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ANTICARIOGENIC ACTIVITY OF GALLS OF *QUERCUS INFECTORIA* OLIVIER AGAINST ORAL PATHOGENS CAUSING DENTAL CARIES

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ABSTRACT: In this study, attempts were made to evaluate the anti-microbial activity of the galls of *Quercus infectoria* Olivier against oral bacteria causing dental caries. Various solvents systems were used to screen the potential of the extract on the clinically isolated organism and reference strains; *Candida albicans* MTCC 183, *Streptococcus mutans* MTCC 497, *Lactobacillus acidophilus* MTCC 10307 and *Staphylococcus aureus* MTCC 1144 using agar-well diffusion method. This was followed by determination of minimum inhibitory concentration (MIC) by using two-fold serial microdilution methods at a concentration ranging between 0.02 mg/ml to 10 mg/ml. The MIC values against each bacterial species was found in the range of 0.16 to 0.31 mg/ml. Minimum bactericidal concentration (MBC) was obtained by subculturing microtiter wells which showed no changes in color of the indicator after incubation. Among all tested bacteria, *S. aureus* was the most susceptible. The clinically isolated *S. mutans* was further analyzed for its ability to form biofilm on the surface of seashells. The biofilm reduction percent assay in microtitre wells was used to quantitatively determine the inhibition in the presence of *Q. infectoria* extract. Maximum reduction was seen at 0.018 µg/ml of methanolic extract. Thus, the galls may be considered as effective phototherapeutic agents for the prevention of biofilm formation by common oral pathogens.

INTRODUCTION: The oral microbial-plaque communities serve as biofilms composed of numerous genetically distinct bacterial types that multiply in close juxtaposition on host substratum. This is made possible via physical interactions such as co-aggregation and metabolic and physiological interactions. There is a sequential organization of the microbial community [1] often initiated by streptococci and antinomies. Numerous microbial interactions including synergism and antagonism are largely affected by diet, oral hygiene, diurnal changes in salivary flow and host defenses [2].

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When this balance is disturbed, acidogenic and aciduric bacteria such as streptococci and lactobacilli increase, leading to demineralization of enamel and dental caries. *Streptococcus mutans* elaborates water-insoluble glucan produced in the presence of sucrose. Lactobacilli have a reputation for inhibiting microbial pathogens by secreting antimicrobial compounds like organic acids, oxygen peroxide, and 'bacteriocins' [3].

There exists a strong association between Lactobacilli in saliva and the occurrence of dental caries with some studies proving that lower levels of salivary Lactobacilli meant reduced chances of developing caries [4]. Secreted Aspartyl Proteinases (SAP) elaborated by *Candida albicans*, one of the major producers of acid in the oral cavity plays an important role in conferring virulence [5]. Staphylococci initiate formation of biofilms with the aid of diverse virulence factors [6].

Biofilm development is inhibited by preventing attachment of cariogenic bacteria, delivery of antimicrobial compounds, manipulation of cell signaling mechanisms, or enhancing host defences. Photochemical extracted from plants used in dental formulations like mouthwash and toothpaste are considerably safe, cost-effective [7] and preferred against chemical-based drugs that come with a lot of side effects [8]. For example, plant-based mouthwashes with 'Triphala' has been one of the best ayurvedic remedies to combat oral diseases especially in children [9]. Plaque control by effective reduction of the supra- and subgingival pathogenic flora is important to prevent the initiation and progression of dental caries. This is mainly done by mechanical means with adjunct therapy including antibiotics and antiseptics [10]. The later is however associated with risks of developing antibiotic resistance, changes in oral microbiota and teeth staining [11]. Alongside established chemicals frequently used in oral prophylaxis and treatment, natural products derived from medicinal plants may represent a suitable alternative, exhibiting less or no side effects.

It is stated that approximately 80% of people residing in developing areas prefer traditional medicines for health care [12]. The galls of *Quercus infectoria* Olivier (Fagaceae) is one of the most popular traditional medicine across Asia against various ailments. Also known as Manjakani, Majuphal or oak apples, these are produced by the Allepo oak tree in response to wasps laying their eggs in the oak bark. When wasps lay their eggs, the oak tree reacts by producing protrusions around the insect egg or larvae; in turn the growing eggs use these galls for shelter and nutrition. In addition to being a natural pesticide for the oak tree, oak galls have been used extensively for the treatment of dysentery, gonorrhea, tonsillitis, internal hemorrhages, impetigo, and menorrhagia [13]. The galls are also used to restore postpartum uterine elasticity and also stimulate the contraction of vaginal muscles [14]. In Indian traditional medicine, it is sometimes used as a constituent of toothpowder for treatment of gum and oral cavity diseases [15]. It may also play a secondary role in reducing other dental problems like caries, extrinsic staining, bleeding gums, and toothache. Through this work, we aim to promote a local and indigenous product for the reduction of dental plaque and rejuvenate the oral cavity with negligible side effects.

MATERIALS AND METHODS:

Preparation of Oak Gall Extract: The galls of *Q. infectoria* Olivier (Fagaceae) used in this study were obtained from Attar Ayurveda, Jaipur, Rajasthan. The dried galls were crushed using a pestle and mortar and then finely powdered in an electric grinder. Twenty grams of gall powder was immersed in 100 ml of the respective solvents: water, methanol, ethanol, ethyl acetate, acetone and hexane and agitated at 200 × g for 6 h, using a rotary shaker. The crude *Q. infectoria* extracts (QIE) were filtered in a Buchner funnel through Whatman filter paper no. 1 to eliminate plant particles followed by evaporation at 40 °C. The dry powder was suspended in distilled water by vigorous agitation at 45 °C, to a final concentration of 10% (w/v). The QIE was sterilized using a syringe filter (0.22 μm pore size) and kept at 4 °C in sterile dark bottles.

Isolation and Characterization of the Clinical Isolate: Demineralized portions of the freshly extracted decayed tooth were obtained from patients with caries from RMV Dental Clinic and Santosh Dental Care, Bengaluru. The specimens were collected in 5 ml of sterile thioglycollate broth in sterile screw-capped vials which served as the transport medium. The specimens were streaked onto Brain Heart Infusion agar for isolation and incubated for 24 h at 37 °C. Each isolate was given a number before its identification and these numbers were maintained throughout. The isolated organisms were identified as Streptococcus based on information in bergeys manual of determinative bacteriology [16]. Distinctive colonial characteristics were noted. Subcultures of isolates were characterized by various biochemical tests namely indole, MR-VP, citrate utilization, sugar fermentation test [17]. The morphology was identified using Gram's staining method [18].

Primary Screening for Biofilm Formation:

Tube Assay: Qualitative assessment of biofilm formation was determined by the tube staining assay following Christensen *et al.* [19]. 3 ml of Brain Heart Infusion broth containing 1% D-glucose was inoculated with a loopful of an overnight culture of Isolate 6 and incubated for 48 h at 37 °C. After

completion of incubation, the tubes were carefully decanted and washed with Phosphate Buffer Saline (PBS) (pH 7.0), dried, and stained with 0.1% crystal violet. Excess stain was removed by washing the tubes with distilled water. The formation of biofilm was confirmed by the presence of attachment (visible film) on the wall and bottom of the tube [20].

Growth Kinetics of the Isolated Strain:

Growth Kinetics of the Isolated Strains was Studied in Planktonic and Biofilm Formation

Planktonic Growth Kinetics: 50 µl of Isolate 6 grown overnight was inoculated into 5 ml of LB broth and the initial OD was taken at 630 nm. The culture was incubated in an orbital shaking incubator at 37 °C with 180 rpm. The OD was measured at intervals of 30 min. The time *vs.* absorbance was plotted to determine the planktonic growth kinetics of the isolated strains.

Kinetics of Biofilm Formation: Overnight broth culture of the Isolate 6 was inoculated into 4 sets of 96 well microtitre plates taking 100 µl each of 1:100 dilution and the absorbance was read using ELISA reader at 570 nm at two-day intervals following similar staining procedures which were used for screening. The OD values were plotted against time intervals to determine biofilm formation kinetics of the isolated strains.

Screening of Anti-Microbial Activity of QIE: Anti-microbial activity of the extracts was determined by following the agar well diffusion method of Okeke *et al.* [21]. In this method, pure clinical isolates of Streptococcus and reference organisms procured from Institute of Microbial Technology, Chandigarh, India namely: *Candida albicans* MTCC 183, *Streptococcus mutans* MTCC 497, *Lactobacillus acidophilus* MTCC 10307 and *Staphylococcus aureus* MTCC 1144 were first subcultured on Nutrient broth at 37 °C for 24 h. *C. albicans* MTCC 183 was cultured in Sabouraud Dextrose Agarat 37 °C under aerobic condition for 24-48 h whereas all bacterial strains were grown and maintained on nutrient agar slants at 4 °C.

The anti-microbial activity of QIE was qualitatively and quantitatively assessed by two different assay methods: 1) zone of growth inhibition (ZOI) by the cup-plate method, and 2) the determination of minimal inhibitory concentrations (MIC) [22] using the suitable growth media for each microbial strain.

Agar Diffusion Method: The cup-plate method was used to evaluate the anti-microbial activity of QIE in various solvents based on its ability to inhibit growth of the microorganism under study [23]. 100 µl of 24 h old inoculums of test organisms at 105 CFU/ml were swabbed on Brain Heart Infusion agar. The media was poured into sterile Petri dishes. Wells of 8 mm diameter were punched into the agar medium and filled with 45 µl of the respective QIE. The plates were incubated at 37 °C for 24 h. 0.2% Chlorhexidine served as positive controls. The zone of inhibition was measured in millimeters. The experiments were performed in triplicates and the mean values of the diameter of inhibition zones with ± standard deviation were calculated.

Determination of MIC and MBC: The MIC of the most effective extracts was determined by using the two-fold serial microdilution method at a final concentration ranging from 10 mg/ml to 0.02 mg/ml [24]. The extracts were added to sterile Mueller Hinton Broth before bacterial suspensions with final inoculums concentration of 10^5 CFU/ml. Each extract was assayed in triplicate. The extracts in broth were used as negative control and the bacterial suspensions were used as positive control. The turbidity of the wells in the microtiter plate was interpreted as visible growth of the micro-organisms.

The MIC values were taken as the lowest concentration of the extracts which showed no turbidity after 24 h of inoculation at 37 °C. The minimum bactericidal concentration (MBC) was determined by the subculture of the well showing no apparent growth in a sterile agar plate. The least concentration showing no visible growth on agar subculture was taken as MBC value.

Inhibition of Biofilm in the Presence of QIE: Shell Assay: The formation of biofilm on shells was studied qualitatively since shells are made of calcium carbonate and can mimic tooth surface. Ridged and smooth shells were sterilized using 2% glutaraldehyde and washed twice with sterile distilled water before use. Isolate [6] grown in BHI broth was inoculated along with QIE. After 6 days

of incubation, the shells were stained with 1% crystal violet for 10 min to allow visualization of biofilm on the tooth surface. Negative control was set up in a similar manner by substituting QIE with PBS [25].

Microtiter Plate Assay: The microtiter plate assay was performed in 96-well polystyrene microtiter plates to quantitatively determine the biofilm formation [26]. BHI broth containing 1% D-glucose was prepared and sterilized by autoclaving. The BHI was inoculated with Isolate 6 and incubated overnight at 37 °C. 33 µL of the broth was transferred aseptically to a 96-well microtiter plate (Tarson, India) followed by the addition of QIE. The plate was then incubated overnight at 37 °C. The wells were washed with 0.85% NaCl 2-3 times and stained with 1% Crystal Violet solution to observe adherence. The optical density was measured at 550 nm using ELISA plate reader. Those microtiter wells containing only sterile LB broth were considered to be the negative controls, while the inoculated wells were positive controls. To determine the inhibition activity, the percentage reduction was calculated using the formula:

$$\text{Reduction percent (RP)} = [(C - B)] - (T - B)] / (C - B) \times 100$$

Where, C = Mean of OD of positive control wells, B = Mean of OD of negative control wells, T = Mean of test wells.

RESULTS AND DISCUSSION:

Preliminary Evaluation of the Clinical Isolate:

Colony Characteristics of the Clinical Isolate: [8] isolates differing in their colony morphologies were isolated from the dental caries samples of three individuals. The characteristics are summarized in **Table 1**. Gram-positive organisms accounted for ~75% of the dental isolates. These were largely found to be streptococci, consistent with literature on biofilm initiation [27, 28]. Early streptococci colonizers are known to adhere to proline-rich proteins and proline-rich glycoproteins found in the pellicle of the plaque [29, 30] where they influence subsequent stages of biofilm formation [31].

TABLE 1: COLONY CHARACTERISTICS OF MICROORGANISMS ISOLATED FROM DENTAL CARIES

Colony character	Colony 1	Colony 2	Colony 3	Colony 4	Colony 5	Colony 6	Colony 7	Colony 8
Size	Medium	Small	Small	Pinpoint	Pinpoint	Pinpoint	Small	Medium
Shape	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular
Colour	White	Cream	White	Cream	White	White	White	White
Consistency	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous
Opacity	Opaque	Translucent	Translucent	Translucent	Translucent	Translucent	Opaque	Opaque
Surface	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
Margin	Entire	Entire	Entire	Entire	Entire	Entire	Entire	Entire
Elevation	Low convex	Low convex	Low convex	Low convex	Low convex	Flat	Low convex	Low convex
Gram nature	Gram-positive cocci in chains	Budding yeast	Gram-negative coccobacilli	Gram-positive cocci in chains	Gram-positive bacilli	Gram-positive cocci in chains	Gram-positive cocci in chains	Gram-positive cocci in chains
Hemolysis pattern of Blood agar	β-haemolytic	γ-haemolytic	β-haemolytic	γ-haemolytic	α-haemolytic	α-haemolytic	γ-haemolytic	β-haemolytic

Biochemical Identification of Clinical Isolates: A systematic process for the identification of bacteria includes assay techniques for fermentation abilities, activities of specific enzymes, and certain biochemical reactions. Qualitative observations made were based on these tests and are as shown in **Table 2**. Isolate no 6 identified as *S. mutans* was used for studies pertaining to biofilm formation.

Primary Screening for Biofilm Formation: Biofilm formation acts as a defensive method during various stress conditions and is able to resist shear forces produced by the action of saliva. Member's strains of the biofilm can also withstand pH changes, nutrient deprivation, oxygen radicals and antibiotics much better than planktonic organisms. Its formation is a highly specific process that is governed by receptor interactions [32] between streptococci and salivary mucin, highly glycosylated proline-rich glycoproteins, α – amylase, and proline-rich peptides [33].

These interactions may be responsible for the formation of biofilm on the shell surface. Co-

adhesion with organisms already attached to the substratum could also be a possibility for those strains that are unable to initiate the biofilm de novo. Out of the eight isolates obtained from the dental samples, only Isolate 6was found to form a biofilm on the inner walls of the test tube when stained with crystal violet **Fig. 1**.

TABLE 2: IDENTIFICATION TESTS FOR THE CLINICAL ISOLATE

Isolate no.	Biochemical tests				Sugar fermentation test			Enzymes		
	Indole test	MR test	VP test	Citrate utilization test	Glucose	Lactose	Sucrose	Amylase	Catalase	Oxidase
1	+	-	+	+	+	+	+	-	-	-
3	+	-	-	-	-	-	-	-	-	-
4	+	-	+	+	+	+	+	-	-	-
5	+	+	-	-	+	+	+	+	-	-
6	+	-	+	+	+	+	+	-	-	-
7	+	-	+	+	+	+	+	-	-	-
8	+	-	+	+	+	+	+	-	-	-

FIG. 1: TUBE ASSAY FOR DETECTION OF BIOFILM FORMATION IN ISOLATE 6

Growth Kinetics:

Planktonic Growth: Isolate 6 in planktonic form showed the following pattern of growth **Fig. 2**.

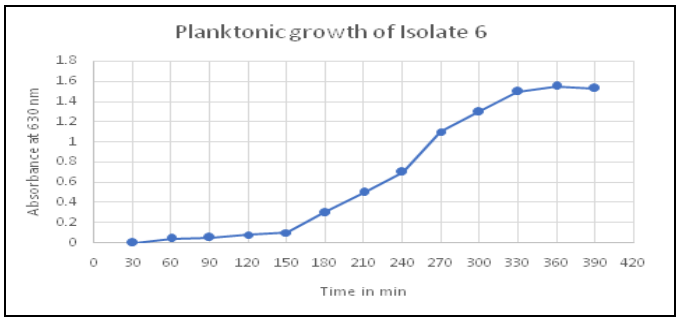

FIG. 2: PLANKTONIC GROWTH CURVE OF ISOLATE 6

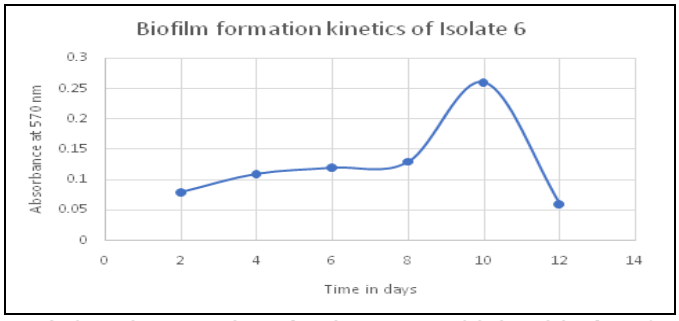

FIG. 3: BIOFILM FORMATION KINETICS OF ISOLATE 6

Kinetics of Biofilm Formation: Biofilm formation by Isolate 6 showed a sharp decline after 10 days due to disintegration **Fig. 3**.

Anti-microbial Activity of QIE on Oral Pathogenic Strains:

Screening of Anti-microbial Activity: Most tradition healers use plant material extracted primarily in water as a solvent. Plant extracts dissolved in methanol is reported to demonstrate greater antibacterial activity compared to aqueous extracts [34]. Nonetheless, acetone is also a useful extractant as it dissolves a host of bioactive compounds with both hydrophilic and hydrophobic components as well as low toxicity to test organisms [35].

Table 3 represents anti-bacterial and anti-candidal activities of extracts with respect to each of the test organisms at concentrations of 200 mg/ml. The anti-bacterial activity was found in all the extracts with methanolic extracts exhibiting maximum zone of inhibition as follows: *S. aureus* (24 mm) and *L. acidophilus* (24 mm) followed by *S. mutans* (22 mm). Ethanolic QIE also demonstrated significant activity. Thus, alcohol is the best solvent followed by acetone compared to the other solvents used in this study. QIE contains broad-spectrum antibacterial compounds making it a potential source of antimicrobial substance. Inhibitory activities of all extracts did not show significant difference towards each microbial species tested.

This can be attributed to the presence of hydrophilic anti-microbial substances which are

extracted out by polar solvents. *C. albicans* were least inhibited by the extract prepared in all solvents while all extracts appeared to react most sensitively against *S. aureus*.

TABLE 3: ANTI-MICROBIAL ACTIVITY OF EXTRACTS OF QIE PREPARED IN DIFFERENT SOLVENTS.

Solvent used for extraction	Zone of Inhibition (mm)			
	S. mutans	***S. aureus***	***L. acidophilus***	***C. albicans***
Water	19	20	18	13
Methanol	22	24	24	18
Ethanol	21	22	22	17
Ethyl acetate	19	17	12	11
Acetone	19	22	20	16
Hexane	16	12	14	11

*After incubation of MH agar plates, inhibition zone of microbial growth around the well filled with each extract was measured (in mm). The tests were performed in triplicate, and the means of the values are shown in Table 3.

Determination of MIC and MBC: The MIC values of methanolic QIE against the oral microorganisms were as observed in **Table 4**. The MIC values against each bacterial species were found in the range of 0.16 to 0.31 mg/ml. The MIC values correlated to the zone of inhibition test result. *S. aureus* appeared to be the most susceptible bacteria with the lowest MIC value. *S. mutans* and *L. acidophilus* displayed same MIC values.

TABLE 4: THE MIC VALUES OF METHANOLIC QIEAGAINST ORAL BACTERIA

Concentration (mg/ml)	Microorganism				Control	
	S. mutans	***S. aureus***	***L. acidophilus***	***C. albicans***	**Positive**	**Negative**
5.00	-	-	-	-	+	-
2.50	-	-	-	-	+	-
1.25	-	-	-	-	+	-
0.63	-	-	-	-	+	-
0.31	-	-	-	+	+	-
0.16	+	-	+	+	+	-
0.08	+	+	+	+	+	-
0.04	+	+	+	+	+	-
0.02	+	+	+	+	+	-
0.01	+	+	+	+	+	-

TABLE 5: THE MBC VALUES OF METHANOLIC METHANOLIC QIEAGAINST ORAL BACTERIA

Strain	Extract concentration (mg/ml)					
	5.00	2.50	1.25	0.63	0.31	0.16
S. mutans	-	-	-	-	ND	ND
S. aureus	-	-	-	-	-	-
L. acidophilus	-	-	-	+	ND	ND
C. albicans	-	-	-	-	+	ND

Inhibition of Streptococcal Biofilm:

Inhibition of Biofilm on Seashell: Isolate 6exhibited mild uniform darkening on the smooth shell surface and random darker spots on the ridged shell after 6 days of incubation. With the addition of crystal violet, the negative control exhibited a high purple intensity while those treated with QIE appeared mildly purple **Fig. 4**. This is suggestive of the fact that these extracts have the capability of inhibiting the growth of the Streptococcal biofilm.

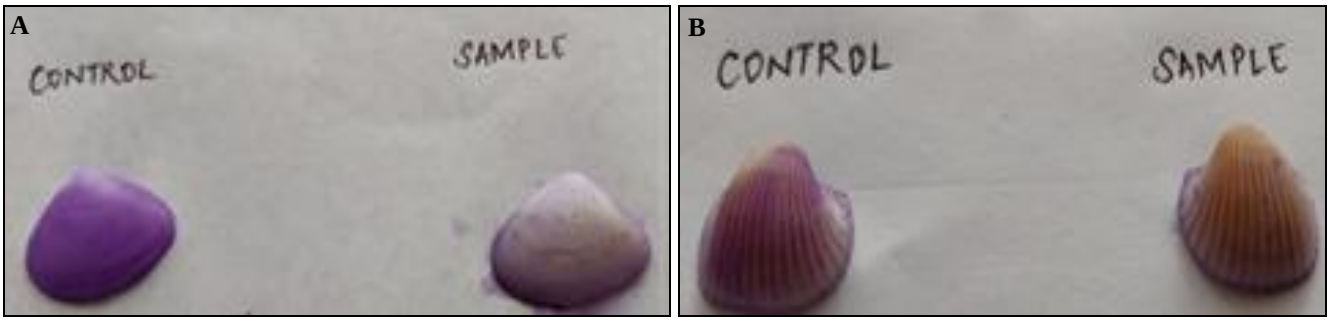

FIG. 4: INHIBITION OF STREPTOCOCCAL BIOFILM BY METHANOLIC QIE ON SMOOTH SHELLS (A) AND RIDGED SHELLS (B)

Inhibition of Biofilm in Microtiter Plates: The reduction percentage of methanolic QIE on Streptococcal biofilm was determined by plotting %RP against concentration QIE in µg/ml **Fig. 5**. The percentage of reduction was found to increase with increasing concentration of QIE extract.

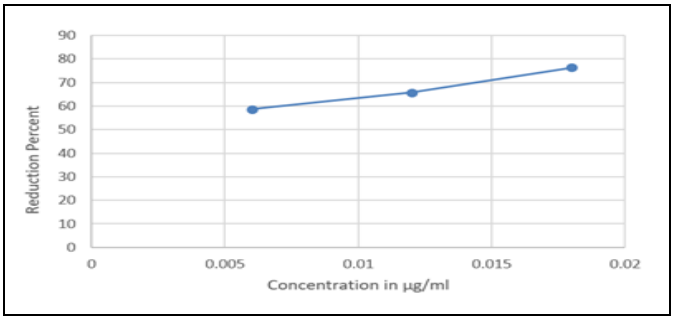

FIG. 5: INHIBITION OF BIOFILM FORMATION BY METHANOLIC QIE ONSTREPTOCOCCAL BIOFILM AS EXPRESSED IN REDUCTION PERCENTAGE

CONCLUSION: This study provides an insight into the usage of *Q. infecoriagall* in traditional treatment of oral disease associated with a bacterial infection. Out of the organisms isolated and characterized by dental caries, *S. mutans* was further analyzed for its ability to produce a biofilm in the presence of sucrose. *Q. infecoriagall* extract in methanol was found to possess potent bioactive that was responsible for inhibiting the growth of commonly seen oral microorganisms and even fared slightly better than chlorhexidine.

Therefore, it can be developed as a commercial product for dental applications. However, further investigations are needed to study the effect of QIE on oral microflora as a mixed culture exhibiting concerted behavioral strategies.

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CONFLICTS OF INTEREST: The authors declared no competing interests.

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