SPECIMEN FORMAT FOR THESES OF THE MONTH

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Title of the thesis	**:**	Unveiling the Anticariogenic Properties of Medicinal Plants and Development of a Polyherbal Dentifrice.
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Abstract within 300 words:

Dental caries, a prevalent multifactorial infection, results from the interaction between acid-producing microorganisms and a diet high in carbohydrates. This study harnesses the potential of a polyherbal remedy that offers a holistic approach to promote overall dental health and combat tooth decay with its natural antibacterial properties. To identify effective oral agents twelve plant extracts, *Achyranthes aspera* root (AAR), *Acalypha indica* leaf (AIL), *Azadirachta indica* leaf (AZL), *Abrus precatorius* leaf (APL), *Barleria cuspidata* leaf (BCL), *Euphorbia hirta* leaf (EHL), *Ficus benghalensis* prop root (FBP), *Piper betle* leaf (PBL), *Psidium guajava* leaf (PGL), *Pongamia pinnata* leaf (PPL), *Tridax procumbens* leaf (TPL), and *Solanum virginianum* fruit (SVF) traditionally used by local tribal populations in Thottakombai Hill, Erode district of Tamil Nadu, were studied. Methanol extracts of AAR, BCL, EHL, FBP, PBL, PGL, PPL, TPL, and SVF demonstrated a higher phytochemical content and exhibited stronger DPPH radical scavenging activity than other plants. Furthermore, clinical plaque isolates, such as *Streptococcus mutans* (SMU), *Streptococcus salivarius* (SSA), *Streptococcus oralis* (SOS), *Streptococcus parasanguinis* (SPSA), *Klebsiella pneumoniae* (KP), *Pseudomonas aeruginosa* (PA), *Acinetobacter baumannii* (AB), and *Candida albicans* (CA) were significantly susceptible to AAR, BCL, EHL, FBP, PGL, and SVF. Hence, further investigation of phytocompounds from these six plants through molecular docking studies revealed a high binding affinity for glucosyltransferase-C, a key virulence factor synthesized by SMU. Consequently, all six plants were optimized for the development of a polyherbal dentifrice (PHDF) in toothpowder tablet form. The PHDF demonstrated low toxicity, and showed the presence of essential minerals proving its safe use. Furthermore, PHDF effectively reduced the pH, and hydrophobicity of SMU, thereby eradicating its biofilm formation and outperforming marketed standards. The network pharmacological approach provided valuable insights into PHDF's mechanism of action, highlighting its potential as a promising natural remedy for the prevention and treatment of dental caries. Thus, this study validates the formulation's strong effect against oral pathogens and suggests a novel, comprehensive approach to oral health care.

i) Major objectives:

1. To assess the phytochemical and antioxidant properties of traditionally used medicinal plants for dental caries.
2. To evaluate the antimicrobial effectiveness of the selected plant extracts against clinical strains of dental plaque-causing pathogens.

3. To identify the potential glucosyltransferase-C inhibitors from the plant extracts against *S. mutans* using molecular docking.
4. To develop and evaluate a novel Polyherbal Dentifrice (PHDF) for dental caries prevention.
5. To assess the *in vitro* antimicrobial efficacy of the formulated PHDF against microorganisms associated with dental caries.
6. To validate the anti-cariogenic effectiveness of PHDF against *S. mutans* to determine targets and mechanisms through network pharmacology

ii) Hypothesis:

The hypothesis set up for the present study is,

- **Null Hypothesis:** The polyherbal dentifrice has no significant effect on the growth or viability of oral pathogens compared to a control group.
- **Alternative Hypothesis:** The polyherbal dentifrice significantly inhibits the growth or viability of oral pathogens compared to a control group.

iii) Methodology:

The root of *Achyranthes aspera* (**AAR**), leaves of *Acalypha indica* (**AIL**), *Azadirachta indica* (**AZL**), *Abrus precatorius* (**APL**), *Barleria cuspidata* (**BCL**), *Euphorbia hirta* **(EHL**), *Piper betle* **(PBL**), *Psidium guajava* (**PGL**), *Pongamia pinnata* (**PPL**), and *Tridax procumbens* (**TPL**), prop root of *Ficus benghalensis* (**FBP**), and fruit of *Solanum virginianum* (**SVF**) were collected in and around Saibaba Colony, Coimbatore, Tamil Nadu, India. The fresh plant specimens were submitted to the Institute of Forest Genetics and Tree Breeding and Botanical Survey of India, Southern Regional Centre, Tamil Nadu Agricultural University Campus, Coimbatore, for authentication with Voucher No: AAR-492/ FRC/ID/FECC/IFGTB/2024, AIL-493/FRC/ID/FECC/IFGTB/2024, APL-501/FRC/ ID/FECC/IF GTB/2024, AZL-495/FR C/ID/FECC/IFGTB/2024, BCL-685/BSI/SRC /5/23/2020, EHL-494/FRC/ID/ FECC/IFGTB/2024, FBP-496/FRC/ID/FECC/ IFGTB/2024, PBL-499/FRC/ID/ FECC/IFGTB/2024, PGL-497/FRC/ID/FECC/IFGTB/2024, PPL- 498/FRC/ID/ FECC/ IFGTB/2024, TPL- 500/FRC/ID/ FECC/IFGTB/2024, SVL- 686/BSI/SRC/5/23/2020.

The collected plant parts were washed in sterile water to remove dust and foreign matter, and then dried in the shade for a few weeks. Then the plant parts were pulverized using an electric blender and kept in sterile glass containers at room temperature for further use. The maceration method was

adopted for the extraction of selected plants. Powdered samples (10g) were weighed and extracted using the polar solvent methanol (1:10) for 24 hours. The resulting solvent-extracted fractions were evaporated to obtain crude extract and stored at 4ºC in an airtight plastic vial.

Phase I

- Methanol extracts from twelve plants were analyzed for Preliminary Phytochemical Screening according to the standard method of Harborne, (1998).
- The quantification of secondary metabolites was carried out for alkaloids, tannins, and terpenoids for the twelve plant extracts.
    - Estimation of Total Alkaloid Content: Ajanal et al. (2012)
    - Estimation of Total Tannin Content: Roghini & Vijayalakshmi, (2018)
    - Estimation of Total Terpenoid Content: Ghorai et al. (2012)
    - Estimation of Total Ascorbic Acid (Vitamin C) Content: Vandervoort & Ludwig, (2002)
- Quantification of Total Antioxidants: The quantification of total antioxidants like phenolics and flavonoids was performed for all twelve plant extracts.
    - Estimation of Total Phenolic Content: Saeed et al. (2012)
    - Estimation of Total Flavonoid Content: Saeed et al. (2012)
- The DPPH (2,2 - DiPhenyl-1-PicrylHydrazyl) Radical Scavenging ability of the twelve plant extracts was assessed based on the method described by Senguttuvan et al. (2014) as presented in Appendix IX.

Antimicrobial Activities of Plant Extracts

- The research was conducted by following the institutional ethical standards. The study on clinical isolates was approved by the Institutional Human Ethical Committee of PSG Pharmacy College (IHEC PSG), Coimbatore, Tamil Nadu with the reference number: PSG/IHEC/2023 Appr/Exp/349 (Appendix X).
- The oral clinical isolates used in this study were obtained from plaque samples of patients visiting PSG Hospital, Coimbatore, Tamil Nadu. The isolates were identified using the matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). A total of eight isolates were used in this study, including *Streptococcus mutans* (SMU), *Streptococcus salivarius* (SSA), *Streptococcus parasanguinis* (SPSA), *Streptococcus oralis* (SOS), *Pseudomonas aeruginosa* (PA), *Klebsiella pneumonia* (KP), *Acinetobacter baumanii* (AB), and *Candida albicans* (CA).

- Oral clinical strains were sub-cultured on BHI (Gram-positive), MHA (Gram-negative), and SDA (fungi), and preserved in LB broth with 20% glycerol for further use (Wijesinghe et al. 2019; Jebashree et al. 2011).
- The Isolate was tested using a well diffusion method (Valgas et al. 2007). The experiment was repeated thrice, and the Inhibition Zone Diameter (IZD) was recorded.
- The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) /Minimum Fungicidal Concentration (MFC) of plant extracts were determined against eight clinical isolates, using broth microdilution in 96-well microtiter plates (Parvekar et al. 2020).
- Biofilm Eradication Potential of Plant Extracts was determined by crystal violet (0.4%) staining (Hayat et al. 2018).

PHASE II

- Retrieval of Active Compounds from Selected Plants: Compounds from six antimicrobial plants were retrieved from IMPPAT and Dr. Duke's databases, screened for drug-likeness (DL $\geq$ 0.18) using MolSoft, and their 3D structures obtained from PubChem for target identification.
- Molecular Docking using GLIDE: Molecular docking was performed using the Schrodinger suite (academic license, Version 2023-1) to predict the binding affinities of the ligand-receptor. The 2D crystal structure of *S. mutans* glucosyltransferase-C (gtfC) was obtained from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB ID: 3AIC) (Friesner et al. 2006; Yuriev et al. 2011).
- Molecular Dynamic Simulation: The previously docked protein-ligand complex structure (PLCS) of the ligand and gtfC was assessed using the Desmond module (Version 7.3) of Schrodinger (Mark & Nilsson, 2001).

PHASE III

- Preparation and Evaluation of Polyherbal Dentifrice (PHDF) Synergistic antibacterial combinations of plant extracts were optimized using MODDE (v13.0) with PLS design, generating 30 combinations. The model identified ratios with low MICs (1.25 mg/mL), which were experimentally validated against *S. mutans* (Mapeka et al. 2024).
- Lyophilized methanolic extracts of six antimicrobial plants were combined with excipients (anti-inflammatory, astringent, analgesic, abrasive, regenerative) selected from literature. The

excipients, extracts, and 10% starch (binder/absorbent) were homogenized and compressed into tablets.

- The resulting polyherbal dentifrice (PHDF) was evaluated for organoleptic, physicochemical, rheological, and anti-cariogenic properties following Ayurvedic Pharmacopeial guidelines.
- PHDF was evaluated for organoleptic properties such as color, odor, taste, texture, and appearance by visual and manual inspection.
- Physicochemical parameters were also assessed, including pH (5 g in 10 mL water using a pH meter), moisture content (loss on drying at 105 °C with LOD apparatus), hardness and thickness (using Monsanto tester and digital caliper), and friability (25 rpm for 4 min, with ≤1% considered acceptable).
- PHDF was assessed for rheological properties including bulk and tapped density, Carr's index, Hausner's ratio, angle of repose (cone method), foamability (foam volume after shaking with water), disintegration (tested in artificial saliva using DT1000), weight variation (20 tablets compared with mean), and stability (stored under varied temperature and humidity for 3 months).
- Cytotoxicity was evaluated using the brine shrimp lethality assay (Artemia salina), where PHDF dilutions (100–1500 µL, 1 mg/mL stock) were tested against 30 nauplii, with mortality recorded up to 24 h and potassium dichromate as a control; data were analyzed using probit regression in SPSS.
- For elemental analysis, acid-digested PHDF samples (HNO_3 :H_2 SO_4 :$HClO_4$, 5:1:1) were subjected to AAS to detect heavy metals (As, Pb, Cd, Hg) and essential minerals (Ca, K, Mg), with each sample analyzed in triplicate and mean values recorded.

PHASE IV

- The anti-cariogenic potential of PHDF was evaluated through multiple assays. Antimicrobial activity was determined by measuring IZD, MIC, and MBC/MFC against clinical isolates, alongside a marketed toothpowder (K.P. Namboodri's) and sodium fluoride as controls.
- The hydrophobic nature of *S. mutans* was assessed using the MATH assay, where bacterial suspensions treated with PHDF (1/8–MIC) were mixed with toluene, and absorbance changes at 600 nm were measured (Prabu et al. 2006).

- Acid production was studied using the glycolytic pH drop assay by treating glucose-containing bacterial suspensions with PHDF and recording pH before inoculation and after 24 h (Prabu et al. 2006).
- Biofilm eradication was tested on polystyrene plates and resin teeth, where *S. mutans* cultures exposed to PHDF (1/8MIC–MIC) were stained, solubilized, and analyzed at 530 nm for inhibition percentage Hayat et al. (2018).
- Morphological alterations in PHDF-treated biofilms were further confirmed using SEM, where treated biofilms grown on glass slides were fixed, dehydrated, sputter-coated, and imaged, with surface coverage quantified using ImageJ software (Wu et al. 2015).

PHASE V

- A network pharmacology approach was applied to screen phytocompounds from PHDF and their targets in dental caries, elucidating mechanisms of action.
- A total of 78 bioactive compounds from six plants were retrieved from PubChem, and their putative human targets were predicted using SuperPRED and Swiss Target Prediction, with duplicates removed and filtered by inference scores.
- Disease-related genes were obtained from GEO, GeneCards, and CTD databases; GEO dataset GSE1629 was analyzed with GEO2R ($p < 0.05$, $logFC \geq 0.5$).
- Overlaps with compound targets were identified using Venny 2.1.0. PPI networks of key targets were generated using STRING (confidence >0.9), and compound–target–pathway interactions were visualized in Cytoscape, with significant interactions ($p \leq 0.05$) mapped onto KEGG pathways.
- Gene ontology (GO) enrichment and KEGG analyses were performed with WebGestalt to clarify the biological roles of PHDF targets.
- Molecular docking was carried out using the Schrödinger suite, where key compounds were docked with MMP3 (PDB ID: 8H78) and CA4 (PDB ID: 5IPZ). Statistical analysis was performed on triplicate data, expressed as mean ± SEM, with Tukey's post hoc test and one-way ANOVA; $p<0.05$ was considered significant.

iv) Findings:

PHASE I

Information about medicinal plants used for treating dental cavities was collected from Thottakombai Hill in the Anthiyur region of Erode district, Tamil Nadu. This data represents

traditional knowledge of plant-based remedies for tooth decay. The selected plants (AAR-*Achyranthes aspera*, AIL-*Acalypha indica*, AZL-*Azadirachta indica*, APL- *Abrus precatorius*, BCL-*Barleria cuspidata*, EHL-*Euphorbia hirta*, FBP-*Ficus benghalensis*, PBL-*Piper betle*, PGL-*Psidium guajava*, PPL-*Pongamia pinnata*, TPL-*Tridax procumbens*, SVF-*Solanum virginianum*) underwent evaluation to assess their potential anticariogenic effects.

Investigation on the qualitative analysis of phytochemical components in the methanol extracts from the chosen plants revealed the presence of various plant-based compounds. The plants *EHL, PBL,* and *PGL* were found to have a greater number of compounds when compared to other plant extracts. Quantification of the highest amount of alkaloids and terpenoids was found to be present in *Tridax procumbens* leaf (52.37±0.011 mg/g and 19.92±0.017 mg/g). The leaves of *Euphorbia hirta, Tridax procumbens, and Barleria cuspidata* exhibited the highest ascorbic acid concentration of 586.6±0.038 mg/g, 370.9±0.300 mg/g, and 309.0±0.007 mg/g, suggesting its potential as a rich source of vitamin C. The leaves of *Psidium guajava*, *Achyranthes aspera*, *Pongamia pinnata,* and *Solanum virginianum* had the greatest phenolic contents (237.7±0.06, 188.66±0.120, 169.43±0.04, 157.50±0.06, 102.74±0.06 mg GAE/g, respectively), while *Piper betle*, *Psidium guajava,* and *Solanum virginianum* fruit had the highest flavonoid contents (228.63±0.08, 201.03±0.121, and 101.912±0.090 mg RE/g, respectively). The extracts of PGL (6.628±6.601 µg/ml), SVF (14.09±5.08 µg/ml), BCL (38.94±0.785 µg/ml), FBP (47.14±20.02 µg/ml), and APL (49.96±0.705) can be classified as extracts with high DPPH° free radical scavenging activity.

The antibacterial screening of the polyherbal crude extracts was performed against *Streptococcus mutans* (SMU), *Streptococcus salivarius* (SSA), *Streptococcus oralis* (SOS), *Streptococcus parasanguinis* (SPSA), *Klebsiella pneumoniae* (KP), *Pseudomonas aeruginosa* (PA), *Acinetobacter baumannii* (AB), *and Candida albicans* (CA). It revealed that all tested bacterial pathogens exhibited a different degree of sensitivity and fluctuated based on the concentration of the extract and the size of the inhibition zone observed for each microorganism tested. Interestingly, the extracts AAR, EHL, PGL, SVF, BCL, FBP, and PBL showed statistically significant ($p<0.0001$) inhibition against the gram-positive isolate *S. mutans* with 24±1.00 mm, 20.3±0.57 mm, 19.6±.20 mm, *and* 18.3±1.52 mm respectively, *S. salivarius* was found to be sensitive towards the extracts SVF, EHL, AAR, PGL, and BCL with 30.3±0.577, 26±4.58, 25.3±0.57, 22.6±1.52, 17.6±1.52 mm zone of inhibition respectively. For *S. parasanguinis*, the

plant extracts EHL, PGL, BCL, and SVF exhibited a strong zone of inhibition of 22.6±1.52mm, 20.3±0.57mm, 20.0 ±1.00mm, 19.3±0.57mm, respectively, and therefore *S. oralis,* the plant extracts PGL, EHL, PBL, AAR, and SVF expressed a 22.6 ±2.52, 21.0±1.00, 20.0±1.00, 19.0±1.00, 18.33±0.57mm zone of inhibition.

The gram-negative bacteria *K. pneumoniae* were significantly sensitive to the extracts, exhibiting the inhibition zone of 18±1.00, 16.6±1.52, 16±1.00, 15.6±0.57, 15.6±0.57 for FBO, SVF, PGL, EHL, and BCL, respectively, while *P. aeruginosa* was resilient to most of the extracts mainly for EHL(27±2.51), PGL(23±2.64), BCL(22.3±3.055), AAR(20.6±1.154), and AIL(20.3±0.577), *A. baumanii* was sensitive to extracts EHL (24±1.00), BCL (23.3±2.08), FBP (23±2.64), PGL (22.6±0.57), AIL (22±1.00), SVF (21.6±1.52), while BCL-(19±1.00), EHL (19±1.00), SVF (19±1.00), and APL (18.3±0.57) exhibited greater inhibition against the fungal strain *C. albicans*. The results of IZD revealed that FBP, EHL, BCL, PGL, and AIL were effective against gram-negative isolates, and the fungal isolate was found to be sensitive towards the extracts BCL, EHL, APL, and SVF.

The minimal inhibitory concentration (MIC) and minimum bactericidal concentration/minimum fungicidal concentration (MBC/MFC) were analysed using microbroth dilution method. Of the eight types of oral bacteria examined, Gram-positive *S. mutans, S. salivarius, S. parasanguinis,* and *S. oralis* were the most susceptible to AAR, BCL, EH, FBP, PG, and SVF which showed minimum inhibitory concentrations of 0.625-5 mg/mL, while the MBC ranged 1.25-10 mg/mL for the same. Gram-negative isolates *P. aeruginosa, K. pneumonieae,* and *A. baumanii* were more sensitive towards the plant extracts EHL, PBL, PGL, and SVF when compared to the other plant extracts with the MIC range of 0.3125-2.5 mg/mL. For *C. albicans*, the MIC value of 1.25mg/mL was noted in the plant extracts EHL, FBP, and PBL followed by the lowest MFC value of 2.5 mg/mL.

The minimum biofilm eradication of the plant extracts AAR, AP, BCL, EH, FBP, PG, and SVF at the MIC level varied from 47.9 % to 94.9 %. The plant extracts EH, SVF, and AAR showed significant ($p<0.001$) results exhibiting the biofilm inhibition at the MIC level against gram-positive agents, SMU, SSA, SPSA, and SOS. EHL could inhibit SMU and SSA biofilm formation, displaying 90% & 94.4 % followed by SVF (87% & 93.2%) and AAR (88.4% & 89.2%), respectively. In contrast, extracts BCL, EH, FBP, and PG demonstrated a significant ($p<0.001$; $p<0.005$)) inhibitory effect of 86.2%- 93.6% at the MIC level (0.002-0.012 mg/ml)

against gram-negative agents KP, PA, and AB. The extracts EHL and BCL revealed promising inhibition of 84.5% and 81.2% respectively, significantly ($p<0.001$) against the fungal pathogen CA.

PHASE II

The docking studies and molecular dynamics (MD) simulations were carried out for the major bioactive compounds reported from six potential plants (AAR, BCL, EHL, FBP, PGL, SVF) to assess their potential in inhibiting gtfC protein (Glucosyltransferase C), which is associated with the *Streptococcus mutans* (SMU) involved in dental caries. 508 bioactive compounds were initially retrieved, of which 78 compounds were selected using a drug-likeness (DL) score for docking studies. The compounds, Guavin A, stachyurin, Guavin C, Guavin D, Amritoside, 10R, Casuaritcin, Isostrictinin, Guavin B, Procyanidin B3, Tellimargrandin-1, Quercetin-3-O-Gentiobioside, Procyanidin B1, Strictinin, Procyanidin B2, Leucocyanidin, and Goreishic acid from PGL displayed a highest docking score of -14.534, -13.44, -13.388, -11.5203, -11.15, -10.938, -10.862, -9.862, -9.827, -9.606, -9.324, -8.802, -8.736, -8.645, -8.027, and -7.041 kcal/mol, respectively, towards gtfC.

Pentagalloylglucose, 2,4,6-Tri-O-galloyl beta-glucose, Myricitrin, and Kaempferol-3-Glucuronide from EHL produced a strong binding affinity of -11.8495, -11.654, -7.88509, and -7.71528 kcal/mol. The SVF compounds, Solasonine, Esculentin, and Matenoside exhibited a docking score of -9.618, -8.251, and -7.194 kcal/mol, respectively. The compounds Proanthocyanidin and 25(S)Ionokesterone, derived from AAR showed a docking score of -8.304 and -7.456 kcal/mol, respectively, followed by Luteolin-7-O-glucoside with -7.984 kcal/mol from BCL, which exhibited a high binding affinity towards the gtfC of *S. mutans*.

Among these, Guavin A from PGL was further evaluated for stability using an MD simulation study, the RMSD graph revealed stability for 80 ns during the 100 ns simulation period. Guavin A interacted with critical amino acids in the gtfC protein, specifically ARG425, ARG538 (chain B), ARG425, ARG538, and LYS549 (chain F) through ionic bonds, hydrogen bonds, and hydrophobic interactions. The ligand properties confirmed stable and intact interactions, indicating Guavin A’s strong potential to inhibit SMU and thereby prevent dental

caries. This suggests Guavin A is a promising candidate for further exploration in dental caries prevention through gtfC inhibition.

PHASE III

The six plants, namely, AAR, BCL, EHL, FBP, PGL, and SVF, were combined in various proportions in the MODDE software. A total of 30 experimental runs using a randomized factorial design were conducted to evaluate combinations of six plant extracts. The PLS model was constructed with an R2 value of 0.945, which signified a high variation in the response (MIC), and a strong fit between the data and the model. The Minimum Inhibitory Concentration (MIC) values varied from 0.3125 to 5 mg/ml and the PLS model was developed with an R^2 value of 0.947, indicating a strong fit between the experimental data and the model predictions.

Selected combinations with a low MIC value of 0.3125 mg/ml against SMU were identified for optimal anti-microbial activity and chosen in the development of the formulation. The optimized MIC levels for these plant extracts showed a notable synergistic effect, enhancing their collective antimicrobial efficacy. Pre- and post-compression evaluations showed that the formulated polyherbal dentifrice (Toothpowder tablet), identified as a PHDF, exhibited good mechanical strength with minimal hardness.

The Atomic Absorption Spectrometric analysis of the formulated PHDF has no such heavy metals as arsenic, cadmium, lead, and mercury, which were found to be present and contained adequate amounts of essential minerals like calcium (7867 mg/kg) and potassium (8209 mg/kg), each contributing to oral hygiene maintenance in various ways. Mortality rate data for the brine shrimp larvae at various observation intervals and concentration levels of PHDF showed that PHDF was less toxic in lower and higher concentrations, with 17% mortality at a 1.5 mg/ml concentration, which indicates that PHDF was considered a safe drug for therapeutic uses. The tablets are designed to release active ingredients upon chewing, facilitating their use for oral hygiene by brushing.

PHASE IV

The evaluation of antimicrobial efficacy, the results for the zone of inhibition of formulated polyherbal dentifrice against tested clinical isolates revealed notable differences in effectiveness. The PHDF achieved substantial inhibition zones of 21.6±1.15mm for *S. mutans,* 21.0 ±1.00 mm for *S. salivarius,* 21.1±0.57 mm for *S. parasanguinis,* 21.6±1.52 mm for S. *oralis*, at 150 µg/ml,

indicating strong antibacterial activity against the *Streptococcus sp*. The Gram-negative isolates, such as *K. pneumoniae*, *P. aeruginosa,* and *A. baumannii,* expressed a ZOI of 22.0±0.00 mm, 23.6±2.3 mm, and 23.6±2.88mm for PHDF, respectively. Likewise, the fungal pathogen *C. albicans* exhibited a 20.6±0.57mm ZOI. The ZOI results expressed impartial inhibition against all the tested clinical isolates, demonstrating effective prevention of bacterial growth.

The results for the PHDF efficacy against eight pathogens are presented with MIC values ranging from 0.3125 to 1.25 mg/mL and MBC/MFC values ranging from 0.625 to 2.5 mg/mL. A decrease in the hydrophobicity index indicated the downregulation of bacterial adhesion to the enamel. Liability of PHDF at 1/8 MIC, 1/4 MIC, 1/2 MIC, and MIC significantly decreased the hydrophobic indices of the *S. mutans* from 66.7%, 64.3%, 50.5%, 28.5%, and 12.4%, which is slightly effective than the standard NaF (30.8%) at the MIC level with a significance range ($P<0.0001$). The activity of PHDF on different MIC values (1/8 MIC, 1/4 MIC, 1/2 MIC, and MIC) on the production of acid by *S. mutans* was examined. The initial pH of all MIC values ranged from 7.3 to 7.5. The pH of the negative control after 24h decreased to 4.0, indicating the acid production of *S. mutans*. PHDF at MIC showed perpetual pH 7.0 even after 24h of incubation, referring to the hindrance of acid production of the cariogenic.

A higher dosage (MIC) of PHDF significantly inhibited 94.87% of the biofilm. The results reported that the 1/2 MIC dose of PHDF eradicated 76.87% and half (50%) of the biofilm formed by the cariogenic *S. mutans,* and the initial doses of 1/8 MIC and 1/4 MIC were able to exterminate only 29.05% and 39.52% of the biofilm, respectively. In addition, the impact of PHDF on the degradation of biofilm was identical to the result obtained with the NaF-positive control, with 91% activity, regardless of the concentration, with $P<0.0001$ significance. The Polyherbal toothpowder tablets are considered a viable alternative to toothpaste for improving oral hygiene, combining effectiveness with ease of use.

The biofilm morphological changes observed by SEM expressed biofilm aggregation in the control sample, after 24h of anaerobic incubation. While the treatment groups, NaF and PHDF, portray the disruption of bacterial biofilms. The formulated polyherbal toothpowder tablets were found to be a better alternative to toothpaste to improve oral hygiene. The formulated polyherbal dentifrice (Tooth Tabs) was found to be more effective against major cariogenic microbes than the marketed product, indicating superior performance in preventing dental caries. From the above results, it was evident that formulated polyherbal toothpowder tablets prevent and cure primary

caries by counteracting the growth of caries-causing agents and biofilm. Based on the results from our *in vitro* studies, the bioactive compounds reported in the six plants AAR, BCL, EHL, FBP, PGL, and SVF exhibited a better antimicrobial efficacy towards cariogenic organisms that were included in the formulation and were selected to examine how the polyherbal and its bioactive components influence dental caries through a network pharmacology approach.

PHASE V

In addition, we explored the interaction and functional analysis of the target genes involved in dental caries. The potential 78 compounds were considered the key active compounds responsible for the strong antibacterial activity. To identify the potential interactions of the components, target fishing was done to screen for effective targets (Homosapiens) for all 78 compounds responsible for dental cavities using the SuperPRED 2022 database, the Swiss Target Prediction database, and the disease databases, namely Malacards, Comparative Toxicogenomics Database (CTD), and Gene Expression Omnibus Database (GEO). Based on the gift and influence score, the targets were filtered, and about 24779 targets were retrieved from Mala cards (56), CTD (14373), and GEO (13298) databases related to dental caries. Once the targets were retrieved from these individual databases, we wanted to identify the key targets common to all these databases. Hence, the targets were integrated using the Venny tool, which identified 9 key targets.

A PPI (Protein-Protein Interaction) network analysis revealed that 9 major proteins were highly interconnected by 29 edges, indicating significant interactions between them. A total of 78 active compounds were linked to 9 putative targets, forming 516 edges, suggesting these compounds interact with multiple biological targets. The overall network analysis revealed 105 nodes and 596 edges, showing a complex interconnected system of active compounds, targets, and pathways involved in dental caries.

These findings suggest that the active biochemical compounds in the formulation may synergistically influence the identified targets involved in dental caries. The pharmacological network analysis reveals that the active compounds in the PHDF target multiple proteins and pathways, creating a “compound-target-pathway” network. This multi-target approach is crucial for its effectiveness. The Polyherbal toothpowder tablets offer a multi-component, multi-target, and multi-pathway strategy to combat primary dental caries. This broad approach is expected to be effective in preventing dental cavities and related conditions. This research highlights the

importance of integrating traditional knowledge with modern scientific approaches to develop effective, natural, and environmentally friendly solutions for oral healthcare.

The medicinal plants utilized in this research exhibited a high phytochemical profile and strong effectiveness against cavity-causing microbes isolated from clinical oral samples. The active biochemical compounds in the formulation may synergistically influence the identified targets involved in dental caries. The Polyherbal toothpowder tablets offer a multi-component, multi-target, and multi-pathway strategy to combat primary dental caries. This broad approach is expected to be effective in preventing dental cavities and related conditions.

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