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"A Comparative Evaluation Of Antimicrobial Efficacy Of Centella Asiatica With Triphala On Biofilm Forming Cariogenic Microorganisms: An In- Vitro Study."

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Abstract:
Background:
Dental caries, a multifactorial disease, is strongly associated with biofilm-forming bacteria such as Streptococcus mutans and Lactobacillus casei. The limitations of synthetic antimicrobials like chlorhexidine—including staining, taste alteration, and microbial resistance—have prompted interest in herbal alternatives.
Aim:
To compare the antimicrobial efficacy of a Mixed Herbal Powder Extract (MHPE) containing Centella asiatica and Triphala with 0.12% Chlorhexidine Gluconate (CHX) against S. mutans and L. casei.
Methodology:
Methanolic and chloroform extracts of Centella asiatica, Terminalia chebula, Terminalia bellerica, and Emblica officinalis were used to prepare MHPE. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined using serial dilution. Biofilm disruption was assessed via scanning electron microscopy (SEM). Statistical analysis was done using SPSS v23.0 with significance set at $p < 0.05$.
Results:
MHPE showed MIC and MBC values of 25.96 µg/ml and 27.03 µg/ml against S. mutans, and 28.85 µg/ml and 29.21 µg/ml against L. casei, respectively. MHPE was significantly more effective against L. casei than CHX ($p=0.001$), while CHX was more effective against S. mutans ($p=0.040$). SEM analysis revealed notable disruption of the biofilm matrix by MHPE, especially in S. mutans.
Discussion:
The enhanced activity of MHPE against L. casei may be attributed to the synergistic effect of phytoconstituents like asiaticoside, tannins, and polyphenols, known for their antimicrobial and anti-adhesive properties. The comparable efficacy of MHPE to CHX highlights its potential as a natural, biocompatible alternative in preventive dentistry.
Conclusion:
MHPE exhibits promising antimicrobial and antibiofilm efficacy against cariogenic bacteria, supporting its use as a natural adjunct in oral healthcare. Clinical studies are needed to validate these findings.
Keywords: *Centella asiatica, Triphala, biofilm, dental caries, Streptococcus mutans, Lactobacillus casei, herbal antimicrobials.*

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I. Introduction:

Herbal medicine has been a crucial element of traditional healthcare, offering natural alternatives to synthetic drugs. Among these, Triphala, a polyherbal formulation in Ayurvedic medicine, has garnered attention for its effectiveness in oral health. Comprising three medicinal fruits—Terminalia chebula (Haritaki), Terminalia bellerica (Bibhitaki), and Emblica officinalis (Amla)—Triphala is known for its antimicrobial, antioxidant, and anti-inflammatory properties, making it a strong candidate for combating oral biofilms and cariogenic microorganisms like Streptococcus mutans and Lactobacillus casei, key contributors to dental caries and periodontal diseases.[1]

Each of the three fruits in Triphala offers distinct pharmacological benefits. Terminalia chebula is rich in tannins, flavonoids, and chebulinic acid, exhibiting strong antimicrobial effects and preventing plaque buildup.[2] Terminalia bellerica contains bioactive compounds such as gallic and ellagic acids, which inhibit bacterial adhesion to oral surfaces, reducing the risk of plaque and tooth decay. [3] Emblica officinalis is a potent antioxidant, abundant in vitamin C, which enhances immune function and reduces oxidative stress, essential for maintaining gum health.[4] Studies have shown that Triphala can reduce biofilm formation, inhibit bacterial colonization, and promote enamel remineralization, making it a promising alternative to synthetic antimicrobial agents in oral care.[5]

In addition to Triphala, other herbal extracts like Centella asiatica, Andrographis paniculata, Juglans regia, and Cymbopogon citratus have also been studied for their antimicrobial properties in dentistry. For instance, Centella asiatica has shown promise in inhibiting oral biofilms and promoting tissue regeneration, while Andrographis paniculata has displayed potent antibacterial activity comparable to chlorhexidine, a commonly used antiseptic.[6,7] Juglans regia (Walnut Husk) and Cymbopogon citratus (Lemongrass) also demonstrate significant antibacterial effects against S. mutans and Lactobacillus, contributing to the prevention of periodontal diseases.[8]

While chlorhexidine is widely used in dentistry to combat cariogenic bacteria, its long-term use can result in adverse effects such as tooth staining and microbial resistance. [9] These limitations highlight the need for alternative, safe, and sustainable treatments. Herbal formulations like Triphala offer biocompatibility, minimal side effects, and a holistic approach to oral health.[10]

This study aims to evaluate and compare the antimicrobial efficacy of Triphala and Centella asiatica against S. mutans and L. casei, assessing the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of these herbal extracts, and analyzing their effects on biofilm disruption through scanning electron microscopy (SEM). [11]

II. Material And Methods:

This in vitro experimental study was conducted at the Department of Pediatric and Preventive Dentistry at Darshan Dental College and Hospital, Udaipur, with the aim to evaluate and compare the antimicrobial efficacy of Mixed Herbal Powder Extract (MHPE) and 0.12% Chlorhexidine Gluconate (CHX) against biofilm-forming cariogenic microorganisms, specifically Streptococcus mutans and Lactobacillus casei.

Source of Data:

The study was performed in collaboration with several institutions: the Department of Pediatric and Preventive Dentistry, Darshan Dental College and Hospital, Udaipur; the Ayurved Evam Herbal Vikas Sansthan Analytical and Research Laboratory, Eklingpura, Udaipur for the preparation of MHPE; and the Department of Pharmacy, Pacific University, Udaipur, where biofilm production was carried out.

Study Design:

The samples for the study were divided into the following groups:

- Group I – Mixed Herbal Powder Extract (MHPE):
  - Subgroup A: Untreated group (both S. mutans and L. casei)
  - Subgroup B: Treated with MHPE (both S. mutans and L. casei)
- Group II – 0.12% Chlorhexidine Gluconate (CHX):
  - Subgroup A: Untreated group (both S. mutans and L. casei)
  - Subgroup B: Treated with 0.12% CHX (both S. mutans and L. casei)

Investigation Methods:

- Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of MHPE on biofilm-forming cariogenic bacteria were assessed using the agar diffusion method.
- The efficacy of MHPE in disrupting bacterial cell morphology was examined using scanning electron microscopy (SEM).

Methodology:

1. ***Collection of Plant Materials:***
   - Extracts of Centella asiatica (herb), Terminalia chebula (fruit), Terminalia bellerica (fruit), and Emblica officinalis (fruit) were sourced from an authorized Ayurvedic vendor.
   - The materials were ground into powder and mixed in a ratio of 70%, 10%, 10%, and 10%, respectively.
2. **Preparation of Mixed Herbal Powder Extract Solvent:** Three extracts were prepared using different solvents:
   - First extract: 50g MHPE with Methanol (10:1 v/w)
   - Second extract: 50g MHPE with Chloroform/Methanol (1:1 v/w)
   - Third extract: 50g MHPE with Chloroform (1:1 v/w) All extracts were incubated, evaporated under reduced pressure, and lyophilized before being stored at -40°C. The final extract was dissolved in 1% DMSO (Dimethyl sulfoxide).
3. ***Evaluation of MIC using Agar Diffusion Method:***
   - A standardized cell suspension of S. mutans and L. casei (200µl, 10^7 CFU/ml) was spread on Brain Heart Infusion Agar (BHI).

- Wells were punched into the agar and filled with different concentrations (1, 10, 25, and 50 µg/ml) of MHPE. The lowest concentration showing inhibition was recorded as the MIC.

4. Evaluation of MBC of MHPE:

- Serial dilutions of MHPE were prepared and mixed with a standardized cell suspension (200µl, 10^7 CFU/ml) of S. mutans and L. casei.
- After incubation, aliquots were plated on BHI agar and incubated overnight at 37°C. The lowest concentration showing bactericidal activity was recorded as the MBC.

5. Preparation of Biofilm:

- A portion of overnight cultures of S. mutans and L. casei (20µl) was added to well plates and incubated aerobically at 37°C.
- After 12 hours, the medium was replaced with fresh BHI broth containing 2% sucrose. This process was repeated three times to encourage biofilm formation. The wells were then washed with sterile distilled water.

6. Effect of MHPE on Bacterial Cell Morphology:

- Biofilms were treated with the MBC concentration of MHPE for 30 minutes at 37°C.
- After treatment, the wells were washed, and samples were fixed using 4% formaldehyde and 1% glutaraldehyde in phosphate-buffered saline.
- The samples were rinsed and dehydrated through ethanol washes (70%, 95%, and 100% ethanol) and examined under a scanning electron microscope (SEM) to assess changes in the morphology of S. mutans and L. casei.

Stastical Analysis:

Descriptive statistics were performed using Statistical Package for Social Sciences Software (SPSS) version 23.0. **Shapiro Wilk test** was used to check which all variables were following normal distribution.Data was found to be normally distributed hence parametric test were used for inferential statistics. Kruskal Wallis test followed by Mann Whitney U test for pairwise post hoc test comparison was used for inferential statistics. Level of statistical significance was set at p-value less than 0.05

III. Results

Table I: Intergroup Comparative Analysis Of Mic Between Streptococcus And Lactobacillus Groups

Minimum Inhibitory Concentration (MIC)	Mean	Std. Deviation	Std. Error Mean	p-value
Streptococcus mutans	25.9625	.93207	.46604	
Lactobacillus casei	28.8450	1.32067	.66033	.012*

*p<0.05 is considered as statistically significant

Table II: Intergroup Comparative Analysis Of Mbc Between Streptococcus And Lactobacillus Groups

Minimum Bactericidal Concentration (MBC)	Mean	Std. Deviation	Std. Error Mean	p-value
Streptococcus mutans	27.0325	.72284	.36142	
Lactobacillus casei	29.2175	1.26939	.63470	.024*

*p<0.05 is considered as statistically significant

Table III: Intragroup Comparison Of Antimicrobial Efficacy Of Mphpe Group

Mixed Herbal Powder Extract			
	Mean	Std. Deviation	p- value
Lactobacilli untreated	106.82240	3.699795	
Lactobacilli MPHPE treated	91.40760	9.728389	0.001*
S mutans untreated	105.50100	9.907832	
Mutans MPHPE treated	99.00440	19.955394	0.001*

*p<0.05 is considered as statistically significant

Table IV: Intragroup Comparison Of Antimicrobial Efficacy In Chx Group

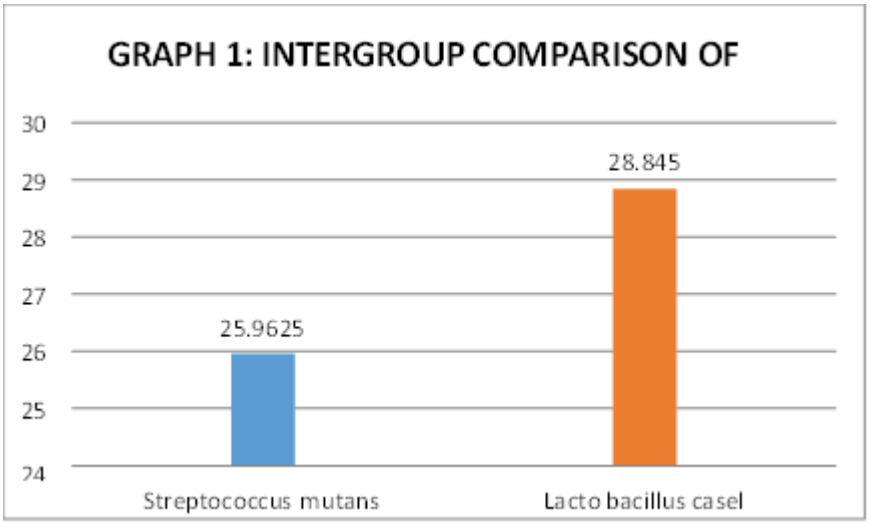
Chlorhexidine			
	Mean	Std. Deviation	p-value
Lactobacilli untreated	106.82240	3.699795	
Lactobacilli CHX treated	106.65420	6.436003	**1.00**
S mutans untreated	105.50100	9.907832	
S mutans CHX treated	84.64620	10.560136	**0.001***

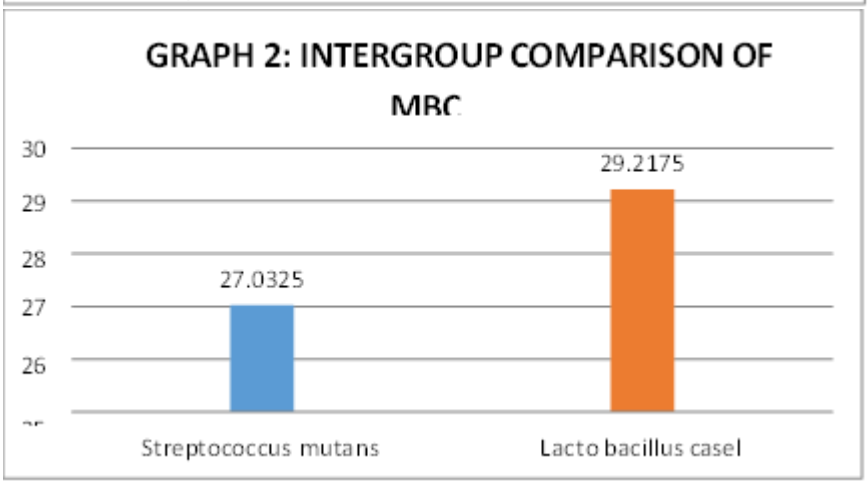
*p<0.05 is considered as statistically significant

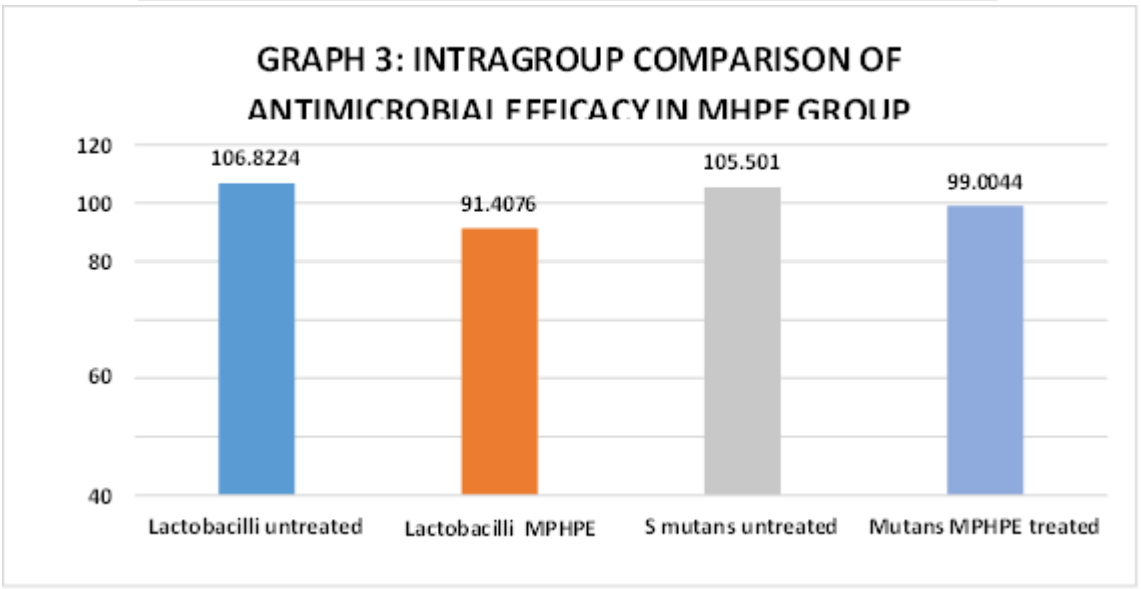
Table V: Intergroup Comparison Of Antimicrobial Efficacy

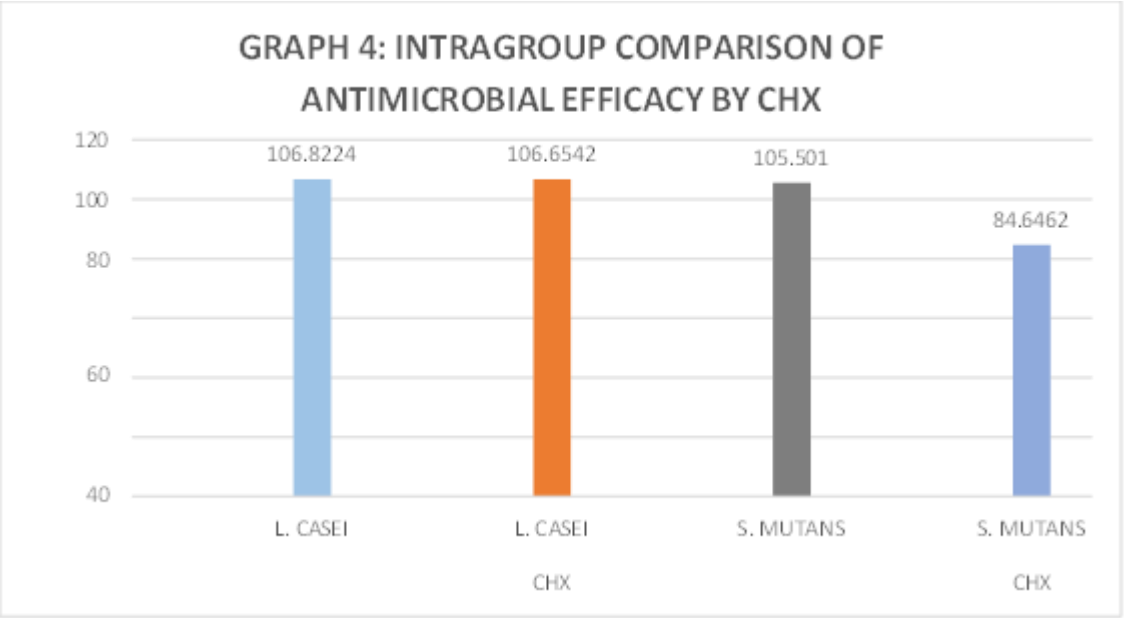
Group	Mean	Std. Deviation	p-value
CHX treated *Lactobacillus*	106.65420	6.436003	

MPHE treated *Lactobacillus*	91.40760	9.728389	0.001
CHX treated *S mutans*	84.64620	10.560136	
MPHE treated *S mutans*	99.00440	19.955394	0.040*









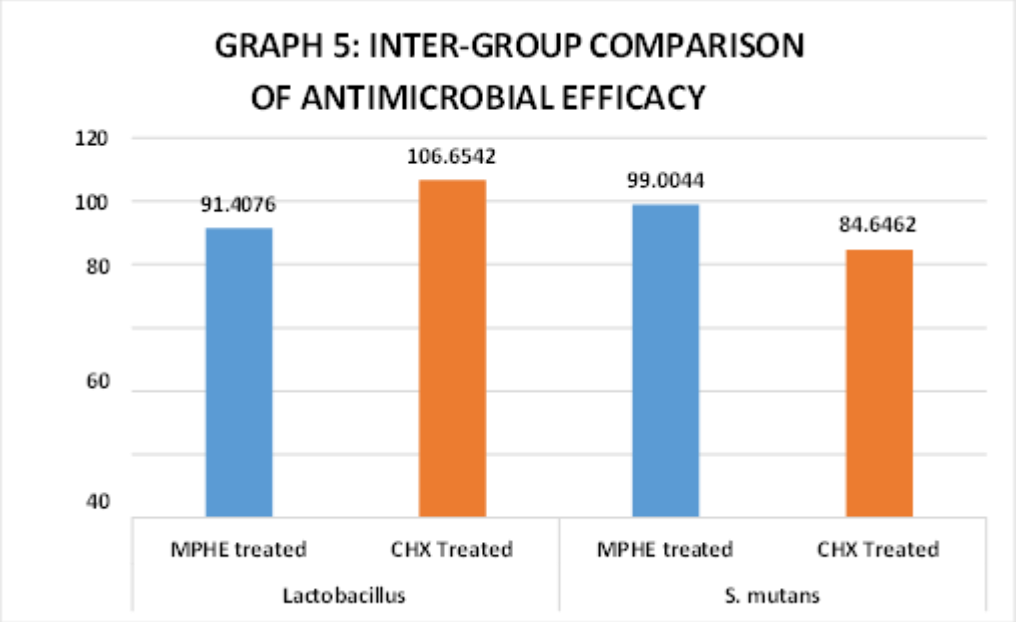


Table I and Graph 1 shows Inter-group comparative analysis of MIC between Streptococcus and Lactobacillus groups.

Comparison of minimum inhibitory concentration of *Streptococcus mutans* and *Lactobacillus casei* were analysed. A statistically significant (p=0.012) difference was observed between the groups with mean values 25.96 ± .93 and 28.85 ± 1.32 in *Streptococcus* and *Lactobacillus* groups respectively and lower mean value observed in *Streptococcus* group.

Table II and Graph 2 shows Inter-group comparative analysis of MBC between Streptococcus and Lactobacillus groups.

Comparison of minimum bactericidal concentration of *Streptococcus mutans* and *Lactobacillus casei* were analysed. A statistically significant (p=0.024) difference was observed between the groups with mean values 27.03 ± 0.72 and 29.21 ± 1.27 in *Streptococcus* and *Lactobacillus* groups respectively and lower mean value observed in *Streptococcus* group

Table III and Graph 3 shows Intragroup Comparison of antimicrobial efficacy in MPHPE group

The effect of Mixed Herbal Powder Extract (MHPE) and Chlorhexidine (CHX) (0.12%) on *Lactobacilli* and *Streptococcus mutans* (*S. mutans*) was analyzed. In the MPHPE group, the mean Lactobacilli count significantly decreased from 106.82 ±3.70 (untreated) to 91.41 ± 9.73 (treated) with a P value of 0.001, indicating

a statistically significant reduction. Similarly, *S. mutans* showed a decrease from 105.50± 9.91 (untreated) to 99.00 ± 19.96 (treated), also with a P value of 0.001, confirming a significant reduction.

Table IV and Graph 4 shows intragroup comparison of antimicrobial efficacy in chx group

The intragroup comparison of antimicrobial efficacy in the Chlorhexidine (CHX) group revealed differing effects on *Lactobacillus* and *S. mutans.* The mean *Lactobacillus* count before treatment was 106.82 ± 3.70, which showed no significant change after CHX treatment (106.65 ± 6.44, p = 1.00), indicating that CHX had minimal effect on *Lactobacillus.* However, the effect of CHX on *S. mutans* was more pronounced. The mean *S. mutans* count before treatment was 105.50 ± 9.91, which significantly decreased to 84.65 ± 10.56 after treatment (p = 0.001), demonstrating strong antimicrobial activity against *S. mutans.*

Table V and Graph 5 shows Intergroup comparison of antimicrobial efficacy

It revealed that a significant difference exists when treated with two different solutions. The mean *Lactobacillus* count when treated with Chlorhexidine (0.12%) (CHX) was 106.65 ±6.43 and when treated with MPHE was 91.41 ± 9.73 and the difference was statistically significant (p=0.001) indicating high MPHE effect on lactobacilli compared to CHX. The mean *Streptococcus* count when treated with Chlorhexidine (0.12%) (CHX) was 84.65 ± 10.56 and when treated with MPHE was 99 ± 19.96 and the difference was statistically significant (p=0.040) and it indicated that CHX has more effect on *S.mutans* when compared to MPHE.

IV. Discussion:

This in-vitro investigation evaluated the antimicrobial potential of a Mixed Herbal Powder Extract (MHPE) composed of Centella asiatica, Terminalia chebula, Terminalia bellerica, and Emblica officinalis. These herbal components were chosen based on their well-documented antibacterial, antioxidant, and anti-inflammatory properties [34]. The study integrates phytochemical extraction, microbiological evaluation, and scanning electron microscopy (SEM) to assess the effectiveness of MHPE against two key oral pathogens: *Streptococcus mutans* and *Lactobacillus casei*.

Selection of Materials and Methods:

The use of organic solvents like methanol and chloroform facilitated efficient extraction of bioactive compounds from plant material. Methanol is widely recognized for its polarity, enabling the extraction of both polar and semi-polar compounds, while chloroform aids in non-polar component isolation. These solvents were combined in different ratios to optimize phytochemical yield. Nitrogen gas was employed to prevent oxidative degradation during processing, and DMSO served as a solvent for dissolving the lyophilized extract due to its biocompatibility and non-interference in microbiological assays.[12]

Microbial cultures of *S. mutans* and *L. casei*, both associated with dental caries progression, were cultivated using Brain Heart Infusion (BHI) agar. Comparative antimicrobial analysis was conducted using chlorhexidine gluconate (CHX), a widely used oral antiseptic with established efficacy against a range of oral microorganisms.[13] SEM analysis was incorporated to visually assess the structural integrity of bacterial cells and biofilms post-treatment.

Minimum Inhibitory Concentration (MIC):

To assess the MIC, standardized bacterial suspensions were exposed to varying concentrations of MHPE. The MIC was determined as the lowest concentration that produced a measurable zone of inhibition. The observed MIC values—25.96 ± 0.93 µg/ml for *S. mutans* and 28.85 ± 1.32 µg/ml for *L. casei*—highlight the extract's potent antimicrobial activity.

These findings are consistent with prior research that underscores the antibacterial effects of Centella asiatica and the Terminalia species. For instance, Kolar et al. demonstrated that several plant-based antimicrobials showed inhibitory effects on Gram-positive pathogens using microdilution assays, affirming the utility of MIC evaluations for natural extracts.[14] In contrast, a study by Tomoyama et al. found higher MIC values for *Staphylococcus* species isolated from orthopedic infections, especially for methicillin-resistant strains, indicating that different clinical isolates may demand higher antimicrobial concentrations.[15] This disparity emphasizes the importance of pathogen-specific studies in evaluating herbal extract efficacy.

Minimum Bactericidal Concentration (MBC):

The MBC test, conducted by serial dilution and subculturing, revealed that MHPE exerted bactericidal effects at concentrations of 27.03 ± 0.72 µg/ml for *S. mutans* and 29.21 ± 1.27 µg/ml for *L. casei*. The slight increase in MBC compared to MIC indicates that while MHPE inhibits bacterial growth at lower concentrations, complete eradication requires marginally higher doses.

A study by Vahabi et al. also supports these findings, reporting significant bactericidal activity of hydroalcoholic plant extracts against oral pathogens using similar methodologies.[16] On the other hand, Kiryakova et al. used broth dilution to determine MICs for *S. mutans* and *S. sanguis*, illustrating methodological differences that influence quantitative outcomes.[17] While our study employed agar-based techniques, broth-based assays might offer a more sensitive measure of antimicrobial potential.

Comparative Efficacy of MHPE and CHX:

The comparative assessment between MHPE and CHX revealed notable differences in strain-specific antimicrobial effects. MHPE demonstrated significantly higher activity against *S. mutans* ($p = 0.040$), while CHX showed superior efficacy against *L. casei* ($p = 0.001$) .[18,19] These results suggest that the polyphenolic and tannin-rich components in MHPE may more effectively disrupt the metabolic processes and adhesion of *S. mutans* biofilms.

Although CHX remains the gold standard in dental antiseptics, its limitations, particularly against biofilm-forming bacteria like *S. mutans*, have been well documented.[20] Previous literature supports this notion; Jiayi et al. observed reduced susceptibility of *S. mutans* biofilms to CHX, suggesting the development of adaptive resistance mechanisms over time . Thus, the inclusion of MHPE could offer a complementary or alternative therapeutic pathway in managing dental caries.

SEM-Based Morphological Assessment:

SEM analysis provided critical visual confirmation of the bactericidal effects observed in MIC and MBC assays. Upon treatment with MHPE, *S. mutans* biofilms exhibited disrupted structural integrity, collapsed cellular membranes, and reduced extracellular matrix, indicating effective biofilm penetration and cellular degradation. These observations are in alignment with a study by AlMatar et al., where green-synthesized silver nanoparticles derived from herbal extracts demonstrated similar disruption in *S. mutans* biofilms.[21]

Interestingly, SEM analysis of *L. casei* post-MHPE treatment showed a mixed response. While biofilm degradation and membrane damage were apparent in some regions, several bacterial cells retained their structural integrity. This implies a relatively reduced susceptibility of *L. casei* to MHPE. Kamal et al. also reported variable effects of polyphenol-rich herbal extracts on *L. casei*, suggesting intrinsic bacterial defense mechanisms that may hinder full susceptibility.[22]

When treated with CHX, *S. mutans* biofilms displayed biofilm disintegration and bacterial membrane damage, though not as extensive as that caused by MHPE. The persistent presence of intact biofilm matrix suggests that CHX may have limited diffusion through dense biofilm layers.[23] In contrast, CHX exhibited a more substantial impact on *L. casei*, with SEM revealing extensive lysis and collapse of cellular structures. This supports previous findings that CHX remains highly effective against *Lactobacillus* strains, although resistance over time remains a concern.[24]

Biofilm Disruption Potential:

Biofilms play a central role in the pathogenesis of dental caries by providing a protective matrix that enhances microbial resistance to antimicrobials. The ability of MHPE to disrupt *S. mutans* biofilms more effectively than CHX is particularly significant. SEM evidence shows considerable disintegration of the extracellular polymeric substance (EPS) matrix, implying that MHPE may enhance the permeability of antimicrobial agents through biofilm layers.

In contrast, CHX, while effective in planktonic cultures and against *L. casei*, demonstrated limited penetration into *S. mutans* biofilms. This phenomenon is consistent with findings from prior studies that emphasize the structural resilience of *S. mutans* biofilms against conventional antimicrobial agents.[1,4,10]

Clinical Implications and Future Directions:

The study highlights the potential of Mixed Herbal Powder Extract (MHPE) as a natural antimicrobial agent in dentistry. It demonstrated efficacy against *Streptococcus mutans* and *Lactobacillus casei*, key pathogens in dental caries. MHPE could be used in oral hygiene products, offering a safer alternative to synthetic chemicals like chlorhexidine, minimizing side effects such as tooth staining. This herbal extract may also help in personalized dental care, providing targeted treatment for patients with high microbial levels, promoting natural, holistic oral health care practices, and reducing reliance on synthetic antibiotics while enhancing sustainability in dental products.

Future research should involve clinical trials to confirm MHPE's effectiveness in human populations and its long-term safety profile. Standardized formulations need development for incorporation into oral care products. Further studies should explore the synergy of MHPE with other antimicrobial agents and its impact on other oral pathogens. Additionally, research into the molecular mechanisms by which MHPE inhibits bacterial growth and biofilm formation could enhance our understanding of its therapeutic potential. Expanding the

spectrum of bacteria tested, including periodontal pathogens, will be critical to assessing MHPE's broader applicability in oral health care.

Limitations:

The study's in vitro nature limits its applicability to real-world clinical scenarios, where complex variables such as oral pH and the microbial community exist. The research focused on only *S. mutans* and *L. casei*, not accounting for other oral pathogens like *P. gingivalis*. Furthermore, while the study determined the minimum inhibitory and bactericidal concentrations, it's unclear if such concentrations can be safely achieved in humans without toxicity. Variability in herbal extract composition could also affect the reproducibility of results. The complexity of biofilms in the oral cavity further complicates the translation of these findings.

V. Conclusion:

The study demonstrates that MHPE, derived from Centella asiatica, Terminalia chebula, Terminalia bellerica, and Emblica officinalis, exhibits significant antimicrobial activity against *S. mutans* and *L. casei*, providing a promising natural alternative to synthetic oral antiseptics. While the extract's effects are promising, further clinical validation is needed to confirm its real-world efficacy and safety. With the potential to reduce microbial resistance and side effects associated with conventional treatments, MHPE could offer a more sustainable, holistic approach to oral health. This extract represents a step toward integrating natural remedies in preventive dental care for long-term oral health benefits.

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