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Sweet Defense: Assessing the Biofilm-Inhibitory Properties of Regional Honey Varieties against Dental Pathogens

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Abstract: Dental caries is a biofilm-mediated disease and remains one of the most prevalent non-communicable oral health problems worldwide. *Streptococcus mutans* plays a key role in the initiation and progression of dental cavities through acid production, enamel demineralization, and biofilm formation. Growing concerns about AMR and the side effects of synthetic agents have raised interest in natural antimicrobial alternatives, such as honey.

- ***Methods:***

An 86 dental samples (42 plaque + 44 caries) were collected from patients in North Chennai, India. Using standard microbiological and biochemical analysis, the bacterial isolates were identified and confirmed to form biofilm. PCR was performed to detect virulence genes (comE and spaP). The antimicrobial activity of four honey varieties, Marthandam, Orange blossom, Sidr, and Acacia honey, was assessed using agar well diffusion, MIC, and MBC assays. GC–MS analysis was performed to identify bioactive compounds in Marthandam honey.

- ***Results:***

Among 86 samples analyzed, 59 isolates (68.60%) were identified as Streptococcus mutans. PCR confirmed the presence of virulence genes comE (~384 bp) and spaP (~670 bp). All honey samples showed concentration-dependent antibacterial activity. Marthandam honey demonstrated the strongest inhibition, followed by Orange blossom honey, while Sidr and Acacia honey exhibited moderate activity. GC–MS analysis identified bioactive compounds, including 5-hydroxymethylfurfural (5-HMF).

- ***Conclusion:***

Marthandam honey demonstrated significant antibiofilm activity against *S. mutans*, suggesting its potential as a natural antimicrobial agent for the prevention and management of dental cavity.

Keywords: *Dental Caries, Streptococcus Mutans, Marthandam Honey, 5-Hydroxymethylfurfural (5-HMF).*

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I. INTRODUCTION

Dental caries is a progressive and chronic, biofilm-mediated infection featured by destruction of dental enamel and composite material resulting from lactic acid produced during the microbial fermentation of dietary carbohydrates (1). Most predominant non-communicable dental diseases globally, affecting around 2.3 billion adults and 560 million children with primary tooth caries. Despite advancement in oral hygienic practices and other controlling measures like fluoride therapy, the global disease burden related to dental cavity continues to remain substantial. The pathogenesis of dental caries is substantially associated with dental plaque biofilm, within which *Streptococcus mutans* plays a pivotal role (2). The *S.mutans* possesses several virulence characteristics, including strong adherence to tooth surfaces, acidogenic and aciduric properties, and the ability to synthesize extracellular polysaccharides that facilitate biofilm formation. These characteristics enable *S. mutans* to persist within the oral environment and contribute significantly to enamel demineralization and caries progression (2).

Although in clinical practice, most dental practitioners recommend the use of antimicrobial mouth rinses and fluoride-based agents for caries management, concerns related to antimicrobial resistance, side effects, and the need for safer preventive strategies have shown interest in organic antimicrobial compounds. Honey has emerged as a promising natural agent due to its broad-spectrum antimicrobial

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properties, which are attributed to factors such as high osmotic pressure, low pH, and the presence of bioactive phytochemicals with antimicrobial activities (3).

However, the antimicrobial activity of honey varies considerably depending on its botanical and geographical origin, and comparative studies evaluating the inhibitory effects of different honey varieties against cariogenic bacteria remain limited (4). This study aimed to detect *Streptococcus mutans* from dental caries samples and evaluate the antimicrobial activity of selected honey varieties (Marthandam, Sidr, Orange blossom, and Acacia). Additionally, PCR-based detection of virulence genes (spaP and comE) and GC–MS analysis of Marthandam honey were performed to investigate the pathogenic potential of the isolates and identify bioactive compounds associated with antimicrobial activity (5).

II. MATERIALS AND METHODS

➢ *Sample Collection and Bacterial Isolation*

Overall, 86 dental specimen consisting of plaque (n = 42) and caries (n = 44) were collected from patients attending local dental clinics in North Chennai, India (Perambur, Vyas Nagar, M.K.B. Nagar, Kodungaiyur, Vyasarpadi, and Patel Road). Before sample collection, the oral cavity was rinsed with sterile water to remove food debris. With the support of dental practitioners Dental plaque can also be collected using a sterile small brush or swab, and the plaque was eluted by scrubbing it in the prepared BHI broth.

➢ *Isolation and Identification of Stretococcus mutans*

The collected clinical samples were inoculated into BHI broth medium and for incubation at 37 °C for 24 hrs under anaerobic conditions using a candle jar. An aliquot of the cultured broth was streaked onto Mitis Salivarius agar plates and anaerobic incubation at 37 °C for 48 hrs. Colonies showing typical morphological characteristics were selected and subcultured on blood agar plates for further identification. Following microbiological procedures, bacterial isolates were identified using colony morphology, staining methods, and biochemical features.

➢ *Biochemical Characterization of S. mutans*

- *Catalase Test*

Catalase activity was determined by adding 3% hydrogen peroxide to bacterial colonies placed on a clean glass slide. A negative catalase reaction is confirmed by no efferscence bubble formation, characteristic of *Streptococcus mutans*.

➢ *Detection of Biofilm Formation*

- *Congo Red Agar Method*

Biofilm formation were evaluated using the Congo Red Agar method. BHI agar broth of 2% sucrose-based and congo red dye of 0.8 g/L was used. The bacterial isolates streaking on pre-prepared plates, further inoculated at 37 °C for 24–48 hours. A dark black colonies confirming the biofilm-producing isolates, whereas red colonies indicated non-biofilm producers.

- *Susceptibility and Sensitivity Testing*

Using Kirby–Bauer disc diffusion method on Mueller–Hinton agar-based plates, susceptibility testing were performed. The bacterial dilution was adjusted to 0.5 McFarland standard prior to inoculation. On inoculation plates with antibiotic discs at 37 °C for 24 h incubation. The inhibition zone were measured and interpreted as sensitive, and resistant based on the standard guidelines.

- *Honey Samples*

Four different types of honey were used in the study: Marthandam multifloral honey, Sidr honey, Orange blossom honey, and Acacia honey, obtained from commercial sources in Chennai, India.

- *Agar Well Diffusion Assay*

Antimicrobial activity of honey samples against *S. mutans* were assessed using the agar well diffusion method. Bacterial suspension on Mueller–Hinton agar plates were inoculated. Wells were prepared using a sterile cork borer, and 30 microlitre of honey solutions (20%, 40%, 60%, and 80%) were added to each well. At 37 °C for 24 hrs incubation of plates, and the inhibition zones were measured in millimeters (mm).

- *Minimum Inhibitory Concentration (MIC)*

Honey samples were analyzed using the broth microdilution technique. Serial dilutions concentration of honey (32, 16, 8, 4, 2, and 1) were prepared in sterile broth. A 100 µL of bacterial suspension standardized to 0.5 McFarland into each well of 100 µL of honey sample and were incubated at 37 °C for 18–24 h.

- *Minimum Bactericidal Concentration (MBC)*

MBC was assessed through inoculating samples of MIC wells, at 37 °C for 24 h incubation and no visible growth onto agar plates. Upon minimal concentration showing no or absence of bacterial colony were measured as the MBC.

- *PCR: DNA Extraction*

Genomic extraction from the bacterial isolate were performed using the QIAamp DNA Blood Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. Bacterial cells were collected by centrifuge technique at 10,000 rpm for 10 minutes, followed by lysis using the provided lysis buffer. Further, released DNA was taken to spin-column purification system supplied with the kit and followed by 50 µL of elution buffer. Using NanoDrop spectrophotometer (Thermo Scientific, USA), the concentration and purity of the extracted DNA were evaluated. The purified DNA was then stored at −20 °C until further use for PCR amplification of target genes.

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- *PCR amplification of spaP AND comE Genes*

PCR amplification was performed to detect the presence of virulence-associated genes (comE and spaP) in Streptococcus mutans. The primers used for amplification were synthesized by Sahagene (Hyderabad, India). The primer sequences used were as follows:

Table 1 Primers Used for Screening spaP and comE Genes

Target Gene	Primers	References
spaP	Forward: 5'-AGCGGCGAGTAAGGTGA-3'	Patel et al., 2013
	Reverse: 5'-ACGGAAGGAGCTGAAGG-3'	
comE	Forward: 5'-AGTGGCTTCTGAGATTCAG-3'	Mohan et al., 2015
	Reverse: 5'-ATTTACAGCAGAGGAGAGG	

PCR amplification on GoTaq® Green PCR Master Mix (Promega, USA) in a thermal cycler. The PCR reaction conditions included an denaturation at 94 °C for 5 min, followed by 29 cycles of denaturation at 94 °C for 30 seconds, annealing at 54 °C for comE and 53 °C for spaP for 20 seconds, and extension at 72 °C for 30 seconds. A final extension step was performed at 72 °C for 8 minutes. The amplified PCR products were analyzed by 1.5% agarose gel electrophoresis and visualized under UV illumination using a gel documentation system. The sizes of the amplified fragments were determined by comparison with a 100 bp DNA ladder used as a molecular size marker.

➢ *GC–MS Analysis*

Among the four honey samples tested, Marthandam honey, which showed the lowest MIC value, was selected for Gas Chromatography–Mass Spectrometry (GC–MS) analysis to identify bioactive volatile compounds responsible for antimicrobial activity.

➢ *Quality Controls*

Streptococcus mutans genomic DNA, like *spa*P and *com*E genes, was used as a positive control. Similarly, nuclease-free water was used as a negative control, instead of template DNA, to assess for contamination.

➢ *Statistical Analysis*

Data were collected in Microsoft Excel and result was analyzed and expressed in frequency and percentage.

III. RESULTS

A total of 86 samples were collected from patients presenting with dental caries in the northern region of Chennai, India**.** The samples pool consists of 42 dental plaque specimens and 44 dental caries specimens, which were subjected to microbiological analysis. The findings revealed a diverse distribution of bacterial species, with *Streptococcus mutans* (68.6%) identified as a predominant organism. Other bacterial isolates are *Escherichia coli* (10 isolates; 11.63%), *Pseudomonas spp.* (8 isolates; 9.30%), *Klebsiella spp.* (5 isolates; 5.81%), *Staphylococcus aureus* (4 isolates; 4.65%), and *Lactobacillus spp.* (2 isolates; 2.33%). These findings revealed that *S. mutans* species were most dominant bacterial species associated with dental caries in the study population (Table 1).

Table 2 Distribution of Bacterial Isolates Recovered from Dental Samples

S. No	Bacterial Species	Number of Isolates (n)	Percentage
1	*Streptococcus mutans*	59	68.60
2	*Escherichia coli*	10	11.63
3	*Pseudomonas* spp.	8	9.30
4	*Klebsiella* spp.	5	5.81
5	*Staphylococcus aureus*	4	4.65
6	*Lactobacillus* spp.	2	2.33
	Total	**86**	**100**

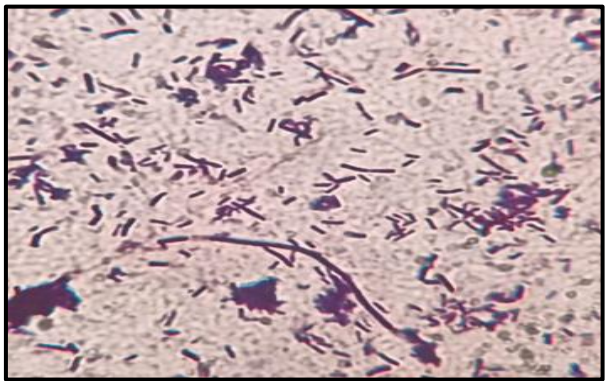

Fig 1 Microscopic Morphology of *Streptococcus mutans* Showing Gram-Positive Cells Observed Under 100× Magnification.

Microscopic examination revealed Gram-positive cocci arranged in chains, consistent with the typical morphological features of *S.mutans* (Figure 1). On Mitis Salivarius agar medium, the isolates produced characteristic blue, raised, mucoid colonies with smooth and glistening surface. The findings suggest of extracellular polysaccharides production and biofilm formation of *S.mutans* (Figure 2). The catalase test findings showed no effervescence upon exposure to 2 drops of 3% hydrogen peroxide, confirming a negative catalase reaction characteristic of streptococci (Figure 3). Furthermore, Congo Red agar assay demonstrated strong biofilm formation, as evidenced by dark-colored colonies, confirming the cariogenic role of the isolates in dental plaque formation (Figure 4).

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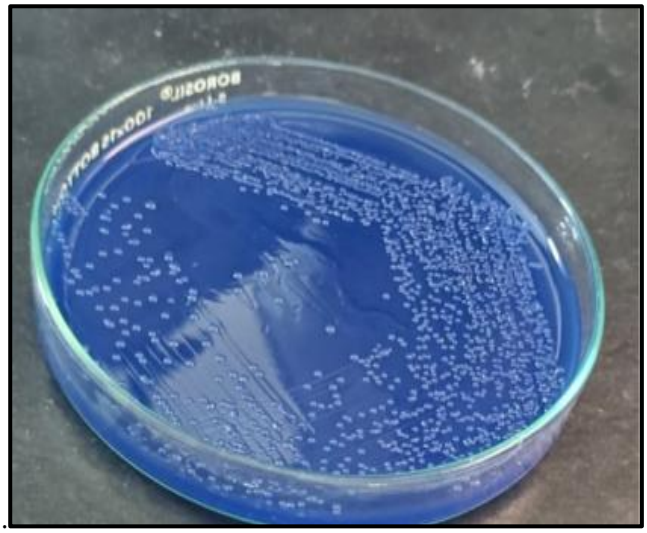

Fig 2 Characteristic Blue Colonies of *Streptococcus mutans* on Mitis Salivarius Agar.

Fig 3 Catalase Test Shows a Negative Reaction for *Streptococcus mutans.*

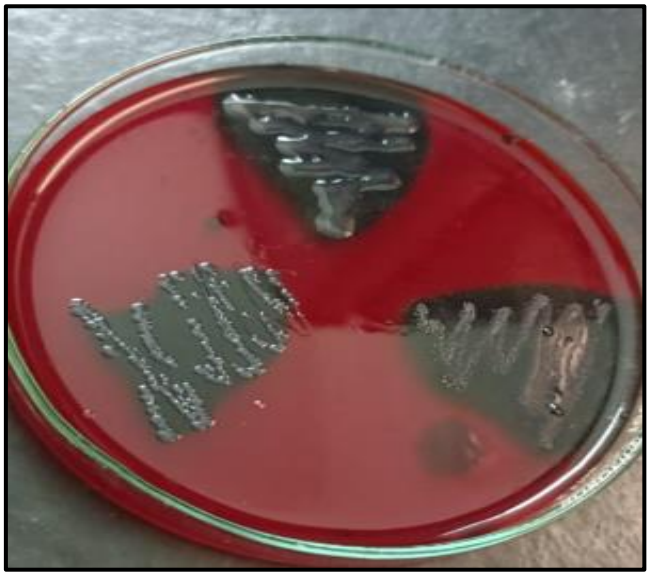

Fig 4 Biofilm-Producing *Streptococcus mutans* on Congo Red Agar.

Molecular analysis using the PCR amplification technique demonstrated the presence of virulence-associated genes *comE* and *spaP* in the *Streptococcus mutans* isolate. Agarose gel electrophoresis of the PCR products revealed clear bands corresponding to the comE gene, which produced an amplicon of approximately 384 bp, while the spaP gene produced an amplicon of approximately 670 bp, confirming successful amplification of both target genes. The positive control showed amplification at the expected band sizes, whereas no amplification was observed in the negative control or no-template control, confirming the specificity of the PCR reaction. These results indicate that the isolated *S. mutans* strain possesses genetic determinants associated with bacterial competence (comE) and adhesion to tooth surfaces (spaP), which are important virulence factors contributing to biofilm formation and dental caries development.

Table 3 PCR Detection of Virulence Genes

Gene Target	Function	Expected Amplicon Size	Detection Status
comE	Genetic competence regulator	~384 bp	Detected
spaP	Biofilm formation and adhesion	~670 bp	Detected

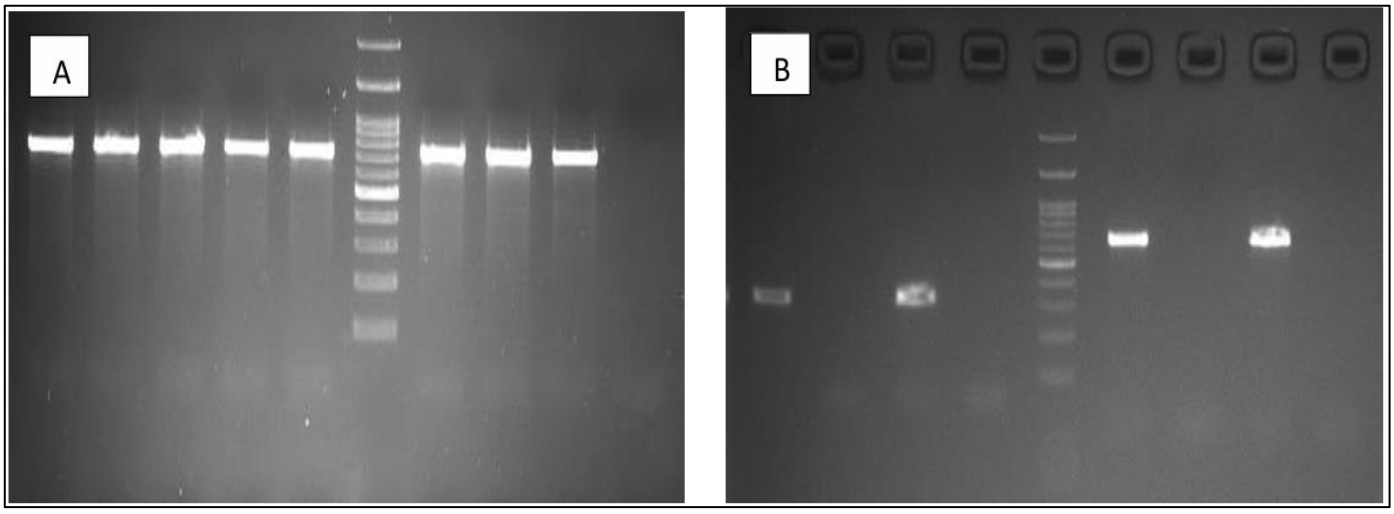

Fig 5 PCR Amplification of comE and spaP Genes in *Stresptococcus mutans*.

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Table 4 Lane Configuration for PCR Detection of Virulence Genes

Lane	Description
1	PC
5	ER25030001
6	DNA Ladder
10	NC

Lane	Description
1	comE – ER25030001 as PC
2	comE – Candida albicans as NC
3	comE – ER25030001
4	comE – NTC
5	DNA Ladder
6	spaP – ER25030001 as PC
7	spaP – Candida albicans as NC
8	spaP – ER25030001
9	spaP – NTC

Antimicrobial susceptibility testing using the Kirby–Bauer disc diffusion method revealed varied complex resistance patterns among the isolates. High sensitivity was observed for rifampicin (84.88%) and penicillin (70.3%), followed by methicillin (69.76%) and bacitracin (52.32%). In contrast, higher resistance rates were noted for streptomycin (48.8%) and erythromycin (41.8%), with moderate resistance observed for methicillin, azithromycin, gentamicin, and penicillin. These findings demonstrate the variability in antimicrobial response and focus on the need for continuous surveillance of antibiotic resistance in cariogenic bacteria.

Table 5 Antimicrobial Susceptibility Profile of *Streptococcus mutans* Isolates

Antibiotic	Disk Potency (µg)	Resistant Isolates (%)	Sensitive Isolates (%)
Amikacin	10	13.5	30.2
Penicillin	10	21.20	70.3
Clindamycin	30	6.8	11.65
Bacitracin	10	8.7	52.32
Erythromycin	15	41.8	38.37
Rifampicin	30	7.3	84.88
Ciprofloxacin	10	8.1	11.63
Streptomycin	10	48.8	22.09
Methicillin	30	27.19	69.76
Gentamycin	30	18.68	5.08
Chloramphenicol	30	11.62	30.02
Azithromycin	30	20.3	48.83

The antimicrobial activity of methanolic extracts of Marthandam multifloral, Orange blossom, Sidr, and Acacia honey was evaluated against *Streptococcus mutans* using both microbroth dilution and agar diffusion methods. The microdilution assay demonstrated a concentration-dependent inhibitory effect, shown by reduced turbidity in microtiter wells (Figure 6 A-E). Consistently, agar diffusion results showed a progressive increase in zones of inhibition with increasing concentrations (20–80 µg/mL) across all samples. Marthandam multifloral honey exhibited inhibition zones ranging from 1.2 to 2.2 mm, while Acacia honey demonstrated comparable and slightly higher activity at the maximum concentration (1.1 to 2.3 mm). Orange blossom honey showed moderate antibacterial effects (0.8 to 1.8 mm), whereas Sidr honey exhibited no inhibition at the lowest concentration but showed measurable activity at higher concentrations (0.6 to 1.7 mm). Overall, these findings confirm a clear dose-dependent antimicrobial effect, with Marthandam and Acacia honey demonstrating comparatively greater efficacy against *S. mutans*.

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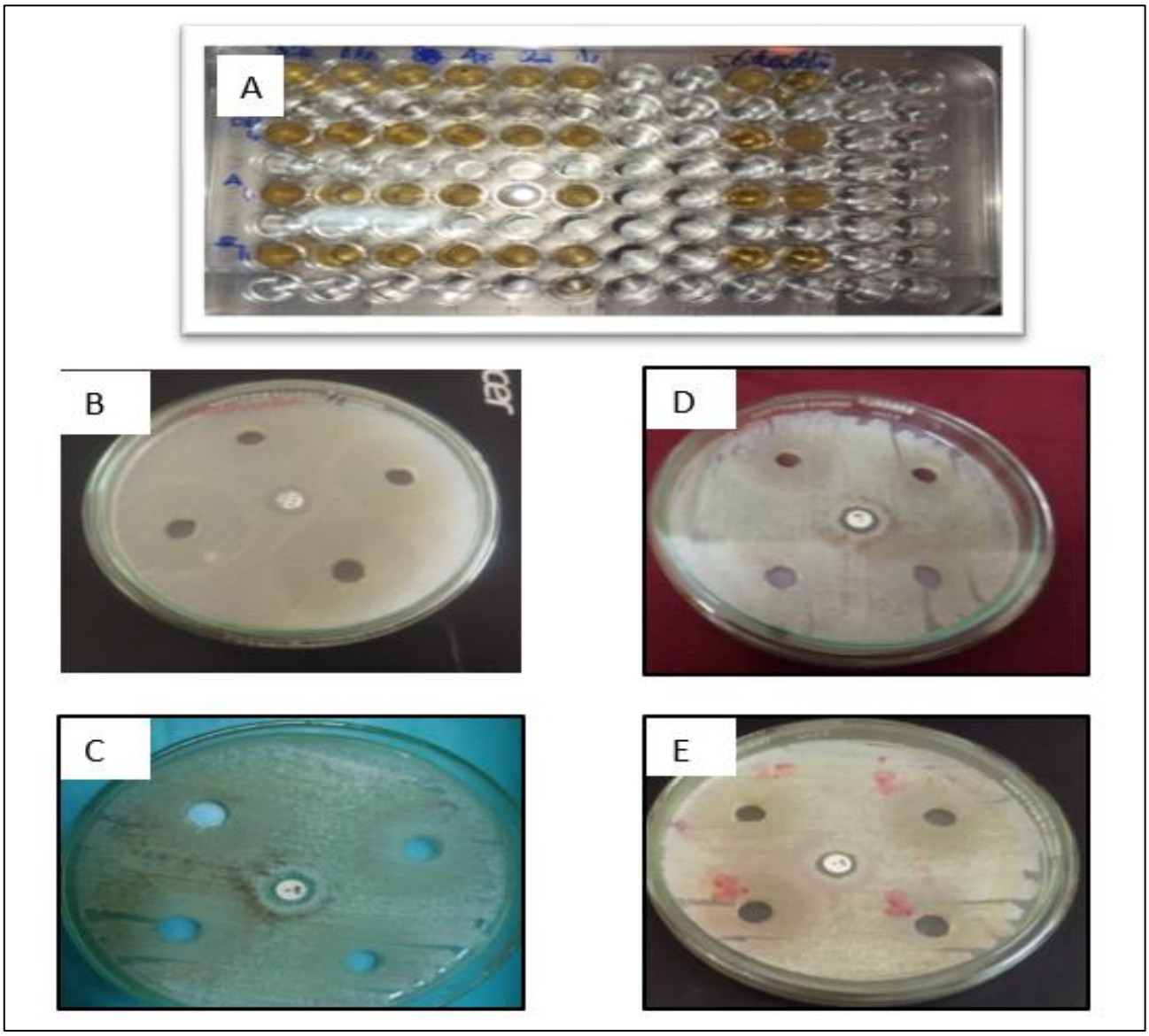

Figure 6 (A-E) Anibacterial activity of honey extracts against *Streptococcus mutans*: A: Microbroth dilution assay for determination of MIC of honey extracts*;* B: Marthandam multifloral honey; C: Orange blossom honey; D: Sidr honey; E: Acacia honey.

Table 6 Comparative Antibacterial Activity of Honey Samples at Varying Concentrations Against *Streptococcus mutans*

S. No	Name of the Honey	Concentrations (µg/ml)			
		20	**40**	**60**	**80**
1.	MARTHANDAM	1.2mm	1.5mm	1.9mm	2.2mm
2.	ORGANGE BLOSSOM	0.8mm	1.2mm	1.6mm	1.8mm
3.	SIDR	No zone	0.6mm	1.5mm	1.7mm
4.	ACACIA	1.1mm	1.5mm	1.8mm	2.3mm

Further chemical profiling of Marthandam honey using GC–MS analysis identified several bioactive compounds, including 5-hydroxymethylfurfural (5-HMF), 4H-pyran-4-one derivatives, cis-dimethyl morpholine, 3-deoxy-D-mannoic lactone, and 1,2,3-propanetriol derivatives. The presence of 5-HMF and related compounds, known for their antimicrobial properties, supports the observed antibacterial activity and highlights the therapeutic potential of honey as a natural and organic agent against cariogenic organisms like *S.mutans*.

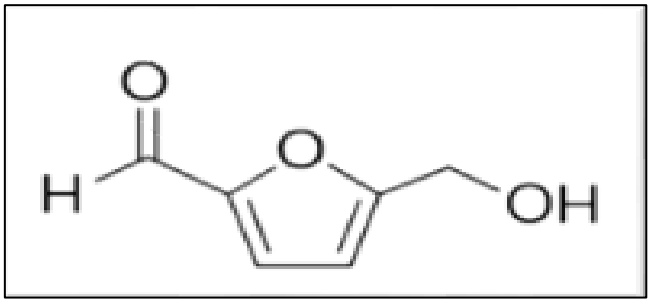

Fig 7 Chemical Structure of 5-HMF

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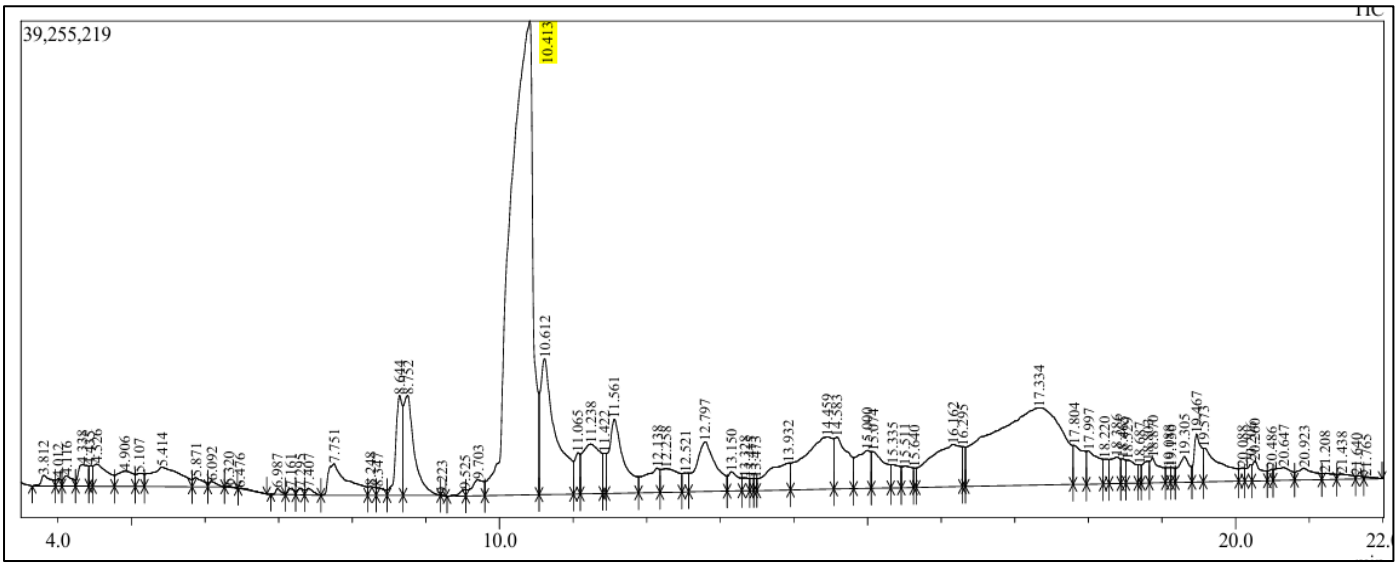

Fig 8 GC–MS Chromatogram of Marthandam Honey

IV. DISCUSSION

The present study evaluated microbial profile of dental caries and confirmed *Streptococcus mutans* as the most prevalent cariogenic species, accounting for 68.6% of isolates, with the addition of other Gram-negative bacilli and other oral bacteria. The findings align with evidence that *S.mutans* are the primary causative organisms for dental caries and a major extracellular polysaccharide-producing species which support in the biofilm formation. As findings from Congo Red Agar confirm the biofilm formation and the presence of comE and spaP virulence genes, they demonstrate high cariogenic potential of the isolated strains, which enhance the genetic competence and adhesion to the tooth surface, resulting in plaque formation (6-7).

Antimicrobial susceptibility analysis of *S.mutans* revealed high sensitivity to antibiotics like rifampicin and penicillin but significant resistance to other notable agents like streptomycin and erythromycin, reflecting the increasing concern of microbial resistance to empirical antibiotics among oral bacteria and focusing the need for periodic and local vigilance to support the empirical management (8). The findings revealed a heterogeneous resistance pattern observed are in line with larger data indicating growing multidrug resistance across the Gram-positive cocci and emphasize the need for importance of empirical therapy and exploring alternative or adjunctive natural agents for management.

The findings of methanolic extracts of honey samples against the *S.mutans* demonstrated a high sensitivity with the lowest MIC concentration with dose-dependent inhibitory activity. Among them, Marthandam honey showed a superior antibacterial activity, whereas Sidr and Acacia honey showed comparatively moderate inhibitory activity against *S. mutans*. MIC ranges for *S.mutans* are in parallel to findings that honey rich in phenolics group or pollen from specific plants like citrus, Satureja spp., and honeydew show high anti-cariogenic and antibiofilm properties. Further evidence suggests that honey possesses broad-spectrum antimicrobial activity against both gram positive and negative bacteria due to multiple physicochemical properties such as high osmotic pressure, acidic pH, hydrogen peroxide production, and flavonoids (9). With studies confirmed that honey possesses high osmotic and hydrogen peroxide properties that interfere with bacterial metabolism and cell membrane integrity. Similarly, Al-Waili et al. (2011) demonstrated that honey-derived bioactive compounds can inhibit several oral pathogens, including *Streptococcus mutans, Streptococcus sanguinis,* and *Lactobacillus species.* In contrast, Acacia honey showed weaker activity, possibly due to lower phenolic presence and variation in floral pollen origin, consistent with previous reports (10).

The molecular analysis conducted in this study further confirmed the presence of virulence genes (spaP and comE) in the isolated *S. mutans* strains, which are associated with bacterial adhesion and biofilm formation (11). These virulence factors play a pivotal in dental plaque development and caries progression. The ability of honey to inhibit *S. mutans* growth may therefore contribute to reducing biofilm formation and limiting the pathogenic potential of these bacteria (12). Overall, the results of the present study indicate that the antimicrobial efficacy of honey against *S. mutans* varies significantly depending on its botanical origin and chemical composition. Among the tested samples, Orange blossom and Marthandam honey demonstrated the most promising antibacterial activity, suggesting their potential application as natural antimicrobial agents for the prevention and management of dental caries (13).

V. CONCLUSION

The present study demonstrated that different honey varieties exhibit varying antibacterial activity against *Streptococcus mutans,* a key pathogen associated with dental caries. Among the tested samples, Marthandam honey showed the strongest antimicrobial activity, followed by Orange blossom honey, while Sidr and Acacia honey exhibited comparatively moderate inhibitory effects. The enhanced antibacterial potential of Marthandam honey as validated by bioactive phytochemicals, particularly 5-

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Hydroxymethylfurfural (5-HMF) identified through GC–MS analysis. These results suggest that Marthandam honey would be promising natural antimicrobial agent on the prevention and management of dental caries.

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- *Conflict of Interest:*
  There is no conflict of interest.

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