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COMPARISON OF THE REMINERALIZATION POTENTIAL OF OYSTER SHELL POWDER, BAMBOO SALT AND CASEIN PHOSPHO-PEPTIDE AMORPHOUS CALCIUM PHOSPHATE (CPP-ACP) - *AN INVITRO STUDY*

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Abstract

Aim: The aim of the study was to compare the remineralization potential of oyster shell powder, bamboo-salt and Casein Phospho-Peptide – Amorphous Calcium Phosphate (CPP-ACP). **Materials and methods:** Forty enamel slabs obtained from impacted third molars were subjected to Vickers microhardness test for evaluating the hardness and SEM-EDAX for elemental quantification of Calcium and Phosphate. Then, the samples were placed in demineralizing solution for 3 days followed by hardness test and SEM-EDAX analysis. They were divided into four Groups(n=10); Group 1- Oyster shell powder, Group 2- Bamboo salt, Group 3- CPP-ACP and Group 4- No treatment. The remineralizing agents were applied onto the enamel slabs for 3 minutes every 12 hours for seven days and left in artificial saliva until next application. After remineralization, hardness test and SEM-EDAX analysis were repeated. **Statistical Analysis:** One-way analysis of variance (ANOVA) and post-hoc Tukey's test with a statistical significance level of $p < 0.05$. **Results:** Oyster shell powder showed statistically significant hardness and Calcium-Phosphate content compared to other groups. **Conclusion** Oyster shell powder had significant remineralizing potential followed by bamboo salt and CPP-ACP. These natural products showed promising remineralizing efficacy which is almost similar or even superior to the currently available proprietary remineralizing agents.

Keywords: Bamboo salt; CPP-ACP; Human Enamel Remineralization; Oyster shell powder; SEM-EDAX; Vickers Hardness Test

INTRODUCTION

Dental caries is a highly prevalent microbiologic disease that causes demineralization of inorganic substances and dissolution of organic substances of the teeth.[1] Though it is multifactorial, the major contributor is the acids generated in the oral cavity by microbial activity, especially lactic acid, which is the predominant end-product of sugar metabolism.[2] There are two aspects which decide the impact of carious activity-Demineralization and remineralization. As acids build-up in the microbial biofilm, the pH drops to the point at which condition the biofilm–enamel interface becomes deficient of minerals leading to demineralization of the tooth.[3] If the saliva is saturated with calcium, phosphate, fluoride, etc, the acids will be neutralized by the buffering action of saliva leading to remineralization. This dynamicity leads to the reversal of the

very early (subclinical) stages of caries.[4] Any imbalance in the demineralization-remineralization cycle commonly leads to loss of calcium, phosphate and other ions from the tooth. The goal of modern dentistry is to provide non-invasive treatment to non-cavitated caries lesions. Thus, the first treatment option for non-cavitated lesions is by the minimally invasive remineralization therapy.[5] One such innovative caries preventive product derived from milk is Casein phosphopeptide – amorphous calcium phosphate (CPP–ACP). It has the capability to remineralize and prevent dental caries progression by releasing calcium and phosphate ions in the oral environment. CPP usually delivers ACP and the former additionally helps the ACP to merge with the dental enamel.[6] On reviewing literature, it is known that many organic and natural products produce compounds which which a variety of microorganisms. Herbal products have active constituents that has both beneficial physiological effect and curative property. One such product is, Triphala, an Indian ayurvedic herbal formulation which shows significant antibacterial activity against *S.mutans* biofilm that helps in curbing the demineralisation activity. Other natural products such as bamboo salt, oyster shell, chicken egg shell, grape seed, green and white tea, milk and milk products, Amukkara, Ashwagandha, Adhimadhuram, are used in different systems of medicines like Ayurveda, Siddha, Unani, Naturopathy, Phytotherapy and Herbal medicine for their therapeutic effects.[7]

Bamboo salt is a very rich source of calcium, potassium and phosphorus.[8] Dentifrice with bamboo salt and sodium fluoride on artificial enamel caries showed a significant increase in the level of the surface hardness and also aided in reducing the net loss of minerals at both the surface and deep areas.[9]

Calcium fluoride compound such as oyster shells, might provide fluoride in a more metabolically active form, since it not only is a practically pure phosphorus-free calcium carbonate but contains many trace elements which may enhance fluoride availability. It contains about 95% of calcium carbonate and forms bone more rapidly than a gold-standard bone substitute material.[10]

Usage of Bamboo salt and Oyster shell for enamel remineralization could be beneficial as they are naturally available sources of calcium. On reviewing the literature, there were no studies that compared and evaluated the remineralization potential of commercially available paste and natural remineralization components. Hence, the aim of this study was to compare and evaluate the remineralization potential of oyster shell powder, bamboo-salt and Casein Phospho-Peptide –Amorphous Calcium Phosphate (CPP-ACP) on demineralized enamel. The null hypothesis is that there will be no significant difference in the remineralization potential of oyster shell powder, bamboo-salt and Casein Phospho-Peptide –Amorphous Calcium Phosphate (CPP-ACP).

MATERIALS AND METHODS

This study was conducted after approval was granted by the Ethical Committee of the institute EC NO: 01041802. Fifteen freshly extracted unerupted Maxillary/Mandibular third molars were collected to procure 40 samples and stored in 0.1% thymol (Sigma Aldrich, USA). Erupted teeth, teeth with caries, fluorosis or other hyper-mineralization defects, teeth with pitting defects, cracks, hypoplastic areas and enamel irregularities were all excluded from the study. Teeth were sectioned 1 mm apical to the cementoenamel junction with diamond disc (D345-190 NTI Flex Diamond Disc,

Kerr,USA) used in micromotor (Waldent, India) run in slow speed. The roots were discarded, and the crowns were used for the study. Four enamel slabs of size 4mm square and 2mm thickness were obtained from a single tooth, one each from the 2 proximal sides and the buccal and lingual surfaces. If an enamel slab underwent fracture during preparation, all the slabs prepared from that tooth were discarded. Each enamel slab was embedded in the resin with the enamel surface facing outwards and exposed (Fig 1). Forty samples were then subjected to baseline evaluation of microhardness and elemental quantification using Vickers microhardness tester (Zwick Roell Indentec, Zwick Inc, Germany) at a load of 25 grams that was applied for five seconds. Five indentations at spacing of 100 microns were taken and the average value was considered the mean baseline micro-hardness for each specimen.

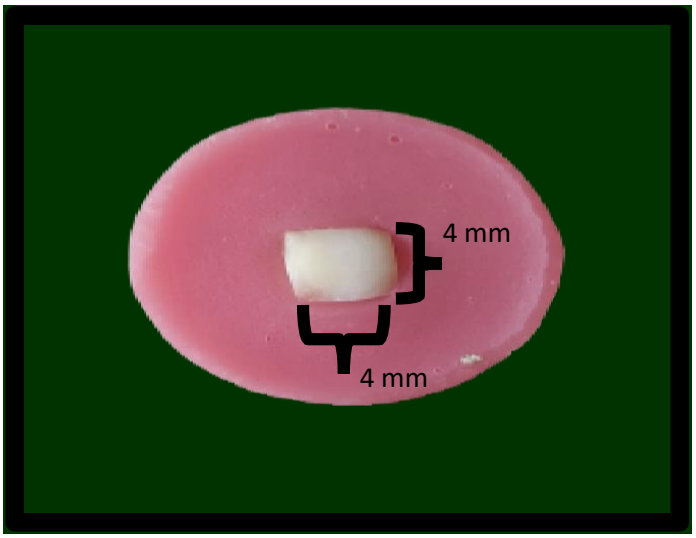


Figure 1: Mounted Enamel Slab In Self-Cured Acrylic Resin

SEM was used to identify the surface topography using an electron beam generated by a Hitachi S-3400N Scanning Electron Microscope (SEM, Hitachi, Tokyo, Japan). The elemental composition was analyzed by Energy Dispersive X-Ray Spectrometer (EDX) with a computer programmed and Quantex IV software. All the specimens were immersed in demineralizing solution for 72 hours to induce artificial carious lesion. The demineralizing solution contains Calcium Chloride (2.2mM), Monosodium phosphate (2.2mM), lactic acid (0.05 M), fluoride (0.2 ppm), adjusted with 50% Sodium hydroxide to a pH 4.5 [Khariwal laboratories, Chennai]. Then, the specimens were washed in distilled water and then subjected to Vickers hardness test and SEM-EDAX.

Then, the forty samples were divided into four groups based on the agents used for remineralization. Group 1 (n=10) Oyster shell powder, Group 2 (n=10) Bamboo salt, Group 3 (n=10) proprietary CPP-ACP (Positive Control) and Group 4 (n=10) No treatment (Negative Control). The oyster shell powder and bamboo salt (Group 1 and 2) were mixed with methylcellulose solution in the ratio 1:1 in a petri-dish with the help of a glass rod. The obtained paste was applied onto the samples using applicator tips and left undisturbed for 3 minutes. For Group 3, the CPP-ACP paste was applied using applicator tips and left undisturbed for 3 minutes. The samples were then rinsed in water for 1 min and stored in artificial saliva for 7 days at 37^0C. For every 12 hours, fresh remineralizing paste was prepared and applied using the same protocol as mentioned above. Before application, the samples were washed twice with distilled

water. Group 4 was not treated with any of the remineralizing agents and the samples of this Group were placed in artificial saliva for 7 days. At the end of 7 days, all the samples were washed thoroughly in distilled water and allowed to dry. The post-remineralization tests (Vickers microhardness test and SEM-EDAX) Were performed.

STATISTICAL ANALYSIS

The obtained values were tabulated in the Microsoft Excel sheet and analyzed using EPI-INFO (version 7.2.2.6, CDC, license: public domain) software. The descriptive data including the mean and Standard Deviation were determined for all the Groups. In order to compare the mean values of dependent variable involving more than 3 Groups, ANOVA was applied once the assumptions are met. Multiple Group comparisons were analyzed by one-way ANOVA followed by post-hoc Tukey's test. The p value of < 0.05 was considered as statistically significant. Significance level is fixed as 5% ($\alpha = 0.05$).

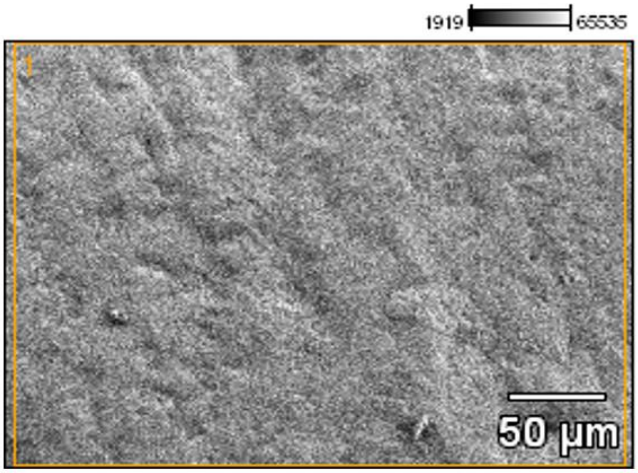
RESULTS

In Vickers microhardness test, the mean baseline values of enamel slabs in Group 1 (oyster shell), Group 2 (bamboo salt), Group 3 (CPP-ACP) and Group 4 (No treatment) were 331.5 VHN, 337.1 VHN, 334.2 VHN and 344.9 VHN respectively. After demineralization, their values were 218.1 VHN, 224.2 VHN, 222 VHN and 219 VHN respectively. There was no statistically significant difference in the baseline and demineralization values among the 4 Groups within the 95% of confidence interval ($p>0.05$). After remineralization, their hardness values were 390.2 VHN, 340.1 VHN, 337.9 VHN and 282.5 VHN respectively. There was statistical significance ($p>0.05$) between all the Groups except Groups 2 and 3 (Bamboo salt and CPP-ACP). Group 1 (Oyster shell powder) had the highest hardness value followed by Group 2 (Bamboo salt), Group 3 (CPP-ACP) and Group 4 (No treatment). (Table 1)

Table 1: Statistical Analysis of the Vickers Microhardness Test Values [Baseline, Demineralization & Remineralization Values]

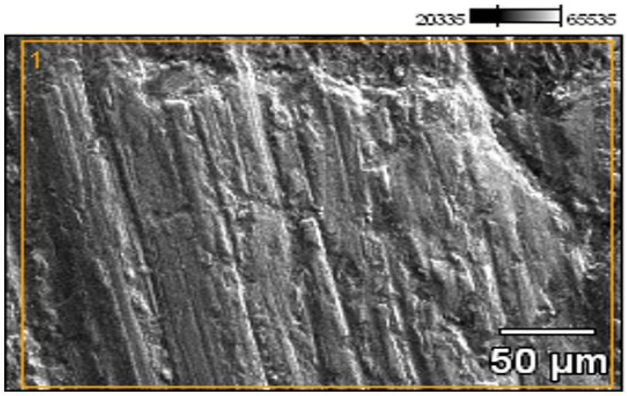
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
					Lower Bound	Upper Bound
Oyster shell powder	10	390.200	21.1755	6.6963	375.052	405.348
Bamboo salt	10	340.100	26.0318	8.2320	321.478	358.722
CPP-ACP	10	337.900	30.1900	9.5469	316.303	359.497
No treatment	10	282.500	26.0096	8.2250	263.894	301.106
Total	40	337.675	45.9980	7.2729	322.964	352.386

The SEM-EDAX results obtained during baseline, after demineralization and remineralization using each Group have been shown in Figures 2,3,4.



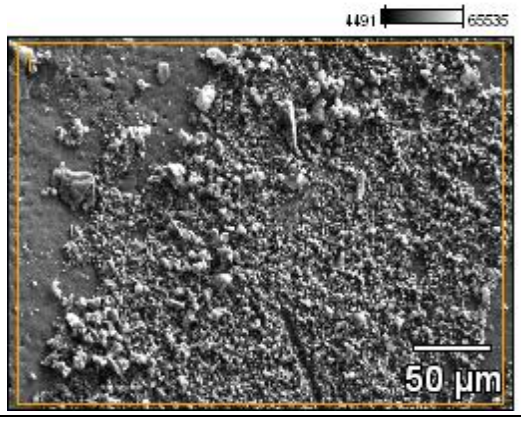
Accelerating Voltage: 15.0 kV
Magnification: 500

Figure 2: SEM– Baseline



Accelerating Voltage: 15.0 kV
Magnification: 500

Figure 3: SEM – After Demineralization

Groups	SEM Images After Remineralization Accelerating Voltage: 15.0 Kv; Magnification: 500x
Group 1	

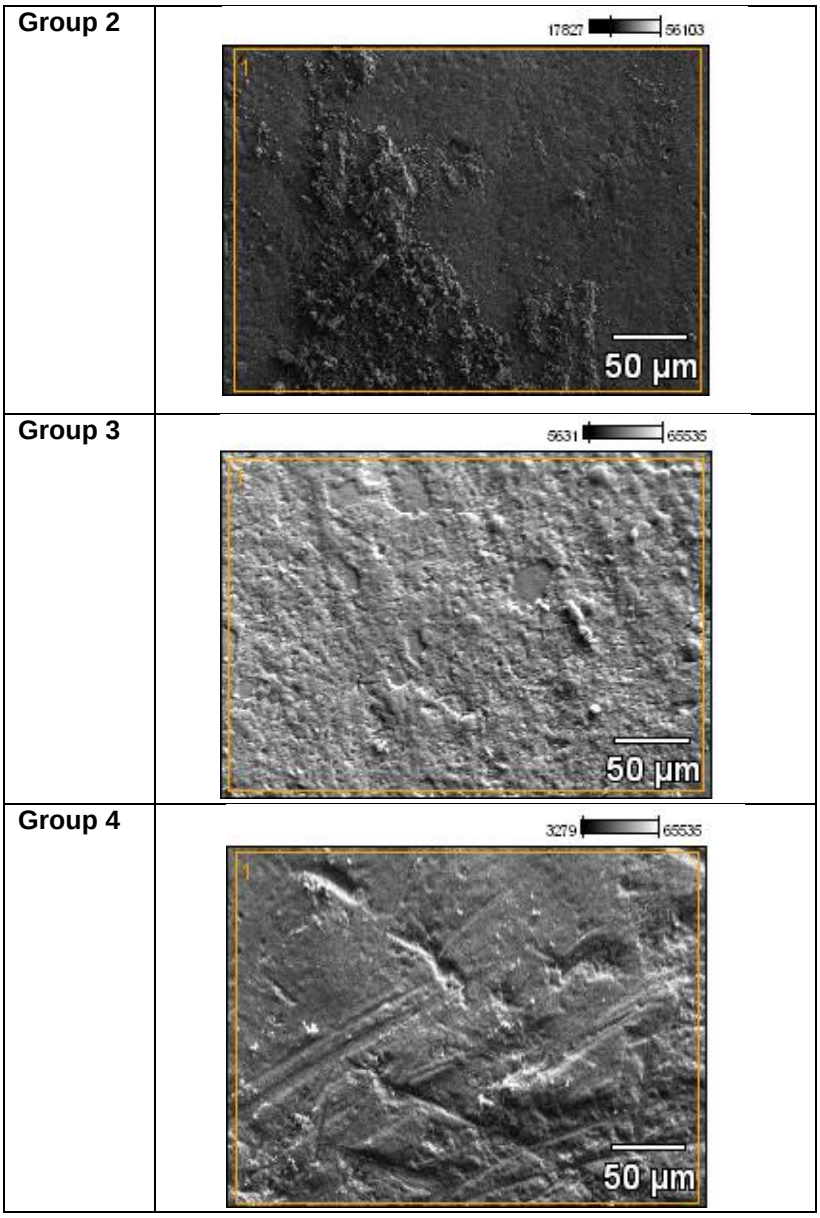


Figure 4: SEM Images – After Remineralization

The mean baseline elemental calcium values of enamel slabs obtained using SEM-EDAX in Group 1 (oyster shell), Group 2 (bamboo salt), Group 3 (CPP-ACP) and Group 4 (No treatment) were 29.809%, 28.242%, 28.065% and 29.279% respectively. After demineralization, their values were 16.656%, 15.281%, 15.909%, 16.028% respectively. There was no statistically significant difference in the baseline and demineralization values among the 4 Groups within the 95% of confidence interval ($p>0.05$). After remineralization, their elemental calcium values were 35.711%, 29.417%, 28.397% and 20.048% respectively. There was statistical significance ($p>0.05$) between all the Groups except Groups 2 and 3 (Bamboo salt and CPP-ACP). Group 1 (Oyster shell powder) had the highest elemental calcium values followed by Group 2 (Bamboo salt), Group 3 (CPP-ACP) and Group 4 (No treatment). (Table 2).

Table 2 : Statistical analysis of Elemental Calcium Content of Enamel Obtained By SEM-EDAX [Baseline, Demineralization & Remineralization Values] Descriptives

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
					Lower Bound	Upper Bound
Oyster shell powder	10	35.7110	3.11764	.98588	33.4808	37.9412
Bamboo salt	10	29.4170	3.33393	1.05428	27.0320	31.8020
CPP-ACP	10	28.3970	4.10277	1.29741	25.4621	31.3319
No treatment	10	20.0480	1.87075	.59158	18.7097	21.3863
Total	40	28.3933	6.43092	1.01682	26.3365	30.4500

The mean baseline elemental phosphorus values of enamel slabs obtained using SEM-EDAX in Group 1 (oyster shell), Group 2 (bamboo salt), Group 3 (CPP-ACP) and Group 4 (No treatment) were 15.085%, 14.77%, 15.233% and 16.061% respectively. There was no statistically significant difference in the baseline and demineralization values among the 4 Groups within the 95% of confidence interval ($p>0.05$). After remineralization, their elemental phosphorus values were increased to 17.372%, 15.287% and 10.639% respectively. There was statistical significance ($p>0.05$) between all the Groups except Groups 2 and 3 (Bamboo salt and CPP-ACP). Group 1 (Oyster shell powder) had the highest elemental phosphorus values followed by Group 2 (Bamboo salt), Group 3 (CPP-ACP) and Group 4 (No treatment). (Table 3)

Table 3 : Statistical analysis of Elemental Phosphorus Content of Enamel Obtained By SEM-EDAX [Baseline, Demineralization & Remineralization Values] Descriptive

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
					Lower Bound	Upper Bound
Oyster shell powder	10	17.3720	1.28835	.40741	16.4504	18.2936
Bamboo salt	10	15.3690	1.52524	.48232	14.2779	16.4601
CPP-ACP	10	15.2870	1.11916	.35391	14.4864	16.0876
No treatment	10	10.6390	1.26986	.40156	9.7306	11.5474
Total	40	14.6668	2.80042	.44279	13.7711	15.5624

DISCUSSION

Diagnosis of early caries lesions and treatment with non-surgical methods such as remineralization, is a contemporary approach that has been well received and adopted. The ultimate goal should be identification of caries susceptibility and risk factors followed by preservation of teeth through minimally invasive techniques.[11] Remineralizing agents can either be fluoridated or non-fluoridated agents. Fluoride increases enamel resistance, increases rate of maturation, remineralization of incipient caries, interferes with microorganisms and improves tooth morphology.[12] Due to its disadvantages like fluorosis, toxicity, the use of non-fluoridated agents like CPP-ACP, Tricalcium Phosphate, Bioactive Glass, Xylitol, un-stabilized Calcium Phosphate with Sodium Fluoride, Arginine Bicarbonate calcium carbonate complex, Calcium carbonate carrier and Ozone came to light in dental practice. In this study CPP-ACP was used as a positive control. The tryptic digestion of milk casein produced multi-phosphorylated CPP which substantially increases the milk protein's solubility and

ability to stabilize calcium and phosphate ions.[13] CPP is a saliva biomimetic but with a significantly greater calcium-stabilizing capacity than salivary proteins due to the higher content of its phosphoseryl residues.[14] The subsurface remineralization pattern produced by CPP-ACP significantly improve the aesthetics, strength and acid resistance of the remineralized white spot lesions (WSL).[15] OS is one of the few potential sources of dietary calcium that has been studied as an alternative treatment for calcium deficiency in medicine.[16] The wide availability and the low cost in addition to its biological–natural origin have made OS highly attractive for the preparation of hydroxyapatite (HA) powders for biomedical applications. OS is naturally composed of $CaCO_3$ (~96%) and trace quantities of other minerals which has made it a suitable bone substitute.[17] A few studies have shown that Bamboo salt (BS) can prevent cavities, reduce plaque and gingivitis, soothe sensitive teeth, fight bad breath, whiten teeth, strengthen tooth enamel, prevent receding gum line and decrease mineral loss.[8,9] The powder quantification was done for oyster shell powder and bamboo salt using XRD for the identification of the major constituents or compounds present in a material. Considering the importance of the surface layer in caries progression, the evaluation of changes in the surface microhardness is done either by Knoops hardness number (KHN) or Vickers hardness number (VHN). According to Chow *et al* in 2000, KHN and VHN show almost the same value.[18] The average hardness value for enamel is in the range of 270–350 KHN or 250 to 360 VHN which is concurrent to the values in this study which was 327.23 VHN. [19] Similarly, the SEM-EDAX values for specimens were measured to know the Calcium:Phosphate ratio at 3 phases - the baseline, after induction of carious lesion/demineralization and after remineralization. Energy dispersive X-rays analysis (EDAX) has been used for elemental analysis at the ultrastructural level. It is one of the effective microanalytical techniques that are used in conjunction with scanning electron microscope (SEM) wherein SEM does the structural analysis and elemental analysis is done by EDAX.[20] In the present study, the specimens were kept in the demineralization solution for 72 hours at 37° C. According to Featherstone in 1988, this created a subsurface demineralization of approximately 150 microns width with a surface layer which is intact, simulating an early enamel lesion.[21] Since CPP-ACP is available in paste consistency, OS powder and bamboo salt were also made into pastes using methylcellulose. Methylcellulose helps to form viscous solutions, improves handling properties and wash-out resistance of the material used.[22] According to the manufacturers, CPP-ACP paste has to be left undisturbed on the tooth surface for atleast 3 minutes. Therefore, the duration of contact of OS and BS remineralizing pastes were also standardized to 3 minutes.[23] The remineralizing gel was applied on enamel blocks twice a day (i.e., for every 12 hours) to simulate the recommended daily oral prophylaxis. The samples were kept in the artificial saliva throughout the study to mimic the in-vivo conditions.

In this study, Oyster shell showed better remineralization property which was reflected by the superior deposition of calcium carbonate compounds and increased surface hardness in comparison to other groups which was statistically significant (p-value<0.05). This is probably the first study testing oyster shell powder with CPP-ACP for remineralization potential as the search for studies with Oyster shell on enamel remineralization did not fetch any previous reports. The remineralisation capability of OS was also better than Bamboo Salt. In bone remineralization studies using OS, it was found that the nacre formation aided in bone deposition.[16] It contains an inorganic mineral phase and an organic matrix, similar to the structure of human bone. The inorganic mineral phase provides mechanical strength, while the organic matrix

imparts osteoconductivity.[24] Its organic matrix also contains biological molecules identical to those found in humans, the bone morphogenetic proteins (BMP) and other molecules which activate osteoblasts through chemical signaling.[25] This also might have been the possible reason for the enamel remineralizing property of OS.

In our study, bamboo salt showed good remineralizing potential on demineralized enamel. One of the reasons for this might be the increased alkalinity and numerous minerals present in BS. According to Jeong and Hyun-Ja in 2011, BS is highly alkaline (pH 10.5). The unique property of bamboo salt is that, it can fully and continuously re-supply the lost minerals to the human body.[26] Another surmise for good remineralizing potential is that BS inhibits glycosyltransferase (gtf) genes of *Streptococcus mutans.* BS also suppressed acidogenicity, bacterial adhesion and the expression of S. mutans biofilm-related gtfB, gtfC, gtfD genes.[27] The last and the important hypothesis for the positive effect of BS on enamel remineralization might be its potency to narrow down the lattice spacing of enamel and the deposition of spherical crystals on the micropores. This possibly increased the enamel hardness and remineralization.[28] The calcium deposition of Bamboo Salt paste was better (29.417%) than CPP-ACP (20.048%) but relatively lesser than Oyster Shell (35.711%). The hardness value of bamboo salt (340.1 VHN) was also better than CPP-ACP (337.9 VHN) but inferior to product from OS (390.2 VHN). CPP-ACP has proven potential to increase enamel hardness and bioavailability of calcium phosphate ions. The added advantage is the acid resistance, which is yet to be proved for OS and BS. CPP – ACP though used as remineralization agents for long, it did not get expected outcome, hence fluoride was added to it later. Though we used CPP-ACP as positive control and it has delivered considerable amount of remineralization potential which was significantly lesser compared to Oyster shell and Bamboo salt. We cannot use CPP-ACP as a yardstick for comparison in future. No treatment Group also had the capability to remineralize to a very limited extent. This is probably because of the buffering action of artificial saliva and the minerals present in it. Thus, the null hypothesis was rejected. Further studies are necessary to know the depth of penetration of calcium and phosphorus ions into the enamel. The acid resistance of these novel materials also remains unknown. In-vivo studies are necessary to know the effect of these products on viable tissues. Also, the effect of OS and BS on the markers which are responsible for odontoblastic activity are yet to be studied.

CONCLUSION

Within the limitations of the study, the oyster shell powder had the best remineralizing efficacy followed by bamboo salt and CPP-ACP. Hence, these naturally available products hold good scope in the near future and also will show promising outcome as remineralizing agents in the field of minimally invasive dentistry.

Conflıct of Interest

No potential conflict of interest relevant to this article was reported

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