



Evaluation of Remineralizing Potential of Cranberry and Chitosan on Demineralized Dentin (An in Vitro Study)

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ABSTRACT

Purpose: To evaluate the remineralizing potential of cranberry extract and phosphorylated chitosan pretreatment on microhardness and chemical structure of demineralized dentin. **Materials and Methods:** A total number of 36 dentin samples were divided into four main groups of 9 samples each according to the utilized treatment after demineralization. In the first group, cranberry extract was used, in the second group, phosphorylated chitosan was used. For the third group, combination of cranberry extract and phosphorylated chitosan was used, whereas in the fourth group, no treatment was carried out (control). All groups were subjected to a pH cycling for 3 days. Five microhardness measurements were performed for each dentin sample at baseline, after demineralization, immediately after treatment, after one month and after three months storage in artificial saliva. Two representative samples from each group were selected for SEM/EDAX analysis. **Results:** Microhardness results revealed significant difference among all treatment groups, cranberry extract + phosphorylated chitosan combination group yielded the highest percentage of change of dentin microhardness, followed by phosphorylated chitosan group, then cranberry extract group. SEM/EDAX confirmed the microhardness results. **Conclusions:** Phosphorylated chitosan and cranberry extract could be considered as an effective natural treatment options to strengthen demineralized dentin.

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INTRODUCTION

Dental caries is a common chronic dental disease with high prevalence rate among children and adults. Around 2.4 billion people with carious lesions have been estimated across the world. Untreated carious teeth may result in aesthetic, functional and psychological problems ⁽¹⁾.

Dentin occupies a significant portion of the human tooth. It contains up to 70% inorganic substance, 20% organic substance and 10% fluid. Dentin organic matrix consists of type I collagen and non-collagenous proteins ⁽²⁾. Demineralization of dentin begins with dissolving of its inorganic substance (Hydroxyapatite crystals) by bacterial acids. With progression of carious lesion, the exposed organic matrix is degraded by proteolytic enzymes. All these alterations cause reduction in mechanical properties of dentin ⁽³⁾.

Medical model of caries management is highly desirable and is one of the corner stones of minimal invasive dentistry. Remineralization process is a natural repair mechanism which occurs under near-neutral pH conditions. Minerals deposition from saliva and plaque within the carious lesion results in formation of larger HAP crystals which are more resistant to dissolution by acids⁽⁴⁾.

Although Fluoride is considered the gold standard in remineralization strategies, it lacks the ability to arrange mineral crystals in an orderly way. Moreover, chronic ingestion of high doses of fluoride may lead to dental fluorosis, skeletal fluorosis in severe cases, where calcification and fractures of ligaments and tendons can occur, leading to reduced joint mobility ⁽⁵⁾.

Natural products have been used for many years and are promising sources for novel therapeutic agents. A majority of the natural products have been studied in the field of oral health focusing on their antibacterial activities but very few have been reported on the demineralization and remineralization of teeth ⁽⁶⁾.

Cranberry (*Vaccinium macrocarpon*) is a phyto-therapeutic agent containing polyphenols, mainly Proanthocyanidins. Proanthocyanidins is naturally occurring plant metabolite that is widely available in fruits and vegetables ⁽⁷⁾. Proanthocyanidins is considered one of the most valuable natural cross-linking agents that enhance bonding to sound and caries affected dentin. It enhances the strength of exposed collagen and increases its resistance to degradation with lytic enzymes ⁽⁸⁾.

Chitosan is a natural polycationic linear polysaccharide. It is originated from chitin by a process called alkaline deacetylation. Chitin is considered a major structural element in the exoskeleton of crustaceans (mainly shrimps and crabs) ⁽⁹⁾.

Chitosan and its derivatives have emerged as a new class of novel biomaterials ⁽¹⁰⁾. phosphorylated chitosan is one of chitosan derivatives, which exhibits bactericidal, biocompatible, osteoinductive and metal chelating properties. Phosphorylated chitosan acts as non-collagenous proteins analogue that is able to mimic the functional domain of natural proteins that regulate the biomimetic remineralization process ⁽¹¹⁾.

Accordingly, this study aimed to evaluate the remineralizing potential of cranberry extract and phosphorylated chitosan pretreatment on micro-hardness and chemical structure of demineralized dentin.

MATERIAL AND METHODS

Preparation of cranberry extract solution

For preparation of cranberry extract solution with a concentration of 0.6% w/v, 600mg of ground cranberry extract powder (Nawah Scientific Research center, Egypt) were dissolved in 100 ml distilled water ⁽⁷⁾. Then by using pH meter, the primary solution pH was recorded as pH = 3, then the pH was adjusted to 7.2 by adding 100 mg of NaHCO₃. Solution was freshly made on a daily basis before its use.

Preparation of Phosphorylated chitosan solution

For preparation of phosphorylated chitosan (Naqaa Foundation for Scientific Research, Egypt), 1g of chitosan powder (Sigma- Aldrich), 10 mL of phosphoric acid and 5 g of urea were added to 40 mL of dimethyl formamide. The mixture was stirred continuously for 1 hour at 150°C by heating in an oil bath. After cooling to the room temperature, the mixture was filtered using filter paper, and the precipitate was thoroughly washed with anhydrous ethanol and distilled water, then was subjected to vacuum drying. 50 mg of lyophilized phosphorylated chitosan were dissolved in 1ml of distilled water. Phosphorylated chitosan with a final concentration of 0.2 mg mL⁻¹ was dissolved in a mineralization medium which consisted of equal volumes of phosphate and calcium containing neutral buffered solutions. Polyacrylic acid (0.350 mg ml⁻¹) was added to the calcium containing solution. 10 mg of sodium azide were added to the solution to inhibit bacterial growth⁽¹¹⁾.

Teeth selection

Ethics committee approval of Faculty of Dental Medicine for Girls Al Azhar University was obtained (REC-OP-21-01). A total of eighteen human sound non carious molars which were collected anonymously were used in this study. All teeth were extracted for therapeutic reasons from patients of age group (20-40). Teeth with fractures, enamel malformations or any other defects were excluded. After cleaning and examination, the selected molars were stored in distilled water at 4°C till testing⁽¹²⁾ where they could be used in this study within one month of extraction. By the end of the study, all teeth were disposed of in a medical waste container.

Sample preparation:

Radicular part of each molar was removed, then the coronal part was sectioned longitudinally into two halves in the mesio-distal direction by a diamond coated disc with 0.3 mm thickness (Buehler, IL, USA) under water coolant resulting into 36 dentin

samples. The samples were mounted in self-cure acrylic blocks using a specially fabricated rounded plastic mold of 10 mm internal diameter and 20 mm height. Dentin surface of each sample was coated with acid resistant nail varnish, leaving 4 mm × 6 mm window of dentin exposed in the middle of the sample surface.

Baseline microhardness test

Dentin microhardness was measured at baseline using Vickers microhardness tester (Wilson TukonTM1102, Germany) with Vicker diamond indenter and 20X lens. The surface of each specimen was subjected to a load of 100 gm for 10 seconds. Three indentations were made on the surface of each sample. These indentations were equally placed over a circle with 0.5mm space between indentations. A built-in scaled microscope was used to measure the diagonals length of the indentations and Vicker values were converted into microhardness values. The Vickers hardness value (HV) was calculated using: $MHV = 1854.4L/d^2$ (Where the load L is in gf and the average diagonal d is in μm)⁽⁵⁾. Three readings were recorded for each sample, and their mean was calculated as the Vickers hardness number (VHN).

Preparation of artificial carious dentin

All the samples were immersed into glass tubes containing 50 ml of demineralizing solution for 72 hours at room temperature⁽¹²⁾. It consisted of 3 mmol/L Ca⁺², 2.2 mM, 3 mmol/L PO₄ and 50 ml/L acetic acid. pH of the solution was adjusted to 5 using NaOH⁽¹³⁾. It was renewed every 24 hours, all the samples were rinsed with distilled water and evaluated again for surface microhardness.

Sample grouping

After demineralization, dentin samples were randomly distributed among four groups according to the applied treatment (n = 9). In the first group, demineralized dentin samples were immersed in test tubes containing 50 ml of cranberry extract

solution for eight days⁽⁵⁾. In the second group, samples were immersed in phosphorylated chitosan solution for eight days. For the third group, samples were first immersed in cranberry extract solution for eight days then were rinsed with distilled water and immersed in phosphorylated chitosan solution for another eight days. While in the fourth group, no treatment was carried out. All solutions were refreshed daily, and the treatment was performed at a constant temperature of 37°C⁽¹¹⁾.

pH cycling

To simulate the oral conditions, all samples were subjected to a pH cycling model, they were cycled three times through an alternating demineralizing and remineralizing regimen. Each cycle consisted of immersing dentin samples in demineralizing solution for one hour, followed by their immersion in a remineralizing solution for twenty three hours⁽¹⁴⁾. The remineralizing solution was consisted of 1.54 mmol/L Ca²⁺, 1.54 mmol/L PO₄, 20 mmol/L acetic acid and 0.308 g ammonium acetate. pH of the solution was adjusted to 7.2 with KCl⁽¹³⁾.

Microhardness evaluation after treatment

Following the pH cycling process, all samples were washed thoroughly with distilled water, then surface microhardness of dentin samples for all groups was measured, where the new indentations were 0.1mm far from the past ones.

Storage of the samples

All samples were evaluated for surface microhardness after one month (T1) and after three months (T3) storage in artificial saliva which was prepared using Na₃PO₄ (3.90mM), KCl (17.98mM), NaCl(4.29mM), MgCl₂(0.08mM), CaCl₂(1.10mM), NaHCO₃ (3.27mM), H₂SO₄ (0.50mM) and distilled water⁽¹⁵⁾. Final indentations were 0.1mm far from the past indentations and the utilized loads were the same as the initial ones (100 gm for 10 seconds). Artificial saliva was replaced every 48 hours⁽¹⁴⁾.

Energy dispersive x-ray analysis

Two representative samples from each group were randomly selected and analyzed using Energy dispersive X-ray spectrometric Analysis (Quanta FEG-250) installed on scanning electron microscope to assess their surface morphology and to determine the calcium and phosphorous content and the change in the levels of these elements after demineralization and after treatment. Applied SEM conditions were 10.1 mm working distance, with an excitation voltage of 20 kV and images were taken at 5000X magnification. Three points from each sample were selected for analysis and their average was calculated. The samples were placed under vacuum and excited to a higher energy state with an electron beam. As the electrons of each element falls back down to its original state of energy it emits X-ray energy at different specified wavelength. Each element identified by known wavelength on the X-axis represented by a peak and the intensity of that peak on the Y-axis determines the amount of elements. These minerals were expressed as a weight percentage⁽¹⁶⁾.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics Version 2.0 for Windows. Data was presented as mean, standard deviation (SD) and percentage. For assessment of data normality, Kolmogorov-Smirnov and Shapiro-Wilk tests were used. The significance level was set at P≤0.05. Two-Way ANOVA was performed for comparing the effect of study variables and their interaction on microhardness of demineralized dentin. One-Way ANOVA followed by Tukey's post-hoc test were used for comparing effect of treatment agents on microhardness and percentage of change of microhardness at each evaluation time. ANOVA repeated measures was performed to compare the effect of evaluation time on microhardness and percentage of change of microhardness within each treatment group. Percentage of change (MH%) of dentin was calculated according to a previous study⁽³⁾ MH% = (final MH-demineralized MH) / (Initial MH-demineralized MH) X 100.

RESULTS

Microhardness results

Effect of treatment agents on percentage of change of microhardness of demineralized dentin at each evaluation time:

Results of the effect of treatment agents on percentage of change of microhardness of demineralized dentin (Table 1 and Fig. 1) revealed that, there was statistically significant difference among all groups. At T0 (immediate), cranberry extract + phosphorylated chitosan combination group yielded the highest mean percentage of change of microhardness, followed by phosphorylated chitosan group, then cranberry extract group, whereas the control group showed the lowest mean percentage of change microhardness with decrease in microhardness values.

At T1(after one month), cranberry extract + phosphorylated chitosan combination group produced the highest mean percentage of change of microhardness, followed by phosphorylated chitosan group, then cranberry extract group, while control group showed the lowest mean percentage of change of microhardness. At T2 (after three

months), cranberry extract + phosphorylated chitosan combination group displayed the highest mean percentage of change of microhardness, followed by phosphorylated chitosan group with the same ranking, followed by cranberry extract group, then control group which showed the lowest mean percentage of change of microhardness.

Effect of evaluation time on percentage of change of microhardness of demineralized dentin within each group:

Results of the effect of evaluation time on percentage of change of microhardness within each group (Table 2 and Fig.2) revealed statistically significant difference in percentage of change of microhardness between different evaluation times within each group. Percentage of change of microhardness in all groups at T2 (after 3 months) was significantly higher as compared to T0 (immediate) and T1 (after 1 month).

SEM/EDAX analysis:

Fig. (3-6) represent the SEM photomicrographs and EDAX elemental analysis of representative sample for each group (after demineralization and immediately after different treatments).

Table (1) Mean±SD and P-value for the effect of treatment agents on percentage of change of microhardness of demineralized dentin at each evaluation time.

	T0		T1		T2	
	Mean	SD	Mean	SD	Mean	SD
Cranberry extract	13.53% ^b	5.78	23.64% ^c	8.60	34.97% ^b	10.26
Phosphorylated chitosan	25.18% ^{ab}	5.12	34.84% ^b	3.98	49.71% ^a	8.06
Cranberry extract+ Phosphorylated chitosan	40.76% ^a	6.12	44.98% ^a	3.88	54.92% ^a	4.43
Control	-3.47% ^b	11.41	17.13% ^c	10.08	28.09% ^b	7.95
P-value	<0.001*		<0.001*		<0.001*	

*: significant at $P \leq 0.05$

Mean values with different superscript lowercase letters within each column are significantly different.

Table 2: Mean \pm SD and P-value for the effect of evaluation time on percentage of change of microhardness of demineralized dentin within each treatment group.

	Cranberry Extract		Phosphorylated Chitosan		Cranberry Extract + Phosphorylated Chitosan		Control	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
T0	13.53% ^c	5.78	25.18% ^c	5.12	40.76% ^c	6.12	-3.47% ^c	11.41
T1	23.64% ^b	8.60	34.84% ^b	3.98	44.98% ^b	3.88	17.13% ^b	10.08
T2	34.97% ^a	10.26	49.71% ^a	8.06	54.92% ^a	4.43	28.09% ^a	7.95
P-value	<0.001*		<0.001*		<0.001*		<0.001*	

*: significant at $P \leq 0.05$

Mean values with different superscript lowercase letters within each column are significantly different.

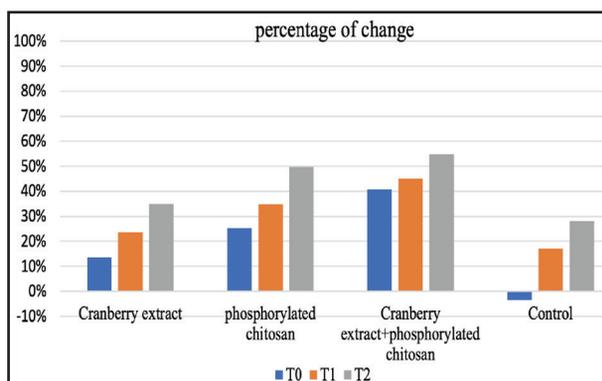


Figure (1) Bar chart showing mean value of percentage of change of microhardness at each evaluation time after different treatments.

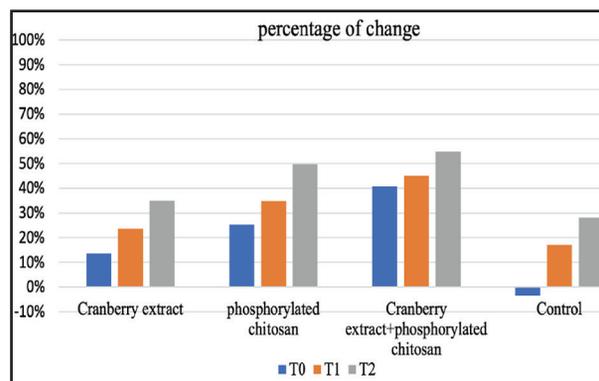


Figure (2) Bar chart representing mean value of percentage of change of microhardness of demineralized dentin within each treatment group at different evaluation times.

- **Cranberry extract group:** SEM showed mineral deposits on the dentin surface and conversion into organized and homogenous structure of dentin organic matrix, EDAX elemental analysis showed Ca (21.6 wt%) and P (10.2 wt%) elements on dentin surface (Fig. 3).

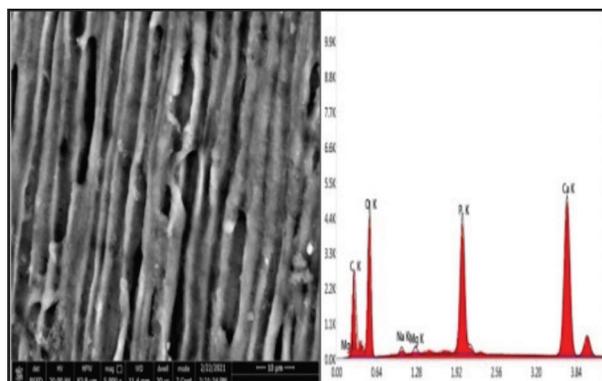


Figure (3) SEM photomicrograph, EDAX elemental analysis of cranberry extract group.

- **Phosphorylated chitosan group:** SEM showed deposition of minerals on dentin surface and highly mineralized dentin matrix with partially occluded dentinal tubules. EDAX analysis showed higher content of Ca (22.1 wt%) and P (11.2 wt%) (Fig.4).

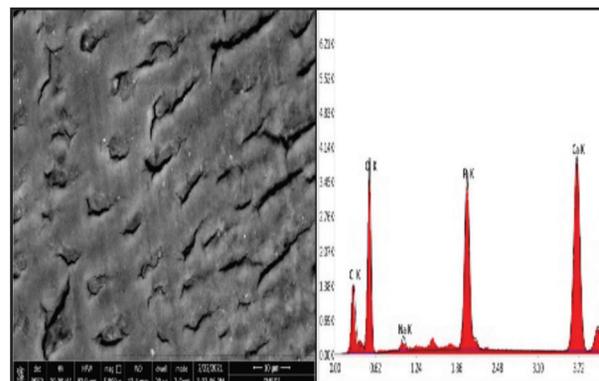


Figure (4) SEM photomicrograph, EDAX elemental analysis of Phosphorylated chitosan group.

- Cranberry extract + Phosphorylated chitosan combination group:** SEM showed minerals depositson on dentin surface with partially obliterated dentinal tubules, EDAX analysis revealed the highest content of Ca (26.2wt%) and P (13.1 wt %) (Fig. 5).

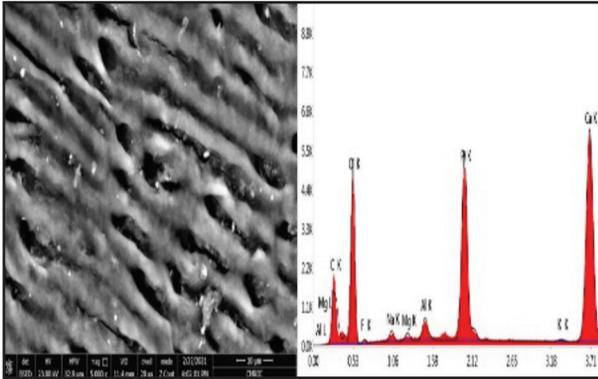


Figure (5) SEM photomicrograph, EDAX elemental analysis of Cranberry extract +Phosphorylated chitosan combination group.

- Control group:** SEM revealed permeable dentinal tubules with no minerals deposition within the collagen network of the intertubular dentin, EDAX analysis showed the lowest content of Ca (15.9 wt%) and P (7.9 wt%) (Fig. 6).

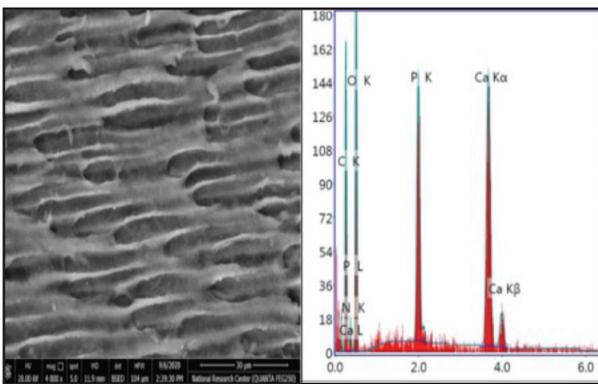


Figure (6) SEM photomicrograph, EDAX elemental analysis of control group.

DISCUSSION

Dental caries is a dynamic process of pathological demineralization and physiological remineralization of teeth ⁽¹⁷⁾. By regulating the mineral balance favourably towards the remineralization, the caries lesion can be arrested or repaired. Because of the little extent of dentinal crystallites and presence of dentinal fluids, dentin demonstrates greater affinity towards the demineralization than enamel ⁽¹²⁾.

Different remineralizing agents and techniques have been researched to achieve newer strategies for dentin remineralization. In recent years, there has been an interest in active compounds that are derived from natural products which may have therapeutic potential in the field of oral health ⁽¹⁸⁾.

Cranberry extract was utilized in this study because it contains polyphenols, mainly proanthocyanidins. proanthocyanidins is dentin biomodifier, it reacts with the exposed collagen matrix and stabilizes the collagen by inducing collagen cross-linking ⁽⁷⁾. It also inhibits matrix metalloproteinases and cysteine cathepsins in dentin, enhancing the stability of exposed collagen matrix against degradation by proteolytic enzymes ⁽¹⁹⁾.

Phosphorylated chitosan was used in this study as a phosphorylated non-collagenous proteins analogue that promotes the self-assembly of type I collagen enhancing dentin mineralization. The phosphorylated functional groups in phosphorylated chitosan could enhance calcium ions chelation which allow the modified collagen matrix to attract more calcium ions, and thus a larger amount of amorphous calcium phosphate is aggregated onto the collagen fibrils ⁽¹¹⁾. Poly acrylic acid was added in the mineralizing solution as a sequestration analogue to enhance intrafibrillar remineralization of dentin. PAA has the ability to stabilize ACP, inhibiting aggregation of liquid ACP nanoparticles into larger particles and transformation into apatite before entering into dentin collagen fibrils ⁽²⁾.

The current study utilized an artificial caries model, in which dentin samples were subjected to demineralizing solution for a period of 72 hours. Following such protocol, a lesion of approximately 150µm depth with a micro hardness values similar to a natural demineralized lesion with similar depth was achieved ⁽¹²⁾.

pH cycling was utilized in this study to mimic the dynamics of minerals loss and gain in natural caries process. The dynamic cycles were performed by sequentially immersing dentin samples in demineralizing solution for one hour and remineralizing solution for twenty three hours for three days. Throughout the day, a 30 minutes pH depression is seen following intake of meals. Thus, the demineralization phase of 30 to 60 minutes is sufficient to simulate the oral conditions ⁽¹⁴⁾.

All samples were stored in artificial saliva to simulate the conditions of the oral cavity. It was proven that artificial saliva is better than deionized water in vitro studies as a storage medium. It could act as a chemical reservoir for calcium and phosphate ions enhancing the remineralizing process ⁽²⁰⁾.

Vickers microhardness test was used to evaluate the microhardness of dentin samples at baseline, after demineralization, immediately after treatment, after one month and after three months storage periods in artificial saliva. This assessment method provides a simple, rapid, and non-destructive test. In addition, it is considered by many researches ⁽²¹⁾. Percentage of change of dentin microhardness was used to assess recovery conducted for each sample through its life track before assessment, after demineralization, after treatment and then after different storage intervals.

Energy Dispersive X-Ray analysis [EDAX] combined with scanning electron microscope [SEM] was used to determine the calcium and phosphorous mineral content and the changes in the levels of these elements after demineralization and after treatment. SEM-EDAX technique represents a useful tool for assessing the elemental distribution in samples ⁽²²⁾.

According to the microhardness results of this study, the highest percentage of microhardness recovery was recorded after treatment with cranberry extract followed by phosphorylated chitosan. This could be explained by the synergistic effect of cranberry extract, as a cross linking agent, that stabilizes collagen matrix together with phosphorylated chitosan, as a phosphorylated NCP analogue, that promotes the self-assembly of type I collagen enhancing dentin remineralization. It could be also due to doubling the treatment time for 16 days not 8 days as was used for each solution separately.

Phosphorylated chitosan treated demineralized dentin group showed significant increase in percentage of microhardness recovery. This could be attributed to the ability of phosphorylated chitosan to mimic the nucleating role of phosphorylated non collagenous proteins to induce mineralization ⁽²³⁾. Moreover, the combination of polyacrylic acid as sequestration analogue and phosphorylated chitosan as a template analogue in the mineralization medium could produce highly ordered intrafibrillar nanoapatite assembly which increase the mechanical properties of dentin ⁽¹¹⁾.

This is in agreement with previous studies ^(11,23) which revealed that phosphorylated chitosan modified the collagen matrix of demineralized dentin thus enhancing mineral crystals nucleation on the collagen promoting dentin remineralization.

Cranberry extract treated demineralized dentin group revealed significant increase in percentage of microhardness recovery, this could be explained by establishment of crosslinks by proanthocyanidins. These cross-links could improve the resistance of dentin surface to deformation by the indenter of microhardness tester ⁽³⁾. The phenolic hydroxyl groups of proanthocyanidins could form hydrogen bond with the amide carbonyl or hydroxyl groups of collagen fibrils, thus enhancing their mechanical properties. In addition, they could enhance collagen stability against proteolytic degradation. This stabilized collagen matrix could serve as a mechanical

barrier, which inhibits the adverse effect of acid and further loss of minerals ⁽¹⁹⁾.

These findings are in accordance with previous studies ^(3,12) which evaluated the remineralizing effect of proanthocyanidin on demineralized dentin and recorded significant increase in dentin microhardness after treatment with proanthocyanidin.

However, the current findings are in contrary with previous studies ^(24,25) which revealed that demineralized dentin treated with proanthocyanidin did not show any significant increase in dentin microhardness. These differences between results could be explained by the different protocols and sources of proanthocyanidin. It has been reported that the results found with proanthocyanidin were strongly dependent on its origin, the solvent used during its extraction, its concentration, and the exposure time ⁽²⁴⁾.

Regarding the control group, results revealed the lowest percentage of change of microhardness among all groups, which might be due to dissolving of minerals and exposure of organic matrix after demineralization. The absence of intra-fibrillar minerals and their optimal interaction with dentin collagen fibrils might have adversely affected microhardness of dentin ⁽¹⁵⁾.

As for the effect of time, results revealed a significant increase in percentage of recovery of microhardness in all groups after one month, and the highest percentage of recovery was recorded after three months storage in artificial saliva. This could be attributed to the synergistic effect of artificial saliva in enhancing the remineralizing process by increasing Ca and P ions precipitation together with the remineralizing effect of each treatment agent in the treatment groups, thus increasing dentin microhardness ⁽²⁷⁾. Although percentage of recovery of microhardness of the control group increased by time, it was lower than other treatment groups. This could be explained by that the artificial saliva may provide an increase in mineral content but without optimal interaction with dentin organic matrix ⁽¹²⁾.

The result of microhardness was in the same line with SEM/EDAX observations which demonstrated that, surface morphology of demineralized dentin after combined treatment with cranberry extract and phosphorylated chitosan showed crystals deposition on dentin surface with partially obliterated dentinal tubules. EDAX results revealed the highest calcium and phosphorous content, which confirms their synergistic effect on increasing the collagen stability and enhancing minerals deposition, thereby promoting remineralization.

SEM photomicrograph of phosphorylated chitosan treated dentin showed deposition of minerals on dentin surface and highly mineralized dentin matrix with partially occluded dentinal tubules. EDAX results revealed higher Ca and P content as compared to cranberry extract treated dentin, this could be due to the effect of phosphorylated chitosan as a phosphorylated NCP analogue.

SEM photomicrograph of dentin treated with cranberry extract showed mineral deposits on dentin surface with organized and homogenous structure of dentin organic matrix. EDAX results showed higher Ca and P content compared to control group. This might be due to the establishment of collagen crosslinks by proanthocyanidins. This cross-linked collagen matrix could act as a scaffold for minerals precipitation ⁽³⁾.

As for the control group, surface morphology of dentin surface showed permeable dentinal tubules with no minerals deposition within the collagen matrix. EDAX results revealed the lowest content of Ca and P which confirms that the minerals were dissolved and the organic matrix was exposed after the demineralization ⁽²³⁾.

CONCLUSIONS

Considering the limitations of this study, Phosphorylated chitosan and cranberry extract could be considered as an effective natural treatment options to strengthen demineralized dentin. Storage time has beneficial effect on the remineralization

potential of the treatment agents. Changes in minerals content and surface morphology correlated well with microhardness of dentin.

RECOMMENDATIONS

Further clinical studies are recommended.

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CONFLICT OF INTEREST

There is no conflict of interest.

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