

Comparative Evaluation of Remineralisation Potential of Grape Seed Extract, Theobromine and Acidulated Phosphate Fluoride with and without Iontophoresis on Extracted Permanent Teeth: An In-vitro Study

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ABSTRACT

Introduction: Early enamel demineralisation is reversible through remineralisation strategies that restore mineral content and enhance enamel resistance. Traditional fluoride therapies, although effective, raise concerns regarding fluorosis and toxicity. Naturally derived agents like Grape Seed Extract (GSE) and theobromine offer biocompatible alternatives. Iontophoresis is a non invasive technique that may enhance agent delivery into the enamel structure.

Aim: To compare the remineralisation potential of GSE, theobromine, and Acidulated Phosphate Fluoride (APF) with and without iontophoresis on artificially demineralised permanent enamel surfaces using polarised light microscopy.

Materials and Methods: This in-vitro comparative study was conducted in the Department of Paediatric and Preventive Dentistry, Sri Aurobindo College of Dentistry, Indore, Madhya Pradesh, India from October 2024 to December 2024. A total of 36 extracted, caries-free human premolars were collected and sectioned longitudinally to obtain 72 specimens, which constituted the final sample size (n=12 per group). Group 1 received APF gel combined with iontophoresis, while Group 2 was treated with APF gel without iontophoresis. Group 3 was assigned theobromine solution with iontophoresis, and

Group 4 received GSE with iontophoresis. Group 5 was treated with theobromine without iontophoresis, whereas Group 6 received GSE solution without iontophoresis. After artificial demineralisation and 8-day pH cycling, iontophoresis (0.5 mA, 4 minutes) was applied in respective groups. Lesion depth and remineralisation were analysed using polarised light microscopy. Data were statistically evaluated using One-way Analysis of Variance (ANOVA) and paired t-test ($p < 0.05$).

Results: APF with iontophoresis showed the deepest precipitation band ($12.33 \pm 2.10 \mu\text{m}$, $p < 0.05$) and the least lesion depth. GSE without iontophoresis had the least remineralising effect (precipitation band: $3.30 \pm 3.51 \mu\text{m}$; lesion depth: $42.91 \pm 4.39 \mu\text{m}$). Iontophoresis significantly enhanced the remineralisation potential of both GSE and theobromine. No significant difference was observed between APF with and without iontophoresis, indicating its high intrinsic efficacy.

Conclusion: APF remains the most effective remineralising agent, regardless of delivery method. However, naturally derived agents like GSE and theobromine exhibit significantly improved efficacy when used with iontophoresis. These findings support the use of iontophoresis-enhanced natural agents that demonstrate potential as safe, minimally invasive alternatives warranting clinical evaluation.

Keywords: Dental caries, Fluorides, Microscopy, Polarisation, Tooth demineralisation

INTRODUCTION

The early phases of enamel demineralisation can be reversed with suitable remineralisation techniques which focus on replenishing lost mineral content, improving the resilience of enamel, and halting the advancement of carious lesions. This process can happen naturally or can be facilitated through various therapeutic interventions.

The pH cycle caries model is a synthetic representation of dental caries that replicates the oral environment by mimicking the processes of demineralisation and remineralisation of teeth, according to Buzalaf MA et al., [1]. While theobromine and GSE have shown remineralisation potential individually, their combined application with iontophoresis remains underexplored, which forms the novelty of the present study.

Fluoride exerts inhibitory action on caries development by decreasing demineralisation and increasing remineralisation [2]. However, its extensive use mainly in the form of dentifrice has contributed to a rising incidence of dental fluorosis, particularly in preschool children, due to chronic ingestion of these products [3].

Among the various fluoride-based treatments, topical APF is widely utilised for enamel remineralisation, yet its use may be limited by certain drawbacks such as reports of unpleasant taste and mucosal irritation in topical fluoride applications generally, according to the American Dental Association (ADA) guideline 2013 [4].

Synthetic hydroxyapatite nanoparticles were found to be about 20-100 nm in size. It has been established by Khetawat S and Lodha S that remineralisation efficacy increases with smaller apatite particle size, as it facilitates deposition of ions from subsurface regions into the outer enamel [5]. Nanohydroxyapatite has been recommended for remineralisation of teeth as it offers a larger surface area and improved wettability compared to traditional hydroxyapatite crystals.

This method, however, can be time-intensive and its effectiveness may vary unpredictably, as clinical fluoride (F) treatments produce only a few hundred nanometres of fluoride deposits, with penetration limited to the outer enamel surface [6]. Furthermore, the presence of a highly mineralised surface on the White spot lesion above the demineralised area may pose a significant obstacle to the remineralisation process occurring within the subsurface of the lesion, according to Schmidlin P et al., [7]. Therefore, better

techniques need to be utilised for the introduction of nanoparticles into tooth structure.

Among non invasive methods of remineralisation, iontophoresis is one of the ideal ways to strengthen the tooth. Iontophoresis uses a mild electric field to actively drive charged ions such as fluoride and calcium toward the oppositely charged electrode, thereby enhancing their penetration and uptake into enamel lesions, as stated by Pauli MC et al., [8].

Although chemicals continue to be widely used today, there has been an increasing interest in natural product diversity lately.

GSE contains 98% proanthocyanidins, which are widely available in fruits, vegetables, seeds, and nuts and is a natural plant metabolite, antioxidant and free radical scavenger, exhibiting potent collagen-stabilising properties that may facilitate remineralisation by enhancing the organic matrix of dentin [9]. One of the essential properties of proanthocyanidin is its ability to strengthen tissues containing collagen by increasing cross-linking of collagen [10,11].

Theobromine, an alkaloid found in cocoa from the methylxanthine family, has sparked interest for its potential to promote hydroxyapatite crystallisation and elevate enamel hardness [12].

Both compounds, being naturally derived, offer an appealing biocompatible alternative to traditional fluoride treatments, particularly in populations seeking non fluoridated options.

Fluoride-assisted natural repair of initial lesions can take place through the modulation of oral hygiene practices and dietary habits; however, this process is contingent upon factors like the quality of saliva and the adherence of the patient to recommended practices. In contrast, non fluoride remineralisation methods are less dependent on these variables and can substantially enhance the structural integrity, aesthetic appearance, and acid resistance of the remineralised lesions. Although these innovative agents are expected to induce remineralisation changes on the enamel surface, there is limited data available to assess their remineralisation efficacy on permanent enamel when iontophoresis is applied.

Therefore, the study aimed to do a comparative evaluation of the remineralisation potential of theobromine and GSE using APF as a control group with and without the application of an iontophoresis device using polarising microscopy, which is also our primary objective. The secondary objectives are as follows: to evaluate the effect of iontophoresis on the remineralisation efficiency of each agent and to analyse the depth of remineralisation achieved by each agent.

The null hypothesis is stated as follows: there is no significant difference in the remineralisation potential of GSE, Theobromine, and APF on enamel lesions; iontophoresis does not enhance the remineralisation potential of any of the tested agents, and there is no significant difference in the depth of remineralisation among the agents.

The alternate hypothesis is stated as follows: there is a significant difference in the remineralisation potential of GSE, Theobromine, and APF on enamel lesions, and iontophoresis enhances the remineralisation potential of one or more of these agents, and there is a significant difference in the depth of remineralisation achieved by the different agents.

MATERIALS AND METHODS

This in-vitro experimental study was conducted in the Department of Paediatric Dentistry, Sri Aurobindo College of Dentistry, Indore, Madhya Pradesh, India. Over a period of three months from October 2024 to December 2024. The study was approved by the Institutional Ethics Committee (IEC) and Scientific Research Committee of Sri Aurobindo Institute of Medical Sciences (SAIMS), Indore, Madhya Pradesh, India, under approval number SAIMS/RC/IEC/136/23. All procedures were conducted in accordance with the ethical standards of the Institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Sample size calculation: The sample size was estimated based on the previous study by Amaechi BT et al. [13]. An effect size of approximately 0.70, derived from the reported mean difference and standard deviation of lesion depth reduction, was used for calculation. Assuming a two-sided significance level (α) of 0.05 and a statistical power of 80% ($\beta=0.20$), the minimum required sample size was 12 specimens per group. Accordingly, 36 extracted premolars were sectioned longitudinally to obtain 72 enamel specimens, which were equally allocated into six experimental groups ($n=12$ per group). The formula used as follows-

$$n=2(Z_{1-\alpha/2}+Z_{1-\beta})^2\sigma^2/(\mu_1-\mu_2)^2$$

Where:

- n =sample size per group
- σ =pooled standard deviation
- $\mu_1 - \mu_2$ =expected mean difference
- $Z_{1-\alpha/2}=1.96$ for a 5% significance level
- $Z_{1-\beta}=0.84$ for 80% power

The study comprised six groups with 12 specimens in each group (total $n=72$ specimens)-

- Group 1 - APF gel with iontophoresis
- Group 2 - APF gel
- Group 3 - Theobromine solution with iontophoresis
- Group 4 - GSE solution with iontophoresis
- Group 5- Theobromine solution
- Group 6 - GSE solution

Inclusion and Exclusion criteria: Inclusion criteria consist of extracted human permanent premolars free from caries, cracks, or hypoplastic defects, whereas exclusion criteria rejected teeth with visible enamel defects or restorations.

Study Procedure

A total of 36 extracted, caries-free human premolars were used in this study. All teeth were visually examined and disinfected in 2% formaldehyde (pH 7.0) for 30 days at room temperature as per standard in-vitro protocols to ensure disinfection and preserve enamel integrity. After storage, the specimens were rinsed with deionised water to eliminate residual disinfectant and ensure a neutral, ion-free surface before the demineralisation procedure.

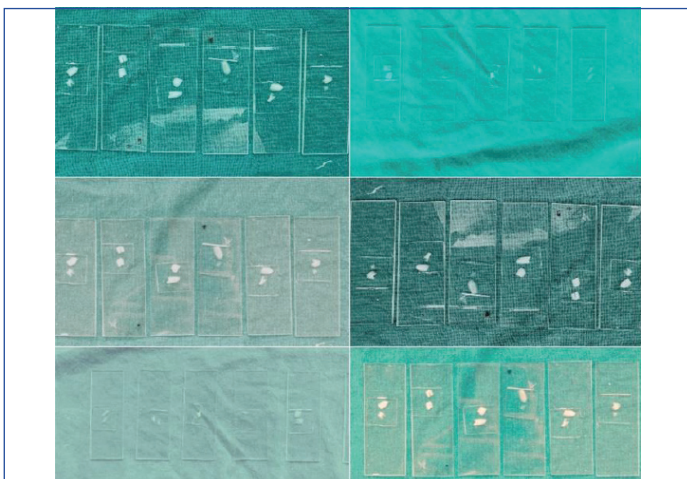
Each tooth was sectioned longitudinally into buccal and lingual halves using a water-cooled diamond saw, obtaining 72 specimens [Table/Fig-1]. One half served as the iontophoresis group and the other as the non iontophoresis group, minimising inter-tooth variability. The specimens were then randomly divided into six groups ($n=12$ per group). Coronal portions were decoronated and ground to obtain a flat enamel surface ($\sim 100 \mu\text{m}$ thickness) using a water-cooled diamond saw. The thickness of each section was confirmed using a digital micrometer, then mounted on glass slides using Dibutylphthalate Polystyrene Xylene (DPX) medium [Table/Fig-2].

Baseline images were captured under a Polarised Light Microscope (PLM) [Table/Fig-3a-f]. All measurements were recorded by an examiner who was blinded to the group allocation of the samples. Coding of the specimens was performed by a second investigator to maintain blinding during data collection and analysis.

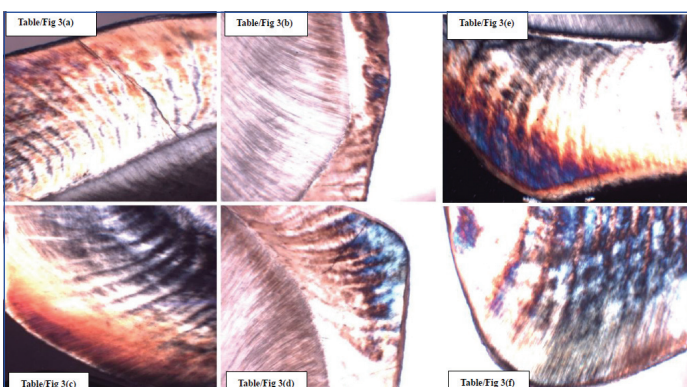
Demineralisation procedure: A demineralising solution (2.2 mM CaCl_2 , 2.2 mM KH_2PO_4 , 50 mM acetic acid, pH 4.6) [14] was prepared in 500 mL deionised water. Specimens were immersed at 37°C for six hours, then rinsed and examined under PLM [Table/Fig-4a-f]. Lesion depths were quantified using ImageJ; the measurement was taken from the enamel surface to the deepest point of the lesion, calibrated in micrometres, to ensure accurate and reproducible quantification [Table/Fig-5].



[Table/Fig-1]: Buccolingual sectioning of tooth.



[Table/Fig-2]: Enamel specimens mounted on glass slides using DPX.

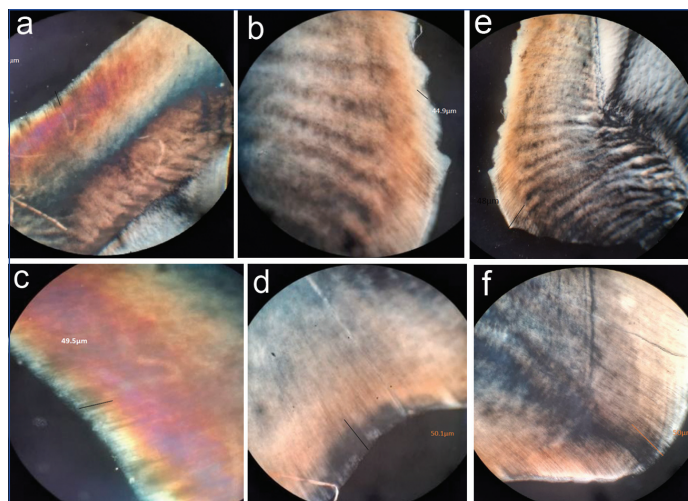


[Table/Fig-3]: Baseline microscopy images of tooth in all groups: (a) Polarising light microscopy baseline image of Theobromine group; (b) Polarising light microscopy baseline image of Theobromine with Iontophoresis group; (c) Polarising light microscopy baseline image of GSE group; (d) Polarising light microscopy baseline image of GSE with Iontophoresis group; (e) Polarising light microscopy baseline image of APF group; (f) Polarising light microscopy baseline image of APF with Iontophoresis group;

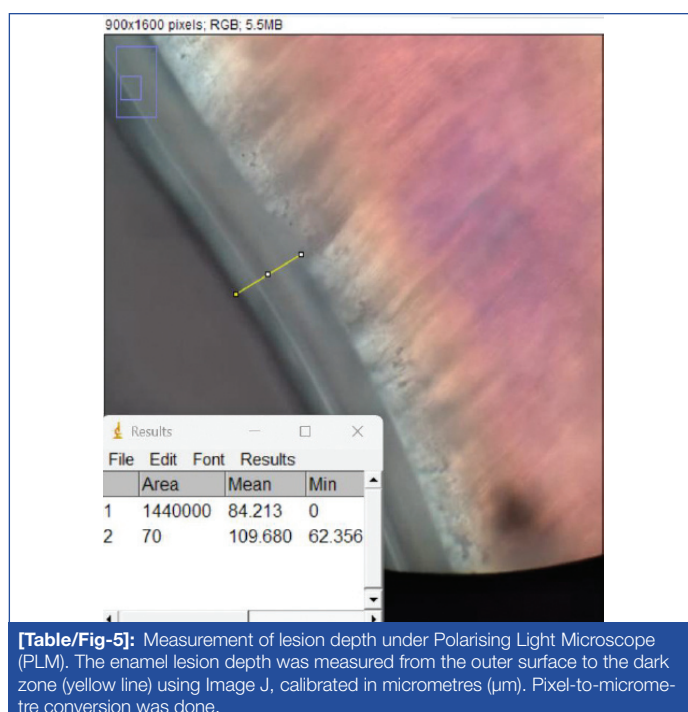
To ensure consistency, all treatment solutions were freshly prepared before use. Artificial saliva served as the remineralisation medium while an acidified buffer acted as the acidic challenge medium to simulate oral conditions.

Preparation of therapeutic agents: Theobromine (purity $\geq 99\%$ from Simson Pharma Limited, Mumbai, Maharashtra, India): 0.192 g theobromine + 0.092 mL 12M HCl in artificial saliva. To ensure complete dissolution, this preparation was done in-house, and no specific reference was used. The GSE (Proanthocyanidins $\geq 95\%$, 400 mg per capsule from INLIFE Healthcare Pvt., Ltd., India): 6.5 g GSE in 100 mL phosphate buffer (0.025 M NaH_2PO_4 and 0.025 M Na_2HPO_4) (pH 7.4) [14].

The APF (Colgate-Palmolive India Ltd., Mumbai, Maharashtra, India) Slurry: 1:3 ratio of 1.23% APF gel and artificial saliva (20 g:60 g). After preparation, they were randomly allocated using a computer-



[Table/Fig-4]: Polarising microscopy images of tooth after demineralisation in all groups: (a) Specimen after demineralisation with Theobromine (44.1 μm); (b) Specimen after demineralisation with Theobromine and Iontophoresis group (44.9 μm); (c) Specimen after demineralisation with GSE (49.5 μm); (d) Specimen after demineralisation with GSE and Iontophoresis group (50.1 μm); (e) Specimen after demineralisation with APF (48 μm); (f) Specimen after demineralisation with APF and Iontophoresis group (50 μm).



[Table/Fig-5]: Measurement of lesion depth under Polarising Light Microscope (PLM). The enamel lesion depth was measured from the outer surface to the dark zone (yellow line) using Image J, calibrated in micrometres (μm). Pixel-to-micrometre conversion was done.

generated random number table (Microsoft Excel, 2025) into the following experimental groups (n=12 per group):

Group 1: APF gel with Iontophoresis; Group 2: APF gel without Iontophoresis; Group 3: Theobromine with Iontophoresis; Group 4: GSE with Iontophoresis; Group 5: Theobromine without Iontophoresis; and Group 6: GSE without Iontophoresis.

APF Gel served as the control group, as it is the standard remineralising agent; hence, no separate untreated control was included. The pH cycling protocol was carried out for 8 consecutive days with six cycles per day [14].

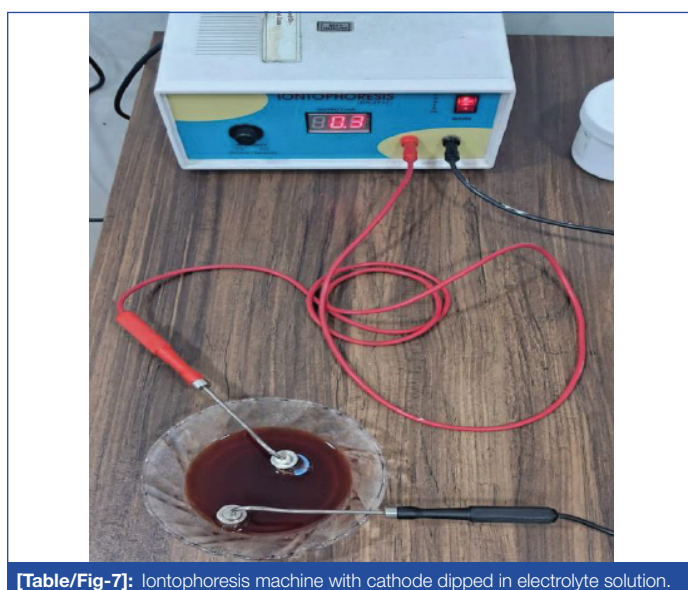
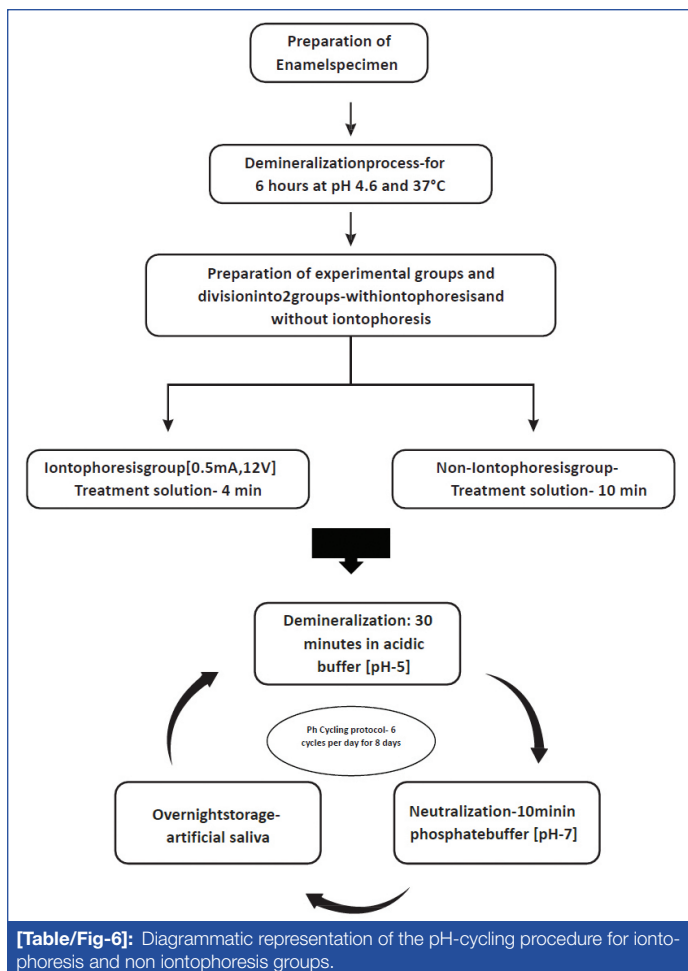
Treatment phase: 10 minutes immersion (4 min Iontophoresis at 0.5 mA, 12V for applicable groups)

Demineralisation: 30 minutes in acidic buffer (pH 5.0)

Neutralisation: 10 minutes in phosphate buffer (pH 7.0)

Storage: Overnight in artificial saliva (pH 7.0-7.4, 37°C) [Table/Fig-6].

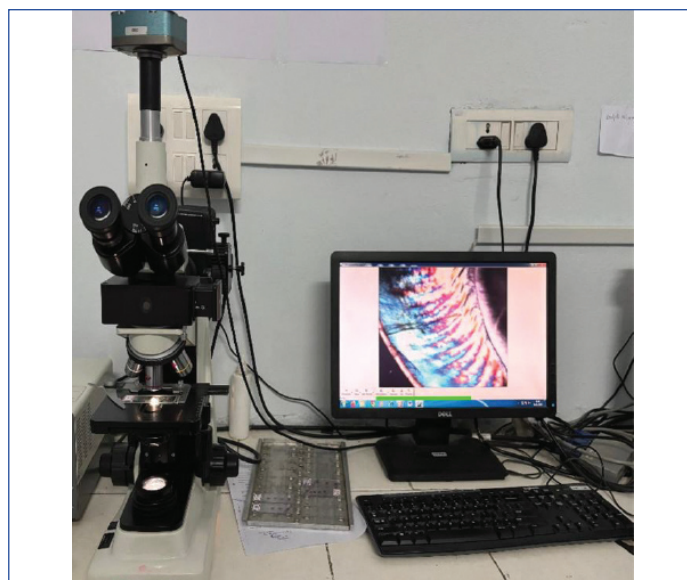
Iontophoresis application: A device (IndeTouch®) delivering 0.5 mA, 12V current was used for four minutes, with the anode on the enamel surface and the cathode in contact with the treatment solution, promoting ion penetration [Table/Fig-7].



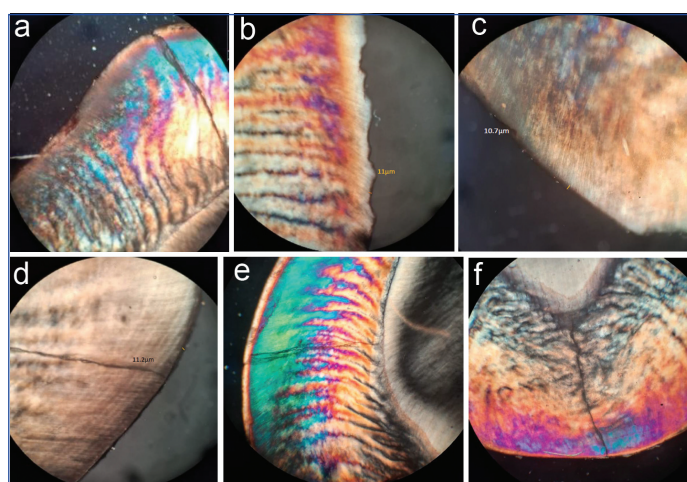
Post-treatment analysis: The following parameters were assessed:

- Demineralisation depth: Distance from the enamel surface to the dark zone, indicating the extent of initial enamel mineral loss.
- Precipitation band depth (post-treatment): Thickness of the remineralising agent deposited on enamel, measured from the enamel surface to the deepest visible extent of the precipitated layer. At baseline, the mean precipitation depth was 0 μm , as no remineralising agent had been applied at this stage.
- Lesion depth (post-treatment): Residual depth of the enamel lesion after treatment, indicating how effective the remineralising agents. All above values are expressed in micrometers (μm).

Specimens were examined under PLM (100 $\times$) to measure lesion depth from surface to dark zone [Table/Fig-8]. Measurements were recorded using ImageJ for quantitative analysis [Table/Fig-9a-f].



[Table/Fig-8]: Polarising Light Microscope (PLM) showing enamel birefringence on screen.



[Table/Fig-9]: Polarising microscopy images of tooth after remineralisation in all groups: (a) Specimen after remineralisation with theobromine (7 μm); (b) Specimen after remineralisation with theobromine and iontophoresis (11 μm); (c) Specimen after remineralisation with GSE (10.7 μm); (d) Specimen after remineralisation with GSE and iontophoresis (11.2 μm); (e) Specimen after remineralisation with APF gel (13 μm); (f) Specimen after remineralisation with APF gel and iontophoresis (15 μm).

STATISTICAL ANALYSIS

All data were entered into Microsoft Excel 2019 (Microsoft Corp., USA) and analysed using IBM Statistical Package for Social Sciences (SPSS) Statistics for Windows, version 26.0 (IBM Corp., Armonk, NY, USA). Descriptive statistics were expressed as Mean $\pm$ SD. The level of statistical significance was set at $p < 0.05$. One-way ANOVA was used to compare inter-group mean values of demineralisation depth, precipitation-band depth, and lesion depth. Paired t-tests were performed for pairwise within-agent comparisons (with vs. without iontophoresis).

RESULTS

Findings showed variation in the degree of remineralisation among the groups, indicating the influence of both the type of remineralising agent and the use of iontophoresis. The demineralisation values were taken as baseline for comparison with the lesion depth reduction seen during remineralisation by the three agents.

Baseline values: The mean demineralisation of the tooth in all the groups is shown in [Table/Fig-10]. No significant difference was observed among groups at baseline ($p > 0.05$), indicating comparable starting conditions before treatment.

Post-treatment values: The mean precipitation depth of the remineralising agents using one-way ANOVA is shown [Table/Fig-11]. A significant difference was found between the groups

Groups	N	Mean±SD (µm)	p-value	F-value
Group 1- (APF with iontophoresis)	12	47.16±2.08	0.08	2.10
Group 2- (APF without iontophoresis)	12	48.82±1.27		
Group 3- (Theobromine with iontophoresis)	12	46.06±1.23		
Group 4- (GSE with iontophoresis)	12	47.84±1.54		
Group 5- (Theobromine without iontophoresis)	12	46.91±1.96		
Group 6- (GSE without iontophoresis)	12	46.21±1.82		

[Table/Fig-10]: Mean baseline demineralisation values (µm) in all groups. No significant differences were observed among the groups at baseline (p>0.05; One-way ANOVA, p=0.08); APF: Acidulated phosphate fluoride; GSE: Grape seed extract; (n=12 specimens per group; each group consisted of teeth treated with or without iontophoresis using different remineralising agents) F=2.10

Groups	N	Mean±SD (µm)	*F value	p-value
Group 1- (APF with iontophoresis)	12	12.33±2.10	30.26	<0.01
Group 2- (APF without iontophoresis)	12	11.91±1.12		
Group 3- (Theobromine with iontophoresis)	12	11.21±0.58		
Group 4- (GSE with iontophoresis)	12	9.90±0.69		
Group 5 - (Theobromine without iontophoresis)	12	8.25±2.87		
Group 6 - (GSE without iontophoresis)	12	3.30±3.51		

[Table/Fig-11]: Mean precipitation depth of the remineralising agent. *F value= F value=F statistic (ratio of between-group variance to within-group variance from ANOVA). Significant differences were observed among groups (p<0.05, one-way ANOVA, F=30.26, p<0.01); APF: Acidulated phosphate fluoride; GSE: Grape seed extract. (n=12 specimens per group; each group consisted of teeth treated with or without iontophoresis using different remineralising agents)

(p<0.05). APF with iontophoresis shows the widest precipitation band value with a mean of 12.33±2.10µm, followed by APF without iontophoresis with the mean value of 11.91±1.12µm, while the narrowest precipitation band was found in GSE without iontophoresis with the mean of 3.30±3.51. This significant value shows that APF with iontophoresis has the highest remineralising property, while GSE without iontophoresis shows the least.

The mean lesion depth between remineralisation agents is compared in [Table/Fig-12]. There is a significant difference between the remineralising agent groups. The highest residual lesion depth (42.91±4.39 µm) was observed in the GSE without iontophoresis group, whereas the lowest residual lesion depth (34.80±1.17 µm) was observed in the theobromine with iontophoresis group. One-way ANOVA demonstrated a statistically significant difference among the study groups (p<0.01). However, as no post-hoc multiple-comparison analysis was performed, superiority between individual groups cannot be conclusively established.

Groups	N	Mean±SD (µm)	*F value	p-value
Group 1- (APF with iontophoresis)	12	35.00±0.42	19.14	p<0.01
Group 2- (APF without iontophoresis)	12	36.93±1.31		
Group 3- (Theobromine with iontophoresis)	12	34.80±1.17		
Group 4- (GSE with iontophoresis)	12	37.93±1.07		
Group 5- (Theobromine without iontophoresis)	12	38.65±3.11		
Group 6- (GSE without iontophoresis)	12	42.91±4.39		

[Table/Fig-12]: Mean lesion depth after treatment across all groups (µm). *F value= F value=F statistic (ratio of between-group variance to within-group variance from ANOVA); named after Sir Ronald Fisher; Values are expressed as mean±SD. Significant differences were observed among groups (p<0.05, one-way ANOVA, F=19.14 p<0.01); APF: Acidulated phosphate fluoride; GSE: Grape seed extract; (n=12 specimens per group; each group consisted of teeth treated with or without iontophoresis using different remineralising agents)

Intragroup comparisons: The comparison of precipitation depth values between remineralising agents with and without iontophoresis

is shown in [Table/Fig-13]. Paired-samples t-tests were used to compare measurements within each group.

Agents	With Iontophoresis (Mean±SD)	Without Iontophoresis (Mean±SD)	p-value	t value
APF	12.33±2.10	11.91±1.12	0.52	0.60
Theobromine	11.21±0.58	8.25±2.87	p<0.05	3.50
GSE	9.90±0.69	3.30±3.51	p<0.001	6.39

[Table/Fig-13]: Intragroup comparison of precipitation band depth (µm) with and without iontophoresis. Paired t-test was used for comparisons within each agent; APF: Acidulated phosphate fluoride; GSE: Grape seed extract; (n=12 specimens per group; each group consisted of teeth treated with or without iontophoresis using different remineralising agents)

- **APF:** No significant difference was observed between APF with and without iontophoresis for precipitation band depth (p>0.05), suggesting similar remineralisation effect.
- **Theobromine:** With iontophoresis (11.21±0.58 µm) showed significantly higher precipitation depth than without iontophoresis (8.25±2.87 µm) (p<0.05).
- **GSE:** With iontophoresis (9.90±0.69 µm) had significantly higher precipitation depth than without iontophoresis (3.30±3.51 µm) (p<0.01).

Lesion depth significantly varied among groups [Table/Fig-14]. Theobromine with iontophoresis showed the greatest reduction. The deepest lesion depth was recorded in the GSE without iontophoresis group, confirming limited efficacy when delivery is not enhanced.

Agents	With Iontophoresis (Mean±SD)	Without Iontophoresis (Mean±SD)	p-value	t-value
APF	35.00±0.42	36.93±1.31	p<0.001	11.67
Theobromine	34.80±1.17	38.65±3.11	p<0.01	4.01
GSE	37.93±1.07	42.91±4.39	p<0.01	3.81

[Table/Fig-14]: Intragroup comparison of lesion depth (µm) between specimens treated with and without iontophoresis for each remineralising agent. Paired t-test was used for intragroup comparison; APF: Acidulated phosphate fluoride; GSE: Grape seed extract; (n=12 specimens per group)

DISCUSSION

The findings of the present study support the alternate hypotheses that there is a significant difference in the remineralisation potential of GSE, Theobromine, and APF on enamel lesions, and that iontophoresis significantly enhances the remineralisation potential of these agents. Furthermore, a significant difference was observed in the depth of remineralisation achieved by the different agents. According to Cochrane in 2010, an effective remineralisation system should ideally provide stabilised bioavailable ions of calcium, phosphate, and fluoride, promoting mineral accumulation in the subsurface rather than merely in the outer layer [15].

Despite fluoride being the gold standard for remineralisation, concerns over fluorosis and fluoride toxicity have been emerging among parents. Hence, the desire for more biocompatible and natural alternatives has led to exploration of novel agents like theobromine and GSE. Iontophoresis is a non invasive technique that utilises a mild electric current to enhance the delivery of therapeutic agents into the deeper layers of enamel. Many individual studies have evaluated the remineralisation efficacy of both agents, but there is a lack of comprehensive data on the potential of these agents with the application of iontophoresis. In the present study, the remineralisation potential of these three agents with and without iontophoresis using polarising microscopy was assessed, thereby contributing valuable insights into minimally invasive, evidence-based caries management strategies.

Studies by Nakamoto T et al., Amaechi BT et al., Taneja V et al., Duraisamy Y et al., and Syafira G et al., have proved theobromine

as a potent remineralising agent [12,13,16-18], however, its effectiveness when combined with iontophoresis has not been well explored. The present study aims to address that gap by evaluating whether combining theobromine with iontophoresis could enhance remineralisation efficacy, especially for fluoride-averse individuals.

Similarly, in research works by Jawale K et al., Benjamin S et al., and Mirkarimi M et al., GSE has been suggested to have protective and remineralising properties for teeth [10,14,19]. Similarly, its potential combined with iontophoresis remains under-researched.

APF has an established role in remineralisation both with and without iontophoresis. Notably, Ragit R et al., confirmed the enhanced efficacy of APF when combined with iontophoresis [20]. Given its widespread clinical use, APF serves as a benchmark for assessing the performance of newer agents.

For the present study, caries-free premolars extracted for orthodontic reasons were selected. Following the methodology of Ragit R et al., each tooth was sectioned into buccal and lingual halves to serve as control and experimental groups [20]. Following demineralisation, enamel surfaces were treated with GSE, theobromine, APF with and without iontophoresis, under controlled laboratory conditions.

The findings align with research by Adam H et al., which demonstrated that artificial saliva could successfully facilitate enamel remineralisation [21]. The pH cycling model described by Xie Q et al., which involves alternating demineralisation and remineralisation phases, with overnight storage in artificial saliva to closely simulate the oral environment were employed [22].

Iontophoresis significantly enhanced remineralisation compared to passive diffusion. The mild current facilitated ionic movement and improved interaction between fluoride, chloride, phosphate, and calcium ions, leading to the formation of CaF_2 , CaCl_2 , and calcium phosphate complexes. In the current study, a current of 0.5 mA was applied for 4 minutes, consistent with earlier research. It's noteworthy that intensities exceeding 1 mA may damage odontoblasts [8].

Birefringent properties of a polarised light microscope make it an excellent tool for visualising enamel and dentin changes. Intact enamel appears highly birefringent; demineralised enamel exhibits reduced birefringence, appearing darker due to mineral loss. Whereas remineralised enamel may show partial birefringence restoration, indicating mineral uptake. ImageJ software was used to interpret the lesion depth.

No significant differences in initial demineralisation values among groups confirmed lesion uniformity [Table/Fig-10]. This ensures that subsequent remineralisation outcomes can be attributed to the treatment effects alone. These findings are consistent with Eronat C et al., and Featherstone JDB [23,24].

A significant difference in precipitation band depth among groups is presented in [Table/Fig-13]. APF with iontophoresis had the deepest precipitation band, followed by APF without iontophoresis- suggesting improved mineral uptake with electrical enhancement. These findings support studies by Chaiwat A et al., which demonstrated the improved efficacy of fluoride-based remineralisation when applied via iontophoresis [25].

In contrast, GSE without iontophoresis had the narrowest band, indicating minimal remineralisation. Pavan S et al., and Amin RA et al., also reported limited penetration of GSE into enamel due to lack of delivery enhancement and lack of collagen hindering its penetration into enamel [9,26].

Lesion depth differed significantly among the study groups. The lowest mean residual lesion depth was observed in the theobromine with iontophoresis group, whereas the highest residual lesion depth was recorded in the GSE without iontophoresis group, indicating comparatively lower remineralisation in the absence of iontophoresis. Although APF demonstrated consistently favourable remineralisation

outcomes, no formal post-hoc intergroup comparison was performed; therefore, definitive superiority of one remineralising agent over another cannot be concluded from the present data. APF was highly effective both with and without iontophoresis, indicating its intrinsic remineralisation capability [Table/Fig-12]. This is supported by Eronat C et al., [23].

Polarising light microscopy images of specimens of theobromine with and without iontophoresis show significantly improved remineralisation over theobromine alone [Table/Fig-9a,b]. Chloride ions increased theobromine's solubility and uptake, maintaining a stable pH. Studies by Nakamoto T et al., and Amechi BT et al., suggest increased crystallinity and enhanced remineralisation in the presence of theobromine [12,13].

While GSE shows superior remineralisation in dentin due to collagen content, its effect on enamel remains moderate. Studies by Mirkarimi M et al., and Xie Q et al., reinforce this by demonstrating GSE's affinity for calcium and its surface-level mineralisation capabilities [19,22].

The results of the present study underscore that while APF can perform effectively without assistance, naturally derived agents like theobromine and GSE show significantly better results when administered through iontophoresis. Iontophoresis has been reported to enhance mineralisation speed up to 15 times compared to diffusion alone, improving agent penetration and kinetic efficiency. Although statistically significant differences were observed, the clinical impact appears modest, as theobromine and GSE with iontophoresis showed remineralisation comparable to APF, indicating their potential as safe, minimally invasive alternatives where fluoride use is limited.

The findings of the in-vitro study offer promising insights for Paediatric and Preventive dentistry, particularly in managing early enamel demineralisation through minimally invasive approaches. Natural agents such as GSE and theobromine demonstrated notable remineralisation, with apparent improvements when combined with iontophoresis; however, formal intergroup post-hoc statistical comparisons were not performed, and these observations should be interpreted cautiously.

Iontophoresis may offer a cost-effective alternative to repeated fluoride treatments by being inexpensive, widely available, and potentially reducing treatment frequency and duration. These agents may serve as safer, child-friendly alternatives to fluoride, particularly valuable in younger children or populations at risk for dental fluorosis. Their non toxic nature and ease of application make them suitable candidates for early intervention, aiming to arrest or reverse white spot lesions before cavitation occurs.

Furthermore, the use of iontophoresis significantly enhances the penetration and efficacy of these agents, suggesting a novel approach for in-office paediatric dental care. This technique offers the potential for faster treatment, reduced chair time, and improved compliance in uncooperative or anxious children. However, it is generally considered safe when performed within current limits. Care should be taken to avoid patient discomfort by preventing excessive current application. The present study utilises a current of 0.5 mA for 4 minutes, which is below levels known to cause odontoblast damage. No adverse effects are expected under these parameters. Incorporating such preventive strategies into clinical practice could reduce the need for restorative treatments, support non invasive caries management protocols, and potentially be integrated into school-based oral health programs. These findings provide a scientific basis for future clinical trials evaluating the long-term effectiveness and acceptability of natural remineralising agents combined with iontophoresis in paediatric populations.

Limitation(s)

While the current study applied a single concentration and duration for each agent, future studies should explore dose-response

relationships for both the natural agents and APF, including variations in concentration, duration of iontophoresis, and frequency of application. This would allow optimisation of remineralisation efficacy and clinical applicability. Furthermore, the study evaluated only a single time-point and did not assess the long-term stability of the remineralisation. It was limited to a single tooth type (premolars), which may restrict the generalisability of the findings to other teeth or clinical conditions. Other limitations of the in-vitro study include challenges in accurately replicating the oral environment, reduced salivary protein concentration, absence of bacteria in the artificial saliva, difficulty in regulating salivary flow rate, application of more intense acidogenic challenges over a shorter duration and absence of inter-examiner reliability. The sample size was limited due to the complexity and precision required in specimen preparation and microscopic analysis.

CONCLUSION(S)

Within the scope of this in-vitro study, APF, theobromine, and GSE all demonstrated remineralisation potential on enamel lesions. Iontophoresis enhanced the efficacy of natural agents, bringing their performance closer to that of APF. These findings indicate that theobromine and GSE, especially when combined with iontophoresis, could serve as effective, minimally invasive alternatives for early enamel remineralisation. However, further in-vivo studies are warranted to confirm these findings and optimise iontophoresis parameters for clinical application.

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