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Evaluation of Manuka Honey Effects on Dental Plaque and Bacterial Load (Clinical Study)

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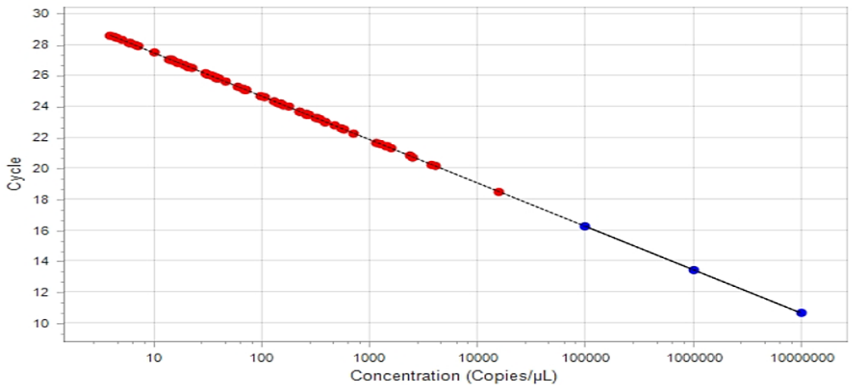
ABSTRACT

Background: Bioactive components of honey offer essential therapeutic properties as antimicrobial and it is linked to several health advantages. The purpose of this study was to examine the effects of manuka honey (MH) mouthwash on plaque (PLI), gingival scores, and *Streptococcus mutans (S. mutans)* bacterial load compared with those of chlorhexidine (CHX) and distilled water (DW).

Methodology: A randomized, double-blind, parallel, and clinical study was designed for the present study. Forty-five individuals between the ages of 20 and 40 years old were chosen randomly and split into the MH, CHX, and DW mouthwash groups. Every person received a bottle at random. The use of mouthwash lasted for 21 days. Before the beginning of using the mouthwash (day zero), for each participants PLI and bleeding on probing (BOP) scores were recorded, the molecular identification and quantification of *S. mutans* was determined. After 21 days, all records were repeated.

Results: All three mouthwash groups were indicated statistically significant decreases in PLI and BOP scores (P<0.001) at the end of 21 day. The reduction in bacterial load was statistically significant in MH group.

GRAPHICAL ABSTRACT



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Introduction

The key etiological component producing periodontal disorders and tooth caries is dental plaque (microbial biofilm). Dental plaque is mostly made up of bacteria and is clinically defined as a structured, strong, and yellow-greyish substance that firmly adheres to intraoral hard surfaces [1], [2]. To put it simply, periodontal disease is a persistent inflammatory illness that destroys the tissues that hold teeth in place. Since tooth loss occurs in its latter stages without treatment, it is one of the most significant health problems [3]. Gingivitis is a mild type of periodontal disease in which the gingival tissue that surrounds the teeth becomes inflamed [4]. Gingivitis is present when there are outward signs of inflammation, such redness, bleeding, swelling, exudation, and to a lesser extent, pain [5]. Gingivitis may progress to periodontitis, which is described by the permanent destruction of tooth-supporting substance, as a result, tooth loss [6], [7]. One of the numerous causes of dental caries, *S. mutans*, is a highly adaptable microbe that may produce pathogenicity determinants that determine its virulence in response to changes in its environment [2, 8], dental plaque contains *S. mutans*, which is often regarded as the most important member of the oral microbiota [9], [10]. Researchers are searching for a safe antibacterial agent with anti-cariogenic activity and few adverse effects [11].

To avoid plaque production and accumulation on the tooth surface, effective plaque control techniques such as toothbrushes, dental floss, and mouthwashes as an adjunctive to these mechanical methods due to a lack of manual skill and motivation [12]. Plaque control is a key component of periodontal therapies, which can be performed mechanically or chemically [13].

The most generally recommended antibacterial and antiseptic agent is chlorhexidine (CHX), making it the "gold standard" of mouthwashes. Unfortunately, it also has some unfavourable consequences, such staining teeth and changing the way food tastes. To counteract these unintended consequences, researchers have been looking for natural remedies. A growing number of people in both urban and rural areas are turning to natural remedies for the prevention and treatment of oral health problems [14].

It is of great medical concern because antibiotic resistance is increasing the prevalence of bacteria and fungi that pose serious threats to public health [15]. Flavonoids, anthocyanins, vitamins C, B and E, phenolic compounds, dietary fibers, carotenoids, and so on are only some of many naturally occurring bioactive components found in fruits, plants, and vegetables [16]. In traditional medicine, crude preparations from the plant's leaves, root, stem, and bark have been used to treat various ailments, including leprosy, malaria fever, smallpox, diarrhea, cholera, intestinal helminthiasis, and respiratory disorders [17]. Honey is a multifaceted substance whose chemical make-up varies widely according to its place of origin and botanical source [18]. It includes an enzyme that, when diluted, generates hydrogen peroxide and it has been shown to have a broad-spectrum inhibitory impact on bacteria and yeasts. Honey peptide isolation reveals promising antibacterial and antifungal properties [19]. Due to its bioactive phytochemical contents, manuka honey (MH) has been demonstrated to display notable quantities of non-peroxide antibacterial capability (which is missing in other commonly used table honey). Manuka refers to the leaves of the New Zealand tea tree, leptospermum scoparium. High concentrations of methylglyoxal (MGO) in MH are responsible for its non-peroxide antibacterial activity. It is often called "healing honey" due to its positive effects on wounds and stomach ulcers. The MIC values of honey fall throughout a broad spectrum, indicating that it has a varied antibacterial effect. Despite indications of its antimicrobial efficiency on non-oral infections, very little is known regarding the specific action of MH against oral pathogens. Therefore, this study aimed to examine how MH mouthwash stacks up against CHX and DW in terms of reducing plaque, gingivitis, and *S. mutans* bacterial load.

Martials and Methods

Subjects: 45 people with gingivitis and plaque buildup were enrolled in the study. The three groups each had 15 participants. Each participant would be randomly allocated to one of the three

research groups in the sequence of their engagement. 15 individuals from group I used mouthwash with 0.2% CHX, 15 participants from group II used mouthwash with 0.2% CHX and 15 participants from group III used a placebo (DW) mouthwash. A standardized questionnaire with questions about the participant's age, gender, and purpose for attending the dentistry clinic was given to each individual under examination. The subject's medical background and past periodontal treatment history were also noted.

Sample Size: G power 3.1.9.7 "program written by Franz-Faul, Universitatit Kiel, Germany" was used to select the sample size, with study power set at 80%, alpha error of probability set at 0.05, correlation between time points set at 0.5, and effect size of F set at 0.2526 "Medium effect size." The study has three primary groups and two time points.

Eligibility criteria: The following requirements had to be satisfied in order for individuals to be eligible to join in this study subjects between the ages of 20 and 40, in good overall health, with more than 20 teeth present and suffering from widespread plaque-induced gingivitis. Patients who have active periodontal disease, cavity caries, need orthodontic treatment, have taken antibiotics within the past four months, require prophylactic antibiotic coverage, have used systemic and/or topical nonsteroidal anti-inflammatory drugs within the past four months, are pregnant or nursing, have had a heart valve replacement, have any chronic illness, have smoked, and/or have used mouthwash within the past four months.

Saliva sample collection: Non-stimulated saliva samples were taken for study participants between 9:00 a.m. and 1:00 p.m., samples were collected before the oral examination by using a spit technique. Then, a letter and a number (before B and after A) were assigned to the sample. Each participant was given a plastic cup and instructed to allow saliva flow into the cup for five minutes in a quiet. After collection, the samples were immediately frozen at -80°C for molecular quantification of bacteria by RTqPCR.

Oral examination: Periodontal health status was recorded through the examination of clinical periodontal parameters (PLI and BOP) by using a periodontal probe of William's graduation.

Preparation of MH mouthwash: Manuka honey (514+ MGO; 15+UMF) mouthwash was prepared based on a previous study conducted in New Zealand [34]. The dilution ratio was (1:3) (V/V) %, meaning that for every 250 ml of MH, it corresponds to 750 ml of DW. MH, CHX, and DW were all put in the same containers. In this way, there is no difference between them and no one solution was biased. The examiner was provided a sequence list of number-coded interventions created by using a random number generator in "Microsoft Excel 2016" to assign the blinded intervention. Therefore, all individuals had an equal chance of being assigned to the interventions sequence.

Randomization and blinding: Due to the double-blind nature of this study, neither the examiner nor the participants were able to determine which intervention could be used. The investigation was completed with decoding. As stated in a prior study, the randomization, blinding, and intervention allocation procedures were followed. An independent third party, who was not connected to the study, carried out simple randomization and coding. The mouthwashes were poured into identical white opaque bottles of totally 300 ml to guarantee blinding of both the participants and the examiner. Each mouthwash group was allocated a random sequential letter (A, B, and C) and decoding was carried out at the conclusion of the study. Microsoft Excel "Microsoft Office 2016, Microsoft Corporation, USA" was used to generate random numbers used to resort the order of the mouthwash groups (A, B, and C) and the participants (N=45) so that each group was received an equal number of participants (N=15) with 1:1:1 allocation. Then, the coded bottles with the mouthwashes were given to the participants together with instructions of use.

Clinical Trial: Participants visited the dental clinic twice during the study period, once on day zero (base line) and the second after 21 days. Before and after the mouthwash use of each participant, PLI and BOP scores were recorded and *S. mutans* was estimated.

Quantification of Streptococcus mutants by using RTqPCR

Promega's reliaPrep™ blood gDNA miniprep technology was used to separate the genomic DNA from saliva samples. Primers targeting the species-specific region on the 16S rRNA were created to identify and quantify *S. mutans* bacteria. The primer quest from integrated DNA technology was used to create and validate the primers used to identify the *S. mutans* 16S rRNA gene, as reported in Table 1.

Absolute quantification is provided by the standard curve. The standard curve technique in the qPCR experiment uses a series of dilutions with a known template copy number. The sample's template concentration (copy µl-1) is calculated by using the standard curve, which is created by using a linear regression of log concentration (copy µl-1) vs. CT. The preparation of eight 0.2 ml tubes began with the addition of 90 l of nuclease free water to each tube. Next, 10 µl of the sample 10*109 copy µl-1 was added to the first tube and a serial dilution was created by transferring 10 µl from the first tube to the second tube and so on. Figure displays the progression of the standard curve response from the tube of 1*107 copy µl-1 to the tube of 1*105 copy µl-1.

Real time PCR amplifications were carried out by using 10 µl volumes containing 5 µl go taq green master mix (2X), 0.5 µl for each primer (10 pmol) and 4 µl of template DNA, as indicated in Table 2.

The real time PCR system was cycled by using the following temperature programme: 5 minutes at 95°C for denaturation, followed by 40 cycles of 95 °C for 20 seconds, 60 °C for 20 seconds, and 72°C for 20 seconds, as demonstrated in Table 3.

Results and Discussion

Table 4 details the socio demographics of the participant groups of the study.

The mean PLI of the research groups at baseline and after 21 days is shown in Table 5 as descriptive statistics of PLI. All groups saw a mean decrease in PLI between the two periods and there was a statistically significant difference between each group (P<0.000). All research groups saw a decline in PLI between two time periods; the mean difference was statistically significant (P<0.018). The percentage change in PLI after MH treatment was (50%), CHX (69%), and DW (39%). Table 6 illustrates the mean difference and intergroup comparisons of mean values of PLI for all pairs of groups at day 21st. Statistically, the comparisons between all groups were non-significant except CHX vs. DW which was a statistically significant (p<0.05).

There was a statistically substantial (59% point) decrease in BOP. Hence, the result was significant (P0.000). For the CHX group, the 79% change in the mean decrease was statistically significant (P<0.0001). Comparatively, the (37%) decrease in the mean for the DW group was statistically significant (P<0.000). Table 1 represents that between the two time points, the BOP reduced in all study groups, with a mean difference that was statistically significant (P<0.002) [7]. Furthermore, the current investigation demonstrated that the mean difference and intergroup comparisons in mean values of BOP for all pairings of groups at day 21st were not statistically significant, except for CHX versus DW which was a statistically significant (p<0.01), as indicated in Table 8.

Quantification of Streptococcus mutans

The mean of *S. mutans* in the MH group was (490.421 ± 113.99) at baseline and (115.65 ±28.57) in 21 days, with a (76%) percentage change that was statistically significant (P<0.001). The mean in the CHX group was (403.22 ±271.67) at baseline and (107.3 ±30.99) in 21 days, with a (73%) percentage change that was statistically insignificant (P>0.05). Likewise, the mean of the DW group at baseline was (1480.2 ±1030.8) and (930.32 ±287.67) in 21 days and the reduction was statistically insignificant (P>0.05) with a (37%) percentage change. The bacterial load changed between two periods among all study groups; the mean difference was statistically significant (P<0.001), as indicated in Table 9. On the other hand, Table 10 represents the mean difference and intergroup comparisons of bacterial load mean values on day 21st for all pairings of groups.

On day 21st, the PLI scores and BOP of the MH, CHX, and DW groups were all significantly lower than the baseline. This was in harmony with another study reported by Singhal *et al.* found that

using MH mouthwash reduced PLI and the mean change was statistically significant. It was compared with chlorhexidine, which reduced the PLI level and the reduction was also statistically significant, but chlorhexidine was more effective in reducing the rate of PLI level [20]. The bactericidal effect of hydrogen peroxide through the production of oxidative damage to membrane, proteins, enzymes, and DNA and the presence of non-hydrogen peroxide MGO chemicals that induce cell death, may account for the antiplaque capabilities of MH. There is a strong relationship between the ''unique manuka factor'' (UMF) categorization and the antibacterial activity of MGO in MH. The comparative studies comparing CHX and DW on clinical parameters and gingival fluid cytokine levels in untreated plaque-associated gingivitis found that the DW group showed a statistically significant improvement in both indices (PI and BOP) after 21 days [27]. The decrease in the placebo group was anticipated because of patients' desire to practice the proper dental hygiene.

The current findings demonstrated a reduction in *S.mutans* in both the MH and CHX groups. Statistically, the decrease was only noticeable in the MH group, not the CHX group. There have been several studies looking into the MH impact on *S.mutans*. However, they all used slightly different approaches. Most studies on MIC and MBC of MH have employed the entire dilution broth method. The PCR-based studies assessing MH's effectiveness against *S. mutans* are lacking, making direct comparisons difficult. The MH effects on *S.mutans* were investigated and compared by Rupesh *et al.* in two groups of children who were otherwise similar. Each group used MH twice daily for 21 days under close observation. Some people used it in addition to their regular tooth-brushing routine, while others continued to use only their regular tooth-brushing routine. Children that took MH saw substantial reductions in salivary *S.mutans* after 10 and 21 days. It is important to note that the 916 UMF honey concentration was used [21].

There was a statistically significant decrease in the number of *S. mutans* count in both groups from baseline on days 7th and 14th of Sruthi's RCT investigation on the effectiveness of MH and CHX mouthwashes on *S.mutans* count in children [28]. Moreover, Schmidlin *et al.* performed an *in vitro* study to prove that MH is effective against three oral pathogens (*S. mutans, Porphyromonas gingivalis, and Aggregatibacter actinomycetemcomitans*) and to compare it with the antibacterial effects of CHX and DW. MH with an UMF value less than 15+ had less bacterial growth-inhibiting capacity than MH with an UMF value more than 15+. MH with an UMF ≥15+ had a much stronger antibacterial impact than the other examined honeys. MH inhibited three species of oral bacteria, all of which were significant statistically decreased. *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* were suppressed more severely than *S. mutans*. Most of the studies are support and consistent with current results. This is due to the fact that MH compounds may have antimicrobial effects.

MH serves as a bactericide by oxidizing cellular components as membranes, proteins, enzymes, and DNA. Honey has this effect because it includes glucose oxidase, an enzyme that, when diluted, creates hydrogen peroxide as a powerful antibacterial agent. D glucono lactone, one of MH's bioactive phytochemical ingredients (also found in umbelliferous plants), lowers pH, while further having natural antibacterial capabilities, making MH shelf-stable without the need of peroxide. The main antibacterial ingredient in it has been identified as MGO, an aldehyde derivative of pyruvic acid. Even at low doses, MGO is hazardous to pathogens, interfering with cell divisions, stopping growth and particularly triggering bacterial DNA destruction.

Most honeys include anti-bacterials, which might also explain why honey is used to heal wounds and other infections. MH, on the other hand, includes a unique component MGO that is not present in other varieties of honey and its extensive study has categorized it as the strongest anti-bacterial discovered in honey, making MH one of the strongest forms of honey.

Juliano and Magrini studied the antibacterial effect of MGO derived from MH on several types of bacteria and Candida, among which was *S.mutans*. MGO's antibacterial activity was evaluated

initially by using standard microbiological techniques (MIC and MBC determinations in a liquid or a solid medium). The MIC values for MGO against Gram+ bacteria (*Staphylococcus. aureus and S.mutans*) were found to be 0.150 mg/mL, whereas the MIC values for Gram- bacteria (*Escherichia coli and Pseudomonas aeruginosa*) were found to be considerably higher. However, MGO was shown to be less effective than other antifungal agents against both yeasts and filamentous fungi (MIC range: 0.63-1.25 mg/mL and 7.5-10 mg/mL, respectively).

Tables and Figures

Table 1: Primer sequences of Streptococcus mutans

Primer's Name	Sequence
S. mutans -Forward.	5`-AGCCATGCGCAATCAACAGGTT-3`
S. mutans - Reverse	3`-CGCAACGCGAACATCTTGATCAG-5`

Table 2: Real time PCR reaction setup

"Master mix components"	"Stock"	"Unit"	"Final"	"Unit"	"Volume1 Sample"
"Master Mix"	2	µM	1	X	5
"Forward primer"	10	µM	0.5	µM	0.5
"Reverse primer"	10		0.5	µM	0.5
"DNA"		10		ng/µl	4
"Total volume"					10
"Aliquot per single rxn"	"6µl of Master mix per tube and add 4µl of template"				

Table 3: Real time PCR program

"Steps"	°C	m:s	Cycle
"Initial Denaturation"	95	05:00	1
"Denaturation"	95	00:20	40
"Annealing"	60	00:20	
"Extension"	72	00:20	

Table 4: Demographic characteristic of participants in three groups of the study

Demographic characteristics	Study groups			P-value
	MH n=15	CHX n=15	DW n=15	
Age (years)				0.830NS
Range	(20-40)	(20-40)	(22-40)	
Mean ± SD	30.9±7.38	30.8±8.32	32.4±5.76	
Sex				1.000NS
Female, f (%)	6 (40)	10 (67)	6 (40)	
Male, f (%)	9 (60)	5 (33)	9 (60)	
Education				0.182 NS
High school, f (%)	6 (40)	6 (40)	7 (47)	
Post high school, f (%)	9 (60)	9 (60)	8 (53)	
Employment				0.528 NS
Employee, f (%)	10 (67)	9 (40)	7 (47)	
Non-employee, f (%)	5 (33)	6 (60)	8 (53)	

Table 5: Mean value of plaque index in study groups before and after mouthwash use

Plaque index	Study groups			ANOVA (P-value)
	MH n=15	CHX n=15	DW n=15	
Before				
Range	(0.50-2.06)	(0.76-2.42)	(1.50-1.99)	0.001**
Mean± SD	1.43±0.41	1.97±0.47	1.48±0.29	
After				
Range	(0.18-1.73)	(0.42-0.88)	(0.38-1.57)	0.018*
Mean± SD	0.77±0.39	0.61±0.13	0.95±0.33	
T-test (P-value)	0.000**	0.000**	0.000**	
Percentage change	50% (decrease)	69% (decrease)	39% (decrease)	

MH: manuka honey; CHX: chlorhexidine; DW: distilled water

Table 6: Intergroup comparisons of mean values of plaque index between all pairs of groups after mouthwash use

Grouping	Mean difference	Tukey's HSD (P-value)
Plaque index		
MH vs. CHX	0.16	0.352 NS
MH vs. DW	0.18	0.266 NS
CHX vs. DW	0.34	0.013*

Table 7: Mean value of bleeding on probing in study groups before and after mouthwash use

BOP	Study groups			ANOVA (P-value)
	MH n=15	CHX n=15	DW n=15	
Before				
Range	(0.40-0.90)	(0.54-1.00)	(0.42-0.93)	0.056NS
Mean± SD	0.71±0.15	0.83±0.13	0.74±0.14	
After				
Range	(0.09-0.64)	(0.06-0.31)	(0.03-0.85)	
Mean± SD	0.29±0.18	0.17±0.06	0.46±0.31	0.002**
T-test (P-value)	0.000**	0.000**	0.002**	
Percentage change	59% (decrease)	79% (decrease)	37% (decrease)	

Table 8: Intergroup comparisons of mean values of bleeding on probe between all pairs of groups after mouthwash use

Grouping	Mean difference	Tukey's HSD (P-value)
BOP		
MH vs. CHX	0.12	0.278 NS
MH vs. DW	0.17	0.800 NS
CHX vs. DW	0.29	0.001*

Table 9: Mean value of bacterial load in study groups before and after mouthwash use

Bacterial load	Study groups			ANOVA (P-value)
	MH n=15	CHX n=15	DW n=15	
Before				
Range	(4.50-1605.0)	(5.80-4060.0)	(3.90-15770.0)	
Mean± SE	490.42±113.99	403.22±271.67	1480.2±1030.8	0.400NS
After				
Range	(5.10-387.2)	(7.10-468.9)	(6.00-3731.0)	
Mean± SE	115.65±28.57	107.3 ±30.99	930.32±287.67	0.001**
Mean Rank				
T-test (P-value)	0.001**	0.144 NS	0.305 NS	
Percentage change	76% (decrease)	73% (decrease)	37% (decrease)	

Table 10: Intergroup comparisons of mean values of bacterial load between all pairs of groups after mouthwash use

Grouping	Mean difference	Tukey's HSD (P-value)
bacterial load		
MH vs. CHX	8.35	0.999NS
MH vs. DW	814.67	0.003**
CHX vs. DW	823.02	0.003**

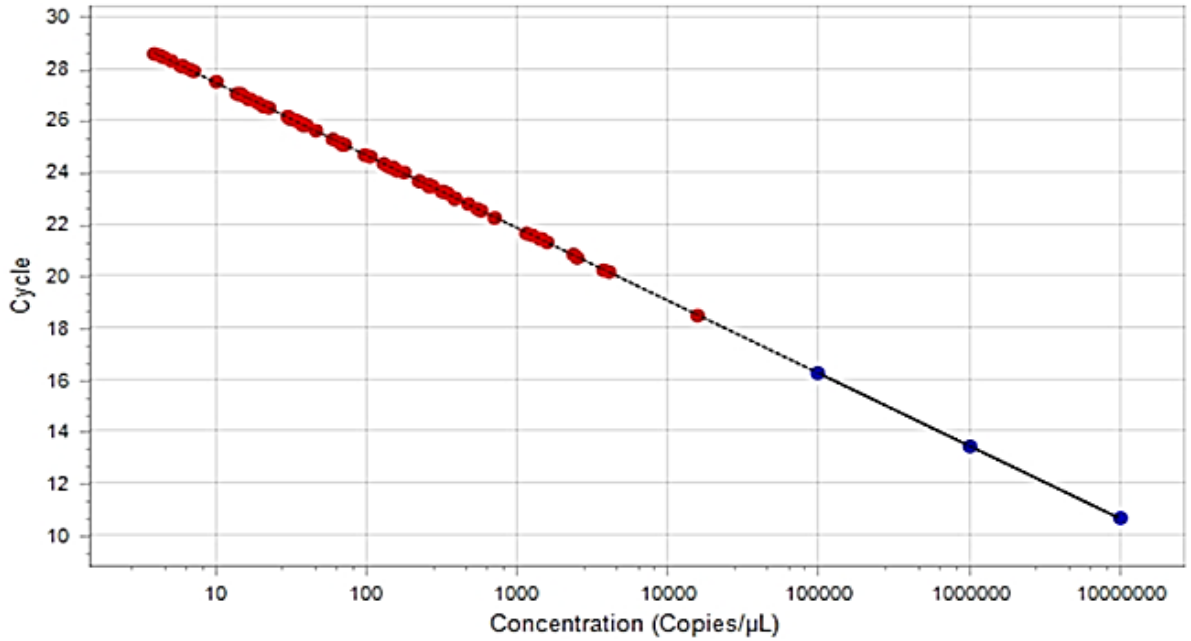

Figure 1: Standard Curve of qPCR

Conclusion

The antibacterial and anti-biofilm properties of MH were proven. In addition, BOP scores dropped. The evidence is overwhelming in favour of using MH as a safe agent that might be used in mouthwash formulas. MH is an appropriate molecule for *S. mutans*' antibacterial defence. We advise the use of MH to enhance dental health.

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Authors' contributions

All authors contributed to data analysis, drafting, and revising of the paper and agreed to be responsible for all the aspects of this work.

Conflict of Interest

The author declared that they have no conflict of interest.

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