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In-vitro Growth Inhibition and Biofilm Dispersion of Caries causing *Streptococcus mutans* by the Natural Extracts of Soil *Streptomyces*

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ABSTRACT

Dental caries is a significant infectious disease of human being caused by multispecies of bacteria. Among the causative agents of dental caries, notable one is *Streptococcus mutans,* which is an obligate parasite. In this study eleven *S. mutans* strains were isolated from carious lesions on Mitis Salivarius Bacitracin agar along with twenty potentially active *Streptomyces* strains were isolated from the soil of agriculture farmlands. The biochemical and physiological characterization along with the molecular identification by 16S rRNA gene sequencing confirmed that caries isolates are pathogenic *S. mutans* and soil isolates belong to the genus *Streptomyces*. The antibiotic resistance profile of *S. mutans* illustrated resistance to optochin. The antimicrobial screening of *Streptomyces* against *S. mutans* showed that these strain are the prolific producers of active agents to inhibit the growth of caries causing *S. mutans*. The natural extracts of *Streptomyces* strains significantly inhibited and delayed the biofilm formation by *S. mutans* and a profound effect was observed in case of the extracts of *Streptomyces* strains HU-09 and HU-10, which delayed biofilm maturation and even decreased biofilm formation with the passage of time. The micro well cytotoxicity assay against *Artemia salina* showed the cytotoxicity of the extracts in the range of 10-35%. The chemical profiling of the extracts was done by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC-UV) to get the indication of the identity of the compounds responsible for antimicrobial activity and biofilm dispersion. Overall the results indicated that natural extracts from these *Streptomyces* strains and their active components can be exploited for the preparation of anti-caries products like mouth washes and tooth pastes *etc*.

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Authors' Contribution
AM isolated and identified *S. mutans* strains, performed the experimental work and wrote the first draft of the manuscript. HMU isolated and characterized the *Streptomyces* strains. ANS and IS supervised the work.

Key words
***Streptococcus mutans*, Dental caries, Biofilm dispersion, Chemical profile, HPLC-UV.**

INTRODUCTION

Oral health is essential for the overall well-being of an individual (Gil Montoya *et al*., 2015). Many disease conditions are associated with oral health such as periodontal diseases, dental erosion, oral cancers, dental fluorosis and dental caries. Dental caries is the most widespread among oral diseases and it high incidence rate is due to poor oral hygiene, poor dietary habits and frequent use of edibles containing sugar (Fejerskov and Kidd, 2009).

The oral cavity is a wet, warm, nutritionally rich and ideal habitat for the growth of microorganisms (Kolenbrander *et al*., 2010). To date, about 280 bacterial species have been cultured from oral cavity and formally named (Dewhirst *et al*., 2010). In 1924 James Kilian Clarke first described the presence of *Streptococcus mutans* (Jakubovics *et al*., 2014) in oral cavity but its role as an etiologic agent of dental caries was first established in 1960 (Fitzgerald and Keyes, 1960). Dental caries is the localized irreversible destruction of susceptible dental hard tissues (Fontana *et al*., 2010). It starts with the shift of commensal oral microbiota, with in the complex biofilms, dominated by cariogenic microorganisms (Metwalli *et al*., 2013). The overall microbiology of dental caries declares it a polymicrobial disease as carious lesions are known to contain a range of *Streptococcal* sp., *Lactobacilli, Actinomyetes, Prevotellae* sp., and occasionally *Candida* yeasts, all of which are acid tolerant organisms (Gross *et al*., 2012). *S. mutans* is often found in elevated levels in plaque, produce abundant acid from dietary sucrose to derive tooth demineralization (Scannapieco, 2013).

Human adult's carious lesion are preferential source of isolating cariogenic strains of *Streptococcus mutans*. The dental health profession can't ever ignore this problem and, therefore, researchers have applied diverse approaches to combat prevalence of caries. Chemotherapeutic agents that especially disrupt biofilm formation and inhibit acid

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formation by cariogenic bacteria seems to be reasonable in preventing caries (Xu *et al*., 2011). Proanthcyanidins from cranberry crude extracts has been evaluated by Koo *et al*. (2010) that significantly reduced biofilm formation on saliva coated model of hydroxyapatite surface. To find out cost effective remedies, use of crude extracts from medicinal plants *i.e.* ginger, garlic, aloe vera, neem, amla, and tulsi have shown promising activity against dental caries pathogens (Jain *et al*., 2015). Taking this scenario in consideration, the potential secondary metabolites from *Streptomyces* can be experimentally checked as anti-caries agent. The filamentous Gram-positive *Streptomyces* are rich source of natural products (Aftab *et al*., 2016). Among 23,000 bioactive microbial secondary metabolites, 45% (10,000) of them are produced by actinomycetes from which ~7,600 compounds are produced by only *Streptomyces* species. Many of these secondary metabolites are potent antibiotics, antitumor and enzyme inhibitors which has made *Streptomyces* to be exploited by pharmaceutical industry (Olano *et al*., 2009). It is well established that each actinomycetes strain has probably genetic ability to produce 10-20 secondary metabolites (Janardhan *et al*., 2014).

This study aimed at exploiting potential secondary metabolites of soil *Streptomyces* against carries originated *S. mutans*. The secondary metabolites of *Streptomyces* are of prime importance due to their beneficial effects on human health (Janardhan *et al*., 2014). The *S. mutans* strains were isolated from carious tooth scrapings and were subjected to biochemical and genetics characterization using 16S rRNA gene sequencing. The *Streptomyces* strains were isolated from the soil of agriculture farmlands and were screened for their antimicrobial potential and suppression of the *S. mutans's* biofilm formation ability, keeping in mind to search the promising natural extracts and active compounds that could be mixed in formulation of oral healthcare products to prevent tooth decay or slow down caries progression.

MATERIALS AND METHODS

Sample collection and selective isolation of Streptococcus mutans *from dental caries*

Dental caries samples of lingual groove lesion were collected in physiological saline (0.85% NaCl) using sterile dentist scraping tool by a trained dentist, with informed consent of patients at Data Darbar Hospital, Lahore, Pakistan. Samples were processed within 2 h using swab culture technique on mitis salivarius agar (Himedia M259) supplemented with 0.2 U/mL bacitracin and 1% potassium tellurite, the plates were incubated at 37°C for 48 h (Gold *et al*., 1973). Polyphasic taxonomic approach was adopted for identification of the isolates and microbiological, biochemical, and physiological properties of the selected strains were determined (Hardie and Whiley, 2006). Genetic characterization included 16S rRNA gene sequencing. Genomic DNA extracted using FavorPrepTM Tissue Genomic DNA Extraction Mini Kit (FATGK001-1), the 16S rRNA gene was amplified following the methods used by Aslam and Sajid (2016). PCR amplicon was purified using FavorPrep GEL/PCR purification kit (FAGCK001) and the gene sequencing was done at the commercial facility of 1st BASE Laboratories Sdn Bhd, Singapore. The gene sequence data was BLAST analyzed at NCBI (www.ncbi.nlm.nil.gov) to determine the % homology of the isolated *S. mutans* strains with those already reported in GenBank. The sequence data was deposited to the GenBank and the accession numbers were obtained.

Antibiotics resistance profile of the S. mutans *isolates from dental caries*

Antibiotic susceptibility was determined by Bauer *et al.* (1966) method. A pannel of ccommercially available selected antibiotics discs including ciprofloxacin (CIP) 5 µg, piperacillin (PRL) 100 µg, oxacillin (OX) 1 µg, ampicillin (AM) µg, bacitracin (B) 10 U, methicillin (ME) 10 µg, amikacin (AK) 30 µg, vancomycin (VA) 30 µg, optochin (OP) 5 µg, amoxicillin (AX) 25 µg and cefpirome (CPO) 30 µg was used. Plates were incubated at 37°C for 24 h. The diameter of inhibitory zones were measured in mm and interpreted according to CLSI (Clinical and Laboratory Standards Institutes) guidelines 2013.

Collection of soil samples and selective isolation of Streptomyces

The soil samples were collected from agricultural farmlands at the university campus (University of the Punjab, Lahore, Pakistan) in sterile polythene bags and were processed within 12 h after collection according to methods described by Sajid *et al*. (2009). The selected strains were characterized for morphological, biochemical and physiological properties. Initially, antimicrobial potential was detected by agar plug method and potentially active strains were kept preserved as glycerol stocks for further screening. Genomic DNA was isolated using methods adopted by Aslam and Sajid (2016) and 16S rRNA gene sequencing was done by the methods described above in the section for *S. mutans* characterization.

Cultivation of Streptomyces *and preparation of cell free extracts*

The selected *Streptomyces* strains streaked on GYM agar were incubated at 28°C for 7 days. The cell free methanolic extracts of the active strains were prepared

by the methods described by Sajid *et al*. (2009). The final dried extract was dissolved in 3-4 mL of methanol and was stored in glass vials at 4°C.

Determination of antimicrobial activity and cytotoxicity

The agar well diffusion assay is a standard and convenient method for determination of bioactivity of *Streptomyces* (Perez *et al*., 1990). Lawn of *S. mutans* and other test organisms including *Staphylococcus aureus* and *Klebsiella pneumonia* on MH agar were made using sterile cotton swabs. The seeded plates were left on slab for 15 min at room temperature and ~40 µL of crude methanolic extract was loaded in each well made with sterile cork borer. Prior to incubation at 37°C for 18-24 h, plates were left at room temperature for 2 h to absorb the extracts. After incubation the diameter of clear hallows around wells *i.e.* zones of inhibition, were measured in mm.

The potential cytotoxic effect of the extracts of selected *Streptomyces* strains was determined by brine shrimp microwell cytotoxicity assay according to the method described by Solis *et al*. (1993). Dried eggs (0.5 g) of shrimp, *Artemia salina*, were cultured in 400 mL of artificial seawater and aerated for 24 to 48 h at room temperature. Only active larvae were dispensed to microtiter plate (wells diameter 1.8 cm, depth 2 cm) filled with 0.2 mL of salt water. Concentration up to 20 µg of each extract in 5 to 10 µL of DMSO was assayed for determination of cytotoxicity and the percentage larval mortality was calculated.

Biofilm formation and dispersion studies, Congo red agar assay

Petriplates containing BHI medium with Congo red dye, supplemented with 20% glucose and without glucose were prepared and the *S. mutans* strains were streaked. The plates were incubated at 37°C for 2 days. The biofilm forming ability of the selected *S. mutans* was checked by formation of black colonies on plates supplemented with glucose (Freeman *et al*., 1989).

Microwell plate method

To determine the effect of natural extracts from *Streptomyces* on biofilm forming ability of *S. mutans* individual wells of sterile, 96 well-flat bottom polystyrene culture plates, were filled with 0.180 mL aliquots of BHI media with extract concentrations as 0, 100, 200, 400, 800 and 1600 µg/mL. To check sterility and non-specific binding of media, only BHI broth served as control. Wells were inoculated with 20µL of fresh inoculum with optical density (OD) set the same for all strains. Culture Plates were incubated at 37°C for 48, 72 and 168 h. After incubation OD was taken at 600nm using microplate spectrophotometer (Biotek instruments, High lands Park, USA). Later the procedure was followed as described by Christensen *et al*. (1985) and OD of the stained adherent bacteria was determined at wavelength of 578 nm.

Chemical profiling of the extracts of Streptomyces

The silica coated aluminium TLC plates were spotted with extracts by superimposing and developed using 10% methanol/dichloromethane (10% MeOH/CH_2Cl_2) solvent system then visualized under UV at 254 and 366 nm to mark the pattern of UV absorbance and fluorescence. Further localization of the components on TLC plates was done by spraying the TLC plates with anisaldehyde/H_2SO_4 and Ehrlich's reagent. The crude extracts were analyzed by high-performance liquid chromatography (Sykum HPLC system) using software clarity. Thermo Hypersil-Keystore column, particle size (5µm) 5, with 25cm length and 4.5 mm ID was used, the methanol and water (95:5) served as mobile phase at flow rate of 1mL/min. Almost 20µL of sample, crude extract in HPLC grade methanol, was injected using micro syringe. Each sample was run for 20 min and UV absorbance was determined at 254 nm. The peaks at different retention times (t_R) were compared with standard UV absorbance data of secondary metabolites.

RESULTS

Taxonomic characteristics of the isolates recovered from dental caries

A total of eight dental caries samples were processed on MSB agar, among them three were found positive for the presence of *S. mutans* on the basis of morphological characteristics and eleven suspected *S. mutans* colonies were retrieved, which were further purified by sub-culturing. The pure cultures were maintained on BHI agar for further investigation. The isolates were labeled as SM-1 to SM-11. All the selected strains were able to utilize sucrose, sorbitol, mannitol and glucose as carbohydrate source and produced acid which was indicated by change in media color, which turned to yellow as compared to the control that persisted as red. All the strains gave flocculation with acetone, ethanol and methanol in dextran production test except the isolates SM-2 and SM-4, which gave turbidity with ethanol and methanol. Tolerance to high concentrations of NaCl confirmed the cariogenic behavior of these isolates. A rapid differentiation of mutans streptococci from *Streptococcus sanguis* by staining with 2, 3, 5-triphenyltetrazolium chloride (TTC) on MS agar was visually detected by change in color of colonies from pale blue to dark pink. The five isolates characterized by 16S rRNA gene sequencing were subjected to NCBI nucleotide BLAST analysis. This showed highest genetic similarity

up to 99% of the caries isolates with *Streptococcus mutans* UA159 strain already reported in GenBank and is a proven pathogenic strain.

Antibiotic sensitivity pattern of the caries isolates

The inhibition zone diameter in mm is the index to assign resistance or susceptible status to a particular isolate. All the isolates were resistant to optochin with evident indication of no clear zones around antibiotic disc. It signified that isolates belong to viridans group of streptococci, while varied results were obtained against bacitracin. Strains SM-1, SM-6, SM-7 and SM-11 were typically sensitive whereas SM-2, SM-3, SM-4, SM-5, SM-8, SM-9 and SM-10 were intermediate resistant to bacitracin. All the strains were sensitive to rest of the nine antibiotics used in the panel. Maximum sensitivity zone of inhibition was manifested by piperacillin and amoxicillin up to 49 mm and 46 mm, respectively.

Taxonomic characteristics of the selected soil Streptomyces

A total of 20 strains, isolated from soil of agricultural farmlands, were purified on GYM agar by repeated sub culturing. These selected strains were assigned the names as HU-1 up to HU-20. The colonies of selected strains were rough and embedded in agar; substrate and aerial mycelium showed pigments ranging in color from white to grey and even pink, yellow and peach. In biochemical and physiological characterization glucose, galactose, lactose, sucrose, arabinose and maltose were used by all the *Streptomyces* strains as sole source of carbon except the strain HU-19 which was unable to use galactose. Fructose was the least utilized sugar as only two strains HU-06 and HU-14 were able to utilize it. Among 20 isolates 6 strains including HU-4, HU-5 and HU-14 up to HU-17 were unable to produce melanin pigment. Three strains HU-3, HU-5, and HU-16 were unable to utilize citrate, on the other hand all strains successfully utilized oxalate and exhibited clear hallows around the growth. In case of urea hydrolysis, 15 strains showed positive result, indicated by pink color in the medium due to the production of ammonia, while the strains HU-4, HU-7, HU-12, HU-15, and HU-16 were negative for urea hydrolysis. Three strains including HU-6, HU-16 and HU-19 were negative for tyrosine hydrolysis. For esculinase enzyme production 13 strains were positive and seven were negative. Five strains among the selected 20 isolates were subjected to 16S rRNA gene Sequencing. NCBI nucleotide BLAST analysis for 16S rRNA gene sequence showed 98-100% similarity of our soil isolates with Atinomycete group and with foremost antibiotic producing genus *Streptomyces*. The GenBank accession numbers for these *Streptomyces* strains are given in Table I.

Table I.- Nucleotide BLAST analysis and GenBank accession numbers of selected *Streptomyces* and *S. mutans* isolates.

Strain	Blast analysis Similarity with others	Similarity (%)	Accession No.
Streptomyces			
HU-09	*Streptomyces puniceus* (AB922839.1)	98	KX641027
HU-10	*Streptomyces* sp. E5N158 (KX279571.1)	99	KX721650
HU-11	*Streptomyces* sp. SE30 (KJ174474.1)	98	KX721649
HU-16	*Streptomyces* sp. JCM28680 (LC133711.1)	99	KX641026
HU-17	*Actinomycete* CXB744 (JF512544.1)	100	KX641028
Streptococcus mutans			
SM-1	*S. mutans* UA159 (NR 074983.1)	99	KR815860
SM -3	*S. mutans* UA159 (NR 074983.1)	99	KR815861
SM -5	*S. mutans* UA159 (NR 074983.1)	99	KR815862
SM -9	*S. mutans* UA159 (NR 074983.1)	99	KR815863
SM -10	*St. mutans* UA159 (NR 074983.1)	99	KR815864

In-vitro *growth inhibition of* S. mutans *and cytotoxicity of* Streptomyces *extracts*

A promising response was observed in *in-vitro* growth inhibition studies against caries causing *S. mutans* strains by the natural extracts of *Streptomyces*. The highest zone of inhibition up to 27 mm was exhibited by the extracts of *Streptomyces* strain HU-10, against *S. mutans* strains SM-1 and SM-4. The extracts of *Streptomyces* strain HU-9 also exhibited promising results by inhibiting the growth of maximum number of *S. mutans* isolates and gave up to 16mm inhibitory zone. The extracts of *Streptomyces* strains including HU-1, HU-4, HU-8, HU-11, HU-12, HU-14, HU-15, HU-16 and HU-20 also exhibited significant activity against caries causing *S. mutans* isolates. While the extracts of *Streptomyces* strains HU-2, HU-3, HU-5, HU-7, HU-13 and HU-17 were substantially inactive against *S. mutans* isolates (Fig. 1). In case of other test organisms like *S. aureus*, the prominent activity was exhibited by the *Streptomyces* strain HU-11, while in the case of *Klebsiella* as test organism maximum zone of inhibition was observed in the extract of *Streptomyces* strain HU-10 (Table II).

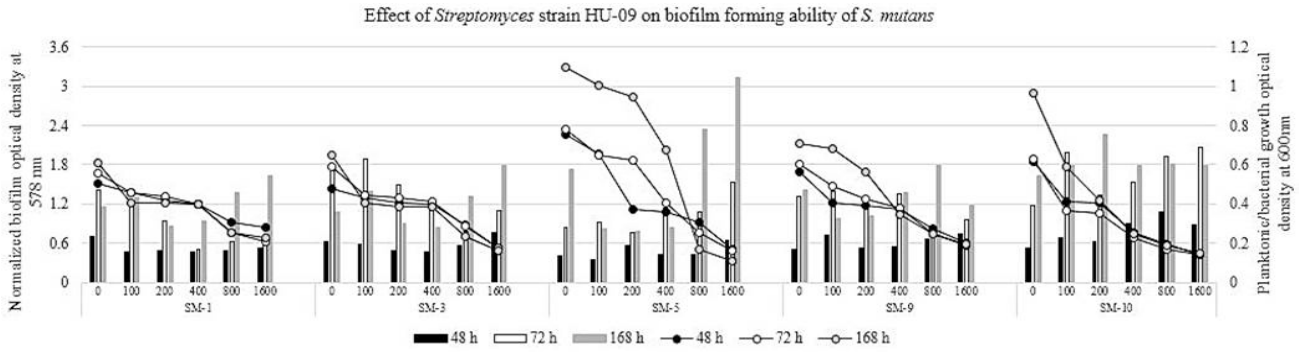

Fig. 1. Normalized biofilm formation by *S. mutans* at 48, 72 and168 rhs (bars) and their respective planktonic growth (lines) under influence of natural extracts at different concentration (0, 100, 200, 400, 800 and 1600 µg/mL) from *Strptomyces* strain HU-9.

Table II.- *In-vitro* inhibition of *S. mutans* by selected *Streptomyces* and %mortality of larvae of *Artemia salina* (cytotoxicity assay).

***Streptomyces* strain**	**Zone of inhibition (mm) against *S. mutans***													**Mortality (%)**
	SM-1	**SM-2**	**SM-3**	**SM-4**	**SM-5**	**SM-6**	**SM-7**	**SM-8**	**SM-9**	**SM-10**	**SM-11**	***S. aureus***	***K. pneumoniae***	
HU-01	8	-	-	-	9	-	-	-	-	16	-	16	12	33.3
HU-02	-	-	-	-	-	-	-	-	-	-	-	17	13	31.13
HU-03	-	-	-	-	-	-	-	-	-	-	-	16	18	15.65
HU-04	-	9	-	10	-	9	11	10	-	11	-	15	14	35.71
HU-05	-	-	-	-	-	-	-	-	-	-	-	18	13	19.35
HU-06	-	-	-	-	-	-	-	-	-	-	-	10	10	21.08
HU-07	-	-	-	-	-	-	-	-	-	-	-	09	09	18.00
HU-08	10	-	8	-	9	-	-	-	10	10	-	19	19	5.55
HU-09	16	13	13	14	15	11	12	10	13	13	12	14	14	12.55
HU-10	27	20	16	27	25	12	12	22	25	16	12	12	20	15.15
HU-11	18	-	-	16	13	13	12	12	13	8	12	20	15	21.05
HU-12	12	-	-	12	-	7	9	8	13	13	13	04	-	11.75
HU-13	-	-	-	-	-	-	-	-	-	-	-	09	14	13.88
HU-14	11	9	8	10	10	9	8	8	8	9	8	03	03	-
HU-15	9	8	9	8	-	-	-	-	8	9	-	07	09	-
HU-16	-	-	7		10	-	-	-	7	9	-	-	10	-
HU-17	-	-	-	-	-	-	-	-	-	-	-	11	-	-
HU-18	-	-	-	-	-	-	-	-	-	-	-	19	15	-
HU-19	-	-	-	-	-	-	-	-	-	-	-	11	11	-
HU-20	15	12	15	15	14	12	13	11	-	11	12	-	12	-

The natural extracts of the selected *Streptomyces* strains were also evaluated for their cytotoxicity against brine shrimp *Artemia salina*. The maximum cytotoxicity was exhibited by the extracts of *Streptomyces* strains HU-04 and HU-01, with larval mortality up to 35% and 33%, respectively. While the extracts of other 7 *Streptomyces* strains exhibited moderate or low cytotoxicity in the range of 10%-20% larval mortality. While only the extract of *Streptomyces* strain HU-8 exhibited larval mortality less than 10% (Table II).

Biofilm formation and dispersion by the natural extracts from Streptomyces

Congo red dye detects the presence of exo-polysaccharides produced by bacteria and turns black when interacts with slime components. *S. mutans* strains streaked on Congo red agar with 20% glucose gave distinctive blackening and while streaked on Congo red agar without glucose appeared as pink.

The effect of *Streptomyces* strains HU-9 and HU-10 on biofilm forming ability of *S. mutans* was evaluated as

both these strains had exhibited significant antimicrobial activity on plates. Planktonic growth was detected at 0, 100, 200 and 400 µg/mL of extract concentrations but growth was significantly reduced at 800 and 1600 µg/mL. Although there was increase in bacterial growth with gradual increase in time (48–168 h). Under the influence of natural extracts from *Streptomyces* strain HU-9, normalized biofilms of *S. mutans* strains SM-1, SM-3 and SM-10 had reached to their maturation phase by 72 h with extract concentrations up to 200-400 µg/mL, respectively. Whereas increasing the extract concentration up to 800–1600 µg/mL had delayed maturation of biofilms by *S. mutans*. Maximum biofilm was formed by *S. mutans* strain SM-5 even at highest concentration (1600 µg/mL) of extract. This notable behavior can be because of initial high planktonic growth of this strain. In case of strain SM-9 auto-aggregation of planktonic cells has overcome the extract effect at 800-1600 µg/mL (Fig. 1). Results from natural extracts of *Streptomyces* strain HU-10 also suggested that maturation of biofilm had been delayed with increasing concentrations of extract for *S. mutans* strains SM-1, SM-9 and SM-10. However inhibitory effect of *Streptomyces* on biofilm forming ability of *S. mutans* strains SM-3 and SM-5 is clear that biofilms again started detachment when extract concentration was increased up to 400 µg/mL (Fig. 2).

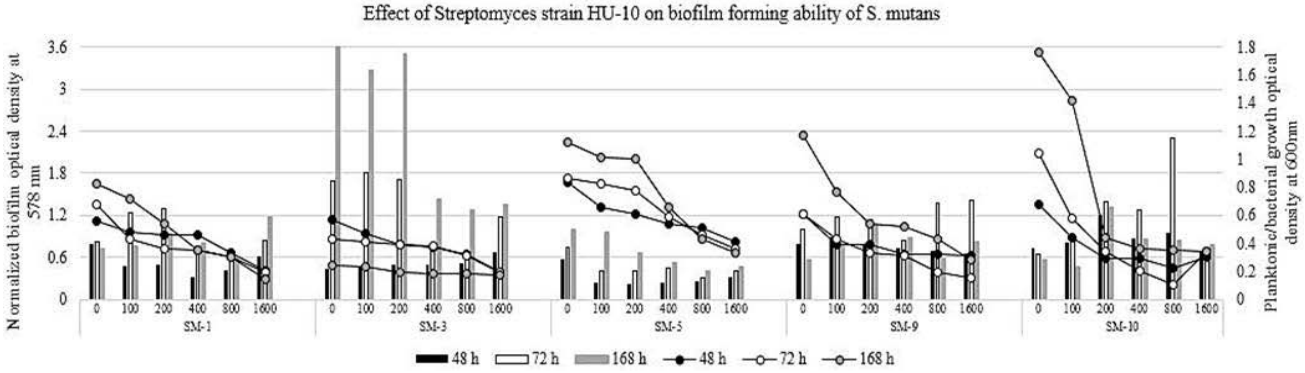

Fig. 2. Normalized biofilm formation by *S. mutans* at 48, 72 and168 rhs (bars) and their respective planktonic growth (lines) under influence of natural extracts at different concentration (0, 100, 200, 400, 800 and 1600 µg/mL) from *Strptomyces* strain HU-10.

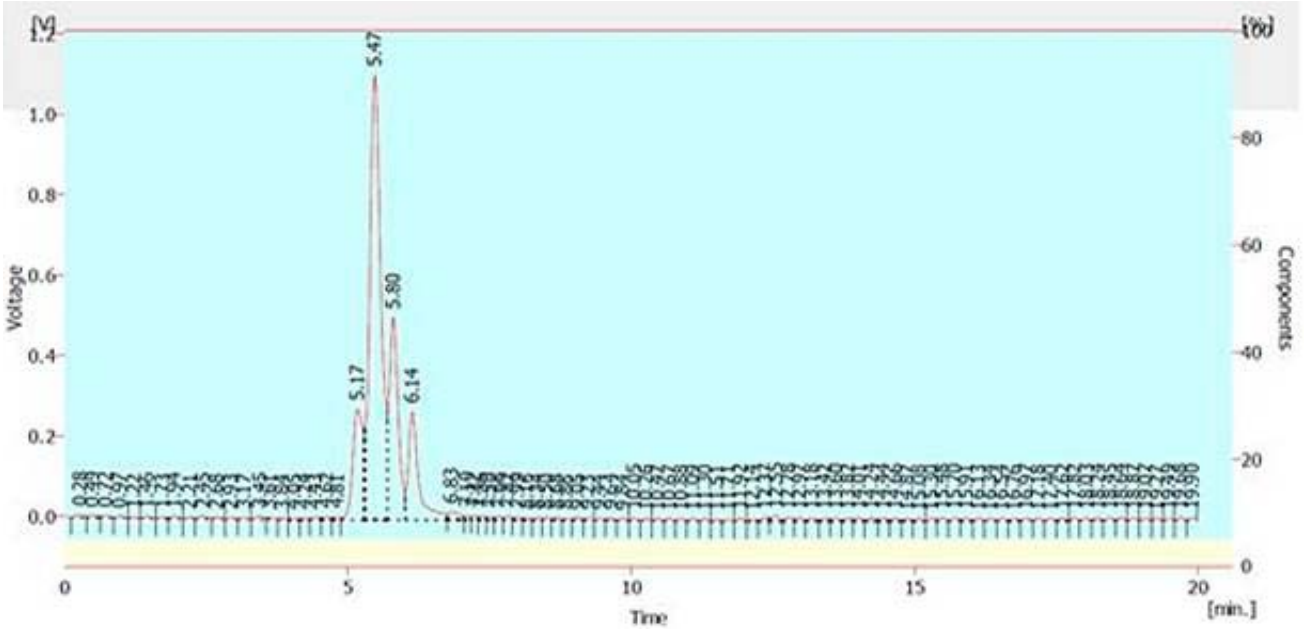

Fig. 3. HPLC-UV chromatogram of strain HU-16. There are major four peaks in chromatogram at different retention times. The fraction of secondary metabolite at retention time 5.47 is abundant in crude extract from this strain.

Chemical profile of the natural extracts of the selected Streptomyces *strains*

An impressive diversity of the chemical constituents present in the extracts of the selected *Streptomyces* strain was observed while analyzed by TLC and HPLC. The TLC developed plates viewed under short (254 nm) and long (366 nm) UV indicated the presence of biologically active components in the extracts of selected *Streptomyces* strains, with intense dark bands against fluorescent green background and fluorescent blue bands against black background, respectively. The TLC plate when stained with anialdehyde/H_2SO_4, the extracts of strains HU-2, HU-3, HU-5, HU-7 up to HU-15, HU-17 and HU-19 indicated the presence of pink and blue bands and different number of colored fractions in the extracts. On the other hand the TLC plate treated with Ehrlich's reagent indicated the presence of bright yellow colored fractions in the crude extracts. The extract of strain HU-2 exhibited maximum number of colored bands. The extracts of strains HU-7 up to HU-13, HU-15 to HU-7 and HU-19 each exhibited 2 bright yellow bands along with other minor fractions of various colors.

HPLC uses retention times (t_R) to differentiate compounds in an extract. HPLC analysis of the extracts of eight strains including HU-1, HU-9 to HU-12, HU-16, HU-17 and HU-20 was done. HU-9 showed three major peaks at t_R 3.316, 3.592 and 3.836 min with peak area in mV.s as 53.5, 14.4 and 7.7, respectively. The extract of strain HU-10 exhibited two major peaks at retention time 5.232 and 5.756 with % peak area up to 67.7 and 16.8, respectively. The chromatogram of the strain HU-16 had four peaks; one at t_R 5.47 had highest peak area up to 47.0 indicating that this might be the most abundant active fraction of extract for antimicrobial activity (Fig. 3).

DISCUSSION

Dental caries is the most prevailing infectious disease among humans and persists as a public health problem worldwide (Alcaraz *et al*., 2012; Selwitz *et al*., 2007). Despite the fact that many therapies have been developed to control caries and many reports are there about caries decline in developed countries yet its incidence is increasing in developing as well as developed countries (Maxood, 2008).

This study deals with the isolation of caries associated *S. mutans,* their *in vitro* growth inhibition and suppression of biofilm formation by the natural extracts from antibiotics producing soil *Streptomyces*. Streptococci are considered to be the fastidious organisms that require extra enrichment of cultural media (Hardie and Whiley, 2006). Although blood agar have always been traditionally used that support growth of *Streptococcus* species but we were unable to identify the mutans streptococci from heterogeneous complex oral flora on blood agar owe to shared α-hemolytic character of the oral flora. Sample culturing on Mitis salivarius agar (MSA) with bacitracin and potassium tellurite came out with best possible results for isolation of *S. mutans* (Gold *et al*., 1973). Number of colonies selected from positive sample culture plate were 5 from sampe-1, 4 colonies from sample-2 and 2 colonies from caries sample 3. *S. mutans* gave pale blue, raised, convex, opaque, undulate and distinctive granular frosted glass appearance (Coykendall, 1989; Facklam, 1977) with a puddle of glistening drop bubble, water soluble glucan produced from sucrose. Biochemical characterization, 16S rRNA gene sequencing, BLAST analysis and phylogenetic tree construction using MEGA software version 6 with neighbor joining method and 500 bootstrap replication value revealed that isolates had highest genetic similarity to the virulent strain of *S. mutans* UA159. As *S. mutans* belongs to viridans group of streptococci that can be readily differentiated from other clinical streptococci, such as *Streptococcus pneumonia,* by their resistance behavior towards optochin and bacitracin, whereas *S. pneumoniae* is sensitive to these antibiotics. So, Antibiotic sensitivity pattern confirmed that caries isolates belong to viridans group of streptococci.

For *Streptomyces* isolation, 10^{-3} dilution of soil sample gave the best results for formation of crowding on agar surface. Out of 20 strains, 4 produced soluble pigments which were diffused into the medium. The comparison of morphological and physiological characteristics with known actinomycetes reported in Bergey's Manual of Systematic Bacteriology (Whitman *et al*., 2012; Atalan *et al*., 2000) suggested that these strains belong to the genus *Streptomyces*. Nucleotide BLAST analysis showed that strain HU-9 had 98% similarity with *Streptomyces puniceus* and HU-10 had 99% similarity with *Streptomyces* sp. E5N158 already reported in the GenBank (Table I).

Biological screening of *Streptomyces* strains proved that these were potent producers of bioactive secondary metabolites (Berdy, 2005; Yilmaz *et al*., 2008) and this study suggested them as good source of anti-caries agents. Among the selected soil *Streptomyces,* 55% were active against *S. mutans* isolates in *in vitro* studies. A strong antimicrobial potential was exhibited by the *Streptomyces* strain HU-10. The cytotoxicity of the strain HU- 10 strain was moderate (up to 15 % larval mortality) and active compounds from it can be exploited as anticaries compounds. The *Streptomyces* strains HU-9 and HU-14 also showed convincing antimicrobial ability by inhibiting 100% isolates of *S. mutans* with moderate cytotoxicity (up to 12.5% larval mortality) against *Artemia salina*.

Artemia has been used as standard model organism to evaluate the cytotoxic potential of therapeutic agents from *Streptomycetes* (Aftab *et al*., 2015). The strains HU-1, HU-4, HU-8, HU-11, HU-12, HU-15, HU-16 and HU-20 were also active against some of the *S. mutans* strains (Table II). About 90% *Streptomyces* strains showed antimicrobial activity against other test organisms including *S. aureus* and *K. pneumonia* (Fig. 1). Potentially active metabolites of *Streptomyces* strains HU-9 and HU-10 had increased biofilm maturation time and even biofilm forming trend was decreased under the influence of increases extract concentrations from HU-10 (Figs. 1, 2).

Chemical screening using TLC and HPLC-UV revealed a unique pattern of secondary metabolites from *Streptomyces*. Staining with anisaldehyde/H_2SO_4 exhibited dark pink bands giving an indication of presence of some quinines and peptides like compounds. Distinct yellow bands appeared when Ehrlich's reagent was sprayed giving an idea of the presence of actinomycins and similar compounds in the crude extracts. The HPLC-UV chromatograms gave a visual representation of the presence of different number of compounds and the relative concentrations of the fractions, separated on the basis of their retention times (t_R), in each of the extract (Fig. 3). The extract of most active *Streptomyces* strain HU-9 showed three major peaks at t_R 3.32 min, t_R 3.59 min and t_R 3.84 min. The component at t_R 3.32 min gave highest peak area, which shows this is the most abundant compound in extract and dispersion of *S. mutans* biofilm might be due to this compound, however the other components in this extract can also be active irrespective of their low concentration in extract.

Potentially active secondary metabolites from *Streptomyces* have been reported as antimicrobial agents against microbial species such as *K. pneumoniae*, Methicillin resistant *S. aureus, Acinetobacter* spp. and *Pseudomonas* spp. (Aslam and Sajid, 2016). Also study from our lab has reported the inhibitory effect of *Streptomyces macrosporeus, Streptomyces vinaceus, Streptomyces erythrogriseus* and *Streptomyces labedae* on biofilm formation of dental plaque isolates, *Acinetobacter schinndleri*, *Moraxella aci* and *Bacillus cereus* (Saleem *et al*., 2015). Based on these finding the study suggest that chemical compounds from *Streptomyces* can be explored for their active components that especially hinder the process of biofilm formation by typical cariogenic pathogen *i.e. S. mutans* that uses glucose dependent pathway for biofilm formation.

CONCLUSION

Over all the study revealed that the selected *Streptomyces* strains are promising source of bioactive compounds which can inhibit the growth and suppress biofilm formation in caries causing pathogens especially in *Streptococcus mutans*. Their distinctive metabolic fingerprinting in chemical screening shows that they are rich source of diverse active secondary metabolites. The natural extracts from these *Streptomyces* strains may be exploited as anti-caries agents and can be used in related products like tooth pastes or mouth washes *etc*.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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