

**COMPARATIVE EVALUATION OF DEMINERALIZATION
INHIBITORY EFFECT AND ANTIBACTERIAL EFFECT
OF COMMERCIALLY AVAILABLE FLUORIDE
VARNISHES –AN IN VITRO STUDY**

**By
Dr. COSMA.S**

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MASTER OF DENTAL SURGERY

PEDIATRIC AND PREVENTIVE DENTISTRY

**SATHYABAMA DENTAL COLLEGE AND HOSPITAL
CHENNAI 600 119**

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AN IN VITRO STUDY ”** is a bonafide and genuine research
carried out by **DR. COSMA. S** under the guidance of **DR.
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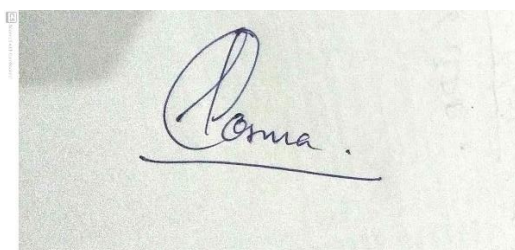
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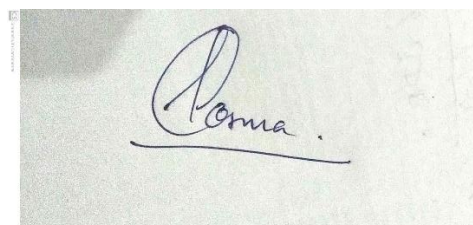
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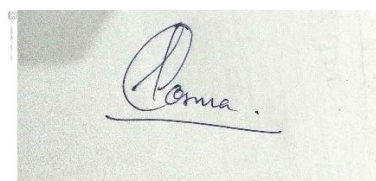
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Dental caries is a complex disease that afflicts a larger proportion of the world regardless of age, gender, race and ethnicity. It has been ranked as the 12th most prevalent condition, affecting around 560 million children worldwide. (1) In Southeast Asian countries, caries prevalence in preschool children is predominantly high (79%). (2) The overall average prevalence of Early Childhood Caries in India is 49.6% with every state contributing above 40%. (3) Several studies have identified the risk factors of Early Childhood Caries, such as sociodemographic factors, dietary factors, oral hygiene factors, and factors related to oral bacterial flora and breastfeeding/bottle feeding. (4) Children affected with Early Childhood Caries not only suffer from tooth pain and infection, but gradually result in a serious systemic infection with severe complications deteriorating children's overall growth, development and general health. In addition, it also poses a challenge for young children to tolerate the lengthy and complicated procedures of restorative treatment. Therefore, a non-invasive and unsophisticated preventive treatment modality is required to tackle the burden of dental caries in young children. (5)

Caries is the result of the disruption of the microbial homeostasis of dental plaque and the consequent breakdown of the mineral equilibrium between enamel and the biofilm fluid. (6) In recent decades, with the better understanding of the ultrastructure and the dynamic nature of enamel caries, there has been a paradigm shift in the management of lesions from an operative restorative approach towards a prevention-remineralization concept. When the pH is below the critical level, dissolution takes place

which is demineralization and contrarily, when the pH is above the critical level for the formation of a respective mineral phase, precipitation occurs which is termed as remineralization. (7) The primary objective of the contemporary preventive management is to shift the dynamic balance of the demineralization-remineralization cycle towards net mineral gain within the lesion, through the provision of bioavailable mineralizing ions.

Different preventive therapies have been analysed to enhance remineralization, and decrease demineralization and, thereby arrest the active carious lesions, which are regarded as a priority for dental services and deliberated more cost-effective when compared to the restorative treatments. (8) Fluoride has been proven to play a primary role in this process and, therefore, it has been employed widely through several vehicles such as water fluoridation, fluoridated toothpastes, and mouthwashes.

Over the years, high fluoride-containing products have demonstrated enhanced remineralization by forming fluorapatite crystals in the tooth. The main effect of fluoridated agents is produced by elevating the levels of fluoride in the oral environment. (9) According to **Page et al.**, five minutes of exposure of various levels of fluoride (up to 1000 ppm) in fluoride delivery system significantly inhibited the demineralization process of enamel. (10) Higher concentrations of fluoride, such as 5000 ppm, have been shown to significantly reduce the risk and incidence of caries among high-risk populations. (11)

Various systematic reviews have suggested that the progression of dental caries could be slowed down or halted non-operatively (12). The application of various topical fluoride agents has been reported to be effective in managing caries.(13) Fluoride varnish is one of the most commonly employed topical fluoride agent. It can adhere onto the tooth surface for a longer duration and act as slow-releasing reservoirs of fluoride. (5) A Cochrane review by **Marinho et al.** concluded that sodium fluoride varnish has a substantial caries-inhibiting effect on primary teeth with an estimated 37% of pooled caries being prevented. (14) To support these results, another review by **Gao et al**, also observed that 5% sodium fluoride varnish is effective in remineralizing early enamel caries. (15)

In spite of all the available evidences, it has been observed that in addition to fluoride, the presence of bioavailable calcium and phosphate in the plaque fluid is also necessary for maintaining the structural integrity of hydroxyapatite crystals and enhance remineralization. While normal saliva does not usually contain an optimal level of fluoride, it has been noticed that saliva is supersaturated with calcium and phosphate ions. Some proteins like statherins, are responsible for stabilizing calcium and phosphate in saliva and preventing their spontaneous precipitation. (16)

Hence, with the aim of increasing the amount of calcium and phosphate in saliva and thereby improve the demineralization prevention efficacy and the possible remineralization potential of the lesion, fluoride

varnishes are incorporated with various forms of calcium and phosphate compositions recently.

One such addition is the combination of calcium and phosphate coated with xylitol to the conventional 5% sodium fluoride varnish. It is expected that xylitol gets dissolved in saliva, releasing the calcium and phosphate ions, to react with the fluoride ions in saliva to form protective fluorapatite on the teeth.

Other addition in the fluoride varnish noted in the literature is addition of functionalized tricalcium phosphate. Clinical studies have shown that the combination of fluoride and functionalized tricalcium phosphate produces stronger, more acid-resistant mineral relative to fluoride alone. In contrast to other calcium-based approaches requiring high levels of calcium and phosphate to drive remineralization, functionalized tricalcium phosphate even at a lower dosage is designed to fit within existing topical fluoride preparations. It also provides a barrier that prevents premature fluoride-calcium interactions and aids in mineralization when applied *via* topical agents.(17) Functionalized tricalcium phosphate works synergistically with fluoride and controls the delivery of calcium and phosphate ions to the tooth substrate during an acidic challenge. Previous studies on fluoride containing functionalized tricalcium phosphate revealed promising results in improving fluoride's activity and enhancing the remineralization of carious lesions. (18)

Since, dental caries is an infectious disease of bacterial origin, it could benefit from the use of topically applied antibacterial agents which generate a favourable ecological shift within the oral environment. The use of antibacterial agents in the treatment of dental caries has been studied for over decades together. (19) Among the bacteria found in dental plaque, *Streptococcus* is known to be the major causative bacterial strain of dental caries. Among them, *Streptococcus mutans* is reported to have close association with dental caries in humans. (20) The use of antibacterial agents has been suggested as a potential method for the reduction, control, and prevention of the accumulation of cariogenic microorganisms.

(21) Fluoride has been indicated as a major contributor in the treatment and prevention of dental caries because of its cariostatic and remineralizing properties. Although the main action of fluoride on the dynamics of dental caries is on de- and remineralization processes that occur on dental hard tissues, it has also been proposed that the fluoride ion can affect the physiology of microbial cells, including cariogenic streptococci, which can thus indirectly affect demineralization. (22) A recent review, however, concluded that fluoride concentrations as found in dental plaque have biological activity on critical virulence factors of *Streptococcus mutans* in vitro, such as acid production and glucan synthesis, but the clinical implications are still not clear. (22)

Considering the recent development of these formulations for sodium fluoride varnishes, the effect in amplifying or debilitating the remineralization effect of fluoride still remains unclear. In addition, there is

a grey area in literature whether the addition of these recent components influences the antibacterial effect of fluoride varnishes .Therefore the present in vitro study has been planned to address these knowledge gaps in literature.

The null hypothesis proposed in this study is that there is no difference in the demineralization inhibitory effect and antibacterial effect of the commercially available fluoride varnishes Vanish™, Embrace™ and Fluorprotector®.

AIM OF THE STUDY

To compare the efficacy of the commercially available fluoride varnishes; Vanish™, Embrace™ and Fluorprotector® in terms of their demineralization inhibitory effect and antibacterial effect.

OBJECTIVES OF THE STUDY

- To evaluate the efficacy of the Demineralization Inhibitory effect of Vanish™ varnish
- To evaluate the efficacy of the Demineralization Inhibitory effect of Embrace™ varnish
- To evaluate the efficacy of the Demineralization Inhibitory effect of Fluorprotector® varnish
- To compare the efficacy of the Demineralization Inhibitory effect of Vanish™ with Fluorprotector®
- To compare the efficacy of the Demineralization Inhibitory effect of Vanish™ with Embrace™
- To compare the efficacy of the Demineralization Inhibitory effect of Embrace™ with Fluorprotector®
- To qualitatively assess the antibacterial effect of Vanish™ varnish
- To qualitatively assess the antibacterial effect of Embrace™ varnish
- To qualitatively assess the antibacterial effect of Fluorprotector® varnish
- To assess if there is any superiority in the antibacterial efficacy among Vanish™, Embrace™ and Fluorprotector®

Lingström P et al (2003) identified that the dental plaque and biofilm are essential for caries lesions to develop, but the mere presence of biofilm is not the sole cause for caries to develop, as additional factors are involved. (23)

Fejerskov et al (2004) claimed dental caries to be a multifactorial disease, in which behavioural, environmental, economic and genetic risk factors work together. (24)

Chu CH et al (2008) revealed that the prevalence of dental caries is significantly higher for children from lower social-economic classes or lower income families, for whom conventional dental care is often either unavailable or unaffordable. (25) Moreover, the lack of dental manpower and the sophisticated dental equipment required makes the conventional restorative care a difficult way to solve this global issue.

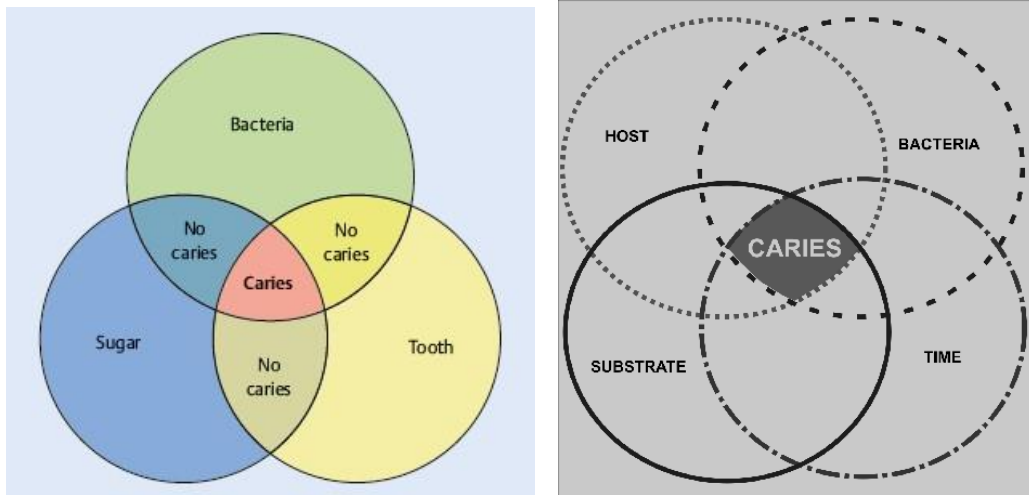
Fejersov and Kidd (2009) have stated that dental caries constitutes the signs and symptoms related to the chemical dissolution of the tooth surface. This process is caused by metabolic events in the biofilm overlying the tooth structure. Dental caries can affect every surface of a tooth where dental plaque is allowed to accumulate over a period of time and mature in a biofilm gradually. (26)

Chu CH et al (2010) stated that the alternative treatments for dental caries of children in disadvantaged communities are mandatory, and professionally applied fluoride therapy has been proposed as one of the effective means for the management of dental caries. Conventional caries management philosophy has changed from the traditional surgical approach to a medical model, and fluoride therapy is now used not only for prevention but also to arrest the already occurred caries lesions. Professionally applied fluoride therapy is a relatively low-cost and easily operated treatment and has been proven to arrest active dental caries. (27)

World Health Organization (2020) has stated that dental caries is a pandemic disease affecting people of all ages, worldwide. The pediatric population is severely affected and the report has concluded that 60-90% of school children suffer from dental cavities. (28) This means that dental caries is prevalent among mankind globally. Poor oral health not only affects the general health on an individual level but can also lead to an excruciating pain and discomfort.

Concepts of dental caries

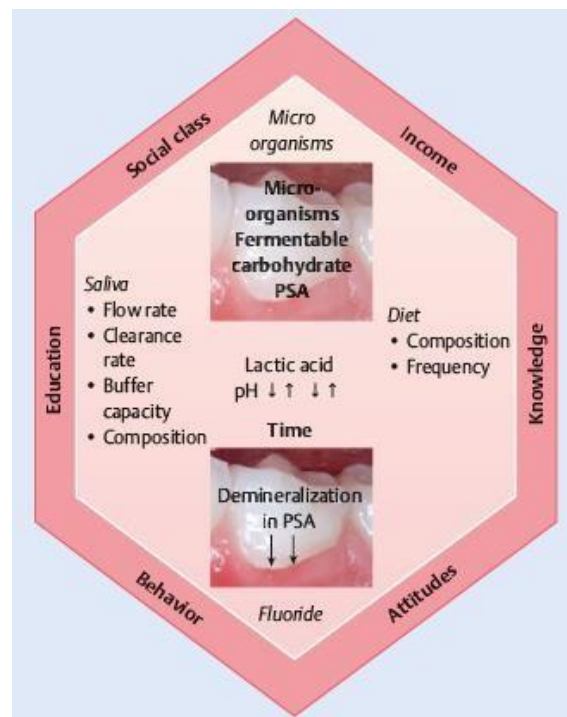
Fejerskov and Kidd (2009) stated that various models have been used to describe the etiology of caries. The famous Venn diagram by Keyes shows the three essential etiological factors responsible for the formation of caries, namely “bacteria,” “tooth,” and “sugar”.



Courtesy: Dental Caries: The Disease and Its Clinical Management

Fejerskov and Kidd 2009.

Later, “time” was added as the fourth essential component by König. Both the above mentioned models have been formulated based on the specific plaque hypothesis. In contrast to these models, Fejerskov and Manji introduced a model to show dental caries as a multifactorial disease.



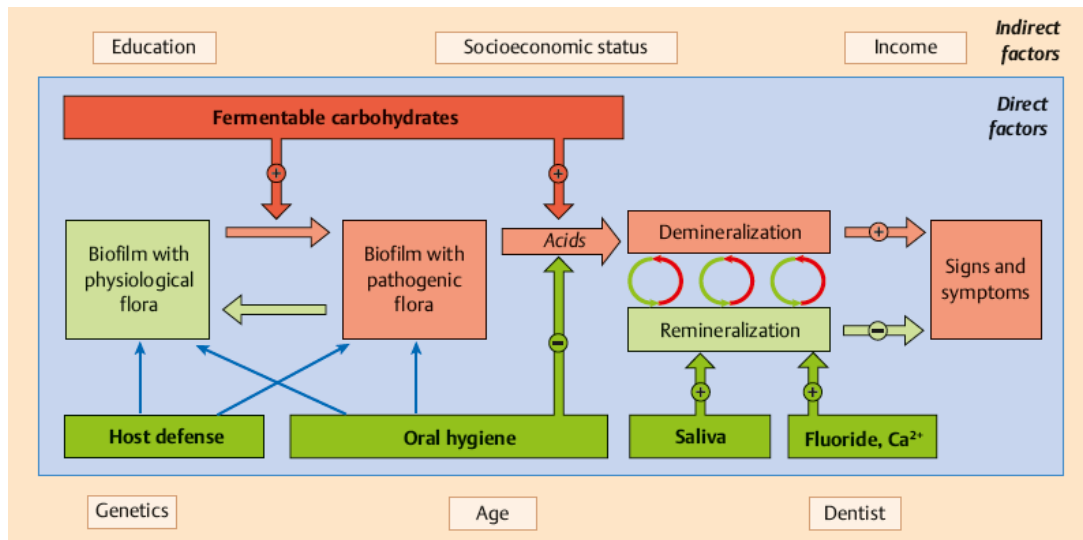
Courtesy: Dental Caries: The Disease and Its Clinical Management

Fejerskov and Kidd 2009.

In accordance to this model, a sugar-rich diet plays a chief role in the etiology and pathogenesis of caries. The frequent consumption of fermentable carbohydrates causes a pathological shift in the oral microflora and promotes the growth of both acidogenic and aciduric species. Consuming fermentable carbohydrates also facilitates potentially cariogenic bacteria such as *Streptococcus mutans* to produce organic acids which demineralize the enamel and dentin which in turn produces the characteristic signs and symptoms of caries.

In addition to the cariogenic bacteria, the protective factors also influence the development and progression of caries. Both the host's defences and the patients' oral hygiene play a role in limiting the growth and metabolism of the oral microflora and thereby acid production. Saliva, with its buffering properties promotes the remineralization of the enamel. The remineralizing effect of saliva can be facilitated by the presence of fluorides and calcium compounds.

Epidemiological investigations have revealed that, in addition to these local, direct factors, other behavioural and socioeconomic factors are also associated with caries. However, the impact of these factors only acts upon the caries process indirectly.



Courtesy: Dental Caries: The Disease and Its Clinical Management

Fejerskov and Kidd 2009.

In the above picture, the consumption of fermentable carbohydrates has been depicted as a pathogenic factor (dark red), and the rest of the factors that locally influence the caries process are shown as protective factors (dark green). Though the influence of these protective factors is indirect, the minimization or complete elimination of protective factors can have a remarkable influence on the caries process. In short, the potential risk factors are increasing the pathogenic component of fermentable carbohydrates as well as reducing or eliminating the protective factors.

According to the current model of caries, the predomination of the protective factors, either prevents caries from developing, or arrests the already existing caries. However, if the pathogenic factors predominate, the disease progresses. This dynamic nature of the caries process enables the disease to be influenced at any stage. Hence, the preventive

strategies available for preventing caries are expected to minimize the pathogenic factors and enhance the protective factors. (26)

Role of Fluoride in caries prevention

Rølla G (1988) stated that following the topical fluoride treatment, the fluoride ions form fluoride-hydroxyapatite and/or calcium fluoride. This effect is dependent on both the pH levels and the fluoride concentration. A lower pH and a high concentration of fluoride ions favour the formation of calcium fluoride. Calcium fluoride particles adhere particularly well to demineralised surfaces as they are more porous. This highlights the fact that high-concentration treatments with fluoride could form oral fluoride reservoirs. (29)

Featherstone (1991) has stated that fluoride inhibits the process of demineralisation of the hard tissue of the tooth and promotes the remineralisation of the surface lesions. In addition, it also causes a reduction in the metabolic activity of the acid-producing cariogenic bacteria. Not only high fluoride concentration applications are advantageous, but, low, yet recurrent exposure to fluoride is also known to be favourable for the tooth, as it helps to shift the equilibrium from demineralisation to remineralisation. (7)

Ogard et al (1994) stated that under acidic conditions, the calcium fluoride releases both fluoride and calcium ions, which prevent the enamel from getting dissolved. (30)

Fejerskov et al (2009) stated that fluoride is one significant tool for preventing and controlling caries, with several proposed mechanisms of action. The most important effects are due to their topical action onto the tooth surface. (26)

Ahovuo-Saloranta et al (2016) The effect of fluoride is observed to be much greater on smooth surfaces and far less on occlusal surfaces. Occlusal surfaces serve to be the focus for fissure sealants or ground fillings. (31)

Role of Fluoride varnishes in caries prevention

Petersen PE et al (2004), suggested, after reviewing 17 clinical studies, that fluoride varnish applications biannually had a caries-preventive effect in both primary and permanent dentition. A meta-analysis of eight clinical studies of Duraphat revealed a mean reduction in caries (38%) and an average reduction of 30%, in permanent teeth, when compared with untreated controls, which proved the caries preventive effect of fluoride varnish. (32)

Beltrán-Aguilar et al (2006) stated that fluoride varnishes were developed with the intention of prolonging the duration of the contact time of the tooth with the fluoride in the varnish. In this way, the fluoride uptake would be both amplified and enhanced. (33)

Poulsen (2009) performed a review which included six clinical trials on the effect of fluoride varnishes, which also confirmed the evidence that fluoride varnishes appear to have a caries-preventive effect on the young permanent dentition, while the effect on caries in the primary dentition remains to be determined. (34)

Marinho et al (2013) conducted a review which included 13 trials which considered primary and permanent teeth. (14) The review noticed an average reduction of around 43%, following the fluoride varnish applications.

Steel et al (2014) stated that fluoride varnish has the credit of easy application and economical intervention and does not require any special equipment or expertise to be applied. (35)

Douglas et al (2016) listed the other delivery systems of topical fluoride treatments including gel, paste, and foam. These treatments are available with varied concentrations of fluoride and versions of these treatments could be applied both professionally and at home. Gels and foams are often applied to the teeth using disposable trays that fit over the teeth. (36)

Studies proving the efficacy of Fluoride varnishes

Marinho et al. (2013) performed a study which included children up to the age of 16 years for analysis of fluoride varnishes. The comparison was done among several fluoride varnishes and control groups who did not

receive any treatment or placebo treatment and based on the results it was proved that the fluoride varnish was efficacious in inhibiting the progression of caries in both primary and permanent dentition. (14)

Jiang et al. (2014) conducted a Randomized Control Trial comparing single time oral hygiene education to parents with hands on parental tooth brushing training with or without fluoride varnish application (semi-annual) for children less than three years of age at a low risk of dental caries. There were no statistically significant differences in the incidences of early childhood caries between the three study groups included in the study. It is to be noted that the study was conducted in an area with effective fluoridation of the public water supply. The authors suggested that the use of fluoride varnish in this locality of low risk children in an area with optimal water fluoridation may not be effective for the prevention of Early Childhood Caries. (37)

Twetman et al. (2015) commenced a review of the fluoride agents which could be applied professionally or self- applied for the prevention and management of Early Childhood Caries in younger children less than three years of age and concluded that the findings of the studies did not provide ample quality evidence that fluoride dentifrice and fluoride varnish were equally effective for the prevention of Early Childhood Caries when compared with no treatment or a placebo. However, the comparison of the effectiveness of fluoride varnish over the fluoride toothpaste was not looked for in the review. (38)

Memarpour et al. (2015) compared no treatment versus oral hygiene information and education versus fluoride varnish plus education. Both the varnish and education alone groups had significantly reduced caries incidence when compared to the no treatment group; however, there were no significant differences reported between the two intervention groups. (39)

Anderson et al. (2016) undertook a non-blinded cluster Randomised Control Trial. Dental clinics were clustered and randomized and the children attending the clinics born in 2010 were included in the study. One of the groups received both topical fluoride varnish and family dental health education and the other group received dental health education alone. No significant difference in the number of dental caries was observed between groups and the authors concluded that there was no enhanced effect with fluoride varnish intervention for the prevention of dental caries. (40)

He et al (2016) conducted a three-arm parallel randomized control trial in patients aged 12 to 25 years who had recently completed orthodontic therapy which was done to check the efficacy of fluoride varnishes and a significant reduction was observed in all the groups when evaluated after six months. However, there was a significantly greater decrease in lesion volume in the varnish group when compared to the film group. Although both fluoride varnish and fluoride film resulted in significant improvements

in white spot lesions, the authors concluded that lesion volume decrease was much greater with fluoride and hence fluoride varnish was determined to be significantly more effective than fluoride film or placebo. (41)

Gao et al. (2016) conducted a systematic review of professionally applied fluoride treatment for preventing and improving dental caries in children. The meta-analysis revealed the overall percentage of remineralization to be 63.6% greater than no treatment and the authors highlighted the effectiveness of fluoride varnish for remineralization of early childhood caries. (15)

Tuloglu et al (2016) compared the efficacy of fluoride varnishes with casein phosphopeptide-amorphous calcium phosphate over other fluoride varnishes in terms of acid resistance in primary dentition. The efficacy was analysed by evaluating their surface micro hardness, lesion depth and structural changes. Within the limitations of the study, the author concluded that fluoride varnish with casein phosphopeptide-amorphous calcium phosphate was more effective in increasing the acid resistance of primary enamel than other fluoride varnishes. (42)

Lenzi et al. (2016) conducted a meta-analysis comparing fluoride varnish with no treatment for the reversal of enamel carious lesions in children of ages three to twelve. Despite a high level of heterogeneity identified in the meta-analysis, the authors concluded that fluoride varnish was an effective product which had the potential to inhibit the progression of enamel carious lesions in both primary and permanent dentitions. (43)

Assessment of demineralization inhibition by fluoride varnishes

Lynch et al (2004) checked the impact of low levels of Fluoride on the demineralization and remineralisation potential of the enamel. The tooth samples were suspended in the unstimulated saliva collected from young adults modified with acidic buffers. The acidic buffer solutions were then analysed for total calcium using atomic adsorption spectrophotometry. For each tooth sample, a calcium demineralisation rate was calculated.

Calcium demineralisation rate in turn reveals the demineralization inhibitory potential of the fluoride dentifrices. Statistical analysis of the results revealed that the fluoride concentrations at 0.014 ppm and above gave significant reductions in demineralisation when compared to controls. No significant reduction in demineralisation potential was seen at 0.009 ppm. (44)

Savas et al (2016) conducted a study to analyse remineralization capacity of fluoride varnishes by different quantitative methods. Buccal window was created on the enamel surface of bovine incisor teeth which was placed in an acidified gel system. The varnish was applied onto the demineralized areas and then analysed after fourth week of application. At the fourth week, the enamel surfaces were tested by surface micro hardness, quantitative light-induced fluorescence-digital, energy-dispersive spectroscopy and laser fluorescence. Laser fluorescence pen measurements showed significant differences at baseline, afterdemineralization, and after the one-week remineralization period. Withregards to the surface micro hardness and quantitative light-induced

fluorescence-digital analyses, statistically significant differences were found. After the 1- and 4-week treatment periods, the calcium and phosphate concentrations and Calcium /Phosphate ratio were higher compared to those of the demineralization surfaces. Based on these observations it was concluded that casein phosphopeptide-amorphous calcium phosphate containing fluoride varnish provides remineralization after a single application and seems appropriate for clinical use. (45)

Nozari et al (2017) performed an in vitro study on the effect of sodium fluoride varnish, nano-Hydroxyapatite Serum and Nano Silver Fluoride on primary teeth enamel remineralization. Incipient caries was induced in primary sound anterior teeth by storing each specimen in demineralization solution for 72 hours. Surface Micro hardness was assessed with Vickers micro hardness tester before and after demineralization and after 10 days of pH-cycling. Statistically significant differences were noted between all groups, however, the highest surface micro hardness values were observed in nano silver fluoride group. (46)

Antibacterial effect of Fluoride varnishes

Deepti et al (2008) conducted a Randomized Controlled Triple Blind Study to check the impact of fluoride varnish on the oral microbe count in saliva of caries free children using Dentocult SM kit and concluded that the Fluor Protector fluoride varnish application showed a statistically significant reduction in the *Streptococcus mutans* count following the varnish application. (47)

Erdem et al (2012) checked the effects of fluoride varnishes on the biofilm formation by *Streptococcus mutans* and *Streptococcus sobrinus* . Based on the results of the study, he concluded that the varnish which had the most antibacterial effects on *Streptococcus mutans* and *Streptococcus sobrinus* biofilms was the combination of Cervitec varnish and Fluoroprotector® when compared to Fluoroprotector® and Bifluoride 12. (48)

Sanchit Paul et al (2014) performed a randomized control trial to check the efficacy of fluoride varnish and chlorhexidine-thymol varnish on modifying the *Streptococcus mutans* levels in human dental plaque. Results revealed that both fluoride varnish and chlorhexidine-thymol have equivocal effect on the streptococcus species. (49)

Sharma et al (2018) evaluated and compared the efficacy of tooth brushing, fluoride varnish, chlorhexidine mouthwash, and a probiotic (Yakult) in reducing the *Streptococcus mutans* counts in saliva. All the groups showed a marked reduction in the total count of *Streptococcus mutans* species with the superior results obtained from the Fluoroprotector® varnish followed by chlorhexidine mouthwash, probiotic (Yakult), and tooth brushing. (50)

Yadav et al (2019) did a randomized control trial to check the impact of three varied compositions of topical fluoride varnishes, with and without oral prophylaxis on influencing the count of *Streptococcus mutans* in

samples obtained from children of age 2-8 years. It was observed that Fluoride varnish with casein phosphopeptide – amorphous calcium phosphate showed a marked reduction of *Streptococcus mutans* count followed by xylitol-containing fluoride varnish and then Fluor Protector. It was also observed that the oral prophylaxis done prior to fluoride application had negligible effect on the efficacy of fluoride varnish. (51)

Ben Khadra et al (2019) checked whether there is any alteration in the count of *Streptococcus mutans* following the application of fluoride and chlorhexidine varnishes. Results proved that there was a significant reduction in *Streptococcus mutans* counts in children's saliva following the application of both the fluoride and the chlorhexidine varnishes. (52)

Need for additional active agents in fluoride varnishes

Johnsson et al (1993) stated that several proteins like statherins play a significant role for stabilizing calcium and phosphate ions in saliva and thereby preventing their spontaneous precipitation. (53)

Larsen MJ et al (2003) stated that though essential, the fluoride component alone is not sufficient enough for the prevention of demineralization and possible reincorporation of minerals into demineralised hydroxyapatite crystals. There is also a need for the presence of bioavailable calcium and phosphate in the plaque fluid for maintaining the structural integrity of hydroxyapatite crystals. Though the normal saliva does not usually contain fluoride in optimal levels needed for

remineralisation, it has been noted that saliva is supersaturated with respect to the calcium and phosphate ions. (54)

Shahmoradi M et al (2017) stated that fluoride varnishes with additional ingredients to enhance its efficacy are available in the market including the ones with added amorphous calcium phosphate, tri-calcium phosphate, casein phosphopeptide stabilized amorphous calcium phosphate and Xylitol coated calcium and phosphate. The study revealed that the added ingredients improved the performance of different fluoride varnishes in terms of the maintenance of the structural and nano mechanical properties of dental enamel. (16)

Addition of functionalized Tri calcium phosphate to fluoride varnishes

Vogel et al (1998) mentioned in his paper that the two different tricalcium phosphates, $\text{Ca}_3(\text{PO}_4)_2$ - α -tricalcium phosphate and β -tricalcium phosphate. α -tricalcium phosphate can exist at high temperatures as high as 1125 °C whereas β -tricalcium phosphate is stable at room temperature. Hence, β -tricalcium phosphate is considered to be the bioavailable form of tricalcium phosphate that could be effectively used in agents for enhancing oral health. It is known that the remineralizing activity of eroded enamel is performed by the calcium and phosphate present in the oral environment. Literature reveals that by using β - tricalcium phosphate, the concentration of calcium in the saliva can be increased. In his study, an increase of calcium in the saliva as

consequence of acidic attacks and an acidic plaque-pH was noted, when 2.5% β -tricalcium phosphate was used as additional compound in chewing gums. Compared to the control, the pH increased (buffering of acidic attacks) along with the concentrations of free calcium and free phosphates, both of which aid in remineralisation process. (55)

Laurence J. Walsh, (2009) claimed that in spite of its advantages, β -tricalcium phosphate when used along with fluoride have pre-mature interactions and form calcium-phosphate complexes and calcium fluoride, thereby the bioavailability of β -tricalcium phosphate and fluoride gets lowered. (56)

Schemehorn et al. (2011) compared the fluoride uptake into the enamel surface from two fluoride varnishes with different sources of calcium phosphate and concluded that amorphous calcium phosphate varnish formulation delivers statistically significantly more fluoride than the formulation containing tricalcium phosphate to both the intact as well as the demineralized enamel. (57)

Karlinsey et al (2012) analysed the impact of a sodium fluoride varnish on the morphology and elemental compositions of sound and demineralized dentin in a pH cycling model. Results from the scanning electron microscopy and energy-dispersive X-ray spectroscopy demonstrated varnish with added tricalcium phosphate (Vanish™) can provide beneficial mineralization relative to the control group for both sound and demineralized dentin substrates. (58)

Alamoudi et al (2013) evaluated the improved effect of the combination of 5% sodium fluoride varnishes with tricalcium phosphate on the micro hardness of primary teeth. The surface micro hardness of the teeth samples was measured by a Knoop indenter before and after treatment and statistically analysed. Based on the findings the authors suggested that the use of an additive including functionalized tricalcium phosphate to a fluoride varnish effectively improves the protective property of the varnish on primary teeth. (18)

Rirattanapong et al (2014) checked the effect of fluoride varnishes containing tri-calcium phosphate sources on remineralization of the initial enamel lesions. Based on the results the authors recommended the use of fluoride varnishes containing tri-calcium phosphate to inhibit progression of the initial primary enamel lesions. (59)

Rirattanapong et al (2016) conducted a study to check the fluoride release from different formulations of calcium phosphate fluoride varnishes on primary teeth including tricalcium phosphate and amorphous calcium phosphate. By the end of three months it was noted that tricalcium phosphate fluoride varnish released more fluoride than the amorphous calcium phosphate fluoride varnish. (60)

Meyer F et al (2018) stated that functionalized β -tricalcium phosphate is recently used in dentifrices and fluoride varnishes along with 5% sodium

fluoride. The rationale behind the addition of functionalized tricalcium phosphate is, in case of functionalized tricalcium phosphate, by milling β -tricalcium phosphate with the organic components, the calcium oxides become 'protected' by the organic materials like fumaric acid/ sodium lauryl sulfate, thus facilitating the calcium and phosphate ions of the β -tricalcium phosphate to coexist with fluoride ions without any premature interactions. When the dentifrice or varnish is applied intraorally, the outer protective coating is removed due to the action of saliva, thus releasing calcium at tooth surface, promoting high bioavailability of fluoride and calcium ions on the surface of the lesion thereby promoting remineralization. (61)

Almaz et al. (2020) performed a randomized control trial to check the antibacterial effect of Fluoride varnish with various added formulations and it was observed that fluoride varnish with added tricalcium phosphate caused the maximum reduction in *Streptococcus mutans* and *Lactobacilli* levels when compared to the other varnishes. (62)

Addition of Xylitol to fluoride varnishes

Söderling et al., (2009) mentioned the mechanism of action behind the anticariogenic property of xylitol which is claimed to be due to the inability of five-carbon sugar to be fermented by *Streptococcus mutans* to produce acids. Addition of xylitol also has the additional benefit of improving the patient compliance. Xylitol in lozenges also stimulates salivary flow, possibly increasing mechanical cleansing activity, delivering minerals to

demineralized enamel, and buffering the plaque acids. The study showed that the resistant strains of *Streptococcus mutans* may be less cariogenic in the habitual xylitol users, as it causes a reduction of insoluble extracellular polysaccharides, thus altering its adherence onto tooth surfaces. (63)

Shen et al (2011) stated that xylitol coated calcium phosphate (Embrace™) varnish resulted in higher remineralization compared to Durashield. indicating the notion that extra calcium and phosphate ions provided by the xylitol coated calcium and phosphate in Embrace™ varnish increases the efficiency of the remineralizing potential. (64)

Moraes et al. (2011) evaluated the antibacterial efficacy of the xylitol component combined in chlorhexidine varnishes and observed that 40% xylitol solution did not provide significant suppression of the *Streptococcus mutans* count. (65)

Garcia Godoy et al (2013) performed a study to check the efficacy of xylitol based dentifrices with and without fluoride on root caries and it was noted that the evaluation of the mean lesion depths showed no statistical difference. (66)

Vongsavan et al (2014) compared the surface micro hardness of the bovine enamel after treatment using fluoride varnish and combination of xylitol and fluoride varnish and noticed that the added xylitol component did not have any additional benefit in vitro. (67)

Cardoso et al (2014) performed a comparative study to check the remineralisation potential of various concentrations of xylitol varnishes and it was noted that surface lesion remineralization was noted on treating with both 10% and 20%. However subsurface lesion remineralization occurred only at 20% concentration. (68)

Cardoso et al (2016) conducted another study, based on the results of the above study, in which the author compared the remineralisation potential of xylitol varnishes with the commercially available fluoride varnishes and concluded that 20% xylitol showed promising results in terms of the remineralizing activity on the surface and subsurface lesions. (69)

Jafari et al. (2018) also suggested that the application of xylitol-based varnishes and casein phosphopeptide-amorphous calcium phosphate varnishes caused a remarkable reduction in the level of *Streptococcus mutans* in the oral cavity. (70)

Gargouri et al (2018) checked the efficacy of fluoride varnishes coupled with Xylitol in terms of their protective effect against demineralization and antibiofilm effect and noticed that the minimum biofilm inhibitory effect was low for xylitol but it was inconsistent when enriched with remineralizing agents. (71)

Jain et al (2019) performed an in vitro study by comparing the remineralizing potential of fluoride varnish with resin modified glass ionomer based fluoride varnish, fluoride varnish with xylitol coated calcium phosphate and conventional fluoride varnish on early enamel lesions and concluded that the remineralizing property of fluoride varnish with xylitol coated calcium phosphate was not as good as those produced by glass ionomer based fluoride varnish. (72)

Yadav S et al (2019) stated that xylitol modifies the growth and metabolism of several bacterial species, but *streptococcus mutans* appear to be the target organism. In addition, the reduction of insoluble extracellular polysaccharides is probably of importance for the xylitol- induced decrease in the counts as well as the transmission of *mutans streptococci*. (51). (5)

The present in vitro study was conducted in the Department of Pedodontics and Preventive Dentistry, Sathyabama Dental college and Hospital, Chennai.

Other facilities involved in the study

- Department of Biochemistry, Sathyabama Dental college and Hospital
- Department of Oral Pathology, Sri Ramachandra Institute of Higher education and Research, Porur
- Department of Oral Pathology, Ragas Dental college and Hospital, Uthandi
- Research Centre, Balaji Dental college and Hospital, Pallikaranai

Materials used in the study

Experimental materials

1. Vanish™ 5% sodium fluoride white varnish, (3M ESPE, St. Paul, MN, USA) (*Fig. 6*)
2. Embrace™ Varnish (Pulpdent corporation, Watertown, MA, USA) (*Fig. 7*)
3. Fluor Protector®, (Ivoclar Vivadent AG, Schaan, Liechtenstein) (*Fig. 8*)

Materials & Equipment for evaluation of Demineralization inhibitory effect

- **Armamentarium used (Fig. 1)**
 - Sterile gloves
 - Sterile tweezers
 - HB graphite pencil (Apsara platinum)
 - Brown duct tape (Wonder cello brown tape)
 - Nail varnish
 - Pipette (10 ml)
 - Ice cube trays
 - OHP/ CD/ DVD permanent marker pen (Luxor)
 - Diamond tipped disc (Strauss Diamond Disc 0.17 mm, Fine grit, Strauss Co., Raanana, Israel)
 - Wax sheet
 - Wax knife
 - Spirit lamp
 - Self-cure acrylic (DPI® Cold Cure acrylic)
 - GDC Caliper Iwanson Metal (CLP1)
 - Glass slide
- Micromotor, (Supreme Labo, CONFIDENT DENTAL EQUIPMENTS PVT LTD, Bangalore) (Fig. 2)
- 40 non carious premolars extracted for orthodontic purposes (Fig. 3)
- Demineralizing solution (Fig. 13)
- Hard tissue microtome (Leica Sp 1600 Saw microtome, Leica Microsystems Nussloch GmbH, Germany) (Fig. 17)

- Polarised light microscope (Olympus BX50 Fluorescence Microscope, Olympus corporation, Shinjuku City, Tokyo, Japan) (Fig. 25)
- ImageJ software (a JAVA based image processing software developed by National Institute of Health)

Materials & Equipment for evaluation of Antibacterial effect

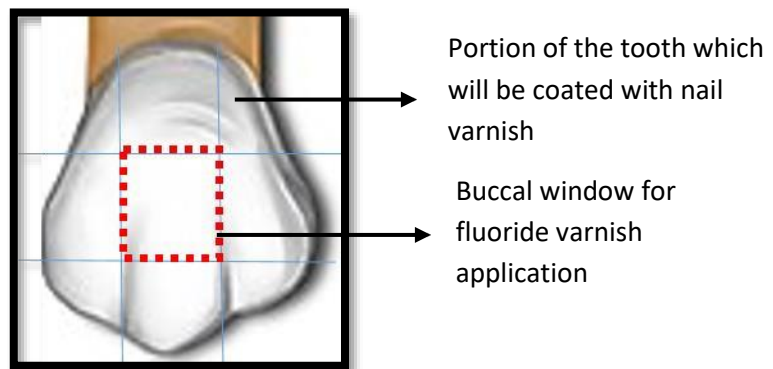
- **Armamentarium used (Fig. 30)**
 - Culture plate
 - Cotton swab
 - Micropipette
 - Wire loop
 - 96 well plate
- Blood agar
- *Streptococcus mutans* strain
- Incubator (Fig. 32)

Methodology

Demineralization inhibitory effect of fluoride varnishes

- **Preparation of tooth sections**

Forty non-carious freshly extracted premolars extracted for orthodontic reasons were selected for the study. The teeth were first cleaned with pumice slurry and polishing brush in a slowly rotating hand piece. The teeth were then thoroughly washed under running water and stored in sterile saline.



Diagrammatic representation of the buccal window on the tooth sample for fluoride application

A buccal window measuring 2 X 2 mm was drawn on the middle third of the buccal surfaces of the enamel portions of the teeth using a HB graphite pencil and was covered using small pieces of duct tape. The remaining portion of the tooth was painted with nail varnish (fig. 5) and was allowed to dry at room temperature. Once dried the duct tape pieces were removed exposing the buccal window for fluoride application.

- **Fluoride pre-treatment of the enamel sections**

The tooth sections were randomly grouped into four groups with ten samples for each group as follows:

Group 1 –Vanish™ 5% sodium fluoride white varnish

Group 2 – Embrace™ Varnish

Group 3 – Fluoroprotector® varnish

Group 4 – No fluoride application - Control

Group 1: The contents of Vanish™ varnish was thoroughly mixed using the applicator brush which was available with the kit as per the

manufacturer's instructions. A thin layer of Vanish™ varnish was then applied on the prepared buccal windows on the buccal surfaces of tooth with the applicator brush using horizontal strokes.

Group 2: A thin layer of Embrace™ varnish was applied on the prepared buccal windows on the buccal surfaces of the tooth samples using the applicator brush provided by the manufacturer. Paint on technique was used for the application of the varnish.

Group 3: A thin film of the Fluoroprotector® varnish was applied on the prepared buccal windows on the enamel portion of the tooth samples with the applicator tip available with the kit. The paint on technique was used for application of the varnish.

Group 4: The prepared buccal windows were left as such without any fluoride intervention.

All the samples were left undisturbed for a period of 24 hours after the fluoride application.

- **Preparation of demineralizing solution**

The demineralizing solution was prepared in accordance to the protocol described by **Patil et al, 2013**. (73) The artificial caries solution was prepared using equal amounts (50 ml) of the following components:

- 2.2 mM calcium chloride ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$)
- 2.2 mM monosodium phosphate ($\text{NaH}_2\text{PO}_4 \cdot 7\text{H}_2\text{O}$)
- 0.05 M lactic acid

The final pH was then adjusted to 4.5 using sodium hydroxide.

- **Immersion of the samples into the demineralizing solution (*Fig. 14*)**

The samples following the 24 hours of drying were then immersed in 3 ml of demineralizing solution in plastic containers for a period of 48 hours at room temperature. At the end of 48 hours the samples were removed from the solution using tweezers for sectioning.

- **Sectioning of the tooth samples**

The root portions of the tooth samples were sectioned using a micromotor with a diamond tipped disc. (*Fig. 15*) The crown portion of the teeth were mounted on acrylic blocks for sectioning using a hard tissue microtome. (*Fig. 16*) A section measuring 100 μm was obtained from each sample using the hard tissue microtome. (*Fig. 18*) The thickness of the sections was confirmed to be 100 μm using GDC Caliper Iwanson Metal. (*Fig. 19*) These sections were then imbibed on glass slides using DPX mounting medium to be observed under the polarised light microscope. (*Fig. 20 - 24*)

- **Measurement of the demineralized zone**

The glass slides with the mounted samples were then analysed using a polarized light microscope. (*Fig. 25*)

Before starting the measurement, the stage micrometre with a 4X objective lens was focused. The sections were photographed under maximum illumination and the photomicrographs obtained. (*Fig 26 - 29*)

These photomicrographs were evaluated using the ImageJ software to measure the demineralized zone. The entire thickness of the enamel and the dark zones were measured using the ANALYZE, SET MEASURE option in ImageJ software expressed in pixels. The unit conversion from pixels to millimetre was done using an online web based software.

The values of the enamel thickness and the dark zones obtained were subjected to statistical analysis.

Antibacterial effect of fluoride varnishes

- **Bacterial strains used**

Streptococcus mutans MTCC 890 was procured from Microbial Type Culture collection and Gene Bank (MTCC), CSIR-Institute of Microbial Technology, Sector 39-A, Chandigarh-160 036, INDIA. This was revived on sheep blood agar and the purity was assessed by gram staining. The strains were inoculated in order to study the antibacterial efficacy.

Inoculation was done by overnight culture of *Streptococcus mutans* MTCC in 10 ml of brain heart infusion agar (Himedia, India) supplemented with sterile human serum (5%) and incubated at 37°C for 1 hour. The turbidity was adjusted to match the turbidity of McFarland standard scale 0.5 to obtain the cell density of 1.5×10^8 cells/ml.

The two widely used techniques to check the antibacterial efficacy of fluoride varnishes are,

1. Disc diffusion susceptibility testing
2. Broth micro-dilution technique

Both the above mentioned techniques were done in this study to check the antibacterial efficacy of the fluoride varnishes.

- **Disc diffusion susceptibility testing**

The disc diffusion method has been widely used to test antibacterial activities of herbal extracts, endodontic medicaments and sealers. It allows direct comparison of conventional medicaments used in dental procedures against the test microorganisms, indicating the medication that has the potential to eliminate bacteria from the region. Standard ciprofloxacin disc (30 µg) was included for comparison. (*Fig. 33*)

Agar well diffusion test, a modification of the disc diffusion test is a more practical and convenient method for analysing the materials which are thixotropic in nature. Owing to the viscous nature of the fluoride varnishes analysed in this study, this modified technique was employed in this study. Using the well diffusion technique, antibacterial resistance can be detected by challenging bacterial isolates with the materials placed in the wells punched on the agar plate that has been seeded with a lawn of bacteria.

Lawn culture of *Streptococcus mutans* MTCC 890 was made on sheep blood agar. Wells of 6 mm diameter was punched using coke borer and 50 µl each of Vanish™, Embrace™ and Fluorprotector® was added in the respective wells and the plates were incubated at 37°C for 24 hours. The assay was done in triplicate. The contents of sheep blood agar include 7% sheep blood plus nutrient agar with a pH of 7.4 ± 0.2 .

- **Minimal inhibitory concentration determination by broth micro dilution technique**

Broth dilution is a technique in which incremental increasing concentrations of the antibacterial agent is added to containers holding identical volumes of broth culture with known density of the test organism. Broth micro-dilution is a modification of the broth dilution and is performed on micro-titre plates with a holding capacity of $\leq 500\mu\text{l}$ per well. In this procedure double serial dilution of an antibacterial agent is prepared in the broth medium and the lowest concentration of the antibacterial agent under defined *in vitro* conditions that completely inhibits visible bacterial growth within a defined period of time is recorded as the minimal concentration.

Minimum inhibitory concentration of Vanish™, Embrace™ and Fluorprotector® were determined as follows. Gradually increasing dilutions of the test solutions were prepared on the respective labelled wells of a micro-titre plate. (Fig. 34) The first well contained the original

concentration of the product tested. Thereafter from the second well, serial dilution of Vanish™, Embrace™ and Fluorprotector was performed.

The last well of each row served as the culture control and no test solution was added. 10 µl of *Streptococcus mutans* suspension was added to all the wells including the culture control (Fig. 34). The plates were incubated overnight at 37°C. Minimal bactericidal concentration of the test solutions (Vanish™, Embrace™ and Fluorprotector) were determined by spot inoculation of 5 µl of the culture from each well of the micro-titre plate onto a Mueller Hinton Agar plate. Minimum concentration of the test solution that completely inhibited (~99%) the growth of the colonies on the Mueller Hinton Agar plate was scored as the minimal inhibitory concentration of the respective test solution. (Fig. 36 - 41)

Statistical analysis

- The mean values of the enamel thickness of the tooth samples in four groups were analysed using the **One-way ANOVA** test initially to check the uniformity of the enamel thickness in all groups for standardization purpose.
- The mean difference values of the demineralization zones of the four groups were analysed using the **One-way ANOVA** test and as the difference was statistically significant further intergroup comparisons were done.
- Pairwise intergroup comparison was done using the **Bonferroni's test** to check the superiority of the efficacy of one material over the other.

Formulae used for analysis

- **Mean**, $\bar{X} = \sum X_i / n$, $i = 1, 2, \dots, n$
- **Standard deviation (SD)** $= \sqrt{\sum (X_i - \bar{X})^2 / n - 1}$
- **One-way ANOVA**

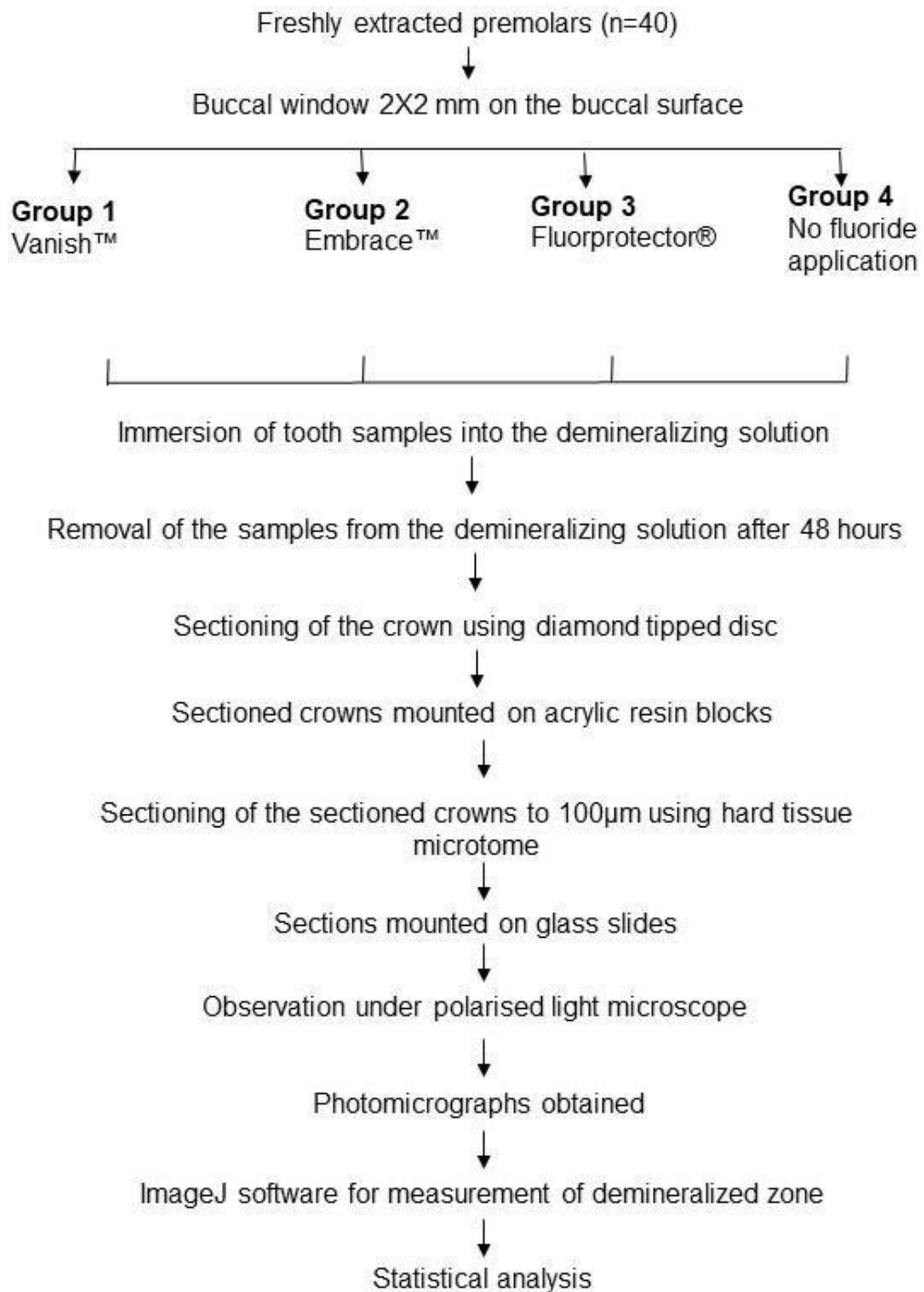
F = Between group variance / Within group variance

- **Bonferroni's post hoc:**

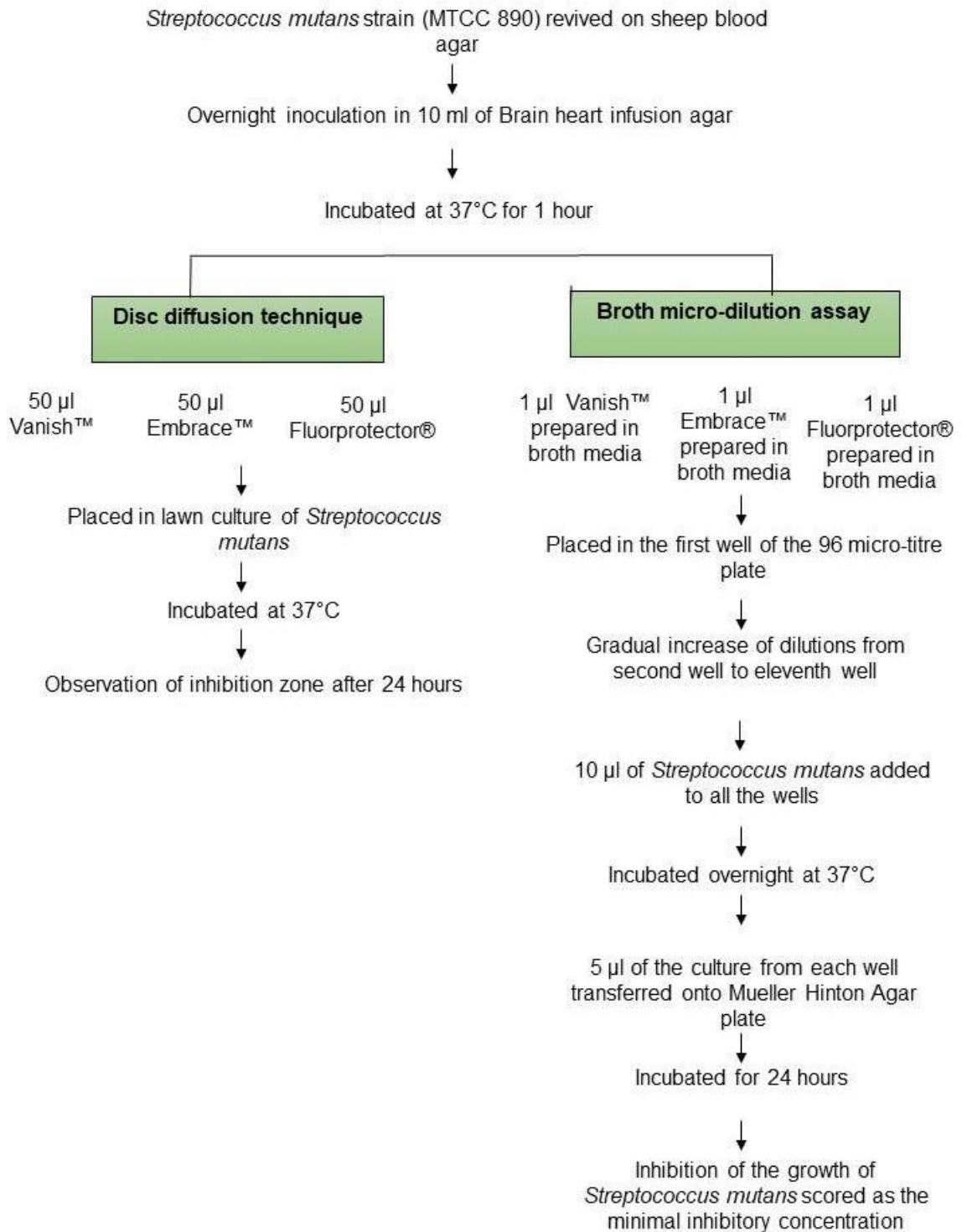
$$s_p^2 = \frac{\sum (n_i - 1)s_i^2}{\sum (n_i - 1)}$$

where n_i is the sample size and s_i is the standard deviation for the i^{th} sample

DEMINERALIZATION INHIBITORY EFFECT OF FLUORIDE VARNISHES



ANTIBACTERIAL EFFECT OF FLUORIDE VARNISHES



Materials and equipment used to check the demineralization inhibitory effect of fluoride varnishes

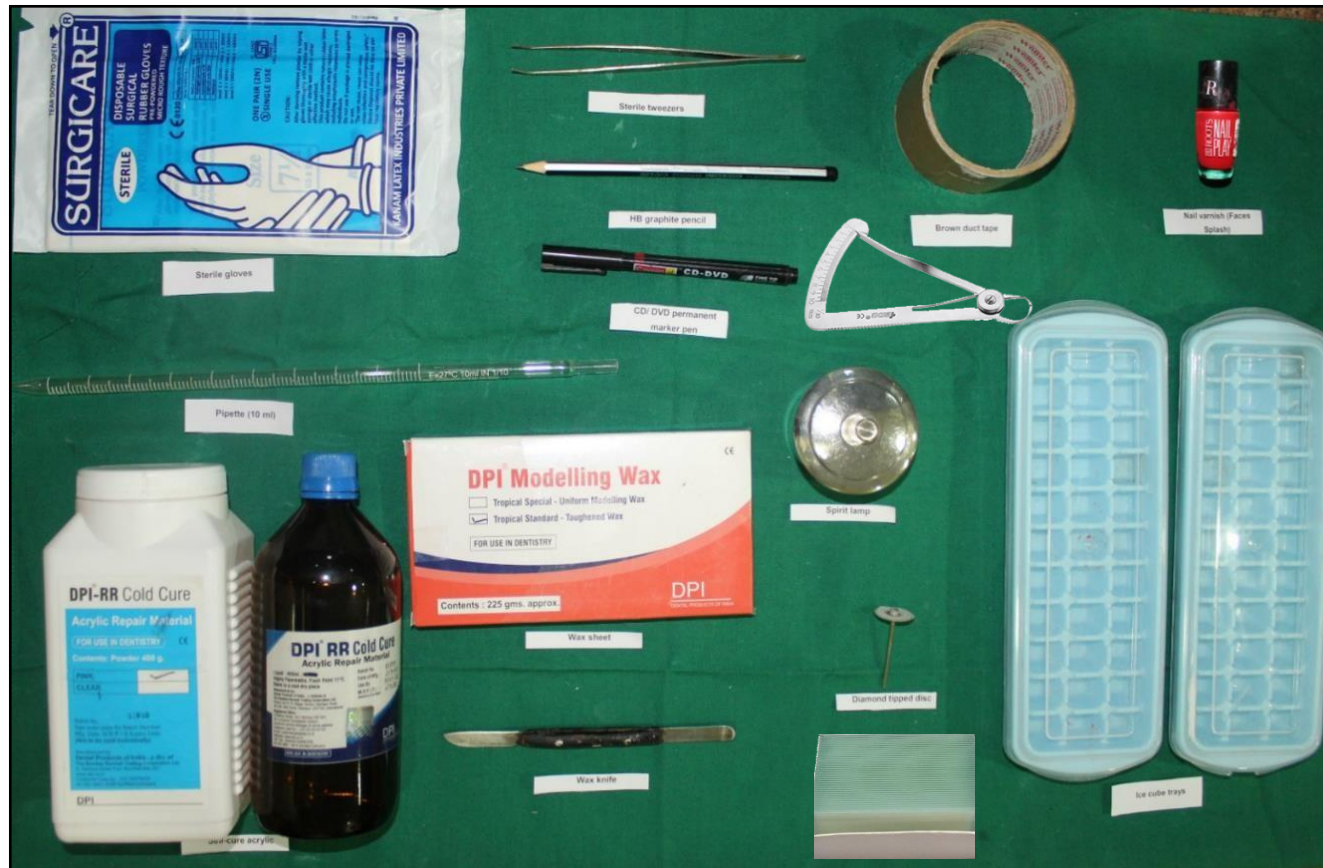


Fig. 1: Armamentarium used for the demineralization inhibitory effect

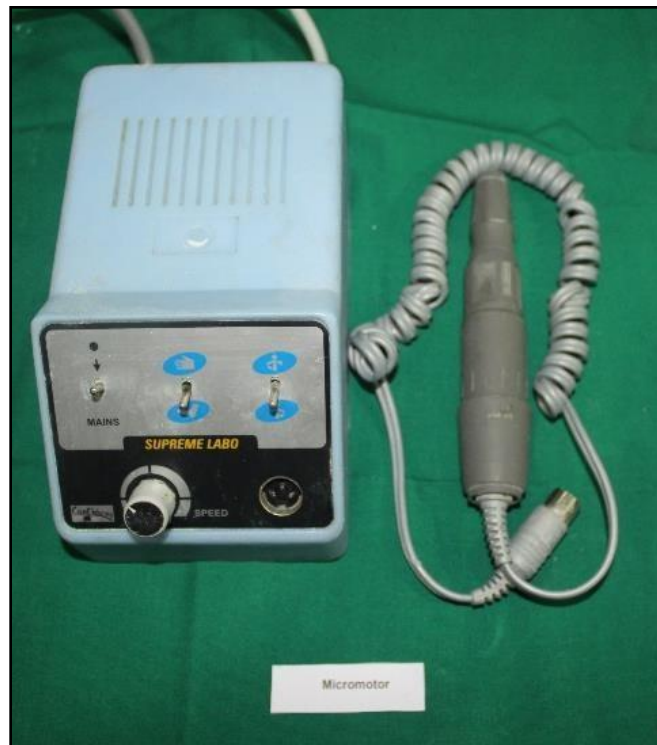


Fig. 2. Micromotor



Fig. 3: Freshly extracted premolars (n=40)



Fig. 4: Buccal window prepared

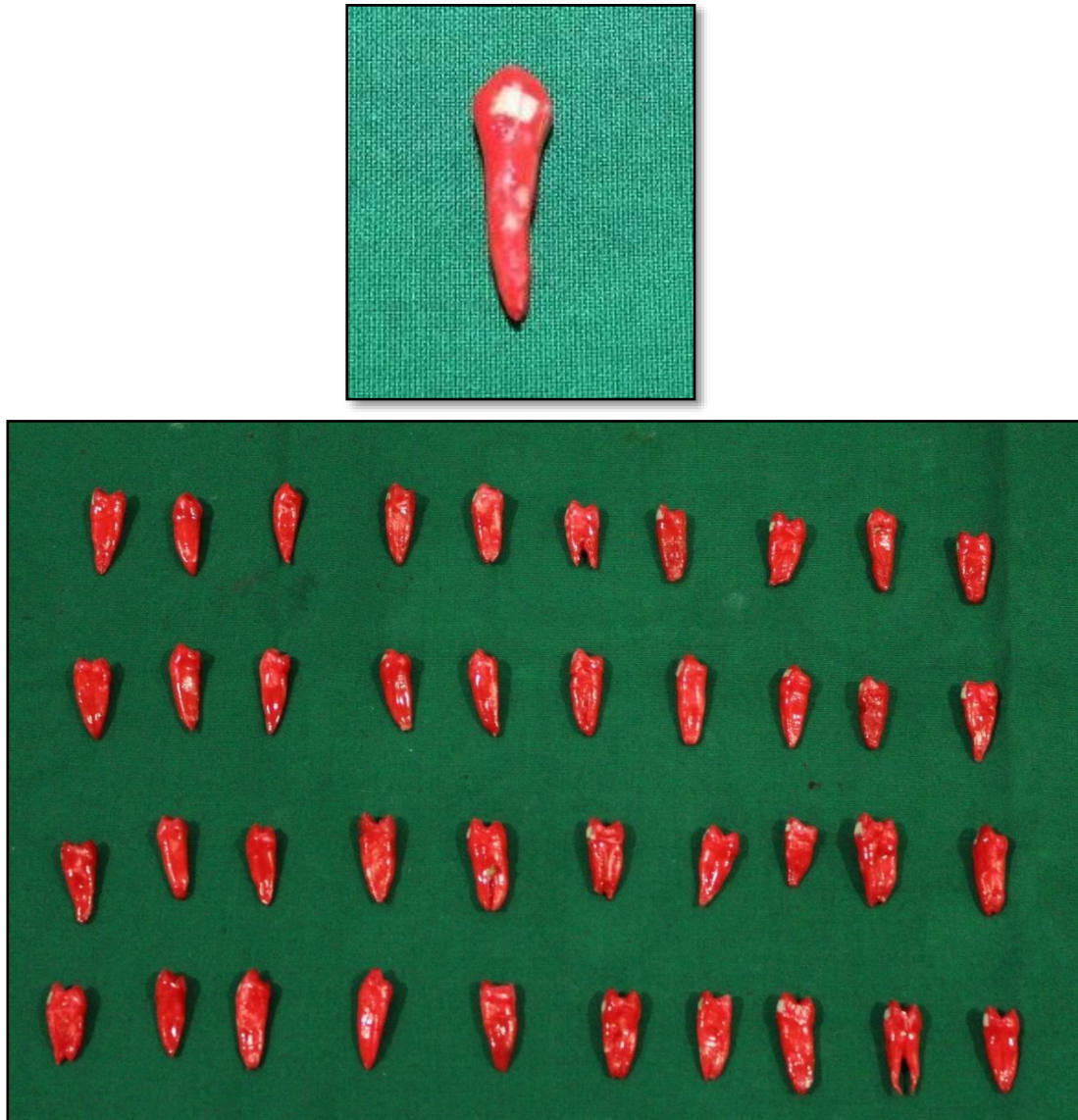


Fig. 5: Nail varnish coated samples excluding the buccal window



Fig. 6: Vanish™ varnish

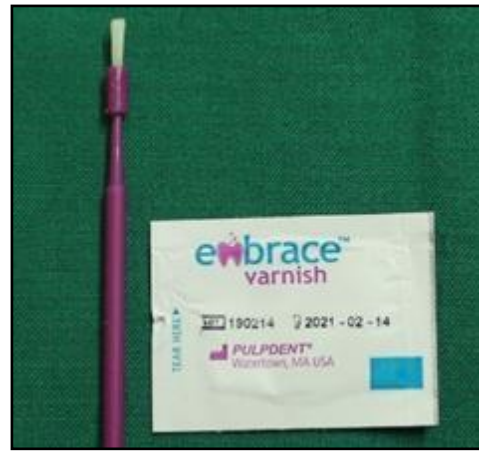


Fig. 7: Embrace™ varnish



Fig. 8: Fluoroprotector® varnish



Fig. 9: Application of Vanish™ varnish



Fig. 10: Application of Embrace™ varnish



Fig. 11: Application of Fluorprotector® Varnish

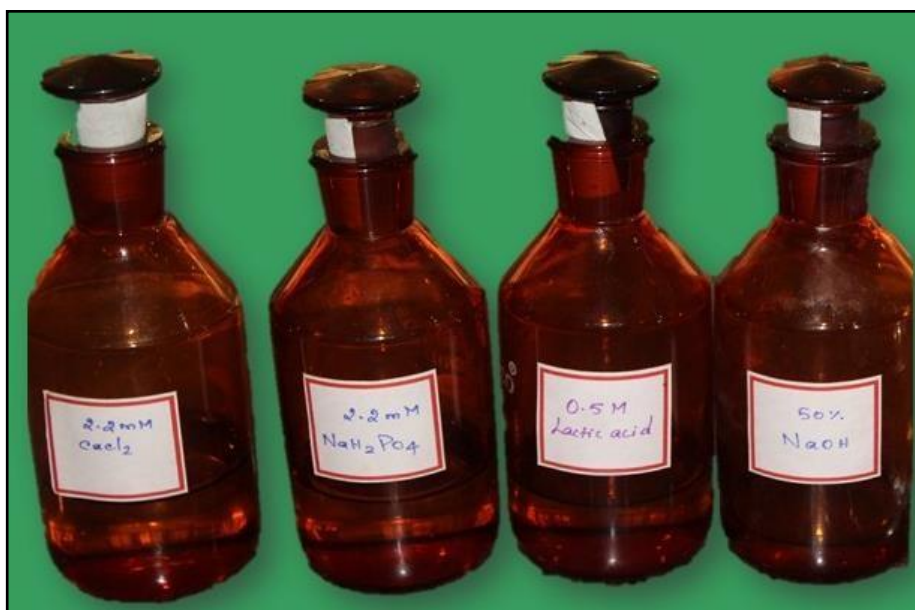


Fig. 12: Agents used for the preparation of demineralizing solution



Fig. 13: Prepared demineralizing solution

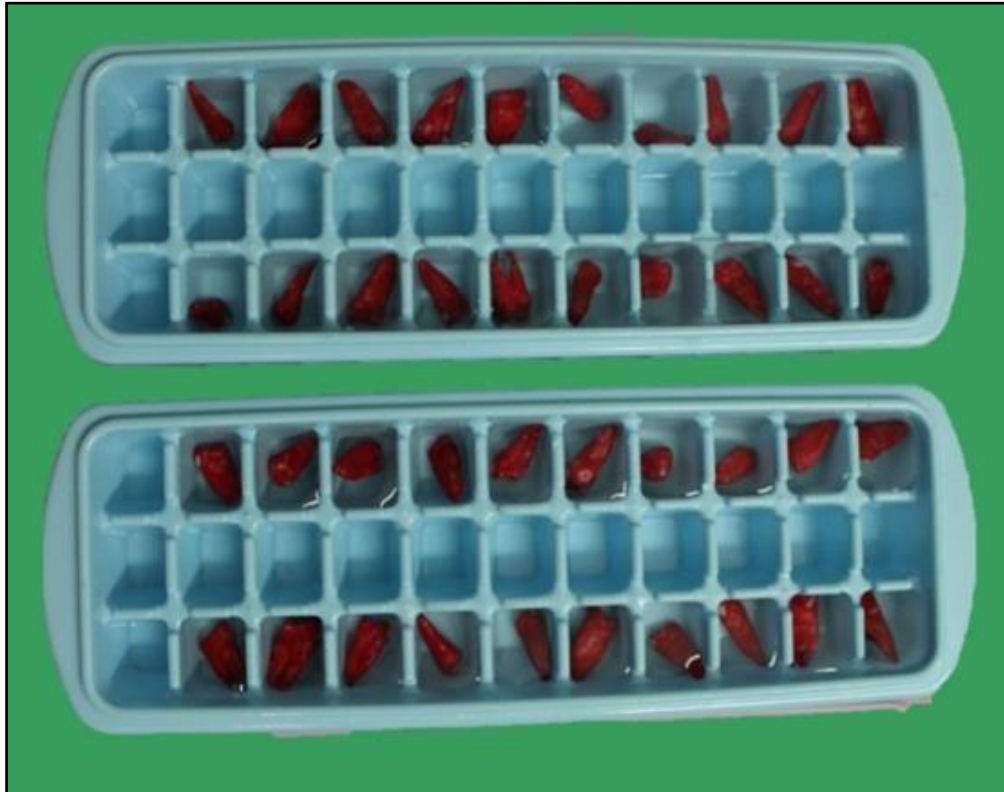


Fig. 14: Immersed tooth samples in the demineralizing solution



Fig. 15: Sectioning of the crown portion using diamond tipped disc



Fig. 16: Crown portion mounted on acrylic block for 100 μ m sectioning



Fig. 17: Hard tissue microtome

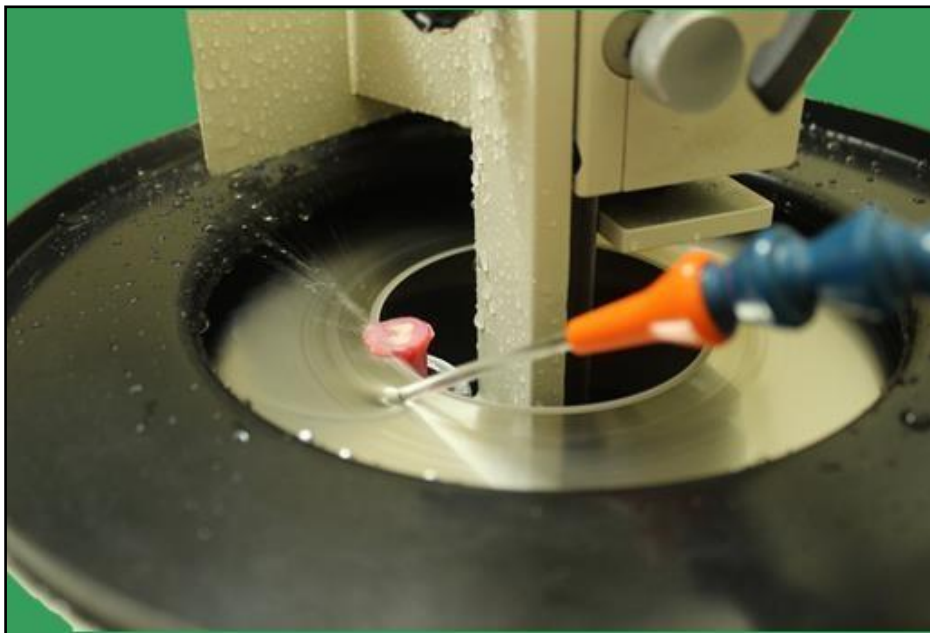


Fig. 18: Sectioning of sample using hard tissue microtome



Fig. 19: Measuring the thickness of the ground sections using GDC calliper

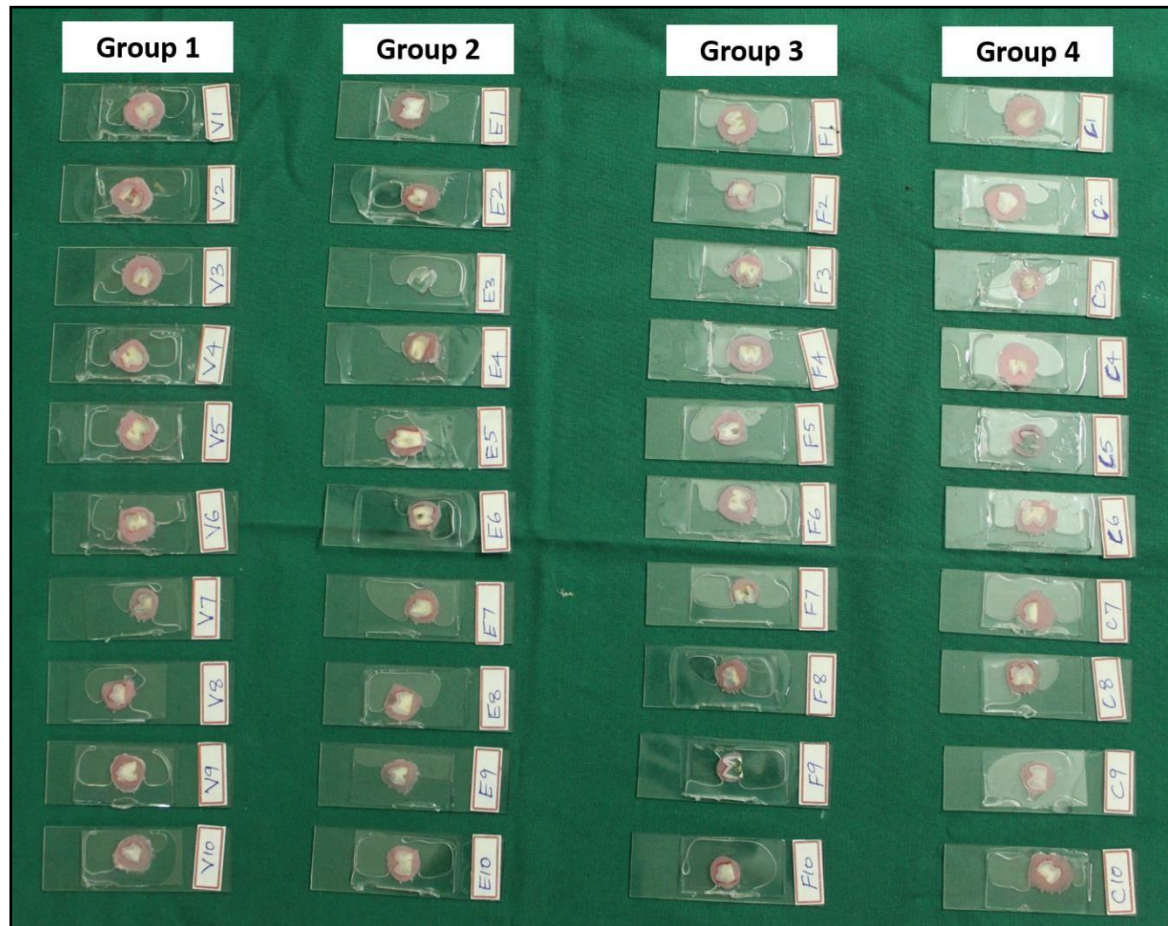


Fig. 20: Tooth sections from all the groups mounted on glass slides

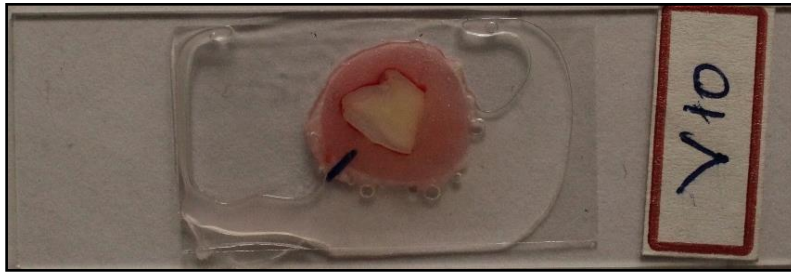


Fig. 21: Tooth section from Group 1 mounted on glass slide



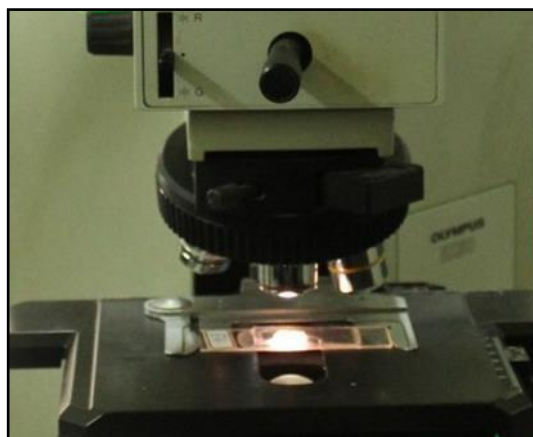
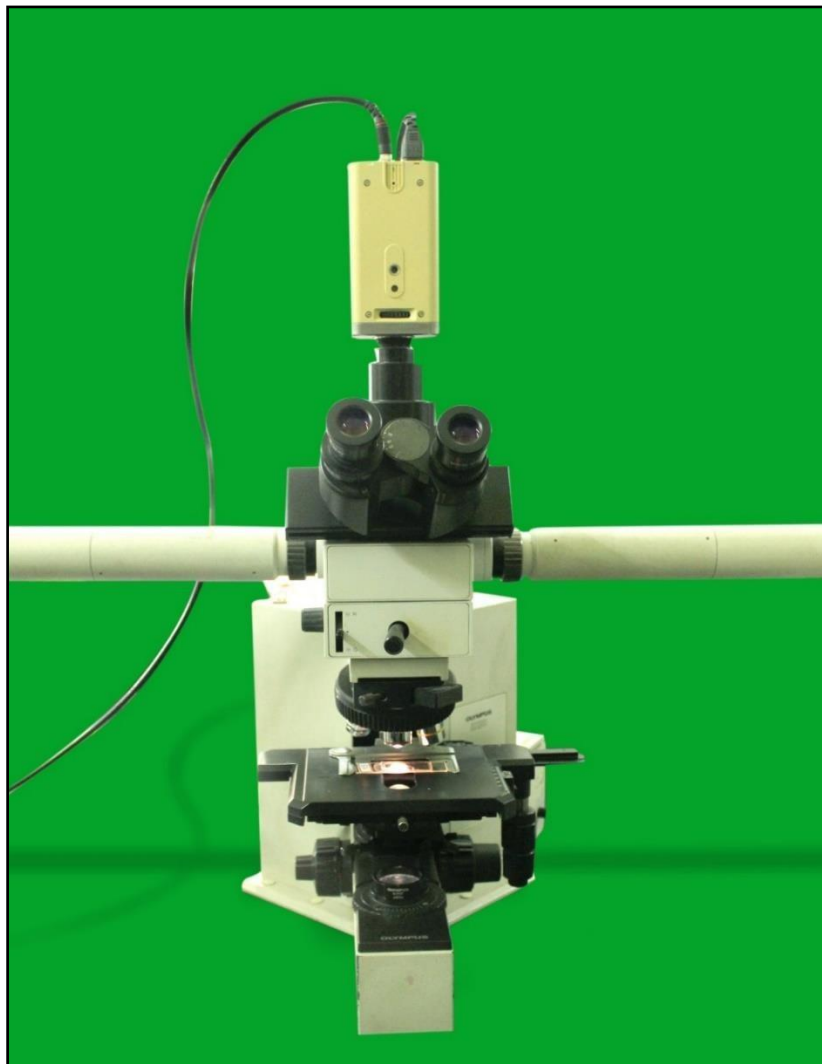
Fig. 22: Tooth section from Group 2 mounted on glass slide



Fig. 23: Tooth section from Group 3 mounted on glass slide



Fig. 24: Tooth section from Group 4 mounted on glass slide



***Fig. 25: Demineralised zone observation under polarised light
microscope***

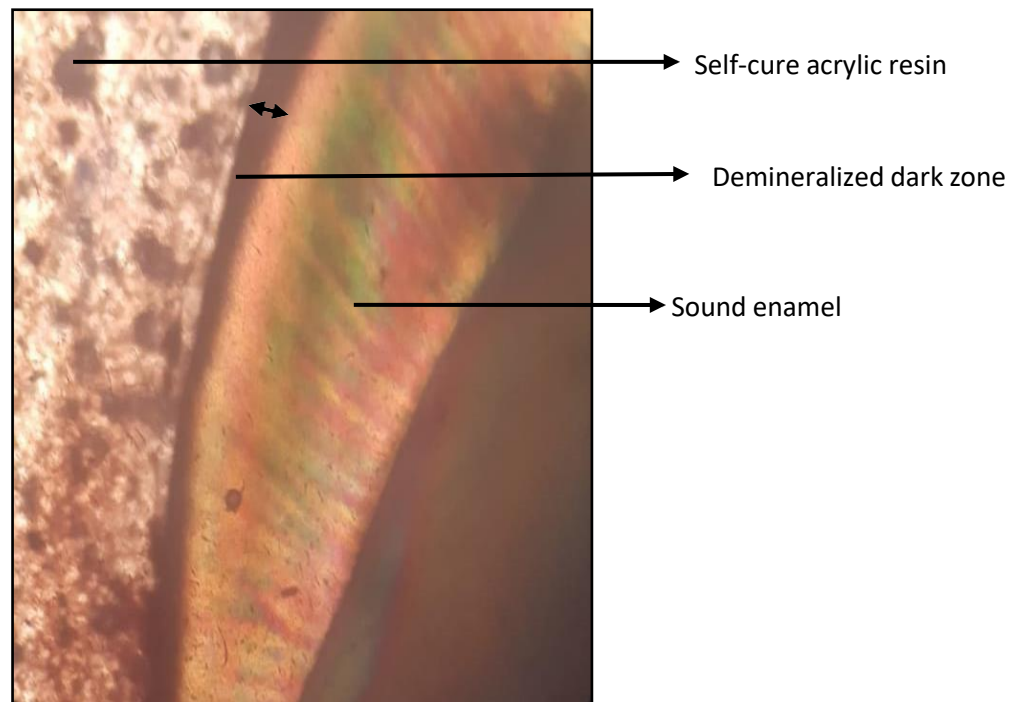


Fig. 26: Demineralised zone (9.84 mm) of Group 1 under polarised light microscopy

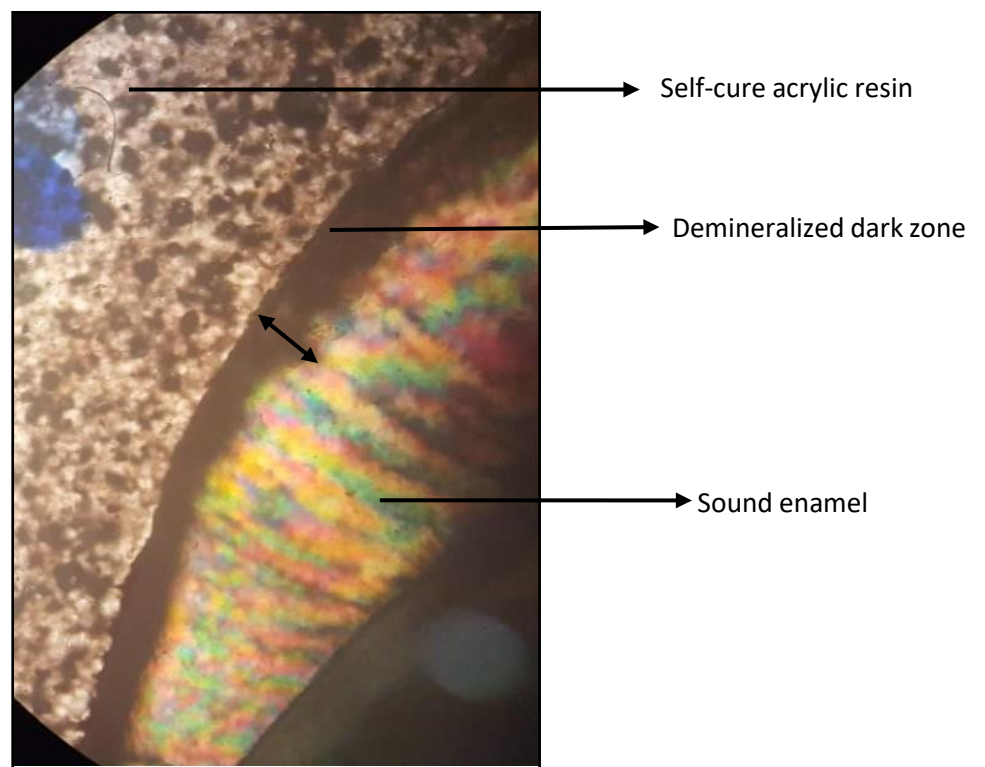


Fig. 27: Demineralised zone (15.89 mm) of Group 2 under polarised light microscopy

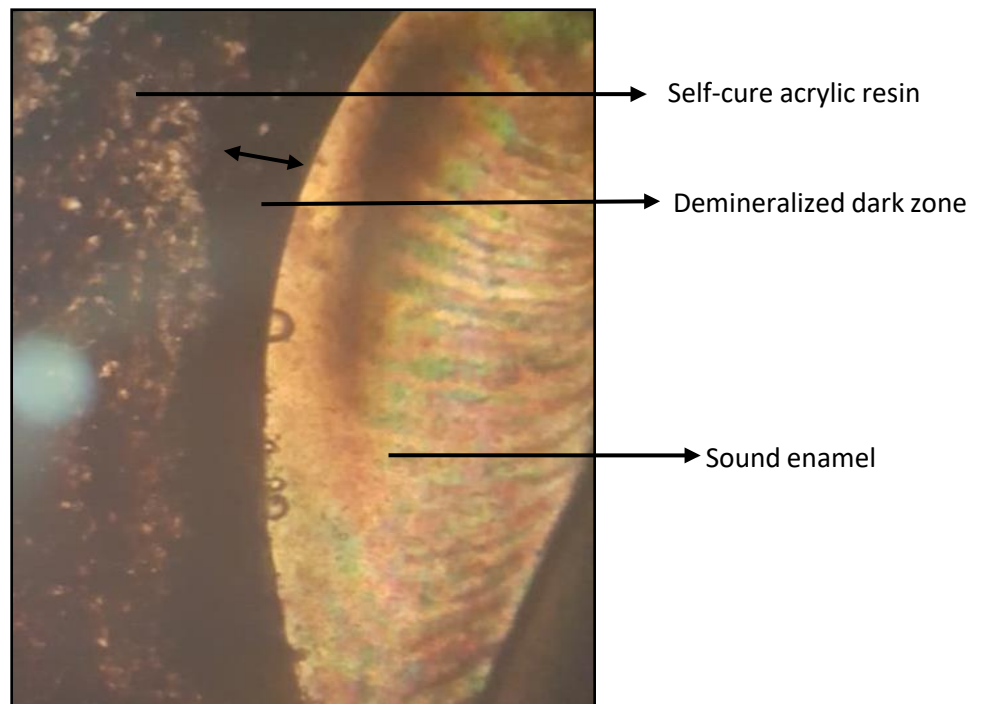


Fig. 28: Demineralised zone (34.16 mm) of Group 3 under polarised light microscopy

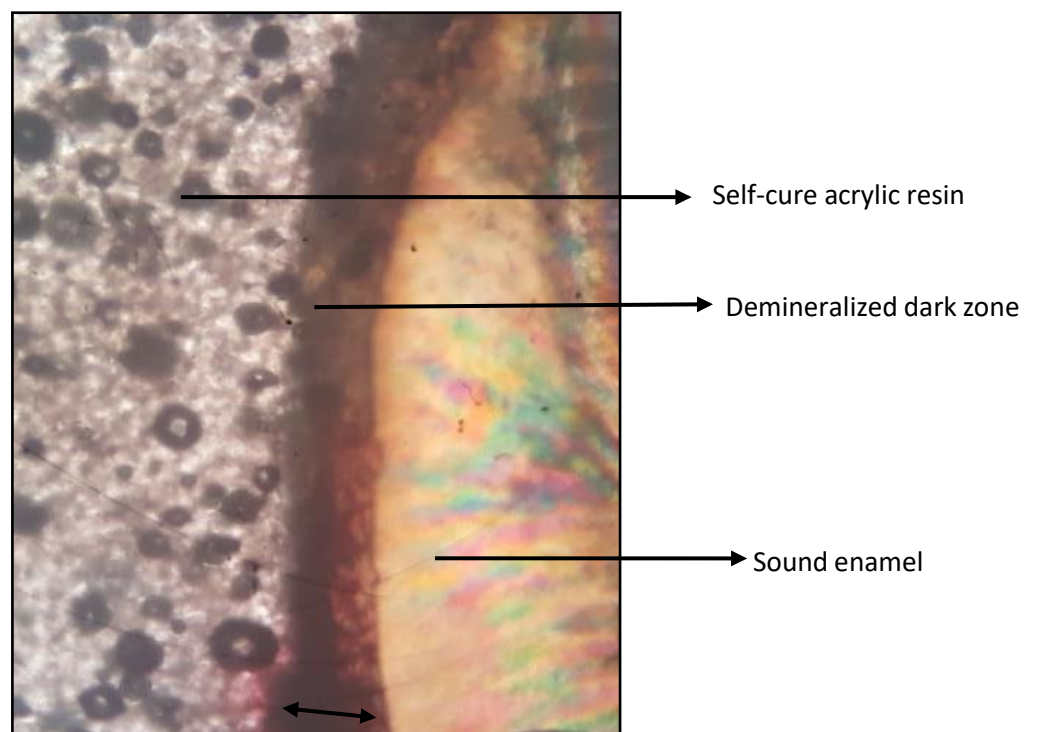


Fig. 29: Demineralised zone (48.44 mm) of Group 4 under polarised light microscopy

**Materials and equipments used to check the antibacterial efficacy of
fluoride varnishes**



**Fig. 30: Armamentarium used for the antibacterial efficacy of the
fluoride varnish**

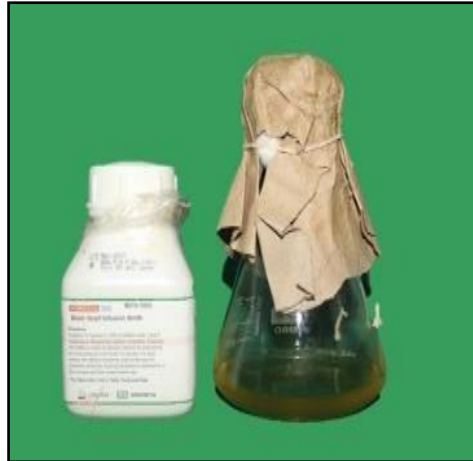


Fig. 31: Brain Heart Infusion agar



Fig 32: Incubator

Disc diffusion susceptibility testing

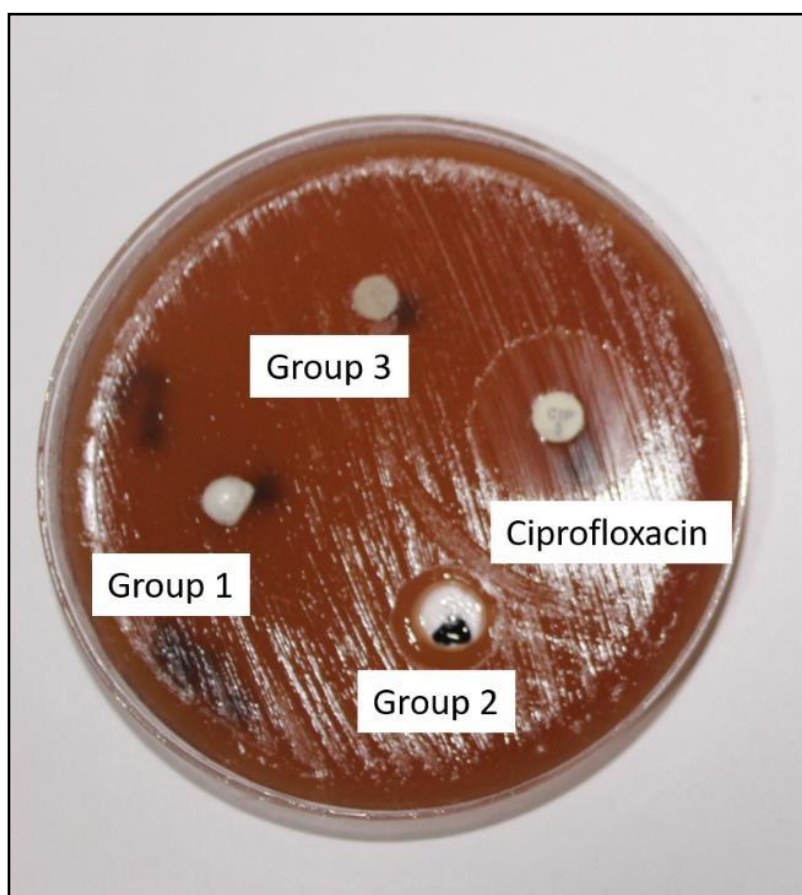


Fig. 33: Disc diffusion method showing the zones of inhibition of the growth of Streptococcus mutans

Broth micro-dilution technique

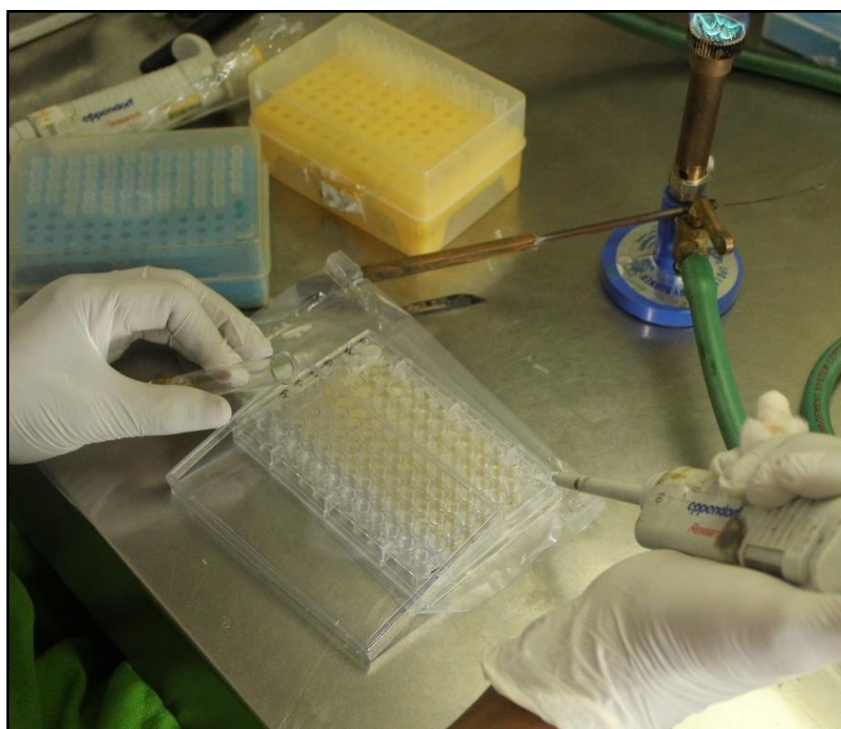


Fig. 34: Transfer of the fluoride varnishes onto the 96 titre well plate

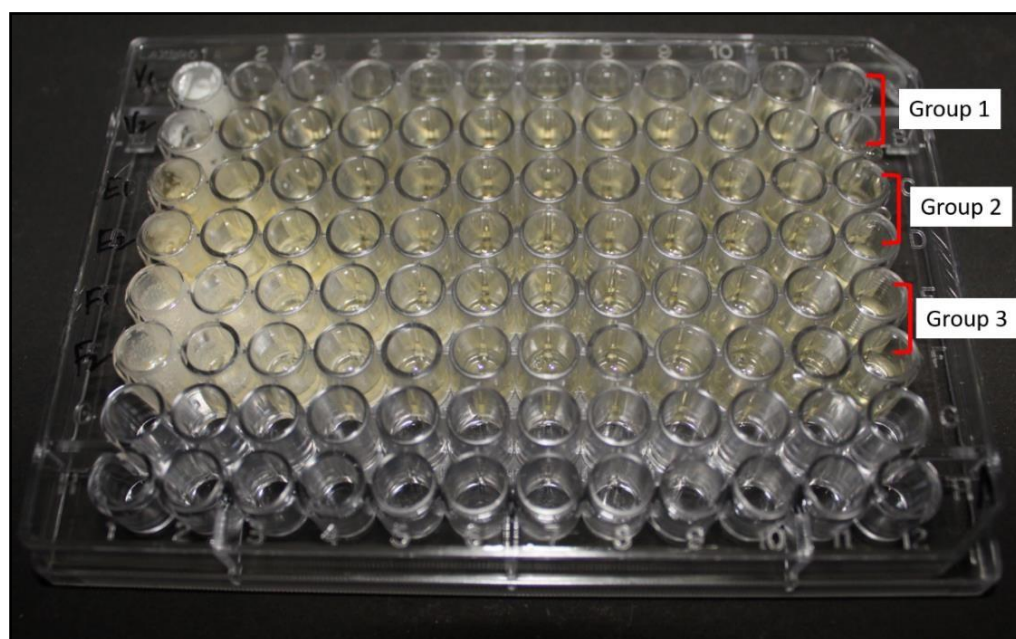


Fig. 35: 96 titre well plate with serial dilution of the fluoride varnishes

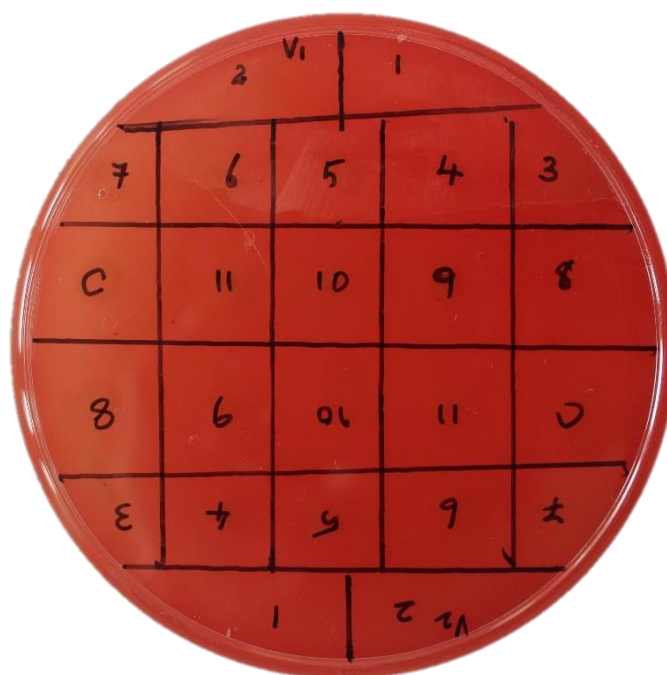
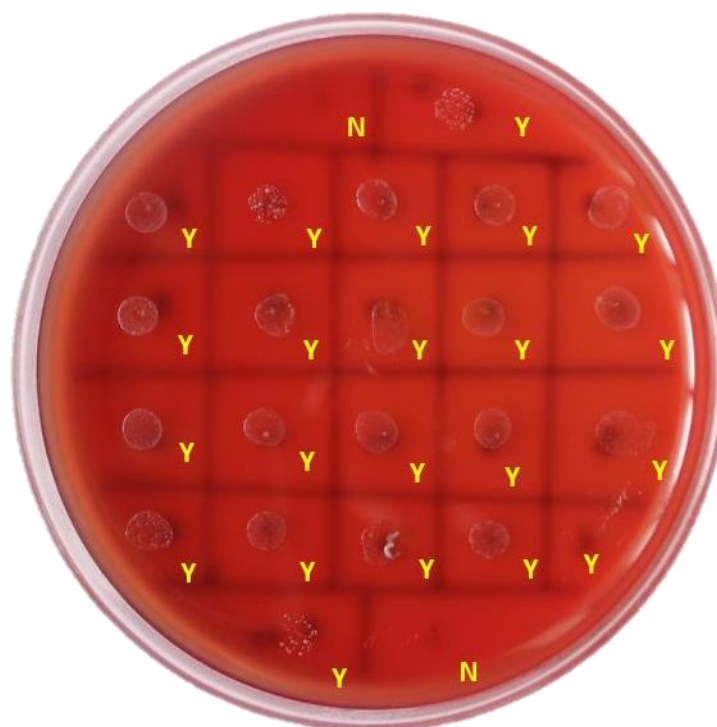


Fig. 36: Cells drawn on the culture plate for diluted concentrations of Vanish™



Y–Growth of *Streptococcus mutans* colonies

N–No growth of *Streptococcus mutans* colonies

Fig. 37: Inhibition of growth of *Streptococcus mutans* attained at 2.5% by Vanish™

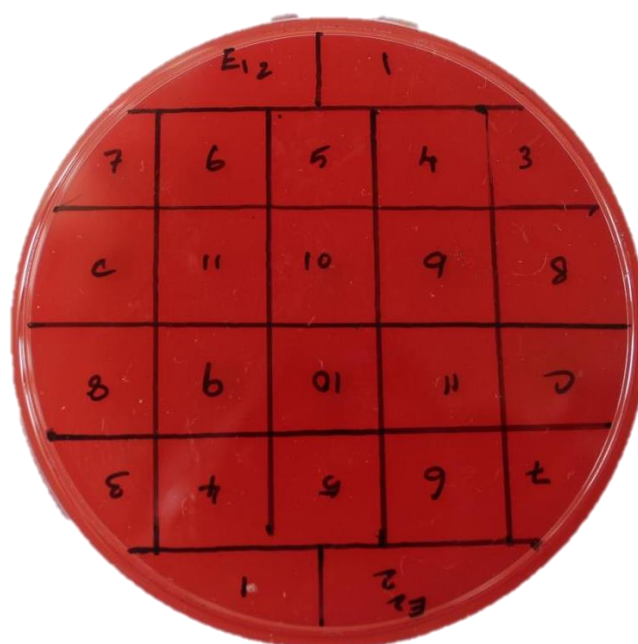
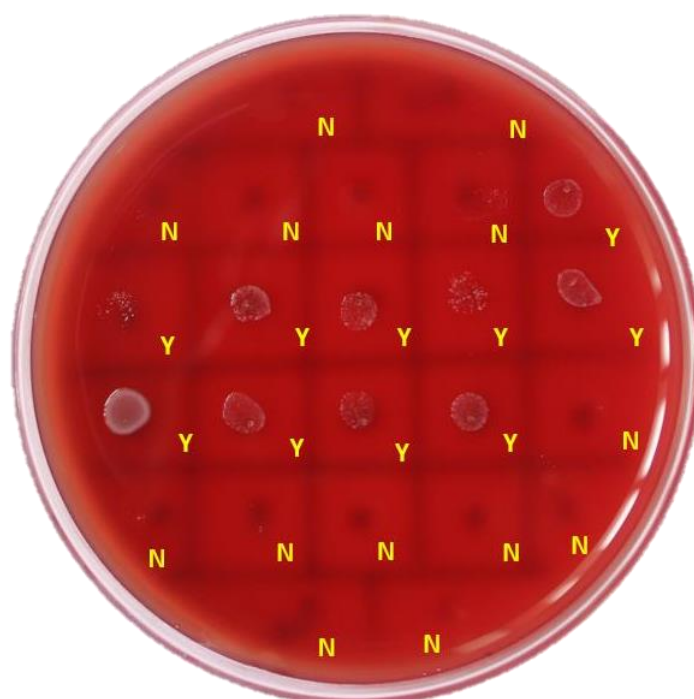


Fig. 38: Cells drawn on the culture plate for diluted concentrations of *Embrace*[™]



Y–Growth of *Streptococcus mutans* colonies

N–No growth of *Streptococcus mutans* colonies

Fig. 39: Inhibition of growth of *Streptococcus mutans* attained at 0.04% by *Embrace*[™]



Fig. 40: Cells drawn on the culture plate for diluted concentrations of Fluorprotector®



Y–Growth of *Streptococcus mutans* colonies

N–No growth of *Streptococcus mutans* colonies

Fig. 41: No inhibition of growth of *Streptococcus mutans* by Fluorprotector®

The present in vitro study was conducted to compare and evaluate the demineralization inhibitory effect and antibacterial effect of two commercially available fluoride varnishes.

Demineralization inhibitory effect

	N	Mean	Std. Deviation	Significance
Group 1 Vanish™	10	55.92	8.49	0.55(p>0.05)
Group 2 Embrace™	10	50.62	11.73	
Group 3 Fluoroprotector®	10	56.67	9.72	
Group 4 No fluoride treatment	10	53.69	10.62	

Table 1: Enamel thickness of the Groups showing the mean, standard deviation and p value

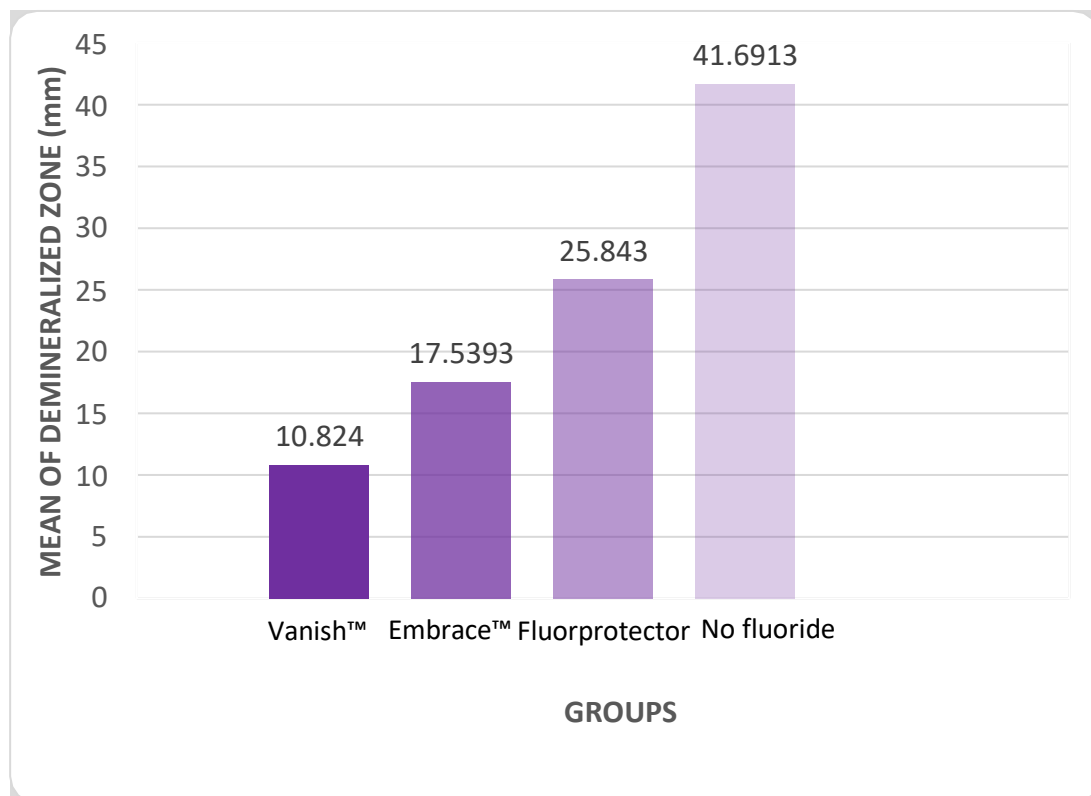
Table 1 shows the mean values of the enamel thickness of Groups 1,2,3 and 4. p value being 0.55, indicates that there is no statistically significant difference between the groups. This also shows that there is uniformity in enamel thickness of all the samples, before subjecting them to the demineralization inhibitory effect. This in turn confirms the standardisation of the samples used in all the groups.

	N	Mean	Std. Deviation	95% Confidence Interval for Mean		ANOVA
				Lower Bound	Upper Bound	
Group 1 Vanish™	10	10.82	4.80	7.39	14.26	0.00
Group 2 Embrace™	10	17.54	7.79	11.97	23.11	
Group 3 Fluorprotector®	10	25.84	10.68	18.20	33.48	
Group 4 No fluoride treatment	10	41.69	11.99	33.11	50.28	

Table 2: Demineralization depths of Groups 1,2,3 and 4 showing the mean, standard deviation and confidence interval

Table 2 shows the distribution of the demineralization depths of Groups 1, 2, 3 and 4 showing the mean, standard deviation and confidence interval. Demineralization depth was the least for Vanish™ (10.82 ± 4.80) followed by Embrace™ (17.54 ± 7.79). Fluorprotector® had a higher demineralized depth (25.84 ± 10.68) when compared to Vanish™ and Embrace™.

ANOVA test showed a statistically significant difference in the demineralization depths between the four groups ($p < 0.05$) and hence, further pair-wise comparison was done using Bonferroni's test.



Graph 1: Graphical representation of the demineralised zones of the four groups

Graph 1 shows the mean values of the demineralization zones of the samples from Group 1 to Group 4. The X axis represents the Groups and the Y axis represents the mean values of the demineralised zone in millimetres.

The demineralized depth for **Vanish™** shows a mean value of 10.82, **Embrace™** shows a mean value of 17.54 and **Fluorprotector®** shows a mean value of 25.84. Group 4 where **no fluoride treatment** was applied showed the demineralized depth to be at 41.69

Group 1 Vanish™	Group 2 Embrace™	Group 3 Fluorprotector®	Group 4 No fluoride treatment
	.678 (C.I -18.26 to 4.83)	.005 (C.I -26.56 to - 3.48)	.000 (C.I -42.41 to - 19.33)
Group 2 Embrace™	Group 3 Fluorprotector®		Group 4 No fluoride treatment
	.313 (C.I. -19.85 to 3.24)		.000 (C.I. -35.69 to - 12.61)
Group 3 Fluorprotector®	Group 4 No fluoride treatment		
	.003 (C.I. -27.39 to 4.31)		

Table 3: Pairwise comparison of the four groups showing p value and confidence intervals

Since, the results of the **One-way ANOVA** had confirmed that there existed a statistical difference in the demineralized zone values among the groups, therefore the pairwise comparison was further done to know the superiority of one group over the other in terms of their demineralization inhibitory effect. **Table 3** shows the pair wise comparison of the groups 1,2,3 and 4 evaluated using Bonferroni's test.

There was no statistically significant difference between Group 1 Vanish™ versus Group 2 Embrace™ and Group 2 Embrace™ versus Group 3 Fluorprotector®. ($p > 0.05$). A statistically significant difference was observed between Group 1 Vanish™ and Group 3 Fluorprotector®.

All groups showed a **statistically significant** difference when compared to **control (Group 4)** ($p < 0.05$), highlighting the efficacy of the three experimental groups in inhibiting the demineralization process.

EVALUATION OF ANTIBACTERIAL EFFECT

The results of the disc diffusion method were inconclusive as the materials did not diffuse satisfactorily owing to their high viscous nature and hence the antibacterial efficacy of the fluoride varnishes were investigated using the broth micro dilution assay.

The Broth micro dilution assay was performed to check the efficacy of the varnishes that completely inhibited (~99%) the growth of the colonies on the MHA plate. The assay was repeated twice for minimizing lab related errors.

		<i>Serial Tube Dilutions</i>											
		1	2	3	4	5	6	7	8	9	10	11	Control
Groups	Group 1	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
	Group 1	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
	Group 2	N	N	N	N	N	N	Y	Y	Y	Y	Y	Y
	Group 2	N	N	N	N	N	N	N	N	Y	Y	Y	Y
	Group 3	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
	Group 3	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y

Y–Growth of *Streptococcus mutans* colonies

N–No growth of *Streptococcus mutans* colonies

Table 4: Minimal inhibitory concentration of the experimental and control groups done using broth micro-dilution assay

Observations from Table 4 show minimal inhibitory concentration values of the experimental groups (Group 1, 2 and 3) checked using broth micro dilution assay.

Table 4 and figure 37 show the inhibition of the target microbe (*Streptococcus mutans*) by Group 1 which was achieved only in the first cell which corresponds to **2.5%**.

Table 4 and figures 39 show the inhibition of the target microbe by Group 2 which was noted up to the seventh cell which corresponds to **0.04%**.

Table 4 and figure 41 reveal no growth inhibition by **Group 3** even in its first dilution.

In light of the above findings, the minimal inhibitory concentration for **Vanish™** is **2.5%** and **Embrace™** is **0.04%**. These findings clearly indicate that **Embrace™** had better antibacterial activity followed by **Vanish™**.

Dental caries is a transmissible microbial disease process caused by the acids released as a result of the bacterial metabolism, which then diffuses into the enamel and dentin, thereby dissolving the mineral component. This cariogenic process is a continuum resulting from repeated cycles of demineralization and remineralization. The demineralization activity commences at the atomic level of the crystal structure of the enamel or dentin and continues to occur until halted, with the end-point being cavitation. Many interventions have been tried to interrupt this continuing process with the aim of arresting or reversing the progress of the carious lesion. (6)

Fluoride is a therapeutic agent which has been studied for decades together in regard to its cariostatic property, and there is ample evidence to prove its ability to reduce demineralization and enhance remineralization. (10,15,36,43,74,75)

The major therapeutic action of fluoride is through topical mechanisms which include

- Inhibition of demineralization at the crystal surfaces of the tooth,
- Facilitation of remineralization at the crystal surfaces (giving an acid resistant surface to the reformed crystals)
- Inhibition of the bacterial enzymes. (7)

Low levels of salivary and plaque fluoride prevent caries by inhibiting demineralization activity and by enhancing remineralization. On the other hand, high levels of fluoride can provide resistance to carious lesion formation and to dental erosion. (44)

Of the various delivery systems available, the use of fluoride varnishes is effective in caries prevention for the paediatric population. (14) Topical fluoride interacts with saliva and forms calcium fluoride on the enamel surface. At a neutral pH, the calcium fluoride compound is stabilized by pellicle proteins and secondary phosphate. When there is a drop in pH levels, calcium fluoride dissolves and releases fluoride ions; therefore, it acts as a prolonged source of fluoride after application. (76,77)

A wide range of fluoride varnishes with varied compositions have been developed to enhance the protective ability of fluoride varnishes. Fluoride varnish containing 5% sodium fluoride (22,600 ppm F) is the widely used topical fluoride that has been preferred for caries management for decades. (15)

After application of fluoride varnish, the main product which gets deposited on the enamel surface and on subsurface carious lesions is calcium fluoride or fluorapatite. (74) The fluorapatite gets permanently bound within the crystalline structure of the enamel, whereas most of the calcium fluoride precipitates on the surface of the enamel. Clinical studies have concluded that fluoride varnishes have the potential to deposit large

amounts of fluoride on the enamel surface. (74,78–81) The amount of fluoride which gets deposited on the demineralized enamel is greater than that present on a sound enamel and tends to be similar in chemical structure to fluorapatite. (82)

Under certain thermodynamic circumstances and in the presence of phosphate, a portion of calcium fluoride can precipitate as fluorapatite during remineralization. This transformation takes place in the physical presence of the varnish. According to **Holmen et al.**, fluoride released from the varnish may produce a redistribution of ions in the body of a carious lesion, creating a favourable gradient of fluoride diffusion, thereby reducing the porosity of the body of the lesion. (83)

Literature reveals ample evidence to substantiate the enhanced benefit of additives added to fluoride like functionalized Tricalcium phosphate and xylitol in inhibiting the demineralization process of carious lesions. (59,84–86) Clinical studies have highlighted the benefit of adding calcium phosphate salts, such as tri-calcium phosphate, inhibiting progression of initial primary enamel lesions better than fluoride varnishes alone. (87) Studies have revealed that the hydrolysis of phosphates results in the formation of calcium hydroxyapatite crystals, and the polymorph of tri-calcium phosphate is capable of expeditious hydrolysis for the formation of hydroxyapatite. (88) This is a remarkable property of tricalcium phosphate as it not only accelerates the formation of hydroxyapatite when

used along with sodium fluoride ions, but also tend to have a greater uptake of fluoride than conventional hydroxyapatite. (88,89)

Primary enamel has a relatively larger amount of structure-less enamel when compared to the permanent teeth making the transfer of fluoride ions across the crystals less pronounced. In this respect, the role of tricalcium phosphate salts may be significant as it is proven to improve the surface micro hardness of teeth as stated by **Alamoudi et al.**(18) In concordance with the above findings, the commercially available fluoride varnish of sodium fluoride with functionalized tricalcium phosphate was included in this study.

Xylitol was also reported to inhibit demineralization by maintaining a higher pH value with super saturation of calcium in saliva. (90) This property is attributed to the capacity of xylitol to form complexes with calcium ions. This in turn inhibits the translocation of dissolved calcium and phosphorus ions from lesions by lowering their diffusion coefficients, thereby inhibiting the demineralization process. (91) Owing to the above findings in the previous studies, a fluoride varnish with Xylitol as an active additive agent was included in this study.

The complexity of the cariogenic process has led to the formulation of various model systems to provide better understanding of the mechanism of demineralization and remineralization processes. These model systems could be broadly classified as biofilm models and chemical

models.(92) Such caries model systems help in the ensuing determination of the clinical benefits of materials analysed in a study. The model used in this study is the simple mineralization model which is categorised under chemical models for artificial carious lesion formation. (93) In literature, 62% of the chemical models have been performed using the simple mineralization technique owing to their simplicity, low cost, efficiency, time saving, reproducibility, and stability. (92)

The enamel thickness of the sections observed did not show any statistical difference across the specimens allotted to each group, indicating the uniformity of the enamel thickness in all specimens.

The demineralization zones were analysed using the polarised light microscope as it proves to be highly beneficial for obtaining a dynamic impression of the relative changes which take place in crystallite/intercrystallite ratio during the carious process. (94) **Fejerskov et al.** 1975, stated that in the observed birefringence, the dark zones observed are interpreted either as representation of demineralization or as an expression of the precipitation of mineral components. (95) The use of polarised light microscopy in the evaluation of the demineralization regions have been tried in several studies and found to be an effective tool for the quantitative measurement of the same. (93,96–98) In this study, it was observed that the mineral content of the dark zone in the experimental groups was much less than that in the Control group, which implied the

demineralization inhibitory effect of the study materials, as observed by Duraisamy et al, 2015. (96)

Demineralization inhibitory effect

Group 1 (Vanish™), Group 2 (Embrace™) and Group 3 (Fluorprotector®) versus Group 4 (No fluoride treatment)

Results of this study showed that the varnishes - Vanish™, Embrace™ and Fluorprotector® inhibit demineralization. This was clearly evident from the huge difference in the demineralization effect of the experimental groups when compared to the control group. This could be attributed to the cumulative fluoride release from Embrace™, as stated by Jablanowski et al. (2012), wherein the fluoride release from various fluoride varnishes were measured at various time intervals until the level was below the limit of detection. (99) These findings are further supported by Patil et al., 2013 which shows that fluoride with tricalcium phosphate was better in remineralizing early carious lesions when compared with casein phosphopeptide amorphous calcium phosphate and its combination with fluoride. (84) The study's results also correlate with recent findings by Gargouri et al. in which xylitol combined with other remineralizing agents improved the protection against demineralization. (71)

Group 1 (Vanish™) versus Group 2 (Embrace™)

On comparing Vanish™ and Embrace™, there is no difference observed in demineralization inhibition potential which highlights the effectiveness of both the additives present in the materials studied. Similar

results were obtained in an in vitro study by **Sahiti et al**, 2020 wherein the demineralization–remineralization of two commercially available fluoride varnishes, one with xylitol and the other with tricalcium phosphate were investigated using DIAGNOdent. The study concluded that both the materials analysed were equally effective as there was no statistically significant difference between the two. (100)

Group 1 (Vanish™) versus Group 3 (Fluorprotector®)

Vanish™ shows better efficacy when compared to Fluorprotector® which manifests the superiority of the sodium fluoride varnish with functionalized tricalcium phosphate in better inhibition of the demineralization process when compared to sodium fluoride varnish. The improved effect might be attributed to the unique property of tricalcium phosphate as it not only accelerates the formation of hydroxyapatite when used in combination with sodium fluoride, but also increases the uptake of fluoride than conventional hydroxyapatite. (88,89)

Group 2 (Embrace™) versus Group 3 (Fluorprotector®)

On comparing Embrace™ and Fluorprotector®, there was no significant difference in the inhibition of demineralisation, indicating that the xylitol component does not improve the demineralization inhibition potential of fluoride varnish. The findings of this study is in accordance to a study by **Amaechi et al** which stated that xylitol, though considered to have a cariostatic effect, its impact on the demineralization – remineralization process is negligible.(101) Similar results were observed

by **Goncalves et al.** 2006 which stated that the addition of xylitol component to fluoride does not have any dominance over conventional fluoride in regard to its demineralization inhibitory effect. (102)

In contrary to these findings, a study by **Miake et al** stated that xylitol does have an influence in the demineralization remineralization activity. (91) The difference in these findings might be attributed to the difference in methodology used for the evaluation of demineralized zones. In the study by Miake, the impact of xylitol was more prominent in the deeper layers (50 – 60 micron) rather than the surface layers (10 micron), which insists that xylitol works efficiently in deeper layers of enamel. (91) Evaluation of such precision would be better achieved with the help of contact microradiography and a high-resolution electron microscope which would have improved the observations of this study.

Future researches could be ameliorated by incorporating the various advancements like Energy Dispersive X-Ray Analysis in analysing the demineralization potential of the varnishes explored, by not limiting the observations to the surface layers but extending to the deeper layers as well. Such modifications could help in better scrutiny of the clinical efficacy of the varnishes researched.

Antibacterial effect

The oral cavity is a complex, dynamic system, which provides shelter for over 700 different bacterial species. Under normal conditions,

the oral bacterial communities live in symbiosis without causing any harm to the host. However, when the equilibrium disrupts, it favours the growth of pathogenic bacteria leading to the occurrence of oral diseases, such as dental caries. (103) Many hypotheses have been laid down for better understanding of the etiology of dental caries, including the ecological plaque hypothesis and the specific-pathogen hypothesis. Out of which, specific pathogen hypothesis implicates a single organism, primarily, *Streptococcus mutans*, as the cause for dental caries. Focusing on the inhibition of this causative pathogen alone could result in the reduction of the severity of disease, and prevent its further progression. (104)

Different formulations of antibacterial agents are available in the global market, including dentifrices, mouthwashes, sprays, and gel forms. In recent years, antibacterial agents have been incorporated into the sustained-release systems, including varnishes for improved benefits. The rationale behind these varnishes is to deliver the therapeutic active agent at the tooth surface for a prolonged period of time. (105) Several clinical trials and in vitro studies have been performed to check the impact of antiseptic varnishes on the oral microbiota, especially the cariogenic ones. (106,107)

The most significant anti-cariogenic property of fluoride is the inhibition of demineralisation and augmentation of remineralisation of early caries lesions. In addition to this, fluoride also has the ability to inhibit acid production by bacteria and cause a reduction in the level of *Streptococcus mutans* species in the oral environment. (44)

Literature shows that under normal conditions, following the acidic challenge, the protons from the extracellular environment diffuse inward crossing the cell membrane which then gets extruded by the enzyme, membrane associated F-ATPase. (108) Hence, F-ATPase serves to protect *Streptococcus mutans* against environmental acid challenge by regulating the pH homeostasis. Sodium fluoride acts by enhancing the proton permeability and reducing the F-ATPase activity of *Streptococcus mutans* biofilm cells, thereby inhibiting their intracellular metabolism. (109) Though efficacious, using fluoride varnishes alone has limited effect on the total salivary levels of *Streptococcus mutans* species as stated by **Bapat et al** (110), **Yadav et al** (51) and **Jafari et al** (70) and hence they are used in combination with other active agents like tricalcium phosphate and xylitol to enhance the overall benefit of the varnish.

The disc diffusion method (or agar well diffusion test) is widely used in the medical laboratories to determine the antibiotic sensitivities of bacterial isolates. The diameter of the inhibitory zones obtained denotes the microbial sensitivity and the quantity of agent released after placement of the material studied. This method was initially chosen as it can be performed rapidly and easily with a large number of specimens. (93) In this study, the experimental varnish products were assessed for their antibacterial activity after 24 hours by measuring the inhibition zones produced against *Streptococcus mutans*.

This disc diffusion method has been widely used in various clinical studies which analysed the antibacterial efficacy of different formulations of sodium fluoride varnishes and was observed to be an effective tool for analysing their bactericidal effect on *Streptococcus mutans*. (14, 17, 25, 29) This same methodology has been incorporated in this study to analyse the inhibitory effect of the experimental groups against the cariogenic pathogen, *Streptococcus mutans* with Ciprofloxacin as the control.

When the zones of inhibition were analysed after 24 hours, Ciprofloxacin showed two clearly distinguishable zones, a bacterial zone, and a completely transparent inhibition zone with no bacterial growth. But the fluoride varnishes showed a translucent semi-inhibition zone, less transparent than the inhibition zone as seen in Fig. 33. This could be attributed to the alcoholic composition of the varnishes which has interfered with the diffusion through the agar plates. (114) The assay was further extended into Broth micro dilution assay as the findings were inconclusive. (70,106) Broth micro dilution assay is an effective alternative to disc diffusion method to check the antibacterial efficacy of the materials which are thixotropic and exhibit inadequate dissolution onto the filterpaper disks.

The minimal inhibitory concentration for Embrace™ varnish was 0.04% and Vanish™ varnish showed inhibition at 2.5%. This difference in antibacterial efficacy might be attributed to their difference in composition of the varnishes. There is a difference in the additional active ingredient

namely, Xylitol coated Calcium and phosphate in case of Embrace™ and tricalcium phosphate in case of Vanish™ varnish, which might have influenced the results of the study. The results of this study are in accordance with **Jafari et al.** 2018 (70), in which sodium fluoride varnish with xylitol as an additive showed the highest antibacterial efficacy when compared to varnishes with tricalcium phosphate. Studies by **Janneson et al** 2002 (115) and **Gargouri et al** (71), 2018 also prove the antibacterial efficacy of Xylitol in remineralizing agents.

Literature shows that, xylitol intake leads to favourable outcomes like reduced incidence of caries and marked reduction of *Streptococcus mutans* levels in oral environment due to the inability of *Streptococcus mutans* to ferment this five-carbon sugar xylitol. (115,116)

The usefulness of xylitol in the prevention of dental caries, especially primary caries, has been well documented in literature. (63,117) The results of this study are analogous with the hallmark studies performed on Xylitol - Scheinin A et al 1975 (118), Kandelman D et al 1988 (120), Isokangas P et al 1988 (121), Kandelman D et al 1990 (86), Mäkinen KK et al 1995,1996 (122,123), Alanen P et al 2000 (124) and Mäkinen KK et al 2000 (119), The expressed antibacterial effect of the functionalized tricalcium phosphate might be attributed to its activity against biofilm formation and planktonic cells of *Streptococcus mutans* as stated by **Monteiro et al.** (125)

Streptococcus mutans species alone has been investigated in this study while the interaction from the other pathogenic bacteria in oral environment has not been discussed. Within the limitations of the *in vitro* condition, study under clinical situations in children is required to confirm the data obtained from this study. More research is needed to assess the effect of the antibacterial agents on other cariogenic bacteria and normal flora in the oral cavity. Correspondingly, the long-term sustainability of the activity of the antibacterial agents incorporated into the fluoride varnish should also be analysed to get a good measure of the products.

The conclusions drawn from this in vitro study were as follows,

- Vanish™ exhibited an increased demineralization inhibitory effect when compared to Fluorprotector®.
- Vanish™ and Embrace™ exhibited a similar demineralization inhibitory effect.
- Embrace™ exhibited an improved demineralization inhibitory effect when compared to Fluorprotector®.
- Embrace™ exhibited better antibacterial efficacy when compared to Vanish™ and Fluorprotector® against *Streptococcus mutans*.

Based on the above findings, it is evident that both Vanish™ and Embrace™ had a better demineralization inhibitory effect when compared to Fluorprotector®. In terms of the antibacterial effect, Embrace™ showed an improved antibacterial effect when compared to Vanish™ and Fluorprotector®.

Encompassing the limitations of the study, fluoride varnishes with active agents like tricalcium phosphate and xylitol could be included in the preventive protocol owing to their versatile properties in influencing the demineralizing activity as well as their impact of the cariogenic pathogens. This could be considered as an effective preventive protocol which could be employed on a regular basis with the motive of reducing the global burden caused by dental caries.

The limitation of the study could be overcome by investigating the varnishes using a biofilm model for artificial caries formation as the microbial aspect is also encompassed unlike the chemical model. Furthermore, the long term sustainability of the additional active agents also remains uncertain which could be explored in future studies.

The present in vitro study was carried out in the Department of Pediatric and Preventive Dentistry, Sathyabama Dental College and Hospital. The study intended to measure the demineralization inhibitory effect and antibacterial effect of commercially available fluoride varnishes with varied active ingredients in addition to fluoride, namely tricalcium phosphate (Vanish™, 3M ESPE, St. Paul, MN, US)), xylitol coated calcium and phosphate (Embrace™, Pulpdent corporation, Watertown, MA, USA) and Fluorprotector® (Ivoclar Vivadent AG, Schaan, Liechtenstein).

Demineralization inhibition

Forty freshly extracted premolars were collected, washed and stored in sterile saline. A buccal window measuring 2 X 2 mm for fluoride treatment was drawn on the middle third of the buccal surfaces of the enamel and the remaining part was thoroughly covered with nail varnish and was allowed to dry at room temperature.

The prepared tooth samples were subjected to fluoride varnish application and categorized as Group 1–Vanish™, Group 2–Embrace™, Group 3–Fluorprotector®, and Group 4–No fluoride application. The fluoride application was done on the designated buccal window as per the manufacturer's instructions and allowed to dry. The samples were then immersed in plastic containers each containing 3 ml of demineralizing solution for a period of 48 hours at room temperature. At the end of 48 hours the samples were removed from the solution. The root portions of

the samples were removed and the crown portions were mounted on acrylic blocks for sectioning. Thin sections measuring 100 µm were obtained using a hard tissue microtome and examined under polarised light microscope. The depth of demineralization was measured using a JAVA based program–IMAGEJ and the value obtained in pixels was converted to mm using an online converter.

Antibacterial effect

Broth micro-dilution assay was performed. Overnight culture of *Streptococcus mutans* MTCC 890 was inoculated in 10 ml of brain heart infusion agar (Himedia, India) and incubated at 37°C for 1 hour. Serial dilutions of the fluoride varnishes were prepared and 10 µl of *Streptococcus mutans* suspension was added to all the wells including the culture control. The plates were incubated overnight at 37°C. The minimal inhibitory concentration was recorded as the lowest concentration of the test solution which did not permit the growth of *Streptococcus mutans*.

Statistical analysis

The values obtained were subjected to statistical analysis using the ANOVA test for intergroup comparison and pairwise comparison was performed using the Bonferroni's test.

Results

Vanish™, Embrace™ and Fluorprotector® exhibited a marked demineralization inhibitory effect in the present in vitro study. The demineralisation inhibitory effect of Vanish™ over Fluorprotector® was statistically significant. However, Vanish™ does not exhibit significant difference in demineralization inhibition when compared to Embrace™. Embrace™ also does not differ in inhibiting demineralization when compared to Fluorprotector®.

The antibacterial efficacy, i.e. the minimal inhibitory concentration values of Vanish™ and Embrace™ were obtained at 2.5% and 0.04% respectively which showcases the better antibacterial efficacy of Embrace™ over Vanish™ and Fluorprotector®.

Conclusion

Based on the results of this study, it has been concluded that both Vanish™ and Embrace™ had a better demineralization inhibitory effect when compared to Fluorprotector® and in terms of the antibacterial effect, Embrace™ showed an improved antibacterial effect when compared to Vanish™ and Fluorprotector®.

Within the limitations of this study, fluoride varnishes with additives such as xylitol and functionalized tricalcium phosphate can be incorporated along with conventional fluoride in the prevention of caries owing to its effective demineralization inhibition and antibacterial effect.

These limitations can be overcome by exploring the deeper layers of the enamel using advancements like Energy Dispersive X-Ray Analysis. Correspondingly the future research could be focussed on analysing the clinical efficacy of these fluoride varnishes using randomised clinical trials to advocate them in larger population to eradicate the global menace of caries.

1. GBD 2015 Disease and Injury Incidence and Prevalence Collaborators. Global, regional, and national incidence, prevalence, and years lived with disability for 310 diseases and injuries, 1990-2015: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet*. 2016 08;388(10053):1545–602.
2. Chen KJ, Gao SS, Duangthip D, Li SKY, Lo ECM, Chu CH. Dental caries status and its associated factors among 5-year-old HongKong children: a cross-sectional study. *BMC Oral Health*. 2017 Aug 31;17(1):121.
3. Ganesh A, Muthu MS, Mohan A, Kirubakaran R. Prevalence of Early Childhood Caries in India – A Systematic Review. *Indian J Pediatr*. 2019 Mar;86(3):276–86.
4. Kirthiga M, Murugan M, Saikia A, Kirubakaran R. Risk Factors for Early Childhood Caries: A Systematic Review and Meta-Analysis of Case Control and Cohort Studies. *Pediatr Dent*. 2019 Mar 15;41(2):95–112.
5. Chen KJ, Gao SS, Duangthip D, Lo ECM, Chu CH. The caries-arresting effect of incorporating functionalized tricalcium phosphate into fluoride varnish applied following application of silver nitrate solution in preschool children: study protocol for a randomized, double-blind clinical trial. *Trials [Internet]*. 2018 Jul 4 [cited 2018 Jul

- 16];19. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6032524/>
6. Featherstone JDB. Dental caries: a dynamic disease process. *Aust Dent J.* 2008 Sep;53(3):286–91.
7. Ten Cate JM, Featherstone JD. Mechanistic aspects of the interactions between fluoride and dental enamel. *Crit Rev Oral Biol Med.* 1991;2(3):283–96.
8. Pulido MT, Wefel JS, Hernandez MM, Denehy GE, Guzman-Armstrong S, Chalmers JM, et al. The Inhibitory Effect of MI Paste, Fluoride and a Combination of Both on the Progression of Artificial Caries-like Lesions in Enamel. *Oper Dent.* 2008 Sep 1;33(5):550–5.
9. Holler BE, Friedl K-H, Jung H, Hiller K-A, Schmalz G. Fluoride uptake and distribution in enamel and dentin after application of different fluoride solutions. *Clin Oral Investig.* 2002 Sep;6(3):137–44.
10. Page DJ. A study of the effect of fluoride delivered from solution and dentifrices on enamel demineralization. *Caries Res.* 1991;25(4):251–5.
11. Tavss EA, Bontá CY, Joziak MT, Fisher SW, Campbell SK. High-potency sodium fluoride: a literature review. *Compend Contin Educ Dent.* 1997;18(2 Spec No):31–6.

12. Borges BCD, de Souza Borges J, de Araujo LSN, Machado CT, Dos Santos AJS, de Assunção Pinheiro IV. Update on nonsurgical, ultraconservative approaches to treat effectively non-cavitated caries lesions in permanent teeth. *Eur J Dent*. 2011 Apr;5(2): 229–36.
13. Duangthip D, Chen KJ, Gao SS, Lo ECM, Chu CH. Managing Early Childhood Caries with Atraumatic Restorative Treatment and Topical Silver and Fluoride Agents. *Int J Environ Res Public Health*. 2017 10;14(10).
14. Marinho VCC, Worthington HV, Walsh T, Clarkson JE. Fluoride varnishes for preventing dental caries in children and adolescents. *Cochrane Database Syst Rev*. 2013 Jul 11;(7):CD002279.
15. Gao SS, Zhang S, Mei ML, Lo EC-M, Chu C-H. Caries remineralisation and arresting effect in children by professionally applied fluoride treatment – a systematic review. *BMC Oral Health* [Internet]. 2016 Feb 1 [cited 2020 Sep 30];16. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4736084/>
16. Shahmoradi M, Hunter N, Swain M. Efficacy of Fluoride Varnishes with Added Calcium Phosphate in the Protection of the Structural and Mechanical Properties of Enamel [Internet]. Vol. 2017, BioMed Research International. Hindawi; 2017 [cited 2020 Dec 4]. p. e7834905. Available from: <https://www.hindawi.com/journals/bmri/2017/7834905/>

17. Karlinsey RL, Pfarrer AM. Fluoride Plus Functionalized β -TCP: A Promising Combination for Robust Remineralization. *Adv Dent Res*. 2012 Sep;24(2):48–52.
18. Alamoudi SA, Pani SC, AlOmari M. The Effect of the Addition of Tricalcium Phosphate to 5% Sodium Fluoride Varnishes on the Microhardness of Enamel of Primary Teeth. *Int J Dent* [Internet]. 2013 [cited 2018 Jul 16];2013. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3679819/>
19. Amin MS, Harrison RL, Benton TS, Roberts M, Weinstein P. Effect of povidone-iodine on *Streptococcus mutans* in children with extensive dental caries. *Pediatr Dent*. 2004 Feb;26(1):5–10.
20. Son J-L, Kim A-J, Oh S, Bae J-M. Inhibitory effects on *Streptococcus mutans* of antibacterial agents mixed with experimental fluoride varnish. *Dent Mater J*. 2020 Aug 2;39(4): 690–5.
21. Attin R, Tuna A, Attin T, Brunner E, Noack MJ. Efficacy of differently concentrated chlorhexidine varnishes in decreasing *Mutans streptococci* and *lactobacilli* counts. *Arch Oral Biol*. 2003 Jul;48(7):503–9.
22. Koo H. Strategies to enhance the biological effects of fluoride on dental biofilms. *Adv Dent Res*. 2008 Jul 1;20(1):17–21.

23. Lingström P, Holm A, Mejäre I, Twetman S, Söder B, Norlund A, et al. Dietary factors in the prevention of dental caries: a systematic review. *Acta Odontologica Scandinavica*. 2003 Jan;61(6):331–40.
24. Fejerskov O. Changing paradigms in concepts on dental caries: consequences for oral health care. *Caries Res*. 2004 Jun;38(3):182–91.
25. Chu CH, Lo ECM. Promoting caries arrest in children with silver diamine fluoride: a review. *Oral Health Prev Dent*. 2008;6(4):315– 21.
26. Fejerskov O, Kidd E. *Dental Caries: The Disease and Its Clinical Management*. John Wiley & Sons; 2009. 641 p.
27. Chu CH, Mei ML, Lo ECM. Use of fluorides in dental caries management. *Gen Dent*. 2010 Feb;58(1):37–43; quiz 44–5, 79–80.
28. WHO | Oral health documents and publications [Internet]. WHO. World Health Organization; [cited 2020 Sep 30]. Available from: http://www.who.int/oral_health/publications/en/
29. Rølla G. On the role of calcium fluoride in the cariostatic mechanism of fluoride. *Acta Odontol Scand*. 1988 Dec;46(6):341–5.
30. Ogard B, Seppä L, Rølla G. Professional topical fluoride applications-
-clinical efficacy and mechanism of action. *Adv Dent Res*. 1994 Jul;8(2):190–201.

31. Ahovuo-Saloranta A, Forss H, Hiiri A, Nordblad A, Mäkelä M. Pit and fissure sealants versus fluoride varnishes for preventing dental decay in the permanent teeth of children and adolescents. Cochrane Oral Health Group, editor. Cochrane Database of Systematic Reviews [Internet]. 2016 Jan 18 [cited 2020 May 1]; Available from: <http://doi.wiley.com/10.1002/14651858.CD003067.pub4>
32. Petersen PE, Lennon MA. Effective use of fluorides for the prevention of dental caries in the 21st century: the WHO approach. *Community Dent Oral Epidemiol*. 2004 Oct;32(5):319–21.
33. Beltrán-Aguilar ED, Goldstein JW, Lockwood SA. Fluoride varnishes. A review of their clinical use, cariostatic mechanism, efficacy and safety. *J Am Dent Assoc*. 2000 May;131(5):589–96.
34. Poulsen S. Fluoride-containing gels, mouth rinses and varnishes: an update of evidence of efficacy. *Eur Arch Paediatr Dent*. 2009 Sep;10(3):157–61.
35. Steel K. How effective is the application of topical fluoride varnish in preventing dental caries in children? a literature review. *Prim Dent J*. 2014 Aug;3(3):74–6.
36. Douglas GVA, Ramsdale MP, Vinall-Collier K, Csikar JI. Using High Fluoride Concentration Products in Public Policy: A Rapid Review of Current Guidelines for High Fluoride Concentration Products. *Caries Res*. 2016 Apr 22;50(1):50–60.

37. Jiang EM, Lo ECM, Chu CH, Wong MCM. Prevention of early childhood caries (ECC) through parental toothbrushing training and fluoride varnish application: a 24-month randomized controlled trial. *J Dent*. 2014 Dec;42(12):1543–50.
38. Twetman S, Dhar V. Evidence of Effectiveness of Current Therapies to Prevent and Treat Early Childhood Caries. *Pediatr Dent*. 2015 Jun;37(3):246–53.
39. Memarpour M, Fakhraei E, Dadaein S, Vossoughi M. Efficacy of Fluoride Varnish and Casein Phosphopeptide-Amorphous Calcium Phosphate for Remineralization of Primary Teeth: A Randomized Clinical Trial. *Med Princ Pract*. 2015 Apr 16;24(3):231–7.
40. Anderson M, Dahllöf G, Twetman S, Jansson L, Bergenlid A-C, Grindefjord M. Effectiveness of Early Preventive Intervention with Semiannual Fluoride Varnish Application in Toddlers Living in High-Risk Areas: A Stratified Cluster-Randomized Controlled Trial. *Caries Res*. 2016;50(1):17–23.
41. He T, Li X, Dong Y, Zhang N, Zhong Y, Yin W, et al. Comparative assessment of fluoride varnish and fluoride film for remineralization of postorthodontic white spot lesions in adolescents and adults over a 6-month period: A single-center, randomized controlled clinical trial. *Am J Orthod Dentofacial Orthop*. 2016 Jun;149(6):810–9.
42. Tuloglu N, Bayrak S, Tunc ES, Ozer F. Effect of fluoride varnish with added casein phosphopeptide-amorphous calcium phosphate

- on the acid resistance of the primary enamel. BMC Oral Health. 2016 Sep 26;16(1):103.
43. Lenzi TL, Montagner AF, Soares FZM, de Oliveira Rocha R. Are topical fluorides effective for treating incipient carious lesions?: A systematic review and meta-analysis. J Am Dent Assoc. 2016 Feb;147(2):84-91.e1.
44. Lynch RJM, Navada R, Walia R. Low-levels of fluoride in plaque and saliva and their effects on the demineralisation and remineralisation of enamel; role of fluoride toothpastes. Int Dent J. 2004;54(5 Suppl 1):304–9.
45. Savas S, Kavrik F, Kucukyilmaz E. http://www.scielo.br/scielo.php?script=sci_abstract&pid=S1678-77572016000300198&lng=en&nrm=iso&tlng=en. Journal of Applied Oral Science. 2016 Jun;24(3):198–203.
46. Nozari A, Ajami S, Rafiei A, Niazi E. Impact of Nano Hydroxyapatite, Nano Silver Fluoride and Sodium Fluoride Varnish on Primary Teeth Enamel Remineralization: An In Vitro Study. J Clin Diagn Res. 2017 Sep;11(9):ZC97–100.
47. A Deepti, Jeevarathan J, Muthu M, Prabhu V R, Chamundeswari. Effect of Fluoride Varnish on Streptococcus mutans Count in Saliva of Caries Free Children Using Dentocult SM Strip Mutans Test: A Randomized Controlled Triple Blind Study. Int J Clin Pediatr Dent. 2008;1(1):1–9.

48. Erdem AP, Sepet E, Kulekci G, Trosola SC, Guven Y. Effects of Two Fluoride Varnishes and One Fluoride/Chlorhexidine Varnish on *Streptococcus mutans* and *Streptococcus sobrinus* Biofilm Formation in Vitro. *Int J Med Sci*. 2012;9(2):129–36.
49. Paul S, Baranya Shrikrishna S, Suman E, Shenoy R, Rao A. Effect of fluoride varnish and chlorhexidine-thymol varnish on mutans streptococci levels in human dental plaque: a double-blinded randomized controlled trial. *Int J Paediatr Dent*. 2014 Nov;24(6):399–408.
50. Sharma M, Pandit IK, Srivastava N, Gugnani N, Gupta M. A Comparative Evaluation of Efficacy of *Streptococcus mutans* Counts in Saliva: An in vivo Study. *Int J Clin Pediatr Dent*. 2018;11(2):94–9.
51. Yadav S, Sachdev V, Malik M, Chopra R. Effect of three different compositions of topical fluoride varnishes with and without prior oral prophylaxis on *Streptococcus mutans* count in biofilm samples of children aged 2–8 years: A randomized controlled trial. *Journal of Indian Society of Pedodontics and Preventive Dentistry*. 2019 Jul 1;37(3):286.
52. Ben Khadra GM, Arrag EA, Alammori M, AlKadi MF. The effect of chlorhexidine-thymol and fluoride varnishes on the levels of *Streptococcus mutans* in saliva in children aged 6-8 years. *Indian J Dent Res*. 2019 Feb;30(1):67–72.

53. M. Johnsson, M.J. Levine, G.H. Nancollas, 1993. Hydroxyapatite Binding Domains in Salivary Proteins - [Internet] [cited 2020 Dec 12]. Available from: <https://journals.sagepub.com/doi/abs/10.1177/10454411930040031601>
54. Larsen MJ, Pearce EIF. Saturation of human saliva with respect to calcium salts. *Archives of Oral Biology*. 2003 Apr 1;48(4):317–22.
55. Vogel GL, Zhang Z, Carey CM, Ly A, Chow LC, Proskin HM. Composition of plaque and saliva following a sucrose challenge and use of an alpha-tricalcium-phosphate-containing chewing gum. *J Dent Res*. 1998 Mar;77(3):518–24.
56. Walsh LJ. Contemporary technologies for remineralization therapies: A review. 11(6):9.
57. Schemehorn BR, Wood GD, McHale W, Winston AE. Comparison of fluoride uptake into tooth enamel from two fluoride varnishes containing different calcium phosphate sources. *J Clin Dent*. 2011;22(2):51–4.
58. Karlinsey RL, Mackey AC, Schwandt CS. Effects on Dentin Treated with Eluted Multi-Mineral Varnish In Vitro. *Open Dent J*. 2012 Oct 5;6:157–63.
59. Rirattanapong P, Vongsavan K, Saengsirinavin C, Pornmahala T. Effect of fluoride varnishes containing tri-calcium phosphate sources on remineralization of initial primary enamel lesions.

- Southeast Asian J Trop Med Public Health. 2014 Mar;45(2):499–504.
60. Rirattanapong P, Vongsavan K, Saengsirinavin C, Jantarakam N. A 3-MONTH STUDY OF FLUORIDE RELEASE FROM DIFFERENT CALCIUM PHOSPHATE FLUORIDE VARNISHES ON PRIMARY TEETH. Southeast Asian J Trop Med Public Health. 2016 Sep;47(5):1098–104.
61. Meyer F, Amaechi BT, Fabritius H-O, Enax J. Overview of Calcium Phosphates used in Biomimetic Oral Care. Open Dent J. 2018 May 31;12:406–23.
62. Erkmén Almaz M, Akbay Oba A. Antibacterial activity of fluoride varnishes containing different agents in children with severe early childhood caries: a randomised controlled trial. Clin Oral Invest. 2020 Jun;24(6):2129–36.
63. Söderling EM. Xylitol, mutans streptococci, and dental plaque. Adv Dent Res. 2009;21(1):74–8.
64. Shen P, Manton DJ, Cochrane NJ, Walker GD, Yuan Y, Reynolds C, et al. Effect of added calcium phosphate on enamel remineralization by fluoride in a randomized controlled in situ trial. J Dent. 2011 Jul;39(7):518–25.
65. Simões Moraes R, Modesto A, Regina Netto Dos Santos K, Drake D. The effect of 1% chlorhexidine varnish and 40% xylitol solution

- on *Streptococcus mutans* and plaque accumulation in children.
Pediatr Dent. 2011 Dec;33(7):484–90.
66. García-Godoy F, Kao LM, Flaitz CM, Hicks J. Fluoride dentifrice containing xylitol: in vitro root caries formation. *Am J Dent*. 2013 Feb;26(1):56–60.
67. Vongsavan K, Surarit R, Rirattanapong P. The combined effect of xylitol and fluoride in varnish on bovine teeth surfacemicrohardness. *Southeast Asian J Trop Med Public Health*. 2014 Mar;45(2):505–10.
68. Cardoso C a. B, de Castilho ARF, Salomão PMA, Costa EN, Magalhães AC, Buzalaf M a. R. Effect of xylitol varnishes on remineralization of artificial enamel caries lesions in vitro. *J Dent*. 2014 Nov;42(11):1495–501.
69. Cardoso C a. B, Cassiano LPS, Costa EN, Souza-E-Silva CM, Magalhães AC, Grizzo LT, et al. Effect of xylitol varnishes on remineralization of artificial enamel caries lesions in situ. *J Dent*. 2016;50:74–8.
70. Jafari K, Hekmatfar S, Fereydunzadeh M. In vitro Comparison of Antimicrobial Activity of Conventional Fluoride Varnishes Containing Xylitol and Casein Phosphopeptide-Amorphous Calcium Phosphate. *J Int Soc Prev Community Dent*. 2018;8(4):309–13.

71. Gargouri W, Zmantar T, Kammoun R, Kechaou N, Ghoul-Mazgar S. Coupling xylitol with remineralizing agents improves tooth protection against demineralization but reduces antibiofilm effect. *Microb Pathog*. 2018 Oct;123:177–82.
72. Jain A, Suprabha BS, Shenoy R, Natarajan S, Rao A. Remineralising Effectiveness of Two Fluoride Varnishes Containing Additives: An In Vitro Study. *Oral Health Prev Dent*. 2019;17(4):385–93.
73. Patil, Namrata. Comparative evaluation of remineralizing potential of three agents on artificially demineralized human enamel: An in vitro study - PubMed [Internet]. [cited 2021 Jan 25]. Available from: <https://pubmed.ncbi.nlm.nih.gov/23716961/>
74. Arends J, Schuthof J. Fluoride content in human enamel after fluoride application and washing - an in vitro study. *Caries Res*. 1975;9(5):363–72.
75. Du M, Cheng N, Tai B, Jiang H, Li J, Bian Z. Randomized controlled trial on fluoride varnish application for treatment of white spot lesion after fixed orthodontic treatment. *Clin Oral Investig*. 2012 Apr;16(2):463–8.
76. Patel PM, Hugar SM, Halikerimath S, Badakar CM, Gokhale NS, Thakkar PJ, et al. Comparison of the Effect of Fluoride Varnish, Chlorhexidine Varnish and Casein Phosphopeptide- Amorphous Calcium Phosphate (CPP-ACP) Varnish on Salivary Streptococcus

mutans Level: A Six Month Clinical Study. J Clin Diagn Res. 2017 Aug;11(8):ZC53–9.

77. Beerens MW, van der Veen MH, van Beek H, ten Cate JM. Effects of casein phosphopeptide amorphous calcium fluoride phosphate paste on white spot lesions and dental plaque after orthodontic treatment: a 3-month follow-up. Eur J Oral Sci. 2010 Dec;118(6):610–7.
78. Koch G, Petersson LG, Gleerup A, Löwstedt E. Kinetics of fluorine in deciduous enamel after application of fluoride-containing varnish (Duraphat). Swed Dent J. 1982;6(1):39–44.
79. Dijkman AG, de Boer P, Arends J. In vivo investigation on the fluoride content in and on human enamel after topical applications. Caries Res. 1983;17(5):392–402.
80. Seppä L, Luoma H, Hausen H. Fluoride content in enamel after repeated applications of fluoride varnishes in a community with fluoridated water. Caries Res. 1982;16(1):7–11.
81. Grobler SR, Ogaard B, Rolla G. Fluoride uptake and retention by sound enamel after in vivo Duraphat application. J Dent Assoc S Afr. 1983 Feb;38(2):55–8.
82. Ogaard B, Rølla G, Helgeland K. Fluoride retention in sound and demineralized enamel in vivo after treatment with a fluoride varnish (Duraphat). Scand J Dent Res. 1984 Jun;92(3):190–7.

83. Holmen L, Ogaard B, Rølla G, Thylstrup A. A polarized light and scanning electron microscope study of the effect of Duraphat treatment on in vivo caries. *Scand J Dent Res*. 1986 Dec;94(6):521–9.
84. Patil S, Sanketh D, Amrutha N. Oral Microbial Flora in Health. *World Journal of Dentistry*. 2013 Dec;4(4):262–6.
85. Rekola M. Correlation between caries incidence and frequency of chewing gum sweetened with sucrose or xylitol. *Proc Finn Dent Soc*. 1989;85(1):21–4.
86. Kandelman D, Gagnon G. A 24-month clinical study of the incidence and progression of dental caries in relation to consumption of chewing gum containing xylitol in school preventive programs. *J Dent Res*. 1990 Nov;69(11):1771–5.
87. Karlinsey RL, Mackey AC, Walker ER, Frederick KE. Surfactant-modified β -TCP: structure, properties, and in vitro remineralization of subsurface enamel lesions. *J Mater Sci: Mater Med*. 2010 Jul;21(7):2009–20.
88. Leamy P, Brown PW, TenHuisen K, Randall C. Fluoride uptake by hydroxyapatite formed by the hydrolysis of alpha-tricalcium phosphate. *J Biomed Mater Res*. 1998 Dec 5;42(3):458–64.
89. TenHuisen KS, Brown PW. Hydrolysis of alpha-tricalcium phosphate in NaF solutions. *Biomaterials*. 1999 Mar;20(5):427–34.

90. Influence of Maternal Xylitol Consumption on Acquisition of Mutans Streptococci by Infants - E. Soderling, P. Isokangas, K. Pienihäkkinen, J. Tenovuori, 2000 [Internet]. [cited 2021 Feb 12]. Available from: <https://journals.sagepub.com/doi/abs/10.1177/00220345000790031601>
91. Miake Y, Saeki Y, Takahashi M, Yanagisawa T. Remineralization effects of xylitol on demineralized enamel. *Journal of Electron Microscopy*. 2003 Nov 20;52(5):471–6.
92. Yu OY, Zhao IS, Mei ML, Lo EC-M, Chu C-H. A Review of the Common Models Used in Mechanistic Studies on Demineralization-Remineralization for Cariology Research. *Dent J (Basel)* [Internet]. 2017 Jun 18 [cited 2021 Feb 15];5(2). Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5806972/>
93. Attiguppe P, Malik N, Ballal S, Naik SV. CPP–ACP and Fluoride: A Synergism to Combat Caries. *Int J Clin Pediatr Dent*. 2019;12(2):120–5.
94. Thylstrup A, Fejerskov O, Larsen MJ. Polarized light microscopy of enamel structure in incisors from newborn infants. *Eur J Oral Sci*. 1976 Oct;84(5):243–54.
95. Fejerskov O, Silverstone LM, Melsen B, Møller IJ. Histological features of fluorosed human dental enamel. *Caries Res*. 1975;9(3):190–210.

96. Duraisamy V, Xavier A, Nayak UA, Reddy V, Rao AP. An in vitro evaluation of the demineralization inhibitory effect of F- varnish and casein phosphopeptide-amorphous calcium phosphate on enamel in young permanent teeth. *J Pharm Bioallied Sci.* 2015 Aug;7(Suppl 2):S513–7.
97. Rajan R, Krishnan R, Bhaskaran B, Kumar SV. A Polarized Light Microscopic Study to Comparatively evaluate Four Remineralizing Agents on Enamel viz CPP-ACPF, ReminPro, SHY-NM and Colgate Strong Teeth. *Int J Clin Pediatr Dent.* 2015;8(1):42–7.
98. Mehta A, Paramshivam G, Chugh VK, Singh S, Halkai S, Kumar S. Effect of light-curable fluoride varnish on enamel demineralization adjacent to orthodontic brackets: An in-vivo study. *American Journal of Orthodontics and Dentofacial Orthopedics.* 2015 Nov;148(5):814–20.
99. Jablonowski BL, Bartoloni JA, Hensley DM, Vandewalle KS. Fluoride release from newly marketed fluoride varnishes. *Quintessence Int.* 2012 Mar;43(3):221–8.
100. Sahiti JS, Krishna NV, Prasad SD, Kumar CS, Kumar SS, Babu KSC. Comparative evaluation of enamel microhardness after using two different remineralizing agents on artificially demineralized human enamel: An in vitro study. *J Clin Transl Res.* 2020 Sep 5;6(3):87–91.

101. Amaechi BT, Higham SM, Edgar WM. Caries inhibiting and remineralizing effect of xylitol in vitro. *J Oral Sci.* 1999 Jun;41(2):71–6.
102. Goncalves NCL a. V, Cury AADB, Simoes GS, Hara AT, Rosalen PL, Cury JA. Effect of xylitol:sorbitol on fluoride enamel demineralization reduction in situ. *Journal of Dentistry.* 2006 Oct 1;34(9):662–8.
103. Kilian M, Chapple ILC, Hannig M, Marsh PD, Meuric V, Pedersen AML, et al. The oral microbiome - an update for oral healthcare professionals. *Br Dent J.* 2016 Nov 18;221(10):657–66.
104. Rosier BT, De Jager M, Zaura E, Krom BP. Historical and contemporary hypotheses on the development of oral diseases: are we there yet? *Front Cell Infect Microbiol.* 2014;4:92.
105. Pinar Erdem A, Sepet E, Kulekci G, Trosola SC, Guven Y. Effects of Two Fluoride Varnishes and One Fluoride/Chlorhexidine Varnish on *Streptococcus mutans* and *Streptococcus sobrinus* Biofilm Formation in Vitro. *Int J Med Sci.* 2012 Jan 7;9(2):129–36.
106. Munshi AK, Reddy NN, Shetty V. A comparative evaluation of three fluoride varnishes: an in-vitro study. *J Indian Soc Pedod Prev Dent.* 2001 Sep;19(3):92–102.
107. Steinberg D, Rozen R, Klausner EA, Zachs B, Friedman M. Formulation, development and characterization of sustained release

- varnishes containing amine and stannous fluorides. *Caries Res.* 2002 Dec;36(6):411–6.
108. Bender ME, Lipin RB, Goudy SL. Development of the Pediatric Temporomandibular Joint. *Oral and Maxillofacial Surgery Clinics of North America.* 2018 Feb;30(1):1–9.
109. Pandit S, Kim J-E, Jung K-H, Chang K-W, Jeon J-G. Effect of sodium fluoride on the virulence factors and composition of *Streptococcus mutans* biofilms. *Archives of Oral Biology.* 2011 Jul;56(7):643–9.
110. Bapat SA, Shashikiran N, Gaugawd S, Gaonkar N, Taur S, Hadakar S, et al. In-vitro Comparison of Anti-Microbial Efficacy and Enamel Microhardness after Application of Two Types of Fluoride Varnishes. *JCDR [Internet].* 2020 [cited 2021 Feb 1]; Available from: https://jcdr.net/article_fulltext.asp?issn=0973-709x&year=2020&volume=14&issue=4&page=ZC05&issn=0973-709x&id=13627.
111. Naorungroj S, Wei H-H, Arnold RR, Swift EJ, Walter R. Antibacterial surface properties of fluoride-containing resin-based sealants. *J Dent.* 2010 May;38(5):387–91.
112. Lewinstein I, Stoleru-Baron J, Block J, Kfir A, Matalon S, Ormianer Z. Antibacterial activity and tensile strength of provisional cements modified with fluoridecontaining varnish. *Quintessence Int.* 2013 Feb;44(2):107–12.

113. Wassel MO, Khattab MA. Antibacterial activity against *Streptococcus mutans* and inhibition of bacterial induced enamel demineralization of propolis, miswak, and chitosan nanoparticles based dental varnishes. *Journal of Advanced Research*. 2017 Jul 1;8(4):387–92.
114. Sankeshwari R, Ankola A, Bhat K, Bolmal U, Rao M. Evaluation of Physical Parameters of Novel Licorice Varnish Versus Fluoride and Combination Varnish: An In-Vitro Study. *Acta Medica Academica*. 2018 Dec 26;47(2):176.
115. Jannesson L, Renvert S, Kjellsdotter P, Gaffar A, Nabi N, Birkhed D. Effect of a triclosan-containing toothpaste supplemented with 10% xylitol on *mutans streptococci* in saliva and dental plaque. A 6-month clinical study. *Caries Res*. 2002 Feb;36(1):36–9.
116. Milgrom P, Ly KA, Roberts MC, Rothen M, Mueller G, Yamaguchi DK. *Mutans streptococci* dose response to xylitol chewing gum. *J Dent Res*. 2006 Feb;85(2):177–81.
117. Suda R, Suzuki T, Takiguchi R, Egawa K, Sano T, Hasegawa K. The effect of adding calcium lactate to xylitol chewing gum on remineralization of enamel lesions. *Caries Res*. 2006;40(1):43–6.
118. Scheinin A, Mäkinen KK, Tammisalo E, Rekola M. Turku sugar studies XVIII. Incidence of dental caries in relation to 1-year consumption of xylitol chewing gum. *Acta Odontol Scand*. 1975;33(5):269–78.

119. Mäkinen KK. The Rocky Road of Xylitol to its Clinical Application. *J Dent Res.* 2000 Jun;79(6):1352–5.
120. Kandelman D, Bär A, Hefti A. Collaborative WHO xylitol field study in French Polynesia. I. Baseline prevalence and 32-month caries increment. *Caries Res.* 1988;22(1):55–62.
121. Isokangas P, Alanen P, Tiekso J, Makinen KK. Xylitol chewing gum in caries prevention: a field study in children. *The Journal of the American Dental Association.* 1988 Aug 1;117(2):315–20.
122. Mäkinen KK, Bennett CA, Hujoel PP, Isokangas PJ, Isotupa KP, Pape HR, et al. Xylitol chewing gums and caries rates: a 40-month cohort study. *J Dent Res.* 1995 Dec;74(12):1904–13.
123. Mäkinen KK, Pemberton D, Mäkinen PL, Chen CY, Cole J, Hujoel PP, et al. Polyol-combinant saliva stimulants and oral health in Veterans Affairs patients--an exploratory study. *Spec Care Dentist.* 1996 Jun;16(3):104–15.
124. Alanen P, Isokangas P, Gutmann K. Xylitol candies in caries prevention: results of a field study in Estonian children. *Community Dent Oral Epidemiol.* 2000 Jun;28(3):218–24.
125. Monteiro JC, Stürmer M, Garcia IM, Melo MA, Sauro S, Leitune VCB, et al. Dental Sealant Empowered by 1,3,5-Tri Acryloyl Hexahydro-1,3,5-Triazine and α -Tricalcium Phosphate for Anti- Caries Application. *Polymers (Basel)* [Internet]. 2020 Apr 12 [cited 2021 Feb 3];12(4). Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7240744/>.

MASTER CHART 1

Compilation of the data representing the enamel thickness and the depth of demineralized zone of the experimental and control groups (expressed in mm).

Sample name	Enamel thickness		Demineralised zone	
	In pixels	In mm	In pixels	In mm
GROUP 1				
V1	249.24	65.944	56.6	14.97
V2	205.01	54.24	37.22	9.84
V3	194.28	51.4	15.01	3.97
V4	273.66	72.4	57.08	15.1
V5	213.47	56.48	70.84	18.74
V6	237.1	62.73	48	12.7
V7	191.47	50.65	42.06	11.12
V8	183.85	48.64	17.03	4.5
V9	191.93	50.78	40.31	10.66
V10	173.67	45.95	25.12	6.64
GROUP 2				
E1	238.84	63.19	49.19	13.01
E2	109.12	28.87	27.78	7.35
E3	147.12	38.92	60.07	15.89
E4	165.68	43.83	137.67	36.42
E5	199.58	52.8	62.43	16.517
E6	213.36	56.45	65.8	17.4
E7	260.93	69.03	89.9	23.78
E8	193.21	51.12	59.55	15.75
E9	174.72	46.22	51.26	13.56
E10	210.88	55.79	59.4	15.716

GROUP 3				
F1	210	55.56	67.27	17.79
F2	245.51	64.95	135.83	35.93
F3	260.5	68.92	131.59	34.81
F4	193.61	51.22	33.94	8.97
F5	233.73	61.84	64.66	17.1
F6	202.71	52.63	64.13	16.96
F7	198.01	52.39	129.11	34.16
F8	140.95	37.29	99.93	26.43
F9	260.54	68.93	91.11	24.1
F10	200.28	52.99	159.45	42.18
GROUP 4				
C1	151.49	40.08	132.77	35.12
C2	290.862	76.95	263.78	69.79
C3	197.226	52.182	161.77	42.8
C4	191.08	50.55	156.02	41.28
C5	210.77	55.76	84.85	22.44
C6	228.77	60.52	144.78	38.3
C7	157.85	41.76	156.67	41.45
C8	200.53	53.05	156.6	41.433
C9	224.59	59.42	183.1	48.44
C10	176.08	46.58	135.57	35.86



SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY

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19th June 2019

Ref: 117/IRB-IBSEC/SIST Dated: 19th June, 2019

Dr. A. Wilson Aruni, PhD; FAMPV
Pro Vice chancellor,
Deputy Chair Person, Institutional
Biosafety & Ethical Committee
(IBEC), SIST

Dr. B. Thayumanavan, MDS
Dean, Sathyabama Dental College
Secretary & Convener
Institutional Review Board &
Institutional Biosafety & Ethical
Committee (IBEC), SIST

To,

Dr. Vishnu Rekha, MDS
Professor & Head,
Department of Pediatric & Preventive Dentistry
Sathyabama Dental College & Hospital
Jeppiaar Nagar, Rajiv Gandhi Road, Chennai -119

This is to inform you that the Institutional Bio-Safety & Ethical Committee has approved your study proposal entitled '**Comparative evaluation of Demineralization Inhibitory Effect and Antibacterial Effect of commercially available fluoride varnishes – an in vitro study**', based on the presentation of the proposal made and recommendations of the Institutional Bio-Safety & Ethical Committee/IRB held on 7th May 2019,

The validity of the Ethical clearance is for one year and you are expected to submit six-month progress and the final report of the study to IHBSEC/IRB

Dr. B. Thayumanavan
Secretary & Convener
IBEC, SIST

Dr. A. Wilson Aruni
Deputy Chair Person
IBEC, SIST