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BIOMIMETIC REMINERALIZATION OF EARLY HUMAN ENAMEL CARIOUS LESIONS WITH DIFFERENT COMMERCIALLY AVAILABLE HERBAL NANOHYDROXYAPATITE TOOTHPASTES: AN IN-VITRO COMPARATIVE EVALUATION

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ABSTRACT

Objectives: To compare the remineralizing capability of three readily available commercial herbal nanohydroxyapatite toothpastes on early human enamel carious lesions using a PH cycling model.

Materials & Methods: Thirty-two caries-free extracted human maxillary central incisors were sectioned cervically about 2mm below cementoenamel junction. Carious-like lesions were induced by immersing the crowns of the samples in acidified gel for 7 days. Samples were then divided into 4 groups (8 samples each); in group I, samples were brushed with BOKA Ela Mint toothpaste, in group II, they were brushed with BOKA Coco Ginger toothpaste, in group III, samples were brushed with BOKA Lemon Lavender toothpaste, and in group IV (control) samples were stored in artificial saliva. The brushing time was 2 minutes twice daily. Using artificial saliva, a pH cycling model was used to simulate remineralization for 45 days. The enamel surfaces of samples were subjected to surface microhardness (SMH) test with a Vickers hardness tester, and the average Ca level was analyzed using energy-dispersive X-ray spectroscopy (EDX). The measurements were done before and after demineralization, and after remineralization. One-way ANOVA, Tukey's test, and paired T-test ($P<0.05$) were used for analyzing the data.

Results: All treatments effectively remineralized the early human enamel carious lesions. BOKA Ela Mint toothpaste showed the maximum increase in SMH and Ca level deposition, while BOKA Lemon Lavender toothpaste showed the lowest.

Conclusion: Nanohydroxyapatite toothpastes may be the optimal for biomimetic remineralization of early human enamel carious lesions.

KEYWORDS*:* Biomimetic Remineralization, Nanohydroxyapatite, Herbal Toothpastes, Enamel Carious Lesions.

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INTRODUCTION

Tooth decay is among the most frequent chronic infectious diseases. Caries dynamic begins when the essential salivary pH (5.5) drops, allowing aciduric and acidogenic bacteria to proliferate. The salivary pH continues to drop by acids produced by bacteria (mostly streptococci and lactobacilli) and sugars in the diet, resulting in loss of surface and underneath enamel minerals causing enamel demineralization[1-5]. Cycles of this dynamic process are repetition of de-remineralization. Once the demineralization process advances over remineralization, carious lesion occurs[2,6]. As the first clinical evidence of tooth decay, white spot lesions arise prior to cavitation[2].

The best strategy to stop tooth decay is to remineralize it when it is still in the early, un-caviated stages. By forming surface coatings that serve as diffusion barriers, remineralizing agents deliver the ions necessary for remineralization[7]. By neutralizing acids, providing antibacterial elements, and serving as a mineral storehouse for phosphate and calcium ions, saliva helps to prevent caries development[2].

The reduction in caries incidence has been associated with tooth brushing habits and fluoride toothpastes[8,9]. Although using fluoride has anti-erosive and remineralizing properties, the line between a beneficial and harmful dose of fluoride is ill-defined[10]. Fluoride over-doses result in tooth fluorosis and induction of streptococcus mutans strains that are resistant to fluoride, hence the search for natural products for enamel remineralization was found worthiest[11]. Natural products have acceptance due to their less side effects, improved patient tolerance, relative affordability, and renewability of nature [12].

Alternative non-fluoridated toothpastes include natural ingredients with antibacterial properties were developed to prevent tooth decay and opportunistic microorganisms in the oral cavity[9,13]. Dental pastes with inorganic ingredients like hydroxyapatite crystals can be used to form synthetic enamel, and to repair the early carious lesions without any need for prior excavation[14,15]. Nanosized hydroxyapatite is comparable to enamel apatite crystals in morphology and structure, so it is a suitable alternative for the natural mineral content of enamel for biomimetic repair[16]. The nanoparticles of 20 nm average size fit well within the nano-defects on enamel surface caused by acidic erosion, and they can firmly adhere to demineralized enamel surface, preventing more acid damage. Compared to fluoride toothpastes, nanohydroxyapatite containing toothpastes revealed comparatively higher remineralization potential [17,18].

Ginger is a natural herbal with antibacterial properties. On the other hand, it exhibits no harmful behavior and 'generally recognized as safe' (GRAS) by the United States Food and Drug Administration (FDA).[19] Non-invasive treatment of early carious lesions with a ginger- containing solution demonstrated a potential for therapeutic benefit and remineralization improvement [20].

Green tea is consisting primarily of catechins like epigallocatechin gallate (EGCG) that suppressed particular virulence factors related to streptococcus mutans and lactobacillus carcinogenicity, ultimately exerting anticaries and anti-erosive properties[21,22]. It is also used to improve oral health condition including periodontal diseases, dental caries, and oral malignancy prevention and regression[23]. The remineralizing potential of green tea is aided by its high fluoride concentration[24]. Xylitol not directly protect enamel or dentin. The mixture of bioactive substances (like green tea and xylitol) in different mint formulations has been studied as an approach for over-the-counter continuous delivery with broad activity[25,26].

The null hypothesis of this study is that no significant differences between the three formulations (Ela Mint, Coco Ginger, lemon Lavender) of herbal nanohydroxyapatite toothpastes in early enamel carious lesions remineralization.

MATERIALS AND METHODS

Ethical approval:

There are no experiments involving humans or animals in this study. It was approved by the Scientific Research Ethics Committee, Suez Canal University (Reference No. 2023/680).

Collection of samples:

Intact caries-free human maxillary central incisor teeth freshly extracted for periodontal problems were collected from oral medicine and periodontology clinic, Faculty of Dentistry, Suez Canal University upon patient's consent. Firstly, all samples (teeth) were autoclaved at 121C° at 15 Ibs psi for 20 minutes, and then were cleaned using pumice-water slurry and alumina paste with electric toothbrush to remove any debris or calculus[27,28]. Secondly, the samples were examined by a trans-illuminator and thirty-two of them without any coronal restorations or enamel malformations were selected[28]. Finally, the selected ones were immersed in 20ml of 0.1% thymol in distilled water at 37C° until they were used[29].

Preparation of samples:

The labial surface of each sample was the target of the study. To facilitate standardization, each sample was sectioned cervically (2mm below cementoenamel junction) with a water-cooled diamond disc (Besqual Dia-Disc NY 11373, USA size: S-22mm) to separate the coronal portion, which was then implanted inside a prefabricated mold made of self-curing acrylic resin keeping the labial surface exposed[29,30]. The labial surfaces were flattened and smoothed by sequential polishing with 600, 800, 1200 grit water proof silicon carbide paper[29]. Furthermore, each labial surface was coated with 4×4 mm adhesive tape, while the rest of surface was coated with double layers of acid-resistant nail varnish ensuring that only in that particular area all brushing techniques and analyses were carried out[31].

Induction of carious lesions:

Early carious-like lesions were induced by subjecting all samples to demineralization for 7 days in an acidified gel system which was renewed daily. The gel with a pH value of 4.5 was achieved by combining 100 mmol/L of sodium hydroxide and 100 mmol/L of lactic acid. Additionally, this solution was forcefully stirred while 6% w/v hydroxyethyl cellulose was added.[32]. After 7 days, all samples were rinsed with distilled water and became ready for evaluation.

Toothpastes used:

Three formulations of herbal nanohydroxyapatite BOKA toothpaste were used in the current study. The ingredients of BOKA Ela Mint toothpaste included (Mint, Green Tea, and Cardamom), BOKA Coco Ginger toothpaste ingredients were composed of (Coconut, Ginger, and Chamomile), while the ingredients of BOKA Lemon Lavender toothpaste were as follow (Positano Lemons, Lavender, and Xylitol). All formulations are 100% fluoride-free.

Preparation of toothpaste slurry/test solution:

A dilution of one part of toothpaste (9 g) to three parts (27 mL) of artificial saliva (the re-mineralizing solution in the study) is thoroughly mixed for 4 min in a laboratory stand mixer until they become homogenous; 4.0 mL per sample of 1:3 dilution is recommended, as this represents the expected dilution that happens when toothpaste products are used regularly. For each group, freshly made slurry should be made immediately before each treatment[28].

Samples grouping & Brushing protocol:

According to the type of each toothpaste used, the samples were split into 4 groups (8 samples each): samples in group I were brushed with BOKA Ela Mint toothpaste (BOKA,USA); in group II they were brushed with BOKA Coco Ginger toothpaste; while those in group III were brushed with BOKA

Lemon Lavender toothpaste; and in group IV (control) the samples were stored in artificial saliva [2.20g/L gastric mucin, 1.45mm $CaCl2·2H2O$, 5.42mm $KH2PO4$, 6.50mm NaCI, 14.94 mm KCI (PH 7.0)]. These formulations of toothpastes were chosen based on their similar excipients and to evaluate only the effective ingredient of each toothpaste. Samples in groups I, II, and III were brushed twice daily/45 days with gentle-rounded bristles of electric toothbrushes from Oral B. Each brushing period was lasted for 2 minutes[31,33].

PH cycling model (45 days):

To mimic the process of de-remineralization in the oral environment as close as possible, a pH cycling model was performed as shown in Table (2). Standard demineralizing and remineralizing solutions were prepared in Department of Organic Chemistry, Suez Canal University's Faculty of Pharmacy according to the compositions shown in Table (1). The demineralizing solution should act as an acid challenge comparable to those produced by oral plaque acids in the mouth, and it was changed twice weekly. The remineralizing solution should be capable of remineralizing early carious lesions. Artificial saliva was the remineralizing solution in all treatment procedures of the study as it was changed three times per week. Throughout the experiment, the solutions were kept in sealed containers at room temperature (25°C)[28].

TABLE (1) Treatment schedule:

Examined surface	No of samples / group	Demineralizing solution	Remineralizing solution	Toothpaste dilution ratio	Diluents	Treatment frequency	Daily Demineralization time (h)	Daily Demineralization time (h)	Tests of evaluation
Human enamel	8	*2.0mmol/L Ca(NO3)2.4H2O *2.0mmol/L KH2PO4 *75.0mMol/L CH3COOH * PH was then corrected to 4.5 using 50% KOH after all components had fully dissolved.	*2.20g/L gastric mucin *1.45mm CaCl2·2H2O *5.42mm KH2PO4 *6.50mm NaCI * 14.94 mm KCI (PH 7.0)	1:3	Distilled water Artificial saliva	2 mins × 2/day for 45 days	6	16	Vickers surface micro-hardness Energy-dispersive X-ray spectroscopy analysis

TABLE (2) PH cycling model:

Time	Treatment
Day1: Storage in a remineralizing solution all day. Then, treatments in remaining days will be as follows:	
2 mins (start 8:00 a.m.) Approximately 1 h to complete all groups	Tooth paste therapy
Wash with distilled water.	
6 h (9:00 a.m.–3:00 p.m.)	Acid challenge (demineralization)
Wash with distilled water	
2 mins (start 3:00 p.m.) Approximately 1 h to complete all groups	Tooth paste therapy
Wash with distilled water	
16 h (from 4:00 p.m. till 8:00 a.m. next day)	Storage in remineralizing solution
Repeat for 44 supplementary days	

The volume of each solution should be 40 mL/ sample for demineralizing solution and 20 mL/ sample for remineralizing one. The solution's pH was checked once daily before treatment[28]. All therapy was performed at 37°C in an incubator.

Surface microhardness (SMH) analysis:

The Vickers SMH of the examined enamel surfaces was measured at three separate times: (T1) pre-treatment (baseline), (T2) post demineralization, and (T3) post treatment (remineralization) using a digital micro-hardness tester (FM-ARS 9000; Future-Tech, Kawasaki, Japan). Three indentations at various points were made on the examined surface of each sample using a Vickers diamond indenter under a 200 g load applied for 15s. The mean of three indentation scores have been estimated and represented as the sample's hardness value[34].

Energy-dispersive X-ray analysis:

Analysis of the elements was performed using energy-dispersive X-ray spectroscopy (EDX) under scanning electron microscope (FESEM, JEOL JSM-6510LV, Japan) to measure the average Ca level (weight %) in each sample. All samples were examined at the three separate times: T1, T2, and T3.

Data analysis:

All data were gathered, tabulated, and analyzed. One-way ANOVA test was applied. When an analysis of variance revealed significance, a Tukey test was performed. Statistical analysis was carried out with IBM® SPSS® Statistics Version 20 for Windows and the significance level was set at $p<0.05$.

RESULTS

1- Surface microhardness (SMH) analysis:

Data were obtained regarding the SMH value of the tooth enamel at each indentation, with the mean values and standard deviation of each group being subsequently calculated.

Enamel SMH mean values at the baseline (T1) & post demineralization (T2): As shown in table 3 & figure 1, SMH mean values were significantly decreased ($p=0.001^*$) after demineralization process in all tested groups with no statistically significant differences were recorded between them ($p>0.05$).

TABLE (3) Statistical analysis of enamel SMH mean values at three-time intervals (T1, T2, and T3) of each group:

Time interval / Groups	Baseline (T1)	Post Demineralization (T2)	Difference (T2-T1)	P value	Post Remineralization (T3)	Difference (T3-T2)	P value
Group I	300.58 ± 27.58	224.76 ± 25.28	76 ± 21.26	0.001*	394.25 ± 47.66	170 ± 56.69	0.000*
Group II	302.14 ± 8.66	250.29 ± 10.48	52 ± 10.01	0.001*	353.14 ± 8.27	103 ± 10.50	0.000*
Group III	273.05 ± 28.07	209.60 ± 28.52	64 ± 28.34	0.001*	275.00 ± 42.94	66 ± 41.80	0.001*
Group IV	289.34 ± 100.55	218.15 ± 57.24	71 ± 61.18	0.001*	237.37 ± 65.44	19 ± 53.29	0.460
P value	0.851	0.076	0.146		0.005*	0.002*	

Statistically significant *P<0.05

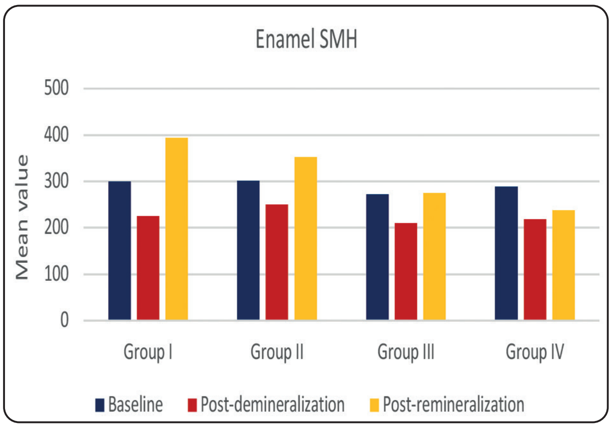

Fig. (1) Bar chart showing enamel SMH mean values at three-time intervals of each group

Enamel SMH mean values post remineralization (T3): The results presented in table 3 & figure 1 revealed that SMH mean values were significantly increased after remineralization (tooth brushing) in groups I, II, and III (p=0.000*, 0.000*, and 0.001*) respectively, while no significant difference was recorded in group IV (p=0.460). One-way ANOVA test revealed significant differences across all tested groups (p<0.05), therefore statistical analysis using pairwise Independent T-test was performed (table 4). On intergroups comparison of the differences (T3-T2), a highly significant difference was recorded when group I was compared with groups II, III & IV. The same results were recorded when group II was compared with groups III & IV, and when group III was compared with group IV.

2- Energy-dispersive X-ray analysis:

The average Ca level (weight %) in each sample was measured (figure 3). The mean values and standard deviation of each group were subsequently calculated.

Ca level (weight %) mean values at the baseline (T1) & post demineralization (T2):

Comparing the changes of Ca level (weight %) mean values after demineralization, the results revealed that they were significantly decreased (p=0.000*) after demineralization process in all tested groups with no statistically significant differences were recorded between them (p>0.05) (table 5 & figure 2).

TABLE (4) Pairwise Independent T-test of enamel SMH mean values between all groups after remineralization:

Groups	Group I	Group II	Group III	Group IV
Group I	---------	0.005*	0.000*	0.000*
Group II	0.005*	----------	0.001*	0.000*
Group III	0.000*	0.001*	------------	0.005*
Group IV	0.000*	0.000*	0.005*	-----------

***Statistically significant P<0.05**

TABLE (5) Statistical analysis of Ca level (weight %) mean values at three-time intervals (T1, T2, and T3) of each group:

Time interval / Groups	Baseline (T1)	Post Demineralization (T2)	Difference (T2-T1)	P value	Post Remineralization (T3)	Difference (T3-T2)	P value
Group I	56.97 ± 2.20	27.85 ± 2.53	29 ± 2.34	0.000*	97.30 ± 1.96	70 ± 2.20	0.000*
Group II	57.87 ± 2.04	29.00 ± 2.73	28 ± 1.18	0.000*	75.99 ± 2.02	51± 1.13	0.000*
Group III	57.07 ± 1.90	27.22 ± 2.60	30 ± 0.89	0.000*	60.47 ± 2.64	33 ± 1.56	0.000*
Group IV	58.47 ± 2.00	27.15 ± 2.24	31 ± 0.77	0.000*	35.18 ± 3.70	10± 0.50	0.512
P value	0.567	0.826	0.365		0.029*	0.003*	

***Statistically significant P<0.05**

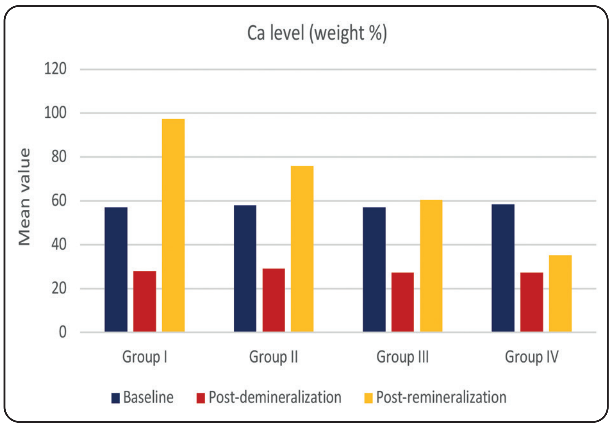

Fig. (2) Bar chart showing mean values of Ca level (weight %) at three-time intervals of each group

Ca level (weight %) mean values post remineralization (T3): After remineralization, the results showed that the mean values of Ca level (weight %) were significantly increased in groups I, II, and III (p=0.000*), while no significant difference was recorded in group IV (p=0.512) (table 5 & figure 2). One-way ANOVA test revealed a considerable significant difference across the analyzed tested groups (p<0.05), therefore pairwise Independent T-test was performed (table 6).

TABLE (6) Pairwise Independent T-test of Ca level (weight %) mean values between all groups after remineralization:

Groups	Group I	Group II	Group III	Group IV
Group I	-----	0.003*	0.000*	0.000*
Group II	0.003*	-----	0.003*	0.000*
Group III	0.000*	0.003*	-----	0.001*
Group IV	0.000*	0.000*	0.001*	-----

****Statistically significant P<0.05***

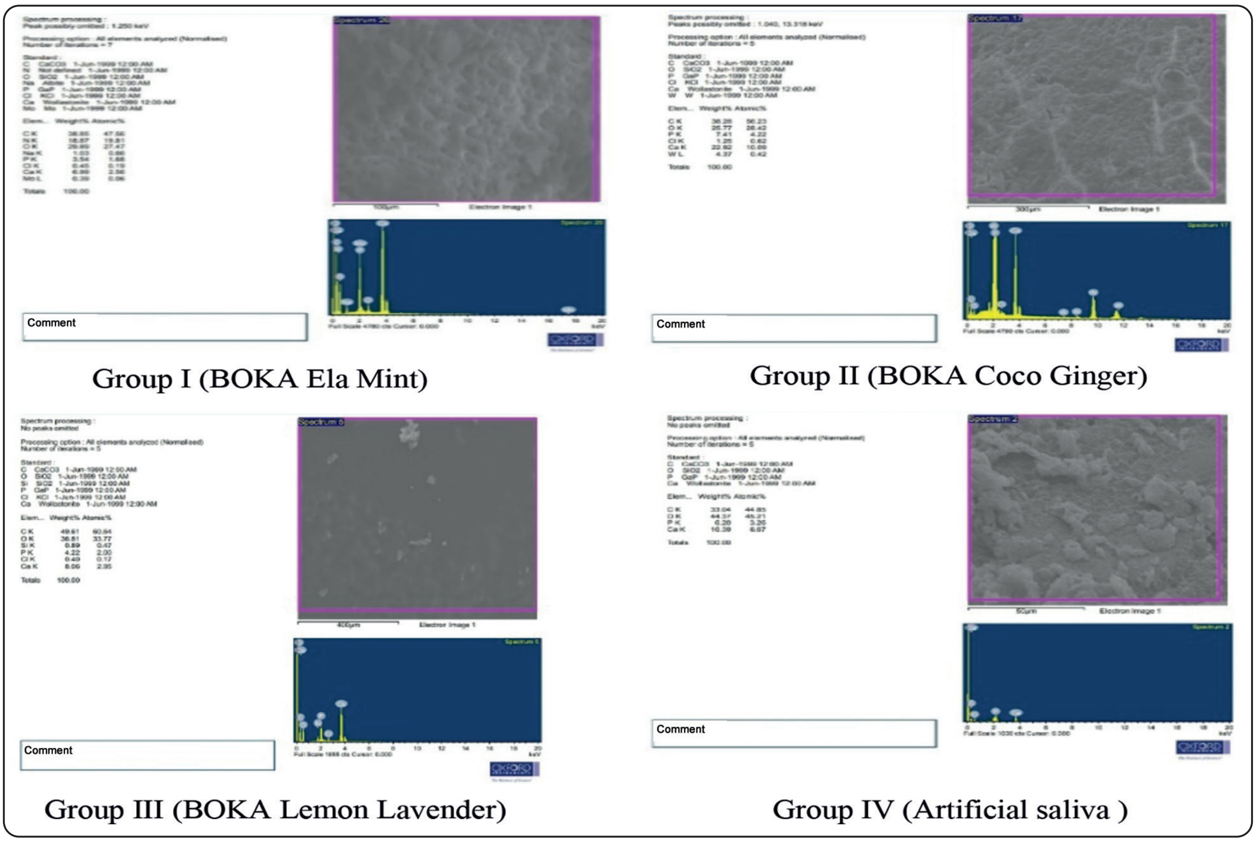

Fig. (3) SEM images & EDX analysis showing increase in Ca level (weight %) in groups I, II, and III after remineralization

DISCUSSION

Dental caries remains a major worry for people worldwide despite substantial advancements in cariology. Initial enamel lesions have intact surfaces, but they have a lower mineral concentration than healthy enamel, which makes the surface less hard[35,36].

Recognizing how crucial the enamel surface layer in caries development, the aim of this experiment was to compare the potential for remineralization of the main elements employed as active ingredients in three commercially available herbal nanohydroxyapatite toothpastes (the first one was BOKA Ela Mint, the second was BOKA Coco Ginger, while the third was BOKA Lemon Lavender) on artificially induced enamel carious lesions in human teeth. This was carried out by using a Vickers microhardness tester to measure the SMH and the Ca level (weight %) with energy-dispersive X-ray spectroscopy (EDX) under scanning electron microscope.

The primary purpose of toothpaste is to prevent tooth decay and promote oral hygiene. The taste, smell, post-brushing feeling, and foaming qualities of toothpastes contribute to the enjoyable experience of brushing teeth and help people maintain this practice. In the current experiment, nanohydroxyapatite was chosen as a biomimetic material as its deposition of calcium/phosphate minerals in enamel resembles hydroxyapatite, however, the crystalline deposition of minerals mimics the natural one by filling the micropores on demineralized enamel[37]. In addition, herbal-based toothpastes showed significant reduction in plaque levels and improving salivary pH without any side effects or using animal products [38-40].

The in-vitro pH cycling model was chosen for the study because it enables exact scientific control with little variation[41]. Scanning electron microscope was chosen for evaluation, since it is one of the most accurate in-vitro approaches to assess the de-remineralization processes by providing incredibly precise images of samples[42]. Moreover, the concurrent EDX analysis was carried out to provide elemental recognition and quantitative information about the composition.

Currently, SMH was recorded by Vickers microhardness tester rather than Knoop's tester, since it's indenter was always preferred due to its conformity with tooth nature. It penetrates about twice as deep as Knoop's indenter making it suitable for small rounded areas, and hard brittle materials with slightly thick sections like that of dental tissues[43,44].

No statistically significant difference was found between all tested groups when samples have been evaluated for baseline SMH and Ca levels. The samples were examined again after the induction of artificial carious lesions. All groups' SMH and Ca level values had significantly decreased without any significant difference between them denoting the demineralizing potential of the acidified gel system used in current study.

To replicate an actual circumstance, the PH cycling model consisted of 6 hours demineralizing cycle to approximate the duration of demineralization process intra orally, and 16 hours overnight remineralizing cycle per day. Additionally, toothpaste was administered twice daily to simulate teeth brushing in the morning and before night. It is important to note that cycle models and in-vivo research are very different from one another. The mouth's acidic conditions could not be perfectly replicated by the pH cycling model, where the pH levels were always determined by many factors like nutrition, oral hygiene practices, fluoride use, and the composition of saliva and biofilm[28].

Artificial saliva was also used as a storage medium for all groups to mimic saliva's capacity to remineralize as it contains inorganic electrolytes (calcium, phosphorus, and fluoride) that are crucial players in the remineralization process[45,46].

The results revealed a statistically significant difference in SMH and Ca level values after the prescribed toothpaste treatment regimen in all tested groups. The elevation of Ca level concentrations was attributed to the higher effect of nanohydroxyapatite in remineralization of early carious lesions, while the rise in SMH values can be explained by the presence of Ca that induce precipitation of apatite by the formation of sub-surface high mineral content [47,48]. Our results came in agreement with Surmeneva et al. (2015) who found that nanohydroxyapatite promote preferential remineralization of only outer layer of enamel carious lesions but full remineralization does not occur, and Wu et al. (2015) who correlated between increasing SMH of demineralized enamel and nanohydroxyapatite application[49-52]. On the other hand, the results showed no statistically significant difference in SMH and Ca level values in group IV (artificial saliva) denoting that artificial saliva alone was not sufficient to remineralize early enamel carious lesions.

Regarding to the statistically significant difference in all the analyzed tested groups, group I (BOKA Ela Mint & Green tea) came in the first with the greatest value, group II (BOKA Coco Ginger) came in second, while group III (BOKA Lemon Lavender) had the lowest score. The highest significance difference in group I was attributed due to the interaction between green tea's tannin, catechin, and caffeine, as well as the synergistic effects of fluoride which could prevent the calcium ions from being emitted in acidic solutions[53]. In addition, the presence of other trace elements such as calcium, zinc, sodium, phosphorus and fluorine in green tea could enhance formation of hydroxyapatite and fluorapatite crystals[54].

Our findings came in agreement with Kato et al. (2012) study which found that green tea reduces enamel wear and enhances its surface quality by increasing its SMH and decreasing its surface roughness, also dentin wear was significantly reduced when ten volunteers were rinsed their teeth with green tea for one minute between each erosive (five minutes, cola drink) and abrasive challenge (30 seconds, tooth brushing)[22,55].

The leaves of green tea contain, on average, 3-4% of alkaloids such as theobromine[56]. When enamel was exposed to acid, the hydroxyapatite crystals start to dissolve. Because theobromine molecules are smaller than the micro-channels created when enamel is dissolved by acid, they can pass through the enamel micro-channels and enter the crystalline structure of hydroxyapatite. The higher electronegativity of oxygen and nitrogen in theobromine molecule (C7H8N4O2) attracts the lower electronegative calcium and phosphate ions and create new crystals of theobromine apatite [Ca10(PO4)6 (OHC7H8N4O2)][57]. This is explained why group I showed higher calcium deposition and remineralizing potential among the other groups.

Moreover, the presence of cardamom seeds among the ingredients of group I toothpaste making it have antimicrobial activity against dental caries, as they are rich in calcium, iron, phosphorous, and volatile oils[58-60]. Similarly, Aneja et al. (2009) found in their study that these traditional seeds not only inhibit the activity of oral flora but also alter salivary pH and hence depicting the anti-cariogenic activity[61].

Concerning of group II results, there was a highly statistical significance difference in both SMH and Ca level values but to a lesser extent than group I. The remineralizing potential of ginger was due to its antimicrobial properties and the high fluoride and calcium contents[62]. These results were confirmed with Gocmen et al. (2016) study which showed that ginger has huge antimicrobial and therapeutic effects which strongly suggests the use of ginger extract as additives in dentifrices[63]. On the other hand, group III results showed the lowest score of statistical significance difference, which was attributed to the acidity of the lemon ingredient in the toothpaste which was not considered as a remineralizing agent, so the remineralization in this group was only related to the effect of the nanohydroxyapatite itself.

CONCLUSION

Within the limitations of this study, the following conclusions could be drawn:

1. Nanohydroxyapatite toothpastes had a biomimetic remineralization potential of early human enamel carious lesions.
2. Extracts of green tea, ginger, and cardamom investigated in the experiment were given positive outcomes for being active substances with an efficient remineralization capacity.

RECOMMENDATIONS

Within the restrictions of this in-vitro experiment, our recommendation is measuring the remineralizing capability of the tested materials in a natural oral environment through in-vivo studies.

AUTHORS CONTRIBUTION

The study's authors all contributed equally.

CONFLICT OF INTEREST

Authors stated they have no competing interests.

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