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Research Article

IN VITRO STUDY: ANTIVIRULENCE EFFECT OF *CINNAMOMUM VERUM* AGAINST *STREPTOCOCCUS MUTANS* - MAIN CAUSATIVE AGENT OF DENTAL CARIES

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ABSTRACT. *Streptococcus mutans (S. mutans*) predominantly resides in biofilms that develop on teeth, commonly referred to as dental plaque. The objective of the study is to explore the antimicrobial and antibiofilm efficacy of the *Cinnamomum verum (C. verum)* plant extract against an isolated strain of *S. mutans.* We assessed the antimicrobial and antibiofilm effectiveness of a natural plant extract derived from *C. verum* against *S. mutans* using a variety of in vitro tests, including the crystal violet biofilm inhibition assay and growth curve analyses, and then quantified extracellular polymeric substances (EPS) using ethanol extract of *C. verum*. At a concentration of 5 mg/mL, the ethanol extract obtained from *C. verum* showed inhibition of *S. mutans*. In the biofilm assay, statistical analysis indicated a 65.25% inhibition of biofilm formation at a subinhibitory dose of 1.25 mg/mL, without impacting the growth of *S. mutans*, and also at 1.25 mg/mL *C. verum* inhibited EPS production of *S. mutans* to 41.55 %. In summary, our investigations revealed that *C. verum* exhibits both antimicrobial and antibiofilm activities against *S. mutans*. The present study found that the ethanol extract derived from *C. verum* has antibacterial properties, and *in vitro* studies show a noteworthy decrease in biofilm formation. *C. verum* could serve as a potent remedy for diverse infections linked to dental cavities, highlighting its potential as a safe and effective alternative treatment option.

Keywords: *Antibiotic resistance, biofilm, Cinnamomum verum, inhibitor, Streptococcus mutans, virulence factors.*

INTRODUCTION

Streptococcus mutans (*S. mutans*) is recognized as the primary causative agent responsible for the development of dental caries, also known as tooth decay or cavities [1]. *S. mutans* primarily resides in dental plaque or biofilms that form on tooth surfaces [2]. These bacteria have the ability to produce both soluble and insoluble glucans from dietary sucrose through the action of glucosyltransferase enzymes (Gtfs). This activity facilitates extracellular aggregation, leading to the creation of a durable biofilm on the tooth surface [3, 4]. The rise and spread of antimicrobial resistance (AMR) among clinical pathogens complicate the treatment and control of infectious diseases [5]. *S. mutans*

exhibit resistance to numerous antibiotics, presenting a notable threat to human health. Antibiotic-resistant streptococci have been associated with various mechanisms, including efflux pumps and alterations in the antimicrobial target [6].

The presence of numerous virulence factors in *S. mutans* designates it as a significant pathogen linked to the initiation of dental caries [7]. The activity of *S. mutans* virulence factors, and the development of biofilms in the oral cavity can be influenced by both environmental conditions and genetic factors [8]. A significant virulence factor in biofilm formation, contributing to the onset of dental caries, is *S. mutans* ability to generate organic acids through various carbohydrate metabolism activities (acidogenicity) and thrive in low pH environments. In addition to that, the ecological environment termed as a biofilm (plaque), which plays a significant role in the pathogenicity of *S. mutans*, is formed by the self-produced extracellular polymeric substances (EPS) matrix comprising proteins, polysaccharides, and nucleic acids [9, 10]. The competence-stimulating peptide (CSP), a quorum-sensing (QS) signaling molecule, initiates a series of events influencing gene expression and diverse physiological responses, resulting in various phenotypic outcomes [11].

Several studies have provided valuable insights into how to reduce or eliminate *S. mutans* cariogenic (cavity-causing) potential by limiting its ability to tolerate various environmental stresses. These strategies include weakening its resistance to oxidative and acidic conditions, changing how it uses carbohydrates, and disrupting its ability to maintain proper metal ion levels within cells [12]. Natural products have emerged as a valuable source of new pharmacological compounds, particularly in the context of AMR. Plant bioactive compounds, can inhibit disease-related gene expression by interfering with virulence factors involved in QS [13].

Cinnamomum verum (C. verum) is a medicinal plant belonging to the family *Lauraceae* and is commonly known as true cinnamon or Ceylon cinnamon [14]. *C. verum*, or true cinnamon, is significant for its culinary uses, potential health benefits including anti-inflammatory and antioxidant properties, traditional medicinal applications, aromatic qualities for aromatherapy, historical role in food preservation, cultural and ritual importance, and its economic value as a high-quality spice [15]. These compounds may act individually or synergistically to inhibit the growth and survival of pathogenic bacteria [15]. The primary goal of this study is to investigate the effect of *C. verum* against *S. mutans* isolate. To the best of our knowledge, *C. verum* anti-QS properties have not been thoroughly studied in relation to tooth pathogens like *S. mutans*.

MATERIALS AND METHODS

In this study, an in vitro experiment was conducted to evaluate the effects of a compound derived from *C. verum*, which was sourced from a native botanical garden in Chennai, Tamil Nadu, India. The authenticity of the plant was verified by a qualified botanist. After extraction from the plant, the leaves were cleaned with water and naturally dried for approximately ten days before being used in the treatment against *S. mutans*.

Solvent extraction

Twenty grams of *C. verum* leaves powder were combined with 100mL of ethanol and distributed evenly across two maceration containers for a period of 48 h, with occasionally shaking using a shaker. After the extraction phase, the resulting suspension

was filtered using No. 1 filter paper (Whatman, Maidstone, England), which was layered over the funnel housing the filter paper with a white muslin cloth. The methanol solvent was then evaporated from the filtrate using a hot water bath maintained precisely at 50°C. The dehydrated filtrate was measured, and the dried substance was weighed before being kept for later use at 4°C.

Identification of chemical composition by GC-MS (Gas Chromatography-Mass Spectrometry)

The GC-MS analysis procedure followed to the method previously described by Sankar Ganesh et al. [16]. Ethanol extract of *C. verum* analysis was performed using Gas Chromatography (GC) and GC-MS (Gas Chromatography-Mass Spectrometry). A Shimadzu GCMSQP-2010 plus detector and an SGE BPX5 fused silica capillary column (30.0 m X 0.25 mm i.d., 0.25 mm film thickness) was used. The oven's temperature program began at 40°C and was held for 1 minute before gradually increasing to 290°C at a rate of 3°C per minute and being held for 10 minutes. Nitrogen was used as the carrier gas at a constant flow rate of 2.5 mL/min. The detector and injector temperatures were set at 290°C.

GC-MS analysis employed an AOC5000 auto injector, maintaining the same capillary column and carrier gas conditions as in GC analysis. The injector and oven temperature profiles mirrored those of GC analysis. Identification of ethanol extract of *C. verum* compounds was achieved by comparing their retention indices (RI), electron impact-mass spectra (EI-MS), and referencing the National Institute of Standards and Technology (NIST) database.

Bacterial strain and growth condition

The clinical samples of *S. mutans* utilized in this study were sourced from Saveetha Dental College and Hospital in Tamil Nadu, India. The bacteria were routinely cultivated aerobically in Luria Bertani (LB) broth culture, provided by HiMedia, India, and maintained at 37°C in a shaking incubator (100 rpm) for 1 day. Previous studies have outlined standard microbiological procedures for identifying genera and species [17]. Distinctive growth patterns were observed on Brain Heart Infusion Agar (BHI agar), Mitis Salivarius-bacitracin Agar (MSB Agar), and several phenotypic tests were carried out, including Gram staining, catalase, oxidase, motility, Hugh-Leifson's oxidative-fermentative (OF), and citrate, and the results were documented. The bacterial cultures were regularly sub-cultured for experimental purposes, and biochemical confirmation was consistently performed on each occasion.

Antimicrobial activity of C. verum.

The antibacterial activity of *C. verum* was determined using the agar well-diffusion method [18]. The bacterial cultures containing *S. mutans* were spreaded onto Mueller Hinton agar (MHA) from HiMedia, Mumbai, India, utilizing a sterilized swab moistened with the bacterial suspension. A sterile cork borer was employed to create a well with an 8mm diameter in the MHA medium, and each well was loaded with 40µL, 30µL, and 20µL of *C. verum*, respectively. Subsequently, the plates were incubated for 24 h at 37°C. Following incubation, the diameter of the inhibition zones was measured using a Vernier calliper with a millimetre scale to assess antibacterial activity.

Antibiotic susceptibility testing

Antimicrobial susceptibility testing (AST) was conducted following the guidelines outlined in the Clinical and Laboratory Standards Institute's (CLSI) 2022 recommendations [19]. The Kirby-Bauer disk diffusion method was employed to assess the susceptibility of *S. mutans* to antimicrobial agents. Briefly, inoculum, consisting of a bacterial culture of *S. mutans*, was evenly distributed on an MHA plate using a sterile swab moistened with the bacterial suspension for testing against metronidazole, ampicillin, rifampicin, cefotaxime, and ciprofloxacin (Hi Media).

Evaluation of minimum inhibitory concentration

The broth microdilution method was employed to ascertain the minimum inhibitory concentration (MIC) of *C. verum* against *S. mutans*. The evaluation encompassed various concentrations ranging from 10 mg/mL to 0.019 mg/mL. MIC for the ethanol extract was determined following established protocols [20]. In brief, microcentrifuge tubes were filled with LB broth, and 10µL of broth culture was added to compare turbidity with 0.5 McFarland units (equivalent to 1.5×10^8 CFU/mL). Following successive serial dilution with *C. verum* extract, all the tubes were incubated at 37°C for a day. After 24 h, 40µL of 2,3,5-triphenyl tetrazolium chloride (TTC) was introduced to the tubes, and the confirmation of results was based on observing any change in colour. The absence of growth (no colour) at the lowest concentration is documented as the MIC. Subsequently, based on the findings, antibiofilm studies at sub-MIC levels were conducted.

Crystal violet biofilm inhibition assay

The effect of the *C. verum* extract on the biofilm formation of *S. mutans* was evaluated using the crystal violet staining assay, as described by Venkatramanan et al. [21]. A microtiter plate was filled with 20µL of an overnight culture of *S. mutans* and 180µL of fresh BHI medium. The extract was then added in a dose-dependent manner (ranging from 2.5mg/mL to 0.004mg/mL), and the plate was incubated for 48 h at 37°C. Subsequently, the biofilm adhering to the surface was dyed using a 0.1% solution of crystal violet (CV), with the removal of planktonic cells achieved through rinsing with sterile distilled water. After a duration of ten minutes, the crystal violet-bound adherent biofilm was washed with 200µL of 70% ethanol, and its concentration was assessed using a UV-Vis spectrophotometer (Biobase BK-D 590 Double beam scanning UV/Vis China) by measuring the intensity of crystal violet at 520 nm, and the treated strains percentage of growth was compared to the untreated control using OD at OD600 nm.

The following equation was employed to calculate the percentage of inhibition:

$$Control\ OD\ 520nm - Treated\ OD\ 520nm / Control\ OD\ 520nm \times 100$$

Bacterial growth curve

The growth of *S. mutans* bacteria was evaluated in the presence and absence of *C. verum* at a concentration of 1.25 mg/mL. The bacterial culture was incubated at 37°C for up to 24 h, with the assessment of cell density conducted at OD 600nm every hour.

Estimation of exopolysaccharide (EPS) in S. mutans

The extraction of EPS followed a previously outlined method [21]. Briefly, the *S. mutans* test culture was cultivated aerobically in BHI broth enriched with 1% sucrose. Ethanol extract of *C. verum* was then applied in a dose-dependent manner, ranging from 2.5mg/mL to 0.004mg/mL, alongside a control, and maintained at 37°C in a shaking incubator (100 rpm) for 24 h. After the incubation period, the cultures underwent centrifugation at 10,000 rpm for 15 minutes. Subsequently, the bacterial pellets were resuspended in 50 mL of high salt buffer, consisting of 10mM KPO4, 7.5mM NaCl, and 2.5mM MgSO4. The bacterial cells underwent separation by centrifugation at 10,000 rpm for 30 minutes. Ethanol was introduced to the supernatant, followed by another centrifugation at 10,000 rpm for 30 minutes. The extracted EPS was then suspended in an appropriate volume of Milli-Q water. To analyze the EPS, 1 mL of precipitated EPS was combined with 1 mL of cold 5% phenol and 5 mL of concentrated sulfuric acid, which produced a red color. The color intensity of both control and treated samples (EPS) was measured at OD490 nm with a UV-Vis spectrophotometer (Biobase BK-D 590 Double beam scanning UV/Vis China). The treated strains percentage of growth was compared to the untreated control using OD at 600 nm.

Statistical analysis

The experiment was repeated three times to ensure accuracy. Statistical significance was assessed for biofilm quantification (using the crystal violet assay), growth curve analysis, and EPS quantification. Microsoft Excel 2018, developed by Microsoft Corporation, was employed to perform statistical analysis for all experiments.

RESULTS AND DISCUSSION

GC-MS analysis

The analysis of the ethanol extract of *C. verum* revealed a total of 15 components, collectively constituting 100% of the extract composition. The primary constituents identified in the ethanol extract of *C. verum* were methoxycinnamaldehyde, terpeneol, and geranyl acetate. The most abundant peaks were observed at retention times of 4.55, 35.45, and 30.25 minutes in the chromatogram (Table 1).

Biochemical characterization and antimicrobial susceptibility

Morphological profiling and the observation of distinct morphotypes were carried out on a regular culture medium for the isolates. *S. mutans* exhibited characteristics of Gram-positive cocci in the Gram staining, along with being catalase-negative, non-motile, and oxidase-negative.

Antimicrobial susceptibility

The measured diameter of the zone of inhibition for the *C. verum* extract is illustrated in Table 2. Our results indicate that *C. verum* exhibits antimicrobial activity against *S. mutans* (Fig. 1).

Table 1. *Chemical composition of the ethanol extract of C. verum (GC- MS)*

Compounds	Retention time (Rt) min	Percentage (%)
Benzyl alcohol	18.23	2.98%
Methyl acetophenone	28.44	1.78%
Borneol	52.00	0.56%
Hydroxy-cineole	41.24	0.62%
Decanoic acid	5.55	4.36%
Cuminyl alcohol	33.54	5.25%
Methoxycinnemaldehyde	4.55	5.36%
Terpeneol	35.45	4.55%
Geranyl acetate	30.25	4.25%
Carveol	39.26	2.85%
Carvone	2.12	2.56%
Octanoic acid	4.26	3.00%
5-methylpropanol	15.23	3.14%
2-methyl propanol	11.24	2.96%
3-methyl propanol	5.26	2.22%

**Retention times (Rt) were measured in minutes (min) using GC-MS. Percentages (%) represent the relative abundance of each compound in the analysed sample.*

Table 2. *The diameter of the zone of inhibition for the ethanol extract of C. verum extract against S. mutans.*

Concentration of *C. verum* (µl)	Diameters of inhibitory zone(mm)
40	19 ± 1.2
30	17 ± 0.9
20	14 ± 0.5

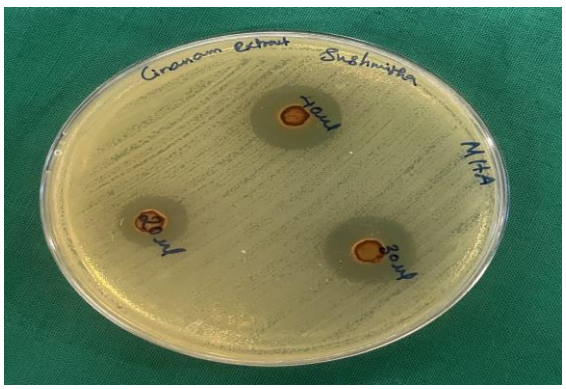

Fig 1. *Antimicrobial activity of the ethanol extract of C. verum extract at different concentration (40µL, 30µL, and 20µL) against S. mutans.*

Antibiotic sensitivity testing (AST)

Antimicrobial susceptibility testing (AST) was carried out in accordance with the Clinical and Laboratory Standards Institute's (CLSI) 2022 Guidelines [19]. In our finding Metronidazole, ciprofloxacin, and rifampicin showed no zone of inhibition, indicating that *S. mutans* are resistant to these drugs (Table 3).

Table 3. *Antibiogram of S. mutans against different antibiotics.*

Sl. no	Antibiotics	*S. mutans*
1	Metronidazole	R
2	Ampicillin	18.3± 1.6
3	Rifampicin	R
4	Cefotaxime	15±1.3
5	Ciprofloxacin	R

**R indicates resistance to the antibiotic. Values represent the mean inhibition zone diameter (mm) ± standard deviation for S. mutans isolates tested.*

At the lowest concentration C. verum inhibited S. mutans

Numerous research teams are presently exploring diverse approaches to formulate novel drugs possessing antimicrobial and anti-biofilm properties essential for addressing dental infections. Effectively treating infections caused by biofilm-forming *S. mutans* poses a considerable challenge, primarily due to bacterial resistance to various drugs, including antibiotics [6]. In this study the antibacterial activity of *C. verum* was assessed using a two-fold serial dilution method, ranging from 10 mg/ml to 0.019 mg/ml. At the concentration of 5mg/ml, we observed inhibition of *S. mutans* growth (Table 4). Our findings indicate that the treatment of *S. mutans* with *C. verum* leads to the inhibition of the bacterial cell wall. In a recent investigation conducted by Kamarehei et al. [22], it was elucidated that the natural compound curcumin (CUR) possesses antibacterial and antibiofilm properties against *S. mutans.* This is achieved through the downregulation of glucosyltransferase enzymes and QS genes. Cui et al. [23] conducted various studies indicating that marine natural compounds, particularly oxazole derivatives and 2-Aminoimidazole (2-AI) derivatives, demonstrate antibacterial effects against *S. mutans*. Hence, our research aims to illustrate sub-MIC of *C. verum* to evaluate its effectiveness against biofilm formation and virulence.

Table 4. *Minimum inhibitory concentration of ethanol extract of C. verum against S. Mutans*

S.no	Two-fold dilution concentration (mg/mL)	Growth [a]
1	10	-
2	5	-
3	2.5	+
4	1.25	+
5	0.625	+
6	0.312	+
7	0.156	+
8	0.078	+
9	0.039	+
10	0.019	+

* *a: The growth measured refers to the presence (+) or absence (-) of visible growth in the microbial culture after exposure to the respective two-fold dilution concentrations (mg/mL) of* C. *Verum*

C. verum inhibited the biofilm of S. mutans

The impact of *C. verum* on the ability of *S. mutans* to form biofilms was examined by employing 0.1% crystal violet dye in a stationary microtiter plate. Spectrophotometric analysis revealed a maximum inhibition of 65.25%, 41.44%, and 24.77% when *S. mutans* was exposed to concentrations of 1.25 mg/mL, 0.625 mg/mL, and 0.312 mg/mL of *C. verum* (Fig. 2A, 2B). Our findings revealed that *C. verum*, at sub-MIC levels, decreased *S. mutans'* QS-dependent biofilm formation in a dose-dependent manner. According to the crystal violet assay, a substantial dose of *C. verum* extract significantly diminished biofilm formation without affecting the proliferation of planktonic cells. The collected data aligns with findings from previous studies [22, 24]. Earlier research demonstrated that *Withania somnifera*, commonly known as Ashwagandha and belonging to the Solanaceae family, reduces biofilm formation at higher concentrations [25]. Propolis essential oil (PEO), a key constituent of propolis, is instrumental in diminishing the overall biomass of biofilms and disrupting the biofilm structure of *S. mutans* [26]. In a recent study Rudin et al. [27] found that the bioactive compound effectively inhibits biofilm formation at a sub- MIC level of 200 µg/ml.

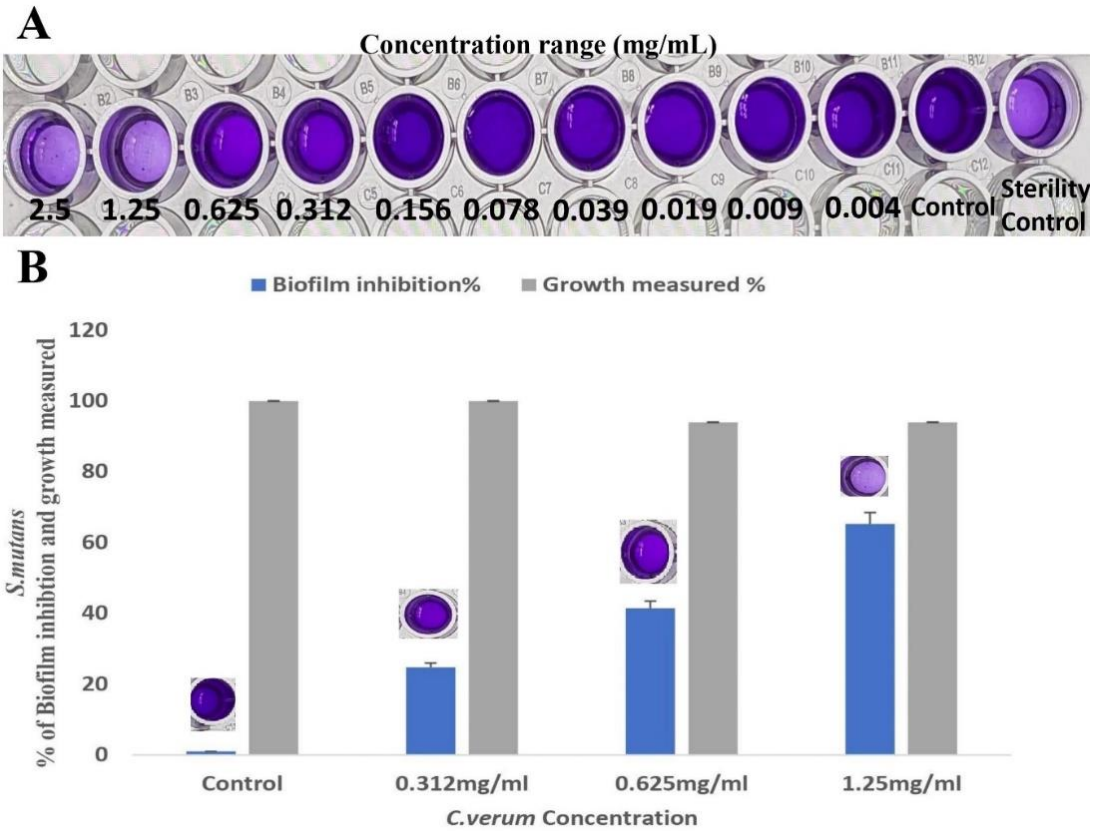

Fig 2. *A: Crystal violet biofilm inhibition assay. Effect of C. verum at sub-inhibitory concentration of 1.25 mg/mL, 0.625 mg/mL, and 0.312mg/mL.;*
B: 65.25%, 41.44% and 24.77% of biofilm inhibition in S. mutans at sub-inhibitory concentration of 1.25 mg/mL, 0.625 mg/mL, and 0.312 mg/mL.

Bacterial growth curve analysis

The growth curve analysis was conducted both with and without the presence of *C. verum*. The results showed that *C. verum* does not inhibit bacterial growth at a concentration of 1.25 mg/mL (Fig. 3). Spectrophotometric analysis demonstrated no discernible difference between the control and treated bacterial cells at 600nm.

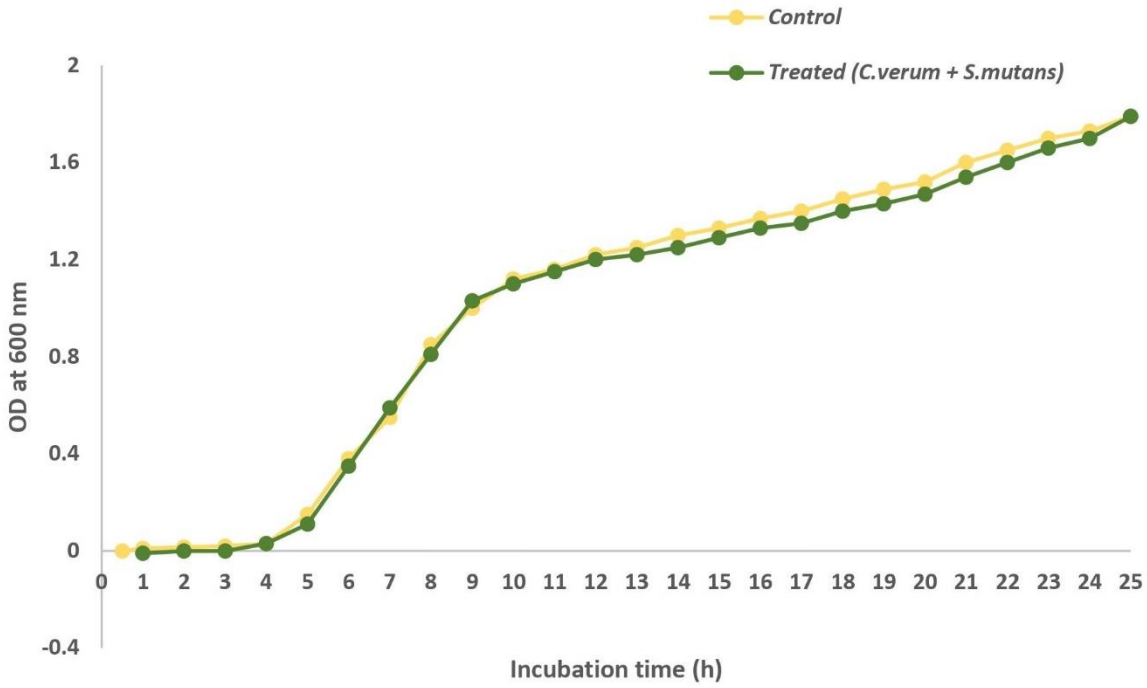

***Fig 3**. Growth curve analysis. S. mutans grown without (control) and in the presence of C. verum extract at the concentration of 1.25 mg/mL.*

Effects of C. verum on EPS of S. mutans

EPS (extracellular polymeric substance) plays a crucial role in both establishing and sustaining the structural integrity of biofilms. The research findings indicated that *C. verum*, at concentrations of 1.25 mg/mL, 0.625 mg/mL, and 0.312 mg/mL, inhibited the production of EPS by *S. mutans* to the extent of 41.55%, 27.77%, and 10.88%, respectively (Fig. 4). This clearly shows that the ethanol extract of *C. verum* reduced EPS production in *S. mutans* and altered the biofilm architecture. Collectively, *C. verum* plays a crucial role in suppressing the QS system in *S. mutans*. In light of these findings, *C. verum* may act individually or synergistically to inhibit the growth and survival of pathogenic bacteria. As a practical recommendation for daily consumption, incorporating *C. verum* into a 200 mL glass of drinking water could potentially achieve the suggested minimum inhibitory concentration (MIC) value of 5 mg/mL as evidenced in the study. However, further research is necessary to identify specific active components that likely possess anti-QS and anti-biofilm-producing properties.

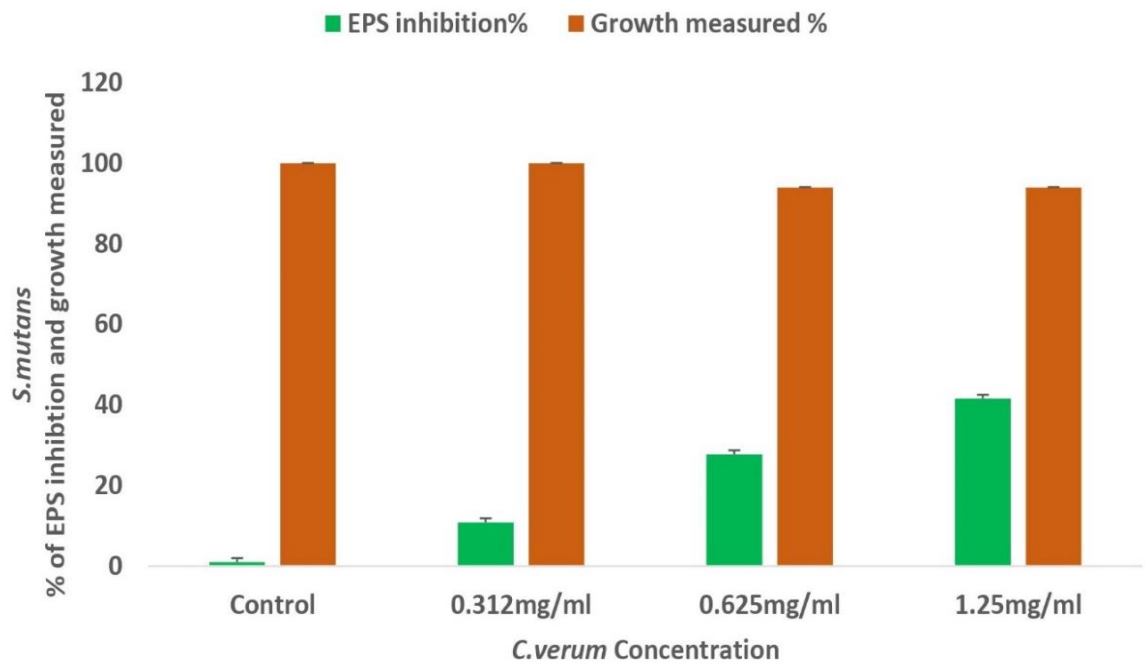

Fig 4. *Graphical representation of EPS inhibition and growth. C. verum inhibited EPS pigment of S. mutans to a level of 41.55%, 27.77%, and 10.88% at sub-inhibitory concentration of 1.25 mg/mL, 0.625 mg/mL, and 0.312 mg/mL.*

CONCLUSION

Our study results suggest that the antibacterial properties and substantial reduction in biofilm formation of the ethanol extract from *C. verum* were evidenced through comprehensive *in vitro* investigations. The results presented not only contribute to our comprehension of *S. mutans* behaviour but also provide opportunities for specific interventions in addressing oral infections. This study adds to the wider domain of AMR and oral health, underscoring the necessity for additional exploration to formulate effective strategies for controlling microbial populations within the oral environment.

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Conflict of Interest. The authors declared that there is no conflict of interest.

Authorship Contributions. Concept: P.S.G., Design: P.S.G., N.N.P., Data Collection or Processing: N.N.P., S.V., Analysis or Interpretation: P.S.G., N.N.P, A.V., Literature Search: P.S.G., N.N.P, S.V., Writing: S.V., NNP, A.V.

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