3.0 MATERIALS AND METHODS

This section displays the materials and methods adopted to develop and prove the anti-cariogenic efficacy of polyherbal toothpowder tablets against oral clinical isolates. The study was carried out five phases.

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PHASE I

3.1. Collection and Authentication of Plant Samples

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/IFGTB/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 (Appendix I).

3.2. Preparation and Extraction of Selected Plants

The collected plant parts were washed in sterile water to remove dust and foreign matter and dried under 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.

3.3. Preliminary Phytochemical Screening

Methanol extracts from twelve plants were analyzed for phytocompounds such as alkaloids, flavonoids, saponins, steroids, tannins, glycosides, terpenoids, starch, cellulose, oil and fat, proteins, carbohydrates, volatile oils, resins, vitamin C, catechins, anthraquinones, and coumarins, according to the standard method of Harborne, (1998) as presented in Appendix II.

3.4. Quantification of Phytoconstituents

The quantification of secondary metabolites was carried out for alkaloids, tannins, and terpenoids for the twelve plant extracts.

3.4.1 Estimation of Total Alkaloid Content

The determination of total alkaloid content was performed by a simple spectrophotometric method by Ajanal et al. (2012) in Appendix III.

3.4.2 Estimation of Total Tannin Content

The quantification of total tannin content in the extracted plant samples was performed based on the method of Roghini & Vijayalakshmi, (2018), in Appendix IV.

3.4.3 Estimation of Total Terpenoid Content

The estimation of total terpenoids was done spectrophotometrically using the method of Ghorai et al. (2012) in Appendix V.

3.4.4 Estimation of Total Ascorbic Acid (Vitamin C) Content

The Vitamin C content was estimated using the standard method of Vandervoort & Ludwig, (2002) in Appendix VI.

3.5. Quantification of Total Antioxidants

The quantification of total antioxidants like phenolics and flavonoids was performed for all twelve plant extracts.

3.5.1 Estimation of Total Phenolic Content

The total phenolic content was quantified by following the method of Saeed et al. (2012) and expressed in milligrams of gallic acid equivalents (GAE) per gram of dried extract, as given in Appendix VII.

3.5.2 Estimation of Total Flavonoid Content

The total flavonoids were expressed as milligrams of rutin equivalents per gram and estimated using the method of Saeed et al. (2012) as in Appendix VIII.

3.6. Estimation of Antioxidant Activity

3.6.1 DPPH (2,2 - DiPhenyl-1-PicrylHydrazyl) Radical Scavenging Assay

The DPPH 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.

3.7. Antimicrobial Activities of Plant Extracts

3.7.1 Ethical Statement

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).

3.7.2 Identification of Cariogenic Isolates from Plaque Samples

The oral clinical isolates used in this 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).

3.7.3 Microbial Culture Media Preparation and Maintenance

Oral clinical strains were sub-cultured on brain heart infusion (BHI) for Gram-positive isolates, Mueller Hinton agar (MHA) for Gram-negative isolates, and Sabroud's dextrose agar (SDA) for fungi. The agar plates were incubated at 37°C for 48 h and purity of the colonies was checked by gram staining. All the isolates were stored in Luria Bertani (LB) broth (pH 5.4) supplemented with 20% glycerol (pH 7) for further study (Wijesinghe et al. 2019; Jebashree et al. 2011).

3.7.4 Antimicrobial Activity of Plant Extracts on Clinical Cariogenic Isolates

The ability of the methanolic plant extracts to inhibit the growth of four gram-positive isolates, three gram-negative isolates, and a fungal isolate was tested using a well diffusion method. Bacterial suspensions (108 CFU/mL) were lawn cultured on a specific agar medium. A 10mm diameter well was punched out with a sterilized cork borer and filled with 25, 50, 100, and 150 µL of plant extracts at 10mg/mL concentration, and then incubated at 37ºC for 24h, followed by 30 minutes of refrigeration. Sodium fluoride (NaF) of 10mg/mL was served as a positive standard, and dimethyl sulfoxide (DMSO) as a negative control

(Valgas et al. 2007). The experiment was repeated thrice, and the Inhibition Zone Diameter (IZD) was recorded.

3.7.5 Determination of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) /Minimum Fungicidal Concentration (MFC)

The MIC and MBC of plant extracts were determined against eight clinical isolates, using broth microdilution in 96-well microtiter plates. Plant extracts (10 mg/ml) were prepared in sterile distilled water with 5% [v/v] DMSO, filter-sterilized, and serially diluted (final concentrations- 10, 5, 2.5, 1.25, 0.625, 0.3125mg/ml) in 100µl of BHI broth supplemented with 0.5% glucose in 96-well polystyrene plates. Then, 100µl of fresh bacterial suspensions (10^7CFU/ml), were inoculated in each well. The wells without bacterial suspension and plant samples served as controls. The plates were kept at incubation for 24h at 37°C and the bacterial growth inhibition was measured by ELISA reader at 630nm. MIC values were analyzed using the resazurin dye indicator that changes color in the presence of bacterial culture at varied concentrations. To identify the MBC of samples, 10µl of the mixture from MIC wells with no growth indicated were recovered and cultured on BHI agar plates followed by incubation for 48h at 37°C. The well with the lowest concentration at which no microbial growth is observed is denoted as MBC. Each experiment was repeated thrice using independent cultures (Parvekar et al. 2020).

3.7.6 Biofilm Eradication Potential of Plant Extracts

Bacterial biofilm was made to form 24 hours before adding plant extracts at MIC to determine its eradication ability. Identified clinical strains (100µl) mentioned in Section **3.7.2** with a concentration of 10^6 CFU/ml were added to each well of a 96-well microtiter plate and incubated at 37°C for 24h facilitating cell attachment and biofilm formation. After 24h, 100µl of each plant extract, sodium fluoride (MIC value) as a positive control, and DMSO as the negative control contained 200µl LB broth was added. Blank wells contained 200µl of LB broth devoid of any bacterial culture. All the treated plates were incubated for 24h and inhibition of bacterial biofilm formation was determined by crystal violet (0.4%) staining. Each assay was performed in triplicate (Hayat et al. 2018) and

biofilm inhibitory percentages were calculated as,

$$Biofilm\ inhibitory\ rate\ (\%) = \frac{Control\ OD - Treated\ OD}{Control\ OD} X\ 100$$

PHASE II

3.8 Molecular Docking

Phase II of the study involved the *in-silico* identification of potential *S. mutans* glucosyltransferase-C inhibitors from the bioactive compounds in selected plant extracts and molecular dynamic simulation of the top-hit compound.

3.8.1 Retrieval of Active Compounds from Selected Plants

The databases IMPPAT (A curated database of Indian Medicinal Plants, Phytochemistry, and Therapeutics), and Dr. Duke's Phytochemical and Ethnobotanical databases were used to collect all compounds from the six potential antimicrobial plants. Collected compounds were screened based on the Drug-likeness (DL≥0.18) score using MolSoft software. The PubChem Database is an online platform for drug-target elucidation, which was used to retrieve 3D molecular structures of the active compounds for drug-target identification.

3.8.2 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). Protein preparation starts by removing water molecules beyond 5Å from heteroatoms, eliminating hydrogens, co-solvents, and existing lead components, followed by selecting respective force fields (OPLS4) and root mean square deviation (RMSD) charges (0.30 Å). Using the receptor grid generation tool, a grid was generated around the active sites with X, Y, and Z coordinates of 10Å (Jacobson et al. 2004). The SDF format of ligand chemical structures of the filtered 78 compounds was downloaded from PubChem and processed with the Ligprep module of the Schrodinger suite. The ligands were neutralized, and desalted, and electron ionization was performed. Molecular

docking was carried out using GLIDE (Grid-based Ligand Docking with Energetics). GLIDE identifies the potential possible ligand locations within the active site region of the receptor. The protein grid file was uploaded as a zip document along with the ligand output file. Docking was performed using Xtra Precision (XP) mode, and high-scoring outputs were analyzed with interaction diagrams to determine the amino acid residues involved (Friesner et al. 2006; Yuriev et al. 2011).

3.8.3 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, to provide receptor-ligand interactions insights under specified thermodynamic conditions (temperature, volume, density, and pressure) for the duration of 100ns. The simulation protocol involved the TIP3P water model solvation complexes, the addition of ions for neutralization, the removal of overlapping water molecules, and the creation of an orthorhombic water boundary box (10Å) (Mark & Nilsson, 2001). Energy minimization was executed at a 1000KJ/mol/nm tolerance. Equilibration of the system was achieved using NVT and NPT ensembles for 100ps. The OPLSE4 force field was employed to develop an MD system for a simulation and performed unrestricted production runs for 100ns simulation time. The various parameters of MDS were analyzed, such as protein- root mean square deviation (P-RMSD), root mean square fluctuation (RMSF), PL contacts, secondary structure elements (SSE), and torsion tree to evaluate stability, compactness, structural fluctuations, and PL interactions in the solvated system.

PHASE III

3.9 Preparation and Evaluation of Polyherbal Dentifrice (PHDF)

3.9.1 Design of Experiments (DOE) Modeling for Synergistic Antibacterial Combinations of Plant Extracts

MODDE (Umetrics Version 13.0) software was utilized to determine the input parameters to achieve desired outcomes. The plant extracts served as six factors with MIC units ranging from 0.3125 to 5 mg/ml. The response (MIC) ranged from 0.0125 to 5 mg/ml with a target MIC value of 1.25 mg/ml. A

randomized control design IV was applied, and the model was fitted with partial least squares (PLS) analysis, by deleting non-significant terms. A total of 30 optimized experimental runs with different ratios of plant extracts were obtained from the design. The worksheet containing plant mixture designs and response models was generated to validate the best model fit. Contour plots illustrated optimal extract combinations that achieved low MIC values. The validity of the model was experimentally confirmed by determining the MIC values against *Streptococcus mutans* (SMU) (Mapeka et al. 2024).

3.9.2 Preparation of Polyherbal Dentifrice

The selected plant methanolic extracts were lyophilized to remove the fluid particles, and an effective combination of the six antimicrobial plants was further selected for polyherbal tooth powder formulation. The excipients that possessed major properties of toothpowder like anti-inflammatory, astringent, analgesic, abrasiveness, and regenerative were selected based on the literature and purchased from the local market were cleaned, dried, and powdered. The excipients, lyophilized antimicrobial agents, and 10% of starch used as a binding agent and an absorbent were mixed homogeneously and compressed into tablets. The prepared polyherbal dentifrice (PHDF) underwent evaluations for organoleptic, physicochemical, rheological, and anti-cariogenic activities according to Ayurvedic Pharmacopeial guidelines.

3.9.3 Evaluation of Polyherbal Dentifrice

3.9.3.1 Organoleptic Evaluation

The powder form of PHDF was examined for its organoleptic and morphological properties like color, odor, texture, and appearance. The color of the tooth powder was visually assessed under normal lighting conditions and odor was determined by smell. Taste, texture, and appearance were evaluated manually.

3.9.3.2 Physiochemical Evaluation

The assessment of the pH, moisture content, hardness, thickness, friability, and quantity of inorganic substances in the herbal tooth powder was conducted for its physical and chemical characteristics.

pH

The pH of a formulated PHDF was determined using a pH meter. A 5g sample was added to a 100 ml beaker followed by adding 10 ml of boiled water, cooled, and the pH of the vigorously stirred suspension was measured.

Moisture Content

The moisture content (loss on drying) is a metric used to determine the amount of water and volatile matter present in PHDF dried under specific conditions. The tooth powder tablet (500 mg) was weighed and then dried in an electronic LOD (helium lamp) apparatus at 105ºC for 5 min. Reduction in the weight after treatment was calculated by the given formula.

$$Moisture\ content = \frac{Weight\ \ of\ original\ powder - Weight\ of\ \ dried\ powder}{Weight\ of\ \ original\ powder}\ X\ 100$$

Hardness of PHDF

The tensile strength of the tablet refers to its hardness (Kg/cm^2) and the force needed to break the tablet through manual compression was measured using a Monsanto harness tester.

Thickness of PHDF

The thickness of each tablet is measured using a digital caliper, which provides information about the tablet variation and facilitates efficient dissolution, comfort, and ease of use.

Friability

Friability is a measure of the tendency of the tablet to break or crumble under mechanical strength. The friability of drugs became determined by way of the usage of the iNWEKA Friability Tester. The initial weight of twenty tablets was taken and introduced into a friability tester that was subjected to 25 rpm for 4 minutes (or runs 100 revolutions). The friability ≤1% is considered acceptable. The final weight was recorded and percentage friability was calculated by,

$$Friability = \frac{Initial\ mass - Final\ mass}{Initial\ mass}\ X\ 100$$

3.9.3.3 Rheological Evaluation

Physical parameters like bulk density, tapped density, foaming test, and angle of repose were observed and calculated for the formulation.

Angle of Repose

A funnel was secured with a clamp and placed above a sheet of graph paper at a particular height. Fifty grams of PHDF powder is slowly poured into the funnel, forming a cone-shaped pile. When the powder peak touched the funnel tip, the maximum angle of the formed heap was measured. The flow property was calculated using the formula:

$$Tan\ \theta = \frac{Height}{Radius}$$

Bulk Density

In a 50 ml graduated measuring cylinder, 20g of PHDF powder was accurately measured and slightly poured. To equalize the upper surface of the powder, the cylinder was slightly dropped at the bottom from a height of 1 inch, and the volume occupied by PHDF was measured and calculated using the formula:

$$Bulk\ Density\ (g/mL) = \frac{Mass\ of\ Granules}{Bulk\ Volume}$$

Tapped Density

20 gm of PHDF was accurately weighed and transferred into a 50 mL graduated measuring cylinder. The granules were tapped mechanically several times by dropping the cylinder from a height of about 2cm. The final volume occupied by PHDF and the tapped density was calculated as:

$$Tapped\ Density = \frac{Total\ weight\ measured}{Tapped\ volume}$$

Carr's index

The compressibility index is directly related to flow rate, cohesiveness, and particle size, and was calculated using the following equation:

$$Carr's\ index = \frac{Tapped\ density - Bulk\ density}{Tapped\ density}$$

Hausner's Ratio

Hausner's ratio signifies the powder flow property and the ratio of the tapped to the bulk density of the powder or granules. It was calculated as:

$$Hausner's\ ratio = \frac{Tapped\ density}{Bulk\ density}$$

Foamability

The foaming index of the toothpowder was assessed by mixing 2 grams with water in a measuring cylinder. The initial volume (V_1) was recorded, then the mixture was shaken ten times and the final foam volume (V_2) was noted.

$$Foamability = V1 - V2$$

V_1=Initial volume of water

V_2=Final volume with foam

Disintegration test

Time taken for breaking down of the tablet into granules or primary powder particles is noticed using disintegration test. The tablet was introduced into the LABINDIA DT1000 tablet disintegration tester. It is assembled with a basket rack and open-ended six transparent tubes attached to a wire screen. The tablets were placed in each tube and the basket was mechanically moved up and down in artificial saliva at a rate of 29 to 32 cycles per minute. The wire screen consistently remains below the fluid level. For standard release tablets, disintegration occurs within 15 minutes.

Weight Variation Test

The weight uniformity test for the tablet is performed by randomly selecting 20 tablets and individually weighing them. The weight of all the tablets is then compared to the average weight to ensure consistency.

$$Weight\ Deviation\ (\%) = \frac{Individual\ weight - Average\ weight}{Average\ weight}$$

Stability Check for PHDF

The stability study was performed as per Ayurvedic Pharmacopieal guidelines. The formulated toothpowder tablet was stored at different temperatures (04±0.5ºC, 25±0.2ºC,30± 2.0ºC) and humidity conditions (0, 60±5%, 65±5% RH) for three months and the changes were noted for appearance, pH, and hardness.

3.9.4 Cytotoxicity Evaluation of PHDF

The cytotoxicity of PHDF was assessed using *Artemia salina* (nauplii) hatched in saline solution. Different volumes (100, 250, 500, 1000, and 1500 µl) of PHDF were diluted in distilled water to prepare a 1 mg/ml stock. A total of 30

shrimps were added to the solution (25mL) and the movement and mortality of the shrimps were monitored at different intervals (1,2,4,6, and 24 hours) using a magnifying lens. The same was followed for negative blank and positive control potassium dichromate (1mg/mL). After 24 h, the mortality rate of the shrimps was calculated through regression probit analysis using SPSS statistical software to measure the toxicity of the extract (Olowa & Nuneza, 2013).

$$Death\ (\%) = \frac{No.of\ Dead\ nauplii}{No.of\ Dead\ nauplii + No.of\ Live\ nauplii} X\ 1005$$

3.9.5 Atomic Absorption Spectrometric Analysis of PHDF

The formulated PHDF was treated for acid digestion, resulting in heavy metal extraction. The acid mixture of nitric acid (HNO_3, 70%), sulphuric acid (H_2SO_4, 65%), and perchloric acid ($HCLO_4$) was prepared in a ratio of 5:1:1. Dried samples were treated with the acid mixture solution and incubated for 10 to 24h at 80°C till a clear, transparent solution was obtained. The supernatant was filtered and separated from the solid residues of the digested samples using Whatman No.39 filter paper at room temperature. Each sample was analyzed thrice to obtain a mean value. Quantitative estimation was analyzed using Atomic Absorption Spectroscopy (AAS) for five heavy metals (Arsenic (As), Lead (Pb), Cadmium (Cd), and Mercury (Hg)) and essential minerals (Calcium (Ca), Potassium (K), Magnesium (Mg)).

PHASE IV

3.10 Anticariogenic Efficacy of PHDF

3.10.1 Determination of IZD, MIC, MBC/MFC

The antimicrobial activity of PHDF, marketed standard (K.P. Namboodri's Toothpowder) and sodium fluoride (NaF) was carried out as previously described in Sections **3.7.4** and **3.7.5.** for all the tested clinical isolates.

3.10.2 Effect of PHDF on the Hydrophobic Nature of *S. mutans*

The Hydrophobic nature of *S. mutans* was evaluated using the microbial adhesion test to hydrocarbons (MATH) (Prabu et al. 2006). *S. mutans* grown in a BHI medium were treated with various PHDF concentrations (1/8 MIC, 1/4 MIC, 1/2 MIC, and MIC), washed three times, and suspended in 3 ml of normal saline

water to achieve an optical density value of 0.300 at 600 nm. Added 0.25 ml of toluene and vortexed for 2-3 mins and equilibrated at room temperature for 10 mins. After toluene and aqueous phase separation, aqueous phase absorbance was measured spectrophotometrically at 600 nm. Strains with a hydrophobic index above 70% were arbitrarily classified as hydrophobic for the isolate *S. mutans.* The hydrophobicity index (%) was calculated using the formula.

$$Hydrophobic\ Index\ (\%) = \frac{Abs.\ Initial - Abs.\ Final}{Abs.Initial} X100$$

Where, Abs = Absorbance.

3.10.3 Effect of PHDF on Glycolytic pH Drop

The acid produced by *S. mutans* was evaluated using a glycolytic pH drop assay (Prabu et al. 2006). PHDF at different MIC values (1/8 MIC, 1/4 MIC, 1/2 MIC, and MIC) was added to the bacterial suspension containing 1% glucose. The initial pH of the bacterial suspension containing the samples was measured before inoculation and after 24 hours of inoculation.

3.10.4 Effect of PHDF on Biofilm Eradication Assay

The eradication potential of PHDF on the bacterial biofilm formed on the polystyrene plate and resin teeth model was estimated according to Hayat et al. (2018). *S. mutans* (5×10^5 CFU/mL) was added into 5 mL of BHI broth with 0.1% sucrose and aerobically incubated at 37°C. Cultures were then treated with or without PHDF (1/8 MIC, 1/4 MIC, 1/2 MIC, and MIC) on resin teeth and 96-well polystyreness plates, incubated at 37°C for 24 h under aerobic conditions. Washed the biofilms formed on the plate and resin teeth with distilled water and treated with 0.4% crystal violet and 0.1% safranin stain respectively for 30s. The stained biofilms were solubilized with 30% (v/v) acetic acid and read at 530 nm. Biofilms were observed, and the inhibitory rate was calculated as follows,

$$Biofilm\ inhibitory\ rate\ (\%) = \frac{Control\ OD - Treated\ OD}{Control\ OD} X100$$

3.10.5 Scanning Electron Microscopic (SEM) Analysis of PHDF Treated *S. mutans* Biofilm

Morphological changes in the PHDF-treated biofilm were observed under

scanning electron microscopy (SEM) as previously mentioned by (Wu et al. 2015). Biofilms of *S. mutans* were grown on glass slides on a 12-multiwell plate and incubated anaerobically at 37ºC for 24h. The slides were then gently washed with phosphate-buffered saline, fixed overnight with 2.5% glutaraldehyde, dehydrated in graded ethanol solutions (10%, 25%, 50%, 75%, and 100%) for 15 min, dried, and sputter-coated with gold palladium before observation. SE mode was followed for evaluating SEM images, and the scanning parameter was set at 20.00 kV. A PHDF-treated biofilm was subjected to a scanning electron microscope at 5.0 kV, high magnification, and 500 nm resolution to evaluate the morphological characteristics and surface structures of *S. mutans* biofilms. The bacterial biofilm formation on the surface was quantified using ImageJ software. Histogram of the image was calculated to determine the biofilm area represented by red pixels. Percentage of the area covered by the biofilm on the glass slide was calculated as follows

$$Remainiig\ Area\ Biofilm\ (\%) = \frac{Biofilm\ area}{Total\ surface\ area} X100$$

PHASE V

3.11 Network Pharmacology Analysis

We employed a network pharmacology approach, to screen phytocompounds and their targets in dental caries, elucidating their mechanism of action. To construct a network, the following parameters were used.

1. Identification and prediction of active compounds and targets using web server.
2. Selection of compounds based on pharmacokinetic parameters i.e., Drug-likeness criteria
3. Genes related to dental caries were identified through human disease databases and compounds targeting these genes were selected
4. Putative targets were further subjected to protein interaction analysis
5. Visualization software facilitated network construction, analysis, and understanding of molecular interactions and mechanisms
6. The binding affinity of highly interacted bio-actives with the key target

protein was analyzed.

3.11.1 Prediction of Active Compounds and Putative Targets

The PubChem web server obtained the 3D chemical structure and SMILES (simplified molecular input line entry specification) for 78 bioactive compounds from the six potential plants in PHDF formulation. Potential human targets of those compounds were identified through target fishing using the Super PRED and Swiss target database. The search parameters were set to generate a maximum of 300 conformations and the option to target human proteins was selected. Default settings were applied for all other search parameters. Replicated targets were removed, and effective targets were filtered using inference scores.

3.11.2 Screening of Disease Targets from Database

Disease targets linked to dental caries were retrieved from the databases, namely the Gene Expression Omnibus dataset, Gene Cards, and Comparative Toxicogenomic Database (CTD). The Gene Expression Omnibus dataset (GSE1629) from the National Centre for Biotechnology Information revealed differentially expressed genes in core pulpal tissue from carious teeth, comparing RNA from 11 carious teeth and two healthy samples. , GEO2R analysis identified key genes ($p < 0.05$, logFC = 0.5), providing insights into disease mechanisms., The online tool, Venny 2.1.0 was used to identify intersecting compounds and disease targets, revealing key potential, polyherbal compounds that contribute to the development of dental caries.

3.11.3 Analysis of Protein-Protein Interaction (PPI) Network

The key targets were submitted to a virtual screening database STRING 11.0 (https://string-db.org/) to understand protein interconnections and their contribution to biological processes. The Protein-protein interaction network was generated with Homo sapiens as the organism and the highest interaction confidence level (>0.9). The network represents proteins as nodes and associated protein-protein interactions as edges.

3.11.4 Construction of Compound-Target-Pathway Network

To gain knowledge on how the active components of PHDF interact with

dental caries targets, a network was constructed using Cytoscape visualization software version (www.cytoscape.org;version 3.7.1). This network consisted of antimicrobial agents, compound target protein interaction, and KEGG pathways. The compound-target network facilitated the analysis of the target mechanism and identification of involved pathways. Statistically significant ($p \leq 0.05$) interactions were defined by the KEGG mapper (https://www.genome.jp /kegg/mapper/) which was used to map the targets involved in the KEGG pathway.

3.11.5 Gene Ontology Enrichment Analysis and KEGG Pathway Annotation

Gene ontology (GO) analysis, which involves biological processes, molecular functions, and cellular components, and, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were conducted to classify the biological attributes of PHDF's potential targets in dental caries using functional enrichment analysis web tool, Webgestalt (http://www.web gestalt.org).

3.11.6 Molecular Docking Studies of Potential Compounds and Key Targets

The highly interacted bioactive compounds in PHDF formulation with the key targets were further evaluated to predict the binding affinity using the Schrodinger suite software. The 2D crystal structure of key targets MMP3 (PDB ID: 8H78) and CA4 (PDB ID: 5IPZ) was obtained from the protein data bank. All the other parameters were performed as mentioned in section **3.8.2**

3.12 Statistical Analysis

Data were expressed as the arithmetic mean±SEM of at least three individual experiments. Statistical significance was analyzed using a Tukey's post-hoc test for two-group comparisons and a one-way analysis of variance for multi-group comparisons. A p-value (<0.05) was considered statistically significant.