilled water. After drying at room temperature for 10 min, crystal violet was finally solubilized using 250 µL of glacial acetic acid (33% v/v) for 15 min and its optical density was determined at 545 nm. The non-inoculated wells were used for sterility check and considered as negative controls. All tests were run in triplicate.

Anti-adhesion assay of Actinobacterial metabolite extracts

To check the effect of extracts on inhibition of biofilm formation, the extracts were added to each well containing 300 μL fresh aliquots of the *S. mutans* (cell density of 0.2 at 600 nm) in nutrient broth supplemented with 1% glucose and 1% sucrose to reach 100 and 300 μg mL^{-1} concentration. After 72 h

of incubation at 37 °C, the spent medium was removed and the wells were washed, fixed and stained according to the crystal violet assay as described previously. The anti-biofilm potential of each extract was expressed as a percentage of inhibition using the formula of (Control OD – Treated OD) / Control OD]×100 (BakkiyarajandKarutha Pandian, 2010). Wells containing bacterial cell suspension in a nutrient medium without any extracts were used as a negative bacterial adhesion control. In addition, the maximum percentage of methanol (16%) used for dilution of the extracts and sterile nutrient broth medium were considered as negative controls. Diuron (Sigma-Aldrich, USA), a commercial anti-biofilm agent, was used as a positive control (1, 10 μg/mL).

Quantitative determination of extra polymeric substance (EPS) content

The inhibitory effect of the extracts (100 and 300 μg/mL) on the synthesis of water-soluble EPS was evaluated by the total carbohydrate assay (DuBois et al., 1956). The control and treated culture medium (10 mL) were grown at 37 °C for 72 h in a nutrient broth medium complemented with glucose and sucrose 1%. After incubation, the culture fluid was separated by centrifugation at 10,000×*g* for 15 min and filtered through 0.2 µm filter paper (MF-Millipore, Germany). The collected supernatant was mixed with a thrice volume of ethanol and maintained for precipitation at 4 °C overnight. Settled EPS was centrifuged at 10,000×*g* for 10 min at 25 °C. Finally, gathered EPS was dissolved in PBS for carbohydrate content determination using the modified phenol-sulphuric acid method. For this, the EPS suspension was mixed with an equal volume of 5% phenol (Merck, Darmstadt, Germany) and five volumes of concentrated H_2SO_4 (Merck, Darmstadt, Germany), incubated for 1 h in the dark condition, and the absorbance was measured at 490 nm.

Bactericidal assay on *S.mutans* biofilm

The bactericidal effect of the extracts was evaluated based on the reduction of tetrazolium salt using WST-1 assay. The suspension of *S. mutans* (cell density of 0.2 at 600 nm) was mixed with 50μL of the extract solution to provide a final concentration of 100 μg/mL in 300μL of the final volume of each well. Bacterial suspensions were removed after 72 h incubation at 37 °C and wells were washed twice with sterile physiological saline. Following the evaporation of excess moisture from each well at room temperature, 100 and 10 μL of fresh nutrient broth medium and WST-1 reagent (Roche, Switzerland) was added to each well, respectively. The suspension was incubated at 37 °C for 2-6 h and the absorbance of probably formed formazan was measured at 450 nm using microplate reader MRP4+ (Heiperion, England). Ciprofloxacin (2 μg/mL) and Actinomycin D (20 μg/mL) were used as positive controls.

Modification of the surface hydrophobicity in the presence of the extract

To assess the Microbial Adherence to Hydrocarbon (MATH), the biofilm culture of the *S. mutans*, were centrifuged after 72 h incubation and pellets were washed twice by saline. The equal volume of toluene was mixed with 1 mL of the adjusted cell suspension with a cell density of 0.4 at 600 nm followed by vortexing for 2 min. Mixtures were allowed to stand at room temperature overnight to enable phase separation. The OD of the aqueous phase was measured and its reduction was taken as a measure of the proportion of the cells which were excluded from the aqueous phase that is equivalent to their hydrophobicity index (HI). It was determined by the equation $[(A_0 - A) / A_0] \times 100$, where A_0 and A are the initial and final optical densities of the aqueous phase, respectively (Serebryakova et al., 2002; ZhangandMiller, 1992).

Determination of MIC and MBC of planktonic *S. mutans*

The minimal inhibitory concentration (MIC) value of the most potent extract on the planktonic growth of *S. mutans* ATCC700610 was assessed following the CLSI method (Humphries et al., 2021). Briefly, seven different concentrations (6.25 to 400 μg/mL) of the extract and ciprofloxacin (0.25, 0.5, 1, 2 μg/mL) as the positive control were prepared in methanol. Thereafter, 20μL of the *S. mutans* suspension (with a cell density of 0.1 at 600 nm) was inoculated into each well and incubated at 37 ^{o}C for 24 h. The bacterial cell suspension and growth medium were used as negative controls.

Hemolytic effect assay

The lytic effect of the metabolic extract with anti-biofilm activity was evaluated on the human red blood cells. The extract at concentrations of 50, 100, 200, 300 and 400 μg/mL was added to 10% human red blood cells previously washed three times with phosphate-buffered saline buffer (PBS). To determine the hemolytic activities, mixtures of blood and the extract (1000 µL) were incubated at 37 °C for 1 h. The absorbance of the supernatants after centrifugation at 16,600×*g* for 10 min was measured at 545 nm. Ferrous sulfate solution (25 mM $FeSO_4$) was used as a positive control serving as the 100% lysis marker.

Cell toxicity assay

Brine shrimp *Artemia salina* larvae were used as a model for primary evaluation of the toxicity of the bioactive actinobacterial extract. The anti-crustacean assay was performed in the 24 well clear polystyrene plate method (Meyer et al., 1982). Larvae hatch solution in 3% artificial seawater (200μL) was added into each well containing 15-20 larvae and various concentrations of the extract (5, 10, 20 and 100 μg/mL). The microplate was incubated at room temperature in dark conditions. In this assay, artificial seawater incorporated with 1% DMSO and potassium dichromate solution (0.5 M) was used as the negative and positive controls, respectively. The mortality rate was calculated after 24 h according to the formula:

$$M = \frac{A-B-N}{G-N} \times N - 0 \qquad Viability = 100 \times (1 - M)$$

Where,

A: Dead larvae number after 24 h N: Dead larvae number in initial M: Dead larvae percentage after 24 h G: Total number of larvae B: Average of dead larvae in negative control after 24 h

Characterization and identification of the potent anti-adhesion producing actinobacterium

The potent anti-adhesion producing strain was characterized based on morphological, physiological and molecular approaches.

The 16S rRNA genes were amplified using a set of universal primers, 9F (AAG AGT TTG ATC ATG GCT CAG) and 1541R (AGG AGG TGA TCC AAC CGC A) (Kumar et al., 2010). Amplified chromosomal DNA obtained from PCR reaction was purified using PCR Purification kit (NucleoSpin® Gel and PCR Clean-up). The 16S rRNA gene sequence of the strain was blasted against the Genbank and EzTaxon databases (Yoon et al., 2017). Phylogenetic analysis was performed using the software package MEGA version 6.0 (Tamura et al., 2013).

Metabolic profiling of the strain with anti-adhesive activity

The identification of specialized metabolites in the extract of the most potent *Actinobacteria* was performed using a UHPLC-ESI-Q-TOF instrument with a Dionex UltiMate 3000 UHPLC connected to a Zorbax Eclipse Plus C18 column (100 × 2.1 mm, 1.8 μm) coupled to a Bruker MaXis IMPACT mass spectrometer. The mass spectrometer was operated in positive ion mode with a scan range of 50–3,000 mz^{-1}. The solvent system of water (A)-acetonitrile (B), each supplemented with 0.1% formic acid, was used for chromatography. A gradient of 5–100% B over 30 min was applied at a flow rate of 0.2 mL min^{-1}. Calibration was performed with 1 mM sodium formate at the start of each run.

Results

Biofilm forming ability of *Streptococcus mutans* ATCC 700610

The biofilm formation of *S. mutans* ATCC 700610 was optimally occurred by growth in nutrient broth medium supplemented with 1% glucose and sucrose which significantly showed higher efficiency on improving the *S. mutans* biofilm formation rather than BHI medium in 72 h. The Todd-Hewitt-2% yeast extract (THY) medium has no positive effect on the strain biofilm formation enhancement (Fig. 1).

Anti-adhesion activity of Actinobacterial strains extract

The capacity of the extracts of the selected marine *Actinobacteria* to attenuate the biofilm formation of *S. mutans* ATCC 700610 is shown in Fig. 2. Five potent metabolite extracts with maximum biofilm inhibition (MBI) activity at the lowest concentration value of 100 μg/mL were selected for further analysis. The biofilm growth of *S. mutans* was inhibited up to 55.6 – 95.1% at the 100 μg/mL by the metabolite extract of *Glycomyces sediminimaris* UTMC 2460.

Effect of the metabolite extract on EPS production of *Streptococcus mutans* ATCC 700610

In addition to biofilm inhibition, a considerable decrease in EPS production of *S. mutans* in the presence of *Glycomyces sediminimaris* UTMC 2460 extract as the most potent strain was detected. The carbohydrate content of the soluble EPS in the presence of 100 and 300 μg/mL of the extract, were 119.52 μg/mL and 61.68 μg/mL, respectively (see Fig. 3). According to obtained data, the crude extract at the concentration of 300 μg/mL showed 66.3% reduction in EPS production of *S. mutans*, as compared to positive control Diuron (10 μg/mL) with the EPS production abatement capacity of 77.4%.

Bactericidal effect of the most potent extract against *S. mutans* in the biofilm state

The WST-1 reagent incubation time of 4 h was found to be the optimum time of incubation since the color intensity did not change after 4 h (data not shown). The cell viability of *S. mutans* decreased up to 19.5 % at the minimum biofilm inhibition concentration of the extract (100 μg/mL) after 4 h of incubation (Fig. 4). Bactericidal effect of the extract was observed during the early phase of growth, with a 1.30 log reduction in metabolic activity after 4 h. Biofilms subjected to the positive control presented a 3.16 log of deduction in metabolic performance at the aforementioned time.

Microbial adherence to hydrocarbon substance in the presence of the extract

In the MATH assay, it was found that the metabolite extract of the *Glycomyces sediminimaris* UTMC 2460 caused cells to become less hydrophobic, which explains at least in part the inhibitory effect of the

extract on biofilm formation. Equal deduction in HI (20 %) was observed between the control Irgarol (1 μg/mL) and the extract (100 μg/mL) that is shown in Fig. 5.

MIC and MBC values of *Glycomyces sediminimaris* UTMC 2460 extract

Recorded data showed that the MIC of the analysis was > 400 μg/mL and growth inhibiting activity was not detected at lower concentrations of the extract. The *Glycomyces* sp. UTMC 2460 extract shows no bactericidal activity against *S.mutans* in the planktonic phase. The MIC of 0.25 μg/mL and MBC of 0.5 μg/mL were recorded for ciprofloxacin (positive control) against *S. mutans*.

Hemolytic activity of the most potent extract

The metabolic extract of *Glycomyces sediminimaris* UTMC 2460 had almost no or negligible hemolysis effect in a range of 1.5 - 8.8 % at the concentrations of 50, 100 and 200 μg/mL after exposure to the human red blood cells. However, at the high dosage of the extract, hemolytic activity was significantly more than the positive control $FeSO_4$ (Fig. 6).

Cytotoxicity of the marine Actinobacterial extracts

The Five organic extracts expressing higher anti-biofilm activity showed no acute cytotoxic effect against *Artemia salina* larvae at the maximum tested concentration (100 μg/mL). After 24 h incubation, all exposed larvae were still alive that manifest the non-toxic effect of the extracts on *Artemia salina* nauplii.

Identification of anti-adhesion producing strain

The sequence similarity of the strain (GenBank accession number KU1741966) was compared with homologous sequences in the data banks of GenBank and EzTaxon. Analysis of 16S rDNA sequences of the strain showed its attribution to the genus *Glycomyces* and introduced as a novel species in the genus under the epithet of *Glycomyces sediminimaris* UTMC 2460 (Mohammadipanah et al., 2018).

In addition, the optimum temperature and pH required for the growth of *Glycomyces sediminimaris* was recorded at 28 ^{o}C and pH 7.5, respectively. The candidate strain had NaCl tolerance, up to 5%.

Characterization of the metabolites in the *Glycomyces sediminimaris* UTMC 2460 extract

The results of UHPLC-ESI-Q-TOF-MS indicated that the diketopiperazine class of natural compounds are the major metabolites in the *Glycomyces sediminimaris* UTMC 2460 extract have molecular formulae and retention times corresponding to the diketopiperazine class of natural products (Supplemental Figure 1S and Table 1S).

Discussions

Investigating the compounds to impair initial attachment of bacterial adhesion to biotic/abiotic surfaces could be the foremost effective strategy against the biofilm formation. The current means of controlling such biofilm infections is to physically remove the biofilm or the infected part and using anti-caries compounds mainly fluoride, chlorhexidine, and xylitol (Cui et al., 2019). Therefore, preventing the initial adhesion of bacteria mainly *Streptococcus* species, instead of attempts to destruct the established biofilms which could be provided by natural anti-adhesive coatings are among the current clinical demand. While only rare studies on dental biofilm inhibition, have focused on marine derived biocompounds (Silva et al., 2020; Stowe et al., 2011), in the present study, marine actinobacterial strains isolated from the Persian Gulf and the Sea of Oman, represented inhibitory metabolites on *S.mutans* biofilm.

In this research, five potent extracts capable of inhibiting the biofilm formation of *S. mutans* comparable to diuron were selected and the extract from marine *Glycomyces sediminimaris* showed the most effective anti-attachment activity with the highest biofilm inhibition value (95.1%) at the lowest concentration (100 μg/mL). The genus *Glycomyces* is a relatively new genus in the phylum *Actinobacteria* and so limited reports demonstrating its potent biological activities are present (Bredholdt et al., 2007; Heidarian et al., 2018; W Li et al., 2018; Qian et al., 2020).

The compound walkmycin C from *Bacillus subtilis* (Eguchi et al., 2011), signermycin B from *Streptomyces* sp. (Watanabe et al., 2012), carolacton from *Sorangium cellulosum* (Sudhakar et al., 2014), vizantin obtained from *Mycobacterium tuberculosis* (Takenaka et al., 2016), outer membrane vesicles (OMV) derived from *Burkholderia thailandensis* (Y Wang et al., 2021), and the crude extract of marine bacterium *Tenacibaculum* sp. 20J (Y Wang et al., 2021) are the reported microbial compounds/extracts against biofilm formation of *S.mutans*. Despite the great potential of microorganisms, especially *Actinobacteria* in bioactive compounds production, these limited reports indicate the microbial capacity has not been well studied. Besides, despite multiple prevention methods currently being used such as mechanical methods, fluoride treatments, and replacing sugars in the diet, the continuation of dental caries and the side-effects of these treatments prove the demand for small molecules compounds which can selectively inhibit *S. mutan*s biofilms through non-microbicidal (antiadhesion and signal interference) mechanisms (Yang et al., 2021).

Diketopiperazines (DKPs) are the smallest cyclic dipeptides ubiquitously found in nature being produced by non-ribosomal peptide synthetases (NRPS) and cyclodipeptide synthases. DKPs are characterized by a heterocyclic piperazine-2,5-dione synthesized from at least two amino acid residues being

biosynthesized by varieties of organisms, including humans, plants, and microorganisms while it is assumed that a higher percentage of Gram-negative bacteria have the potential to produce diketopiperazine structural types compared to Gram-positive bacteria (W Fenical, 1993). Important biological activities contributed to diketopiperazine are not only limited to anti-tumor, anti-viral, anti-fungal, anti-bacterial, anti-prion, anti-hyperglycemic and glycosidase inhibition activities (P de CarvalhoandAbraham, 2012) but also their proposed action in modulating bacterial communication, LuxR based quorum sensing (QS) process and finally in the clinic to control the biofilm infections, indicate the prominent perspective potential of this class of compounds (Sun et al., 2016). The production of diketopiperazine compounds from microorganisms including marine microorganisms has been reported (Song et al., 2021) and its production in the members of *Glycomyces* spp. other than *G. sediminimaris* has not been reported previously (Sheida Heidarian, 2018).

Diketopiperazines are reported in studies as potential compounds to control biofilm infections acting as anti-biofilm compounds (P de CarvalhoandAbraham, 2012) which also inhibit biofilm formation in *S. mutans*. Anti-bacterial and quorum quenching properties of the six out of nine diketopiperazines identified in this extract have already been investigated. These include compounds cyclo-(leucyl-prolyl), cyclo-(phenylalanyl-prolyl), cyclo-(prolyl-valyl), cyclo-(valyl-phenylalanyl), cyclo-(prolyl-tyrosyl) and cyclo-(leucyl-valyl) (Abed et al., 2013; Alshaibani et al., 2017; J Li et al., 2011; P de CarvalhoandAbraham, 2012; RyanandDow, 2008). Among them, the anti-biofilm and quorum-sensing inhibitory activity of the compounds cyclo-(Phe-Pro), cyclo-(Trp-Ser), cyclo (Pro-Leu), cyclo(Pro-Val) and cyclo(Ala-Val) on biofilm forming bacteria (*Serratia marcesce*ns, *Pseudomonas aeruginosa, Bacillus amyloliquefacien*s and *Escherichia coli*) and *Chromobacterium violaceum* (reporter strain for screening QS) has previously reported by (Sulieman et al., 2018), (Sun et al., 2016), (J-H Wang et al., 2016), and (Abed et al., 2013), respectively. Recently, the anti-biofilm activity of 75 synthetic cyclic peptides was assessed against *S. mutans* (Simon et al., 2019). The obtained results of our study suggest that the inhibition effect of *Glycomyces sediminimaris* extract probably be attributed to its dominant cocktail of diketopiperazines.

The inhibition potency of the introduced extract in this study implies the lower quantity required of the compound for attaining the same inhibitory effect in comparison with prevalent dentistry fluoride compounds and other potent biocompounds which have been investigated recently.

Furthermore, the impact of *G. sediminimaris* metabolites on the viability of *S. mutans* cells in a biofilm state showed 20% reduction in metabolic activity without any anti-bacterial effect on the planktonic state of *S. mutans*. Considering the anti-adherence activity of a biocompound is independent of its ability to interfere in cell metabolic pathways, this inhibitory performance could be accompanied by metabolic reduction activities and biofilm formation inhibition without affecting the cell viability. In addition to the inhibition of cell attachment, *G. sediminimaris* extract may be capable of attenuating the integrity of the mature biofilms through diminishing the cellular metabolic activity and even can be considered as an adjuvant compound for the eradication of formed biofilm in infectious oral diseases.

The metabolite extract of *G. sediminimaris* extract has no anti-bacterial effect on the planktonic state of *S. mutans*. It seems that the mechanism of action of the extract is predominantly related to adherence and biofilm formation inhibition and it has a negligible negative impact on the proliferation of bacterial cells in the planktonic state while it only diminishes the bacterial cell metabolism up to 20% in the biofilm structure without significant anti-bacterial effect in the biofilm phase. Thus, the type of mediated biofilm inhibition by this extract is not through the inhibition of cell division, which implies its non-toxic effect on oral microflora and the balance of oral ecosystem

The high activity of *Glycomyces sediminimaris* extract in inhibiting the *S. mutans* adhesion on polystyrene surfaces by decreasing the surface protein hydrophobicity was comparable with other studies directing to the mechanism underlying the inhibitory effect of *G. sediminimaris* extract on attachment of *S. mutans*

It is possible that the Diketopiperazines interconnects in cell surface proteins reducing the net hydrophobicity of the cell surface. Hence, the presence of extract may modify their hydrophobicity as a strategy to defer the initiation of biofilm formation or decrease the affinity of the surface proteins to attach to the surface.

Further, abating exopolymeric substances (EPS) production as a pivotal substance in maintaining the coherency of the biofilms (WatnickandKolter, 1999) and resistance of the cells to a variety of external tension (Wai et al., 1998) not only inhibits the biofilm matrix formation but also will not protect the internal cells of the biofilms from physical or chemical stresses including the antibiotics or biocides. The highly potent anti-adherence extract of *G. sediminimaris* can also contribute to the inhibition of EPS production by *S. mutans* and, inhibitors of EPS synthesis hold promise for the development of functional anti-biofilm agents for prophylaxis or therapeutic purposes.

Concludingly, this new attributed biofilm inhibition activity by a new species in the *Glycomyces* genus demonstrated that *G. sediminimaris* metabolites can be a competent candidate, not only for biofilm inhibition treatments but also, other unexplored bioactivities that can be discovered from this newly described species in further studies. Furthermore, since the efficacy of diketopiperazine compounds produced by different taxonomic resources is verified in hampering the biofilm formation, the presence of this compound in this non-toxic extract that has not been reported previously, can be considered as a potential candidate as an additive to the anti-septic mouthwash solutions for biofilm suppression, plaque formation and even reduction of other biofilm-forming pathogens on various types of implants.

Declarations

Acknowledgments

The authors would like to thank Dr. Mahdieh Shirzad for the help during the experimental phase.

Statements and Declarations

Competing Interests

The authors declare that there is no conflict of interest.

Funding information

This study was partially funded by the University of Tehran, Iran .

Data availability statement

The data that support the findings of this study areavailable from the corresponding author upon reasonablerequest.

References

1. Abdel-Razek AS, El-Naggar ME, Allam A, Morsy OM, Othman SI (2020) Microbial natural products in drug discovery. Processes 8: 470. https://doi.org/10.3390/pr8040470
2. Abed RM, Dobretsov S, Al-Fori M, Gunasekera SP, Sudesh K, Paul VJ (2013) Quorum-sensing inhibitory compounds from extremophilic microorganisms isolated from a hypersaline cyanobacterial mat. J Ind Microbiol Biotechnol 40: 759-772. https://doi: 10.1007/s10295-013-1276-4
3. Almasi F, Mohammadipanah F, Adhami HR, Hamedi J (2018) Introduction of marine-derived *Streptomyces* sp. UTMC 1334 as a source of pyrrole derivatives with anti-acetylcholinesterase activity. J Appl Microbiol 125: 1370-1382. https:// doi: 10.1111/jam.14043
4. Alshaibani MM, Zin N, Jalil J, Sidik NM, Ahmad SJ, Kamal N, Edrada-Ebel R (2017) Isolation, purification, and characterization of five active diketopiperazine derivatives from endophytic *Streptomyces* SUK 25 with antimicrobial and cytotoxic activities. J Microbiol Biotechnol 27: 1249-1256. https:// doi: 10.4014/jmb.1608.08032
5. Bakkiyaraj D, Karutha Pandian ST (2010) In vitro and in vivo antibiofilm activity of a coral associated actinomycete against drug resistant *Staphylococcus aureus* biofilms. Biofouling 26: 711-717. https:// doi: 10.1080/08927014.2010.511200
6. Bakkiyaraj D, Sivasankar C, Pandian SK (2012) Inhibition of quorum sensing regulated biofilm formation in Serratia marcescens causing nosocomial infections. Bioorg Med Chem Lett 22: 3089-3094. https:// doi: 10.1016/j.bmcl.2012.03.063
7. Beloin C, Ghigo J-M (2005) Finding gene-expression patterns in bacterial biofilms. Trends Microbiol 13: 16-19. https:// doi: 10.1016/j.tim.2004.11.008
8. Bredholdt H, Galatenko OA, Engelhardt K, Fjærvik E, Terekhova LP, Zotchev SB (2007) Rare actinomycete bacteria from the shallow water sediments of the Trondheim fjord, Norway: isolation, diversity and biological activity. Environ Microbiol 9: 2756-2764. https:// doi: 10.1111/j.1462-2920.2007.01387.x

9. Chen L, Ren Z, Zhou X, Zeng J, Zou J, Li Y (2016) Inhibition of *Streptococcus mutans* biofilm formation, extracellular polysaccharide production, and virulence by an oxazole derivative. Appl Microbiol Biotechnol 100: 857-867. https:// doi: 10.1007/s00253-015-7092-1
10. Cui T, Luo W, Xu L, Yang B, Zhao W, Cang H (2019) Progress of antimicrobial discovery against the major cariogenic pathogen *Streptococcus mutans*. Curr Issues Mol Biol 32: 601-644. https:// doi: 10.21775/cimb.032.601
11. De Simeis D, Serra S (2021) Actinomycetes: A never-ending source of bioactive compounds—An overview on antibiotics production. Antibiotics 10: 483. https:// doi: 10.3390/antibiotics10050483
12. DuBois M, Gilles KA, Hamilton JK, Rebers Pt, Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 28: 350-356. https://doi.org/10.1021/ac60111a017
13. Eguchi Y, Kubo N, Matsunaga H, Igarashi M, Utsumi R (2011) Development of an antivirulence drug against *Streptococcus mutans*: repression of biofilm formation, acid tolerance, and competence by a histidine kinase inhibitor, walkmycin C. Antimicrob Agents Chemother 55: 1475-1484. https:// doi: 10.1128/AAC.01646-10
14. Eshed M, Lellouche J, Banin E, Gedanken A (2013) MgF 2 nanoparticle-coated teeth inhibit *Streptococcus mutans* biofilm formation on a tooth model. J Mat Chem B 1: 3985-3991. https:// doi: 10.1039/c3tb20598c.
15. Fenical W (1993) Chemical studies of marine bacteria: developing a new resource. Chem Rev 93: 1673-1683. https://doi.org/10.1021/cr00021a001
16. Fenical WB (2020) Marine microbial natural products: the evolution of a new field of science. J Antibiotics 73: 481-487. https://doi.org/10.1038/s41429-020-0331-4
17. Golberg K, Pavlov V, Marks RS, Kushmaro A (2013) Coral-associated bacteria, quorum sensing disrupters, and the regulation of biofouling. Biofouling 29: 669-682. https://doi: 10.1080/08927014.2013.796939
18. Heidarian S, Mohammadipanah F, Maghsoudlou A, Dashti Y, Challis GL (2018) Anti-microfouling Activity of *Glycomyces sediminimaris* UTMC 2460 on Dominant Fouling Bacteria of Iran Marine Habitats. Front Microbiol 9: 3148. https:// doi: 10.3389/fmicb.2018.03148
19. Hozzein WN, Mohany M, Alhawsawi SM, Zaky MY, Al-Rejaie SS, Alkhalifah DH (2021) Flavonoids from marine-derived actinobacteria as anticancer drugs. Curr Pharm Des 27: 505-512. https://doi: 10.2174/1381612826666201216160154
20. Humphries R, Bobenchik AM, Hindler JA, Schuetz AN (2021) Overview of changes to the clinical and laboratory standards institute performance standards for antimicrobial susceptibility testing, M100. J Clin Microbiol 59: e00213-00221. https:// doi: 10.1128/JCM.00213-21
21. Kumar V, Bisht GS, Institu S (2010) An improved method for isolation of genomic DNA from filamentous actinomycetes. Int J Eng Technol Manag Appl Sci, 2: 2.
22. Li J, Wang W, Xu SX, Magarvey NA, McCormick JK (2011) *Lactobacillus reuteri*-produced cyclic dipeptides quench agr-mediated expression of toxic shock syndrome toxin-1 in *staphylococci*. PNAS 108: 3360-3365. https://doi.org/10.1073/pnas.1017431108

23. Li W, Liu C, Guo X, Song W, Sun T, Duan L, Wang X, Zhao J, Xiang W (2018) *Glycomyces tritici* sp. nov., isolated from rhizosphere soil of wheat (Triticum aestivum L.) and emended description of the genus *Glycomyces*. Antonie Van Leeuwenhoek, 111: 1087-1093. https://doi.org/10.1007/s10482-017-1011-7
24. Meyer B, Ferrigni N, Putnam J, Jacobsen L, Nichols Dj, McLaughlin JL (1982) Brine shrimp: a convenient general bioassay for active plant constituents. Planta Medica 45: 31-34. https:// doi: 10.1055/s-2007-971236
25. Mohammadipanah F, Atasayar E, Heidarian S, andWink J (2018) *Glycomyces sediminimaris* sp. nov., a new species of actinobacteria isolated from marine sediment. Int J Syst Evol Microbiol 68: 2357-2363. https:// doi: 10.1099/ijsem.0.002847
26. Nithya C, Pandian SK (2010) *Bacillus pumilus* of Palk Bay origin inhibits quorum-sensing-mediated virulence factors in Gram-negative bacteria. Res Microbiol 161: 293-304. https:// doi: 10.1016/j.resmic.2010.03.002
27. Osherovich L (2012) Keeping biofilms at bay. SciBX: Science-Business eXchange, 5. https:// doi: 10.1038/scibx.2012.860
28. P de Carvalho M, Abraham W-R (2012) Antimicrobial and biofilm inhibiting diketopiperazines. Curr Med Chem 19: 3564-3577. https:// doi: 10.2174/092986712801323243
29. Pénez N, Culioli G, Pérez T, Briand J-F, Thomas OP, Blache Y (2011) Antifouling properties of simple indole and purine alkaloids from the Mediterranean gorgonian Paramuricea clavata. J Nat Pro 74: 2304-2308. https://doi.org/10.1021/np200537v
30. Qian L, Duan L, Lin J, Yang Y, Song J, Wang X, Zhao J, Xiang W (2020) *Glycomyces albidus* sp. nov., a novel actinobacterium isolated from rhizosphere soil of wheat (*Triticum aestivum* L.). Int J Syst Evol Microbiol 70: 3096-3104. https:// doi: 10.1099/ijsem.0.004131
31. Radaic A, Kapila YL (2021) The oralome and its dysbiosis: New insights into oral microbiome-host interactions. Comput Struct Biotechnol J 19: 1335-1360. https://doi.org/10.1016/j.csbj.2021.02.010
32. Rath S, Bal SCB, Dubey D (2021) Oral biofilm: development mechanism, multidrug resistance, and their effective management with novel techniques. Rambam Maimonides Med J 12. https:// doi: 10.5041/RMMJ.10428
33. Romero M, Martin-Cuadrado A-B, Roca-Rivada A, Cabello AM, Otero A (2011) Quorum quenching in cultivable bacteria from dense marine coastal microbial communities. FEMS Microbiol Ecol 75: 205-217. https:// doi: 10.1111/j.1574-6941.2010.01011.x
34. Rosas-Piñón Y, Mejía A, Díaz-Ruiz G, Aguilar MI, Sánchez-Nieto S, Rivero-Cruz JF (2012) Ethnobotanical survey and antibacterial activity of plants used in the Altiplane region of Mexico for the treatment of oral cavity infections. J Ethnopharmacol 141: 860-865. https:// doi: 10.1016/j.jep.2012.03.020
35. Ryan RP, Dow JM (2008) Diffusible signals and interspecies communication in bacteria. Microbiol 154: 1845-1858. https:// doi: 10.1099/mic.0.2008/017871-0

36. Serebryakova E, Darmov I, Medvedev N, Alekseev S, Rybak S (2002) Evaluation of the hydrophobicity of bacterial cells by measuring their adherence to chloroform drops. Microbiol 71: 202-204. https://doi.org/10.1023/A:1015154406214
37. Sharma D, Misba L, Khan AU (2019) Antibiotics versus biofilm: an emerging battleground in microbial communities. Antimicrob Resist Infect Control 8: 76. https:// doi: 10.1186/s13756-019-0533-3
38. Silva A, Silva SA, Carpena M, Garcia-Oliveira P, Gullón P, Barroso MF, Prieto M, Simal-Gandara J (2020) Macroalgae as a source of valuable antimicrobial compounds: Extraction and applications. Antibiotics 9: 642. https:// doi: 10.3390/antibiotics9100642
39. Simon G, Berube C, Voyer N, Grenier D (2019) Anti-biofilm and anti-adherence properties of novel cyclic dipeptides against oral pathogens. Bioorg Med Chem 27: 2323-2331. https:// doi: 10.1016/j.bmc.2018.11.042
40. Song Z, Hou Y, Yang Q, Li X, Wu S (2021) Structures and Biological Activities of Diketopiperazines from Marine Organisms: A Review. Mar Drugs 19: 403. https:// doi: 10.3390/md19080403
41. Stepanović S, Vuković D, Dakić I, Savić B, Švabić-Vlahović M (2000) A modified microtiter-plate test for quantification of staphylococcal biofilm formation. J Microbiol Methods 40: 175-179. https:// doi: 10.1016/s0167-7012(00)00122-6
42. Stowe SD, Richards JJ, Tucker AT, Thompson R, Melander C, Cavanagh J (2011) Anti-biofilm compounds derived from marine sponges. Mar Drugs 9: 2010-2035. https:// doi: 10.3390/md9102010
43. Sudhakar P, Reck M, Wang W, He FQ, Dobler IW, Zeng A-P (2014) Construction and verification of the transcriptional regulatory response network of Streptococcus mutansupon treatment with the biofilm inhibitor carolacton. BMC Genomics 15: 1-22. https:// doi: 10.1186/1471-2164-15-362
44. Sulieman F, Ahmad A, Usop G, andKuang LC (2018) Diketopiperazine from marine bacterium *Pseudoalteromonas ruthenica* KLPp3. J Biol Res 91. https://doi.org/10.4081/jbr.2018.7197
45. Sun S, Dai X, Sun J, Bu X, Weng C, Li H, Zhu H (2016) A diketopiperazine factor from *Rheinheimera aquimaris* QSI02 exhibits anti-quorum sensing activity. Sci Rep 6: 39637. https:// doi: 10.1038/srep39637
46. Takenaka S, Oda M, Domon H, Ohsumi T, Suzuki Y, Ohshima H, Yamamoto H, Terao Y, Noiri Y (2016) Vizantin inhibits bacterial adhesion without affecting bacterial growth and causes *Streptococcus mutans* biofilm to detach by altering its internal architecture. Biochem Biophys Res Commun 480: 173-179. https:// doi: 10.1016/j.bbrc.2016.10.021
47. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Bio Evol 30: 2725-2729. https:// doi: 10.1093/molbev/mst197
48. Wai SN, Mizunoe Y, Takade A, Kawabata S-I,Yoshida S-I (1998) Vibrio cholerae O1 strain TSI-4 produces the exopolysaccharide materials that determine colony morphology, stress resistance, and biofilm formation. Appli Eviron Microbiol 64: 3648-3655. https:// doi: 10.1128/AEM.64.10.3648-3655.1998

49. Wang J-H, Yang C-Y, Fang S-T, Lu J, Quan C-S (2016) Inhibition of biofilm in *Bacillus amyloliquefaciens* Q-426 by diketopiperazines. World J Microbiol Biotechnol 32: 143. https:// doi: 10.1007/s11274-016-2106-4
50. Wang Y, Hoffmann JP, Baker SM, Bentrup KHz, Wimley WC, Fuselier JA, Bitoun JP, Morici LA (2021) Inhibition of *Streptococcus mutans* biofilms with bacterial-derived outer membrane vesicles. BMC Microbiol 21: 1-12. https:// doi: 10.1186/s12866-021-02296-x
51. Watanabe T, Igarashi M, Okajima T, Ishii E, Kino H, Hatano M, et al (2012) Isolation and characterization of signermycin B, an antibiotic that targets the dimerization domain of histidine kinase WalK. Antimicrob Agents Chemother 56: 3657-3663. https:// doi: 10.1128/AAC.06467-11
52. Watnick PI, Kolter R (1999) Steps in the development of a Vibrio cholerae El Tor biofilm. Mol Mirobiol 34: 586-595. https:// doi: 10.1046/j.1365-2958.1999.01624.x
53. Yang S, Zhang J, Yang R, Xu X (2021) Small Molecule Compounds, A Novel Strategy against *Streptococcus mutans.* Pathogens 10: 1540. https:// doi: 10.3390/pathogens10121540
54. Yoon S-H, Ha S-M, Kwon S, Lim J, Kim Y, Seo H, Chun J (2017) Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. Int J Syst Evol Microbiol 67: 1613-1617. https:// doi: 10.1099/ijsem.0.001755
55. Zhang Y, Miller RM (1992) Enhanced octadecane dispersion and biodegradation by a Pseudomonas rhamnolipid surfactant (biosurfactant). Appl Environ Microbiol 58: 3276-3282.

Figures

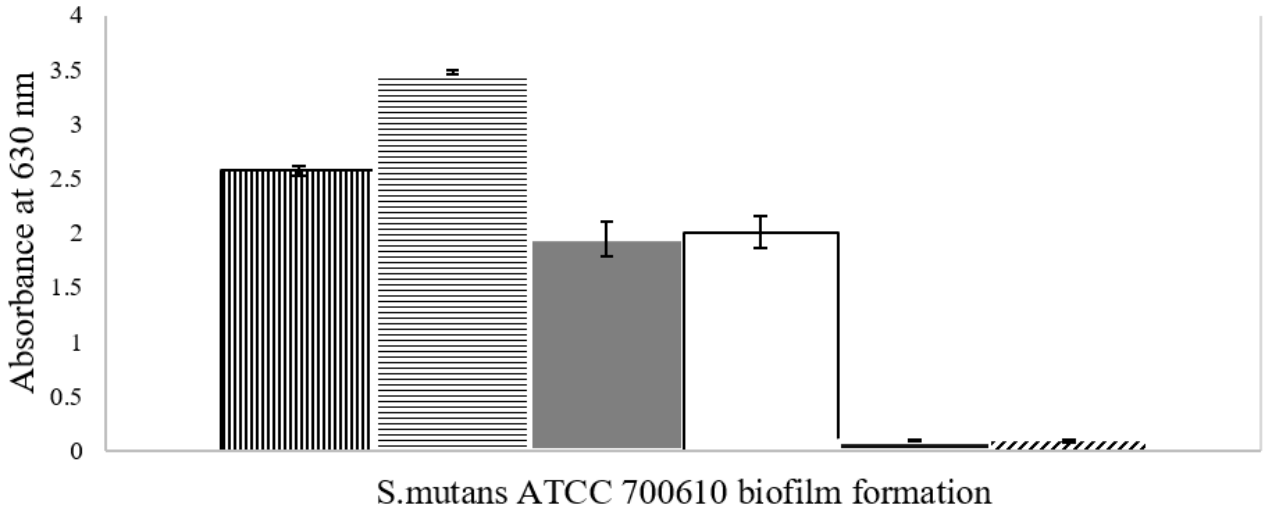

Fig. 1 Evaluation of different nutrient media in enhancing the *in vitro* biofilm formation of *Streptococcus mutans* ATCC 700610. Two different levels of inoculums [15 (■,■,▥) and 50 (▤,□,▨) μL] were used in the experiment

Figure 1

Please See image above for figure legend.

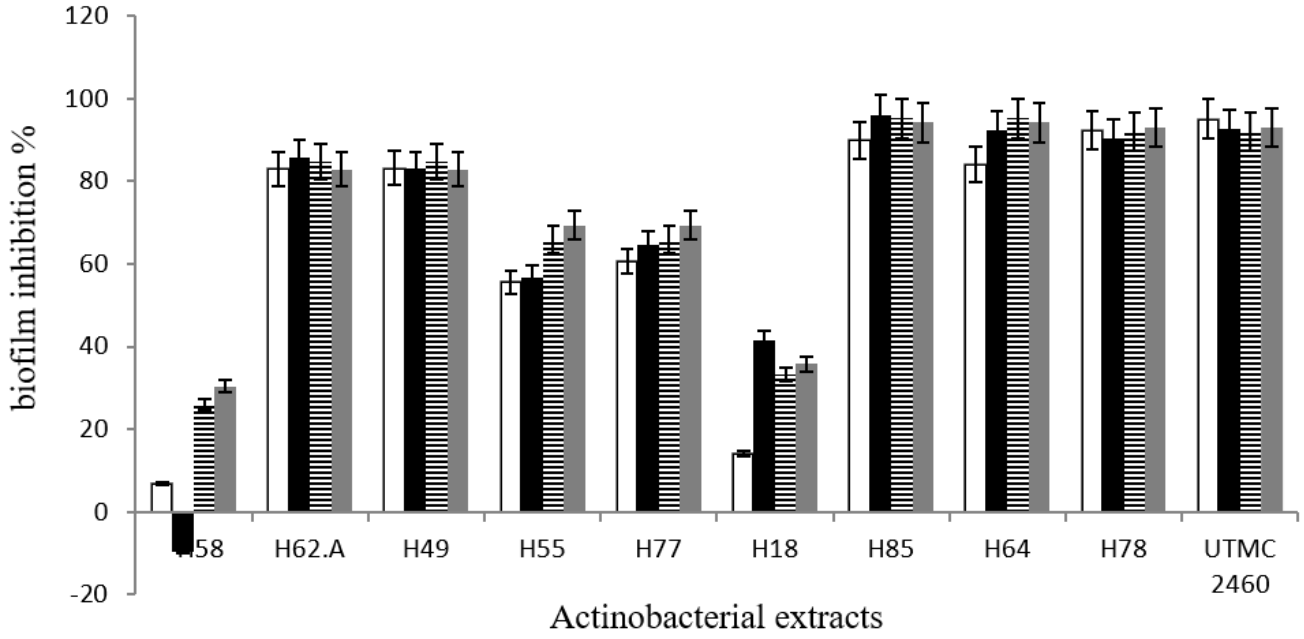

Fig. 2 Anti-adhesion screening of 10 selected marine Actinobacterial extracts on *S. mutans* biofilm formation at [100 (□) and 300 (■) μg/mL] concentrations. Five extracts with maximum anti-attachment characteristic (list them) compared with diuron at two different concentration of [1 (▤) and 10 (■) μg/mL] were elected for further analysis

Figure 2

Please See image above for figure legend.

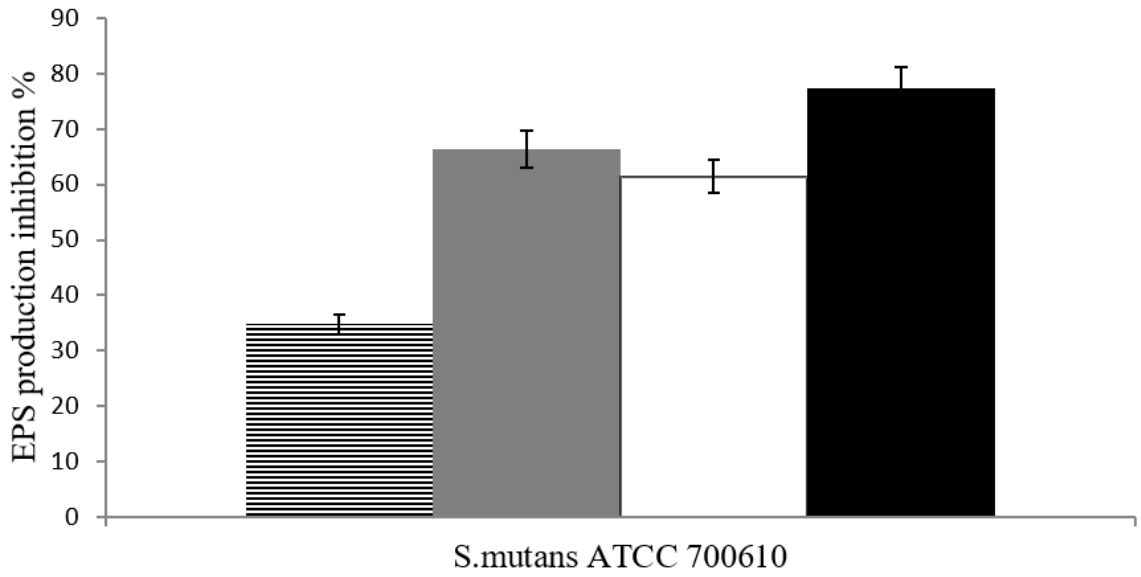

Fig. 3 Inhibitory effect of *Glycomyces sediminimaris* extract on EPS production. *Streptococcus mutans* ATCC 700610 EPS production was limited to 34.7 % and 66.3 % in presence of 100 (▤) and 300 μg/mL (■) of the metabolite extract , respectively. Diuron at two different concentrations of [1 (□) and 10 (■) μg/mL] caused 61.4 % and 77.4% reduction in production of EPS by *S. mutans,* respectively

Figure 3

Please See image above for figure legend.

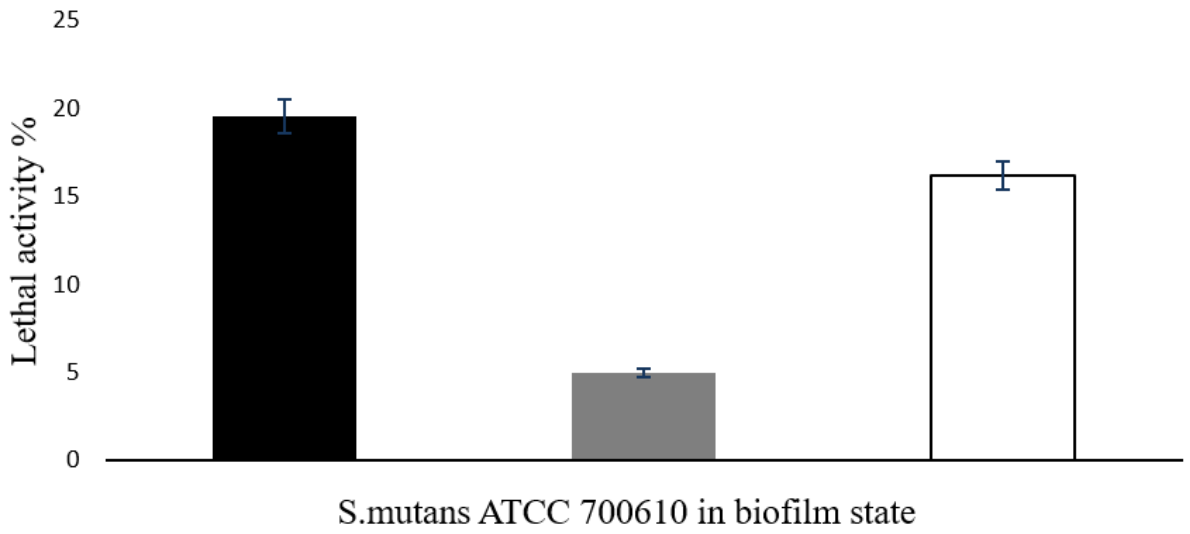

Fig. 4 The lethal activity of the extract on *S. mutans* cells in biofilm state. Bactericidal effects of *Glycomyces sediminimaris* extract at the lowest biofilm inhibition concentration 100 μg/mL (■) in comparison to bactericidal antibiotic Actinomycin 20 μg/mL (■) as the positive control. An equal cell viability reduction compared to Ciprofloxacin 2 μg/mL (□) was observed

Figure 4

Please See image above for figure legend.

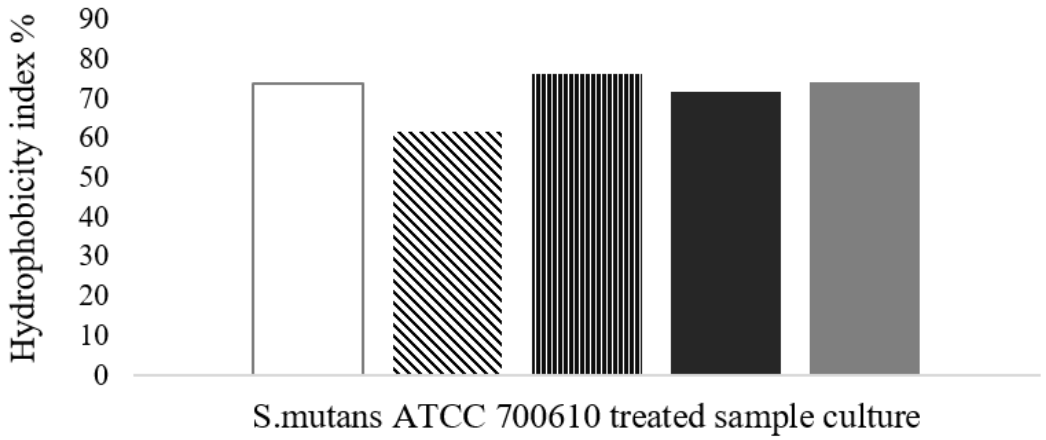

Fig. 5 The adherence tendency of *Streptococcus mutans* ATCC 700610 to a hydrophobic surface. *S. mutans* cell surface hydrophobicity was decreased up to 7 % and 20 % in presence of [100 (□) and 300 (▧) μg/mL] of *Glycomyces sediminimaris* extract, respectively. At the concentration of 100 μg/mL of the extract, the hydrophobicity index was approximately similar with positive controls of diuron 1 (▥) and 10 (■) μg/mL and irgarol 1 (▩) μg/mL

Figure 5

Please See image above for figure legend.

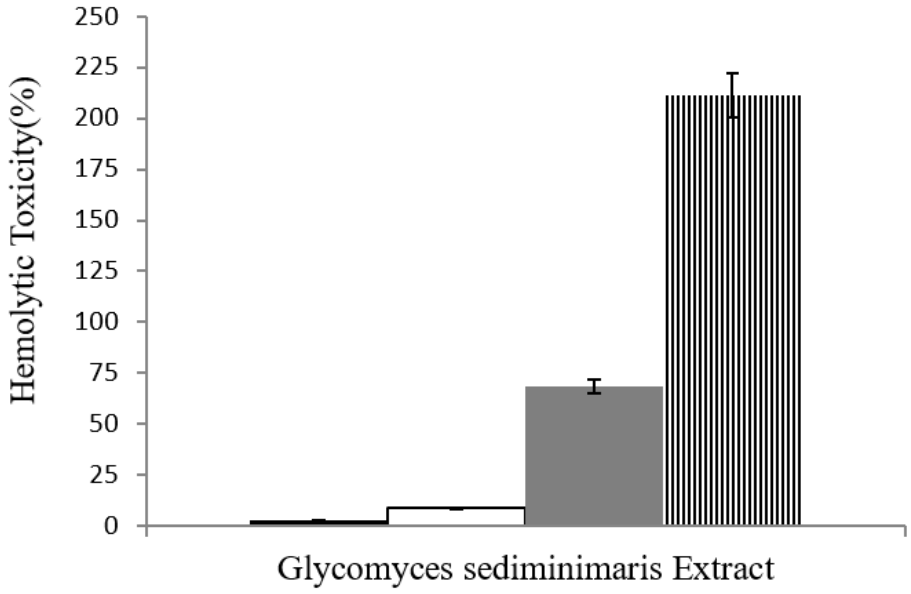

Fig. 6 Hemolysis toxicity of *Glycomyces sediminimaris* metabolite extract on human red blood cell. At the effective anti-adhesive doses of extract [100 (■), 200 (□) and 300 (▩) μg/mL) almost no or low hemolytic effects was detected. The overdose of the extract 400 (▥) μg/mL induced the cell lysis

Figure 6

Please See image above for figure legend.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryInformationSI.docx