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Research Protocol

Dentistry Section

Enamel Remineralisation and Antimicrobial Efficacy of a *Withania somnifera*, *Ficus religiosa*, *Salvia rosmarinus*-based Polyherbal Gel: A Research Protocol

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ABSTRACT

Introduction: Dental caries is a multifactorial and chronic disease. *Streptococcus mutans* and Lactobacillus species are the primary microorganisms responsible for dental caries and enamel demineralisation. Caries can be prevented by fluoridated products like Casein Phosphopeptide- Amorphous Calcium Phosphate (CPP-ACP) or non fluoridated products. CPP-ACP can be used as a remineralising agent. Fluoride can also be used only as a remineralising agent. This article focuses on *Withania somnifera*, *Ficus religiosa*, and *Salvia rosmarinus*, which have all the three properties- antibiofilm, antimicrobial and remineralising.

Need of the study: Dental caries is a significant oral disease due to bacterial biofilms and enamel demineralisation. Traditional chemical medicaments such as fluoride have drawbacks of toxicity and resistance. Therefore, safer natural substitutes are desired. *Withania somnifera*, *Ficus religiosa*, and *Salvia rosmarinus* have antimicrobial, antioxidant, and remineralising activities. But they have to be studied in combination as a polyherbal gel for enamel. Therefore, the present study aims to assess the antimicrobial, antibiofilm, and remineralisation capability of this plant formulation, providing a biocompatible and effective means to prevent dental caries.

Aim: To evaluate the antimicrobial, antibiofilm and remineralisation efficacy of *Withania somnifera*, *Ficus religiosa* and *Salvia rosmarinus*-based polyherbal gel on enamel.

Materials and Methods: An in-vitro study will be conducted in the Department of Paediatric and Preventive Dentistry, Datta Meghe Institute of Higher Education and Research, Wardha, Maharashtra, India, from December 2025 to December 2026. In the present study, teeth extracted for orthodontic purposes or teeth samples with non cavitated lesions suitable for remineralisation assessment will be selected. Total teeth will be 70, with two groups each containing 35 teeth, n=35. Group 1: *Withania somnifera*, *Ficus religiosa*, *Salvia rosmarinus*. Group 2: Control group CPP-ACP. Antimicrobial efficacy will be determined against oral pathogens with the agar diffusion and broth microdilution techniques. Antibiofilm activity will be evaluated using biofilm inhibition and disruption assays. The remineralisation ability will be examined by scanning electron microscopy. A paired t-test will be applied to compare biofilm reduction before and after treatment within the same group. An independent t-test will be used to compare outcomes between two independent groups (polyherbal gel and control-CPP-ACP). The p-value of ≤0.05 will be considered statistically significant.

Keywords: Biofilm, Dental caries, Phytotherapy, Plant extracts

INTRODUCTION

Dental caries results from dietary sugar, the host's oral environment, and the action of cariogenic bacteria, primarily *Streptococcus mutans*, which produce acids that demineralise tooth enamel [1]. It involves dissolution of enamel apatite crystals with loss of calcium and phosphate; if untreated, it progresses to cavitation and weakens the tooth surface [2].

In recent years, the philosophy of minimally invasive dentistry has been increasingly accepted, focusing on healthy tooth structure and early diagnosis and treatment of caries. Non-invasive remineralisation is one of the most significant practices in this philosophy, and aims to arrest or reverse early enamel caries by replenishing lost minerals [3]. Apart from conventional remineralising agents such as fluoride and calcium phosphates, there has been a growing emphasis on the use of herbal products for the preventive measures and treatment of caries. These products of animal, plant, or microbial origin have been checked for their excellent health benefits, antimicrobial, and anti-inflammatory properties. Natural products such as polyphenols, essential oils, and bioactive peptides are being checked for their ability to inhibit cariogenic bacteria and induce enamel repair. This aligns with a current trend in healthcare, the application of biocompatible treatment options, which is the future of natural products in caries management strategies [4].

Fluoride systems are used in dentistry to deliver fluoride to tooth surfaces. It is available in both topical and systemic forms. At-home use of low-level fluoride can be through toothpastes, gels, and mouth rinses [5]. Routine use of low-concentration topical fluoride prevents dental caries by promoting remineralisation. Topically applied fluoride concentrates salivary fluoride and inhibits the solubility of enamel by increasing the formation of fluoroapatite. Furthermore, fluoride varnish can be shown to suppress bacterial metabolism and therefore prevent caries [5].

CPP-ACP came into the market as a remineralising toothpaste in 1998 as an attempt at escaping some of the flaws in fluoride. It contains milk protein CPP-ACP nanocomplexes. The combination of CPP and fluoride causes a synergistic action. Having a high saturation level with bulky minerals was believed to boost remineralisation of caries lesions as well as suppress cariogenic bacteria colonising dental surfaces [6].

CPP-ACP has been considered particularly useful since CPP stabilises phosphate and calcium ions against precipitation while ACP provides a reservoir for such remineralising ions [7]. CPP-ACP is a milk-derived protein complex that stabilises calcium and phosphate ions as ACP. It binds to dental plaque, enamel, and oral soft tissues, delivering calcium and phosphate for remineralisation. CPP can also

carry fluoride ions (CPP-ACPF), enhancing fluoride penetration into demineralised enamel and acting as a buffer to reduce acid damage while promoting subsurface enamel remineralisation [8].

Medicinal plants have been used for centuries in treating oral or systemic diseases with oral symptoms. Herbal mouthwashes and dentifrices are preferred since they contain no alcohol or colour and provide effective antimicrobial and anti-inflammatory action through phytochemicals. *Withania somnifera*, or the Queen of Ayurveda, is purported to have potent anticarcinogenic properties. Based on experimental laboratory findings, ashwagandha systematically possesses phytochemicals, trapino, withananine, alkaloids, and choline that possess anti-angiogenic properties that may inhibit tumour vascularisation [9]. It has inhibitory action on *S. mutans*. It consists primarily of mono and disaccharides in a methanolic extract, with a small quantity of alcohol sugars and some organic acid groups. These substances have both bacteriostatic and bactericidal actions at much higher concentrations. The extract possessed significant inhibitory activities on *S. mutans* at the optimal Minimum Inhibitory Concentration (MIC). This proves that, to a certain approximate tolerance level, this herbal extract may therefore inhibit the cariogenic activity against pathogenic bacteria [9].

The ethanol extract from the bark of *F. religiosa* was found to have modest antifungal efficacy against the opportunistic pathogenic yeast *Candida albicans* [10].

Salvia rosmarinus essential oil was found to inhibit the growth of *E. coli*. It has an inhibitory action on *S. mutans*. Moreover, it possesses several beneficial properties, including anti-oxidant, antiviral, antibacterial, anti-inflammatory, and anti-mutagenic activities. Due to low toxicity and few side-effects, rosmarinic acid has potential as a therapeutic agent. In addition, rosmarinic acid has significant antiviral, antibacterial, and anti-inflammatory properties, which imply that it can be employed in treating infections and inflammatory conditions [11].

The present research aimed to evaluate a polyherbal gel with CPP-ACP as a control agent.

Primary Objectives

- To check the antimicrobial, antibiofilm and remineralisation efficacy of *Withania somnifera*, *Ficus religiosa* and *Salvia rosmarinus*-based polyherbal gel on enamel;
- To evaluate the remineralisation efficacy of CPP-ACP on enamel.

Secondary Objectives

To compare the efficacy of polyherbal gel and CPP-ACP on enamel.

REVIEW OF LITERATURE

Interest in drugs derived from medicinal plants has significantly increased over the last decade. As there are some drawbacks to conventional products used in dentistry for a long time, including immune suppression, hypersensitivity, allergic reactions, and resistance, some of which are mutagenic and cytotoxic, a shift to herbal products is necessary [12]. Therefore, the need to convert to herbal products led many researchers to experiment on different medicinal and herbal plants. The current research aimed to provide a natural, plant-derived antimicrobial agent for preventing oral diseases against the backdrop of the global rise in antimicrobial resistance.

Gunther M et al., demonstrated the use of *Salvia rosmarinus* extracts as an herbal adjuvant, offering an alternative to synthetic chemicals for caries and periodontitis. The extract showed a marked effect on both aerobic and anaerobic bacteria, exhibiting bactericidal activity, with Colony-Forming Units (CFU) significantly reduced after treatment compared to pre-treatment levels [11].

Sienkiewicz M et al., found *Salvia rosmarinus* essential oil affects the growth of E. coli. A series of studies also testified to the efficacy of *Salvia rosmarinus* against S. aureus [13].

Hickl J et al., utilised in-vitro models for the first time to determine the action of different plant extracts, including *Salvia rosmarinus*, on oral microbes. The authors concluded that the growth of all the tested bacteria was severely suppressed by rosemary extract. Apart from reducing total bacterial numbers and viable counts, the antimicrobial activity of *Salvia rosmarinus* extract was also demonstrated by the total number of bacterial species present, which substantially reduced after treatment with the extract [14].

Sharma H et al. reported in an in vitro study that the 67% ethanolic stem extract of *Ficus religiosa* exhibited antibacterial activity against primary plaque colonisers only after 48 hours, producing a mean Zone of Inhibition (ZOI) of 2.6±0.54 mm. There was no significant activity in the extract at 24 h or 72 h, indicating that the antibacterial action was only transient. Findings revealed that although F. religiosa exhibited some antimicrobial properties, the effectiveness was low and short-lived, attributed to the fact that unrefined ethanol extraction may fail to provide all the effective phytochemicals. The researchers suggested conducting studies on methods of extraction, concentration, and human testing to understand antimicrobial purposes [15].

Dausage P et al., found that, in comparison to normal saline, ashwagandha preparations were superior for ion diffusion across the dentinal tubules over an extended period (up to 168 hours). For intracanal application, it can be used as an ideal vehicle for calcium hydroxide [16].

Abd El Aziz PM et al., illustrated that the richness of ashwagandha, in terms of biologically active compounds and minerals, has been well documented; elemental analysis has supported this with a high calcium and phosphorus content. Alkaloids and steroidal compounds in ashwagandha possess intense antibacterial, anti-inflammatory, and antioxidant activity. These molecules can bind and precipitate macromolecules, such as bacterial enzymes involved in bacterial metabolism. 0.5% ginger, ashwagandha, and maca are potent remineralising agents for early enamel carious lesions and possess antibacterial properties against *Streptococcus mutans*. They are a cheap and safe substitute for mouthwash and fluoride. They serve as essential minerals and phenolics. He also used laser fluorescence to confirm the remineralisation of early enamel carious lesions [17].

The antibacterial activity of the ancient Indian medicinal plants is tremendous. F. religiosa aqueous extracts exhibited marked antibacterial activity against isolated bacteria, which are primarily associated with dental caries [18]. *Ficus religiosa* helps in wound healing and acts as a disinfectant. It can also be used as a dye and applied as a varnish. *Salvia rosmarinus* has an antifungal effect; it acts against Candida albicans and can also be used with titanium dioxide and zinc oxide nanoparticles. It also has better dentin adhesive properties. The main property of *Withania somnifera* is its antibacterial, mainly against S. aureus, E. faecalis, E. coli, and P. aeruginosa. Also, Ashwagandha is water-soluble, and Rosemary can be both water and oil-soluble, so the combination of these three products forms an hydrogel. Due to their capacity to treat oral diseases, these medicinal plants should be utilised. Therefore, this study is different from other studies. Thus, the study aims to evaluate the antimicrobial, antibiofilm and remineralisation efficacy of *Withania somnifera*, *Ficus religiosa* and *Salvia rosmarinus*-based polyherbal gel on enamel.

Null Hypothesis

The polyherbal gel does not exhibit any significant antimicrobial, antibiofilm, or remineralisation effects on enamel when compared with control groups.

Alternate Hypothesis

The polyherbal gel exhibits significant antimicrobial, antibiofilm, and remineralisation effects on enamel when compared with control groups.

MATERIALS AND METHODS

An in-vitro study will be conducted at Datta Meghe Institute of Higher Education and Research, Wardha, from December 2025-December 2026. Ethical clearance was obtained with the ethical clearance no.- DMIHER(DU)/IEC/2025/552. Informed consent was obtained before commencing the study.

Inclusion criteria:

1. Teeth extracted for orthodontic purposes;
2. Teeth samples with non cavitated lesions;
3. Teeth of children aged 10-14 years.

Exclusion criteria:

1) Teeth samples with fractures, restorations, or other defects unrelated to caries;
2) Teeth with fluorosis or eroded teeth;
3) Teeth with root caries or teeth exposed to fluoride varnish or bleaching agents.

Sample size calculation:

$n \geq \{Z\_(1-\alpha/2)+Z\_(1-\beta)\}^2 \times (\sigma_1^2+\sigma_2^2/r)/(\mu_1-\mu_2)^2$

Remineralisation potential (Mean) in Group 1 (μ_1): 3.10 (17)

Remineralisation potential (Mean) Mean Group 2 (μ_2): 2.40 (17)

Standard deviation in Group 1 (σ_1): 0.88 (17)

Standard deviation in Group 2 (σ_2): 1.17 (17)

Ratio (r, Group 2/Group 1): 1

$\sigma_1^2 + (\sigma_2^2/r)=0.7744+1.3689=2.1433$

$Z_{\alpha/2}=1.96$ (for 95% confidence level)

$Z_{\beta}=0.84$ (for 80% power)

$(1.96+0.84)^2=(2.8)^2=7.84$

$$n=\frac{7.84\times2.1433}{(0.70)^2}$$

$(0.70)^2=0.49$

$7.84\times2.1433=16.80$

$$n=\frac{16.80}{0.49}$$

$n=34.29$

Sample size per group: 35

A total of 70 teeth will be required. 35 teeth will be in Group 1: *Withania somnifera*, *Ficus religiosa*, *Salvia rosmarinus*, and the remaining 35 teeth will be in Group 2: Control group CPP-ACP.

Study Procedure

Source of plant material: Plants of Ashwagandha, Peepal and Rosemary will be taken from the Ayurvedic college of Datta Meghe Institute of Higher Education and Research, Wardha.

Exact composition of polyherbal gel:

1. *Withania somnifera* extract- Antimicrobial, anti-inflammatory- 2% w/w
2. *Ficus religiosa* leaf extract- Antibacterial, antioxidant- 2% w/w
3. *Salvia rosmarinus* (Rosemary) extract- Antimicrobial, antibiofilm- 2% w/w
4. Propylene glycol- Humectant- 5% w/w
5. Methyl paraben- Preservative- 0.1% w/w
6. Distilled water- Vehicle
7. Carbopol 940- Gelling agent- 1% w/w
8. Triethanolamine- pH adjustment- 0.5–1%

Preparation of polyherbal gel:

First leaves of Ashwagandha, Peepal and Rosemary will be collected. The gel formulation is prepared by first dispersing Carbopol 940 in 50 mL of distilled water to form the base. Each herbal extract is then dissolved in propylene glycol to create the herbal phase. This mixture is gradually incorporated into the hydrated Carbopol gel with continuous stirring to ensure uniformity. Methyl paraben, dissolved separately, is added as a preservative. The pH of the formulation is adjusted to 6-7 by the dropwise addition of triethanolamine, which neutralises the Carbopol and yields a clear gel. Distilled water is then added to bring the final weight to 100 g. The formulation is homogenised using a magnetic stirrer or homogeniser until a smooth, uniform gel is obtained. Finally, the gel is transferred into sterile airtight containers and stored at room temperature for subsequent use.

Quality Control

Quality control tests will be carried out for macroscopic evaluation- colour, odour and texture; microscopic evaluation- identification of diagnostic plant structures.

Quality Evaluation of the Polyherbal Gel

The prepared gel will be evaluated for pH, viscosity, spreadability, homogeneity, drug content uniformity, and stability testing (25°C and 40°C).

OUTCOMES

1. For antimicrobial testing, the pathogens will be cultured on agar plates. Antimicrobial efficacy of the polyherbal gel against cariogenic microorganisms assessed by ZOI. The agar well diffusion method will be used, diameter will be recorded in mm using a vernier calliper. MIC protocol will be- dilutions of gel extract prepared in broth, both positive and negative controls will be taken, with the positive control being a bacterial suspension without extract, and the negative control will be broth. Incubated for 24 hours at 37°C.
2. Antifungal testing will be done by culturing Candida albicans on culture plates and then by the agar diffusion method.
3. For antibiofilm testing, the treated biofilm will be examined using a Scanning Electron Microscope (SEM). Antibiofilm efficacy will be assessed by biofilm formation reduction using the crystal violet assay. Reduction percentage will be calculated by using the formula- {OD (control) – OD (test)/OD (control)}×100 [19].
4. Remineralisation efficacy assessed by change in enamel surface microhardness by using the Vickers Microhardness test. Measurement intervals will be at baseline, Post-demineralisation and post treatment- 7 and 14 days. It will be calibrated using a reference block before testing.
5. SEM analysis- Imaging parameters will be magnification 1000, 3000 and 5000, and resolution will be high resolution mode.
6. SEM-EDX- lesion depth will be standardised by immersing all the samples in the same demineralising solution for the same duration. Elemental analysis of Ca and P will be evaluated, and Ca/P ratio will be calculated for each sample.
7. For stability and handling properties, pH, viscosity, and shelf-life will be evaluated.
8. Handling properties like spreadability will be evaluated by the two glass slides method, and acceptability will be evaluated by a 5-point Likert scale.

STATISTICAL ANALYSIS

Statistical analysis will be performed using SPSS software (Version 30). A paired t-test will be applied to compare biofilm reduction before and after treatment within the same group. An independent t-test will be used to compare outcomes between two independent groups (polyherbal gel and control-CPP-ACP). A p-value ≤0.05 will be considered statistically significant.

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