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Bioactive Remineralization of Human Enamel using Piper Betle Leaf Extract and its Additives

Sanjna Santhosh Menon

Post Graduate Student, Department of Conservative Dentistry and Endodontics, AB.Shetty Memorial Institute of Dental Sciences,Mangalore

Abstract

The role of the betel leaf has been described since ancient times in various books of Ayurveda mainly due to its curative properties and been used for the treatment of various disorders and stimulated anti-oxidative, anti- mutative, and detoxifying properties. Betel quid chewers have cancerogenic potential seen in many parts of rural areas in India. This study proves that when used in correct proportions [betel leaf, areca nut and betel lime], acts as natural means for protection against caries and non -carious lesions as it re-hardness the tooth enamel naturally.

Aims: Comparative evaluation of Piper Betel leaf Extract and its additives like Betel Lime and Areca catechu on the enamel surface quantitatively using Energy Dispersive X-ray Analysis [EDAX] and qualitatively by Scanning Electron Microscopy [SEM] analysis

Methods and Material: Enamel sections from the buccal and palatal surfaces of human premolars were subjected to surface treatment using extracts: G2(BL)-Betel leaf, G3(BLM)- Betel leaf and Betel lime and G4(BLAN)- Betel leaf, betel lime and areca nut for 15min for seven days.(G1)Sound enamel stored in artificial saliva. After pH cycling, surface morphology were observed using the scanning electron microscopy and the elemental composition using Energy Dispersive X-ray Analysis

Statistical analysis used: Data were analysed using nMaster Software version 2 with Kruskal Wallis ANOVA.

Results: Enamel surface treated with Betel leaf, betel lime and areca nut recorded significantly higher Fluoride (F%-72.85), Chloride (Cl%-72.8), Carbon (C%-45.6), Phosphorus (P%-81.3) when compared to control group.

Conclusions: Betel leaf along with additives provides anticariogenic effect by enhanced fluoride concentration along with rehardening of enamel all though slight alteration of enamel surface was observed due to micro-prism crack formation.

Keywords: Betel leaf, Enamel, Betel Lime, Areca Catechu, Fluoride

Introduction

Natural herbs have been utilized for generations in medicine and food across the world. Piper betle leaf also known as betel leaf is one such important herb.[1] that are consumed along with areca nut, catechu, mineral-slaked lime and flavoring substances.[2] Several epidemiological studies have demonstrated that betel chewers have a lower incidence of dental caries than non- chewers.[3] No studies were available on the effect of Piper betle leaves with their additives on the composition and texture of the enamel surface. Hence, this study was designed to evaluate the effect of Piper betle leaf Extract and its

additives on enamel surfaces.

Subjects and Methods

Sample Selection

28 Human premolars extracted for orthodontic purposes were collected from the Department of Oral and Maxillofacial Surgery and selected based on the inclusion and the exclusion criteria and digital radiographs of these teeth were taken to check for any carious lesions. Intact permanent maxillary premolars with no evidence of caries were included in the study were as teeth that had caries, restorations, hypoplastic conditions, fractures and developmental anomalies were excluded.

Preparation of Solution

a) Piper betel leaf, Areca nut, and Betel lime extracts

Extracts consisting of 15 % Piper betle leaf, 7 g Areca catechu/nut, and 2.5 g Betel lime were prepared using a maceration process using 80 % ethanolic extract as shown in (Figure 1).[4,5]

Figure 1. Preparation of 15% Betel Leaf and Extracts from 2.5 g of Betel Lime(Slaked lime) and 7 g of Areca Catechu

b) Composition of Artificial saliva solution

NaCl(Sodium Chloride)– 0.08%
KCl(Potassium Chloride)- 0.12%
$MgCl_2$ (Magnesium Chloride) – 0.01%
K_2HPO_4 (Potassium Biphosphate) – 0.03%
$CaCl_2$ (Calcium Chloride) – 0.01%
CMC-Na (Sodium Carboxymethyl Cellulose)– 0.10%
IEW(Ion Exchanged Water)- 99.6%
pH – 6.57 (Figure 2)[6]

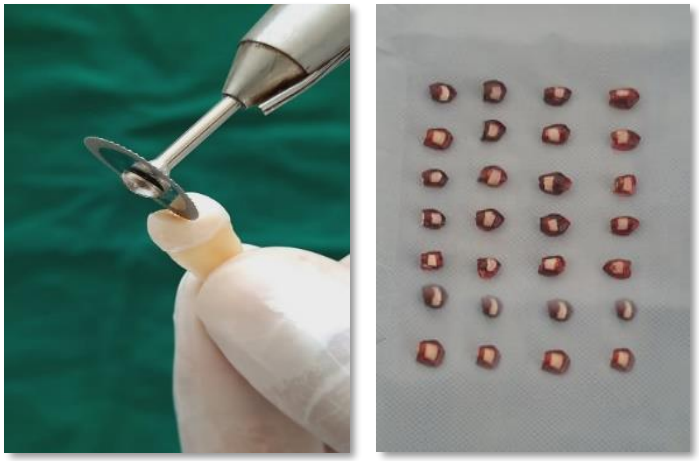
Figure 2- Preparation of Artificial saliva

Preparation of the Specimens

All the twenty-eight samples were autoclaved according to Occupational Safety and Health Administration (OSHA) guidelines, 2004.[7,8] The specimens were cleaned of soft tissue and calculus using an Ultrasonic device. 28 extracted premolars were then sectioned in a buccolingual orientation using a circular diamond disc (Dentsply) attached to micromotor handpiece (NSK, Japan) to obtain enamel sections from the buccal and palatal surfaces of the teeth using a circular diamond disc with water coolant to give a total of 56 samples. All the sectioned specimens were divided into four groups of seven specimens each for surface treatment as shown in (Figure 3).

G2-15% Betel leaf extract (BL) for 15min for seven days, G3- 15% Betel leaf and Betel lime extract (BLM) for 15min for seven days, G4- 15% Betel leaf, Betel lime, and areca catechu extract (BLAN) for 15min for seven days. G1 (Control group) – wherein no surface treatment was done and stored in artificial saliva throughout the study.

Figure 3. Preparation of sectioned enamel samples followed by surface treatment



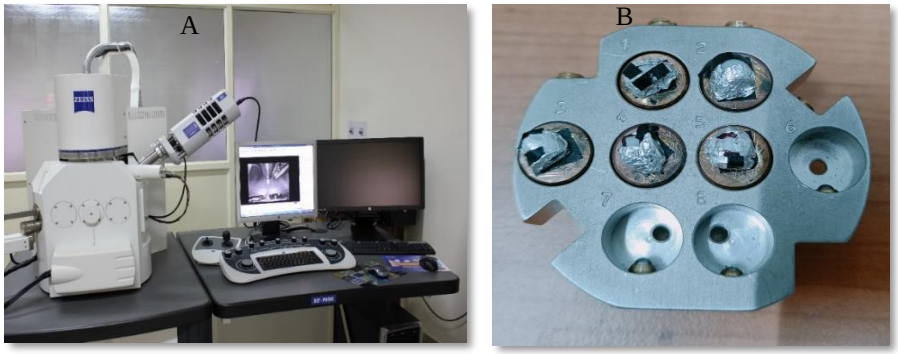
pH-cycling

To mimic the oral conditions, samples were exposed to a pH-cycling model based on reverse (Remineralizing) immersion in acid buffer (50 mM acetate, 1.3 mM KH_2PO_4; 2.25 mM $CaCl_2.H_2O$, 130 mM KCl; at pH-4.5) for two cycles of one hour per day for 21 days, with the remaining 22 hours in artificial saliva. (pH-7.2).Samples were then rinsed for two minutes with de-ionized water, dried by blotting and then kept at 37°C for 24 hours before testing.[9]

Scanning Electron Microscopic Analysis(SEM study)

The surface of enamel specimens was qualitatively analyzed using a scanning electron microscope (SEM) (JEOL, JSM-6360A, Tokyo, Japan) operated at a 10 kV acceleration voltage for changes in surface texture, Integrity, and pattern and then quantitatively evaluated for elemental analysis using Energy Dispersive X-Ray Analysis(EDAX) shown in (Figure 4.A).Tested samples were dried, mounted on aluminum stubs, placed in a vacuum chamber, and sputter coated with a Silver layer(Class One System), and then observed under a scanning electron microscope shown in (Figure 4.B).

Figure 4. (A) Scanning electron microscope & (B) Sectioned enamel samples placed on aluminium stubs for sputtering



Statistical analysis

The Sample size of 28 was calculated using nMaster Software of version2 with a standard deviation of 7.15 and a margin of error 13 at 95% confidence level and 80 % power. Data was analysed using Kruskal wallis ANOVA with p value less than 0.05 being statistically significant**.**

Results

Data obtained were compared and statistically analyzed using the Kruskal-Wallis test.The level of significance was set at p=0.05.p-value < 0.05 was considered to be statistically significant and p-value > 0.05 was considered to be statistically non-significant.When the comparison of weight % of elements in each study group was done, (Table 1) samples in G4 showed the highest values of mineral and elemental composition (47.22) followed by samples in G3 (45.20) and then G2(43.46) and were statistically significant. Samples in (G1) control group (40.08) showed the lowest elemental and mineral composition values and were statistically significant.

Table 1. Comparison of Weight % of elements in each study group

Group	Element	N	Mean (SD)	Range	Median (Q1-Q3)
Group 1	C(Carbon)	7	10.26 (6.34)	5.5 - 23	7.3(5.8- 12.3)
	O(Oxygen)	7	30.29 (11.68)	12.5 - 43.6	31.6(18.3- 43.6)
	P(Phosphorous)	7	18.69 (3.33)	15.3 - 23.7	20.2(15.3- 20.4)
	Cl(Chloride)	7	0.99 (0.34)	0.6 - 1.7	0.9(0.8- 1)
	Ca(Ca	7	39.06 (8.57)	27.8 - 50.4	40.5(27.8- 46.4)

Group 2	C	7	6.94 (1.64)	4.9 - 10.2	6.5(6.2- 7.5)
	O	7	34.24 (8.28)	17.2 - 44.1	35(33.6- 37.6)
	F	7	0.96 (0.38)	0.4 - 1.5	0.9(0.6- 1.2)
	P	7	19.46 (2.65)	16.8 - 25.2	18.9(18.5- 19)
	Cl	7	0.13 (0.34)	0 - 0.9	0(0 - 0)
	Ca	7	37.61 (6.25)	32.2 - 51.4	36.1(35.1- 36.3)
Group 3	C	7	9.56 (2.08)	4.9 - 11	10.2(10.2- 10.2)
	O	7	32.60 (7.27)	16.2 - 37	35(35- 35)
	F	7	3.29 (5.47)	1.2 - 15.7	1.2(1.2- 1.3)
	P	7	19.31 (3.13)	16.5 -26.2	18.5(18.5- 18.5)
	Cl	7	0.19 (0.49)	0 - 1.3	0(0- 0)
	Ca	7	37.16 (6.33)	33.1 - 51.4	35.1(35.1- 35.2)
Group 4	C	7	13.43 (2.02)	9.2 - 14.8	14.6(12.6- 14.6)
	O	7	37.84 (5.56)	30.9 - 43.6	35.6(33.6- 43.6)
	F	7	0.51 (0.09)	0.4 – 0.7	0.5
	P	7	16.67 (2.08)	15 - 20.5	16(15- 18.2)
	Cl	7	0.51 (0.09)	0.4 - 0.7	0.5(0.5- 0.5)
	Ca	7	29.10 (5.98)	25.6 - 38	25.6(25.6- 37.7)

When the comparison of (weight)wt % of elements between the study groups were compared shown in (Table 2), Ca % was found to be highest in G1(77.5) and lowest % in G2 (BL-73.1), where $p<0.05$ P% was found to be highest in G4 (BLAN-81.3),where $p>0.05$ and lowest % in G3 (BLM-73.7). Cl % was found to be highest in G4 (BLAN-72.8) and lowest % in G2 (BL-14.4),where $p<0.05$. F % was found to be highest in G4 (BLAN-72.85) and lowest % in G1(0),where $p<0.05$. C % was found to be highest in G4 (BLAN- 45.6) and lowest % in G3 (BLM- 42.5), $p<0.05$. O % was found to be highest and similar in G1 and G2 (BL –69.47), but lowest in G3 (BLM-67.5),where $p>0.05$.

Table 2. Comparison of Weight % of elements between the study groups

Element	Group	N	Mean(SD)	Range	Median		Kruskal Wallis Test	
						Percentage %	Chi-Square	p-value
Ca	G1	7	39.06 (8.57)	27.8 - 50.4	40.5(27.8- 46.4)	77.55	7.11	<0.001*
	G2	7	37.61 (6.25)	32.2 - 51.4	36.1(35.1- 36.3)	73.1		
	G3	7	37.16 (6.33)	33.1 - 51.4	35.1(35.1- 35.2)	75.9		
	G4	7	29.10 (5.98)	25.6 - 38	25.6(25.6- 37.7)	76.5		

P	G1	7	18.69 (3.33)	15.3 - 23.7	20.2(15.3-20.4)	78.5	5.68	0.13(NS)
	G2	7	19.46 (2.65)	16.8 - 25.2	18.9(18.5-19)	77.2		
	G3	7	19.31 (3.13)	16.5 -26.2	18.5(18.5-18.5)	73.7		
	G4	7	16.67 (2.08)	15 - 20.5	16(15-18.2)	81.3		
Cl	G1	7	0.99 (0.34)	0.6 - 1.7	0.9(0.8-1)	68	16.12	0.001*
	G2	7	0.13 (0.34)	0 - 0.9	0(0 - 0)	14.4		
	G3	7	0.19 (0.49)	0 - 1.3	0(0- 0)	14.6		
	G4	7	0.51 (0.09)	0.4 - 0.7	0.5(0.5-0.5)	72.8		
F	G1	7	0 (0)	0 - 0	0(0- 0)	0	24.48	<0.001*
	G2	7	0.96 (0.38)	0.4 - 1.5	0.9(0.6-1.2)	64		
	G3	7	3.29 (5.47)	1.2 - 15.7	1.2(1.2-1.3)	21		
	G4	7	0 (0)	0 - 0	0(0- 0)	72.8		
C	G1	7	10.26 (6.34)	5.5 - 23	7.3(5.8-12.3)	44.6	11.36	0.01*
	G2	7	6.94 (1.64)	4.9 - 10.2	6.5(6.2-7.5)	44.6		
	G3	7	9.56 (2.08)	4.9 - 11	10.2(10.2-10.2)	42.5		
	G4	7	13.43 (2.02)	9.2 - 14.8	14.6(12.6-14.6)	45.6		
O	G1	7	30.29 (11.68)	12.5 - 43.6	31.6(18.3-43.6)	69.4	2.48	0.48(NS)
	G2	7	34.24 (8.28)	17.2 - 44.1	35(33.6-37.6)	69.4		
	G3	7	32.60 (7.27)	16.2 - 37	35(35-35)	67.5		
	G4	7	37.84 (5.56)	30.9 - 43.6	35.6(33.6-43.6)	69.2		

Scanning Electron Microscopy Study

SEM image of intact enamel stored in artificial saliva showed smooth, intact surface with a homogenous appearance shown in (Figure 5a) along with Elemental analysis by EDAX (Figure 5b) whereas in G2, SEM image of enamel surface treated with BL showed mineral deposition in amorphous layer with thick interrod regions and globular- like appearance shown in (Figure 6a) along with Elemental analysis by EDAX (Figure 6b) In G3, SEM image of enamel surface treated with BLM showed scaffolding deposits with cluster-like structures seen as amorphous clumps shown in (Figure 7a) along with Elemental analysis

by EDAX (Figure 7b) and in G4, SEM image of enamel surface treated with BLAN showed micro-cracks shown in (Figure 8a) along with Elemental analysis by EDAX (Figure 8b).

Figure 5.a) Structural analysis of enamel surface by SEM, after being placed in artificial saliva – 1000X b) Elemental analysis of enamel surface by EDAX, after being placed in artificial saliva – 1000X (G1)

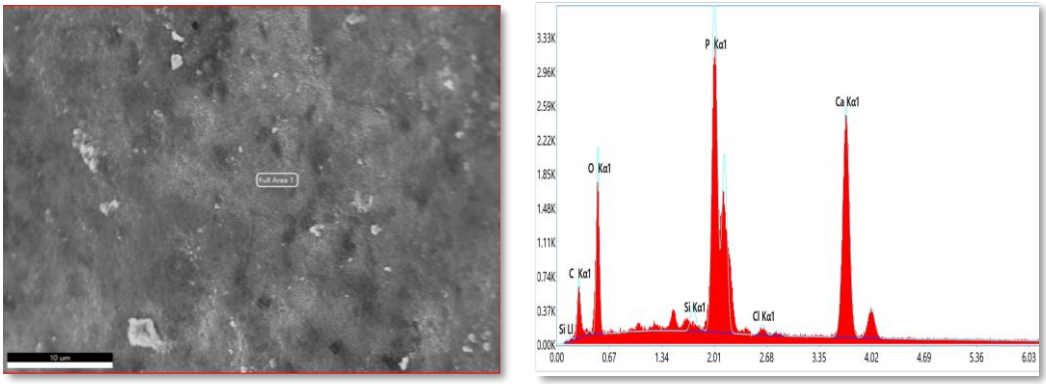


Figure 6.a) Structural analysis of enamel surface by SEM, after surface treatment with 15% Betel Leaf – 1000X b) Elemental analysis of enamel surface by EDAX, after surface treatment with 15%Betel Leaf– 1000X (G2)

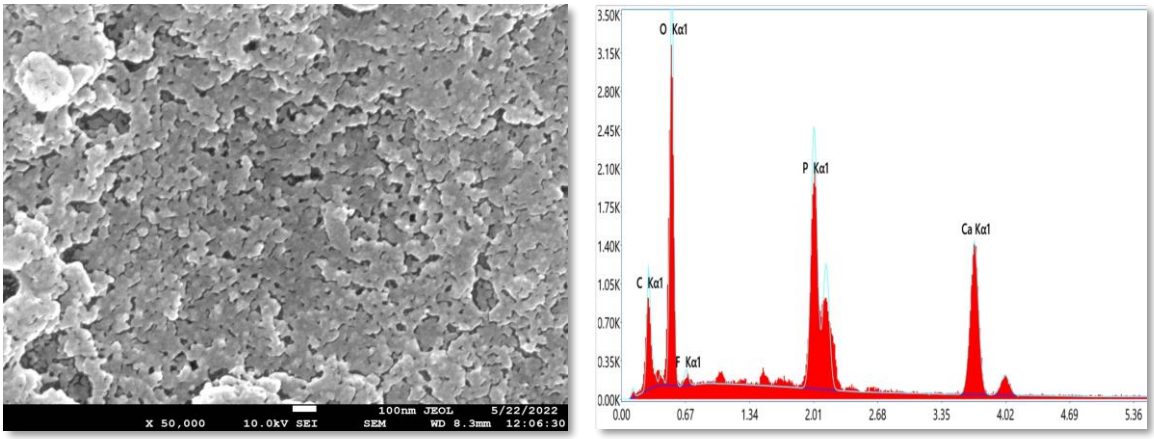


Figure 7.a) Structural analysis of enamel surface by SEM, after surface treatment with 15% Betel Leaf and Betel Lime – 1000X b) Elemental analysis of enamel surface by EDAX, after surface treatment with 15% Betel Leaf and Betel Lime - 1000X (G3)

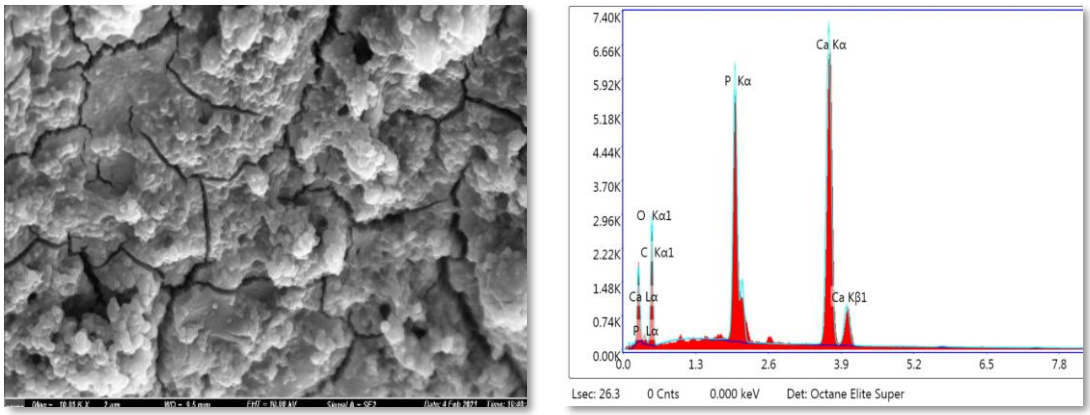
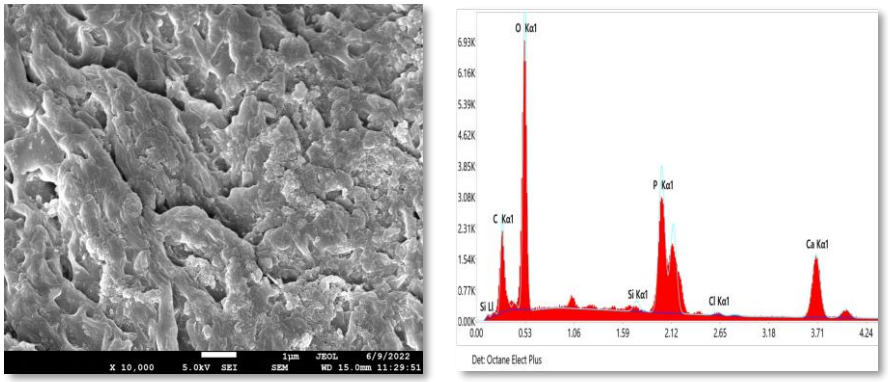


Figure 8.a) Structural analysis of enamel surface by SEM, after surface treatment with 15% Betel Leaf, Betel Lime and Areca Nut – 1000X b) Elemental analysis of enamel surface by EDAX, after surface treatment with 15% Betel Leaf, Betel lime and Areca Nut- 1000X (G4)



Discussion:

In the present study, EDX spectra of the intact enamel samples (G1) were examined to record the baseline composition followed by an SEM study of surface characteristics to be compared with the experimentally treated groups. The Ca % (77.5) was significantly higher in comparison to all the groups which was statistically significant. P% was higher in comparison to G2 (77.2) and G3 (73.7) but lower in comparison to G4 (81.3). The basic mineral component of mature enamel made of a complex of calcium and phosphate exists as hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ which imparts properties such as hardness to resist tooth substance loss through abrasion, attrition as well as resistance to demineralization by dental caries and erosion.[10]

Whereas in G2, the Ca % was lower compared to G1 (77.5) and G4 (76.5) marginally greater when compared to G3 (75.2) which was statistically not significant. The P % was also lower compared to G1 (78.5) and G4 (81.3) which was statistically significant but marginally greater when compared to G3 (73.7).The increase of Ca(Calcium) and P(Phosphate) in G2 is attributed to the presence of minerals like Ca (55 mg) and P (65 mg) in Betel Leaf composition reported by Mohanapriya S et al.[11] F % was significantly higher in comparison to G1(0) and G3 (21) but lower in comparison to G4 (72.85 %), which was statistically significant. The presence of Cl(Chloride) and F(Fluoride) in G2 samples could be attributed to the composition of the betel leaf extract which according to A.Jain et al reportedly contains 3.9 µg/g chloride and 4.3 µg/g fluoride.[12] However, it may be variable depending on the fluoride concentration of the soil used for growing the betel crop.[13] Marginal variation in C(Carbon) and O(Oxygen) could be attributed to the changes in the microscopic structure of enamel such as decreased water and carbonate content, increased hydroxyl ion content, pyrophosphate establishment, and protein dissolution in the samples.[14]

The presence of fluoride was noted in both G2 and G3 samples revealing the anti-cariogenic property of betel leaf as reported by Fu et al,[15] due to formation of fluoroapatite crystals and influences the rate of dissolution of enamel without altering the solubility of minerals thus enhancing the anticariogenic effect.[16] Piper betel also contains phytochemicals such as phenols, flavonoids, and tannins, which could increase collagen crosslinks by strengthening collagen-based tissues even though mature enamel is known to be a substrate free of collagen.[17,18]

In the EDX analysis of enamel samples treated with Betel Leaf extract along with Betel Lime (G3), the presence of Ca and P in the treated samples could be attributed to the incorporation of Ca and P from betel lime extract which also consists of Ca $(OH)_2$ as reported by Nair et al.[19] The discoloration seen in Betel Leaf chewers could be triggered by orthoquinone polymers which when combined with slaked lime, may increase the pH of the oral environment due to which in response to attrition, there is an ample supply of sclerosed dentine, which may provide additional protection against microbial invasion. Hence these stains, which frequently coat the surfaces of teeth, may act as a physical barrier to tooth demineralization since the presence of tannin in betel leaf has antimicrobial properties that contribute to its cariostatic role.[20]

Enamel samples treated with Betel Leaf extract along with Betel Lime and Areca catechu (G4), showed the highest mineral, halide, and elemental content which is attributable to the components of areca nut itself that might have contributed to the elemental composition of enamel samples.[21] The presence of chloride ions also accounts for more than 60% of the ionic strength of saliva and can be strongly linked to enamel rehardening.[22]

In the present study, SEM has been used to evaluate surface changes on enamel at micro-structural level because of its wide range of magnification that are easy to interpret and three-dimensional in nature, which may be more appealing to the human eye, whereas EDAX is a common analytical technique used for providing measurements of elements on the surface semi-quantitatively, within the surface layer of 1-2 microns thickness.[23]

SEM micrographs for the enamel surface of intact samples stored in artificial saliva (G1) (Figure 5a) showed smooth and intact surfaces with a homogenous appearance which was in agreement with the findings by Worawongvasu et al.[24] In comparison to G1, SEM observation for samples treated Betel Leaf extract (G2-BL) (Figure 6a) exhibited mineral deposition in the amorphous layer which was accentuated in a continuous manner along deep infiltration of the rod ends in certain areas wherein this deposition exhibited fused globules along with a globular- appearance with a deficiency process of fusion in some areas similar to study conducted by Younis SH et al on Moringa leaves.[25] SEM observation for samples treated Betel Leaf extract and Betel Lime (G3- BLM) (Figure 7a) showed scaffolding deposits on the enamel along with coating depositions of insoluble complexes on the enamel surface seen as amorphous clumps which were in similar findings to study conducted by Moshy et al using Agarose hydrogel on enamel.[26] SEM micrographs for samples treated with Betel Leaf extract, Betel Lime, and Areca catechu (G4-BLAN) (Figure 8a) showed micro-cracks after subjecting to surface treatment along with disrupted prismatic structures which were in similar findings to a study conducted by by Cherian et al using remineralizing agents like Tri Calcium Phosphate on enamel .[27]

Artificial saliva was used as a storage medium during the experimental procedure to stimulate the intraoral conditions and since it cannot mimic the remineralization and demineralization process inside the intra-oral environment, the specimens in this study were subjected to a pH cycling procedure to further simulate intra-oral conditions.

CONCLUSION:

Within the limitations of the present study, it could be concluded that; except control group, all the experimental groups exhibited remineralizing potential. Amongst the test groups, G4 (BLAN) showed highest mineral, elemental and halide content.Thus clinical application of Betel leaf extract along with added additives such as Betel lime and areca nut/catechu can be beneficial in providing anticariogenic effect by enhancing the fluoride concentration along with rehardening of enamel by additional supply of calcium, phosphorus and chloride ions even though it causes slight alteration in the surface morphology of enamel resulting in discoloration due to certain micro-prism crack formation.

Limitations

Even though an in-vitro model has the benefit of providing extremely well-controlled experimental conditions, for conclusive results, additional studies that closely simulate in-vivo conditions, followed by long-term clinical trials, are recommended.

Clinical significance

This is the first study that we are aware of that compares the morphological and elemental composition of these three variants (BL, BLM and BLAN) on human enamel.

Conflict of Interest:

There are no conflicts of interest in the present investigation.

Authors' Biography

I am currently working as Restorative dentist and endodontist in reputed clinic in Kerala.

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