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An In Vitro Study of Nanohydroxyapatite-Fortified Toothpaste on Tooth Remineralization: Microhardness, Mineral Content, and Biocompatibility

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ABSTRACT

Nanohydroxyapatite (nano HA) has gained broad interest not only in orthopedy but also in dentistry. In dentistry, nano HA has been developed as a biomimetic agent for oral care due to the similar chemical composition and particle size with hydroxyapatite crystals of enamel and dentin matrix. Being explored as a biomimetic agent, nano HA has been developed for remineralization and, thus, protection of early dental erosion. This study aimed to evaluate the effect of toothpaste containing nano HA on remineralization and their biocompatibility for personal oral care. The remineralization was evaluated by surface microhardness and mineral content changes in extracted human premolar teeth following toothpaste application. The biocompatibility of toothpaste was evaluated through lactate dehydrogenase (LDH) activity and interleukin-1alpha assay using HEK293T cells. The microhardness of specimens increased for all toothpaste formulas indicating the effectiveness of remineralizing dental caries. The linear correlation of elemental percentage changes indicates the ability to improve both teeth's calcium and phosphor content. Moreover, the LDH assay showed that most toothpaste formulas exhibit a low degree of cytotoxicity considerably as a safe oral product. The inflammation study (interleukin-1alpha determination) showed no acute irritation in most formulas with the same score of negative control (untreated with toothpaste).

Keywords: Cytotoxicity, dentin, inflammation, mechanical recovery, oral care product

1. INTRODUCTION

Human quality of life is closely associated with oral health as an essential indicator of people's general health [1]. Oral health has been defined as a patient's condition of being free from diseases that may impact the well-being, discomfort, and pain [2]. Oral diseases and disorders have been the primary concern that could disturb patients' well-being and self-esteem [3], resulting in several socioeconomic and psychological consequences [2, 4]. Dental diseases are humans' most prevalent chronic diseases, creating substantial public healthcare and economic burden [5, 6]. Dental caries is a major public health problem, affecting people of all ages, over 90% in some countries [6, 7]. The main cause of dental caries is the demineralization and net mineral loss of dental hard tissues, leading to unbalance of a physiological equilibrium in the oral environment [8]. This condition has been strongly affected by cariogenic bacteria activity and changes in salivary pH [6, 8]. The early stage of tooth demineralization is in the surface then invades deeper levels of tooth lesions (dental caries) [6]. Naturally, saliva performs caries protection due to their supersaturation with Ca^{2+} and PO_4^{3-} ions but in a slow process. As a result, the saliva insufficiently remineralizes the lesions without additional agents [4]. Therefore, it would be beneficial to formulate daily dental care to prevent dental caries containing remineralizing agents.

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Biomimetic systems are gaining attention as one of the new caries remineralization technologies [4]. Synthetic hydroxyapatite (HA) ceramics is among considerable materials for oral care products due to their use in various applications, including in preventative, therapeutic, and regenerative therapies [6]. Hydroxyapatite is well-known to be biomimetic ingredient for oral care product due to the similar chemical composition to the apatite crystals of tooth minerals [8–12]. As used in toothpaste, hydroxyapatite acts as a reservoir of calcium and phosphate ions and elevates them on tooth surfaces by maintaining a topical state of supersaturation to tooth minerals [13]. Moreover, biomimetic HA particles with apatite teeth crystals have an advantage in binding to the damaged enamel surface and filling the porous surface irregularities, restoring surface integrity [14].

Hydroxyapatite; in the form of micro- and nano-crystalline; has been widely used in several oral care products such as toothpaste, dentifrice, mouthwash, and mouth rinse [15]. The efficacy of microcrystalline hydroxyapatite-fortified toothpaste was trialled clinically demonstrating their ability in preventing dental caries and remineralizing the initial caries lesions [16]. As being explored as a biomimetic oral care agent, the similarity of synthetic HA to the target substance, such as enamel and dentin has been an important prerequisite. An important prerequisite for a biomimetic oral care agent is its similarity to the target substance, tooth enamel [17].

Nano-sized particles of hydroxyapatite have gained prominence as a bionic material due to the morphology and crystal structure to the apatite crystals of tooth enamel [6, 11, 18, 19]. An in vitro study revealed nano HA's ability to repair damaged enamel by adhering to the enamel surface, resulting in tooth remineralization [17, 18]. Those in vitro studies provide evidence for a new surface/enamel layer as an interaction of nano HA with the enamel surface. Thus, the exploration of nano HA-fortified toothpaste formulation has been widely studied as an oral care product. A previous study formulated 20 kinds of toothpaste fortified with nano HA and C. *Aeruginosa* oil as an active ingredient [20]. The study reported that most toothpaste formulas have the potential to decrease caries prevalence regarding remineralization and antibacterial/antibiofilm activity. The tooth remineralization activity in the previous study was evaluated by the morphological changes of extracted human premolars following toothpaste applications. Nevertheless, the quality of human dentin enamel accounts not only for the microstructure and morphology of the remineralized tissue but also for its mineral content and mechanical properties [11]. Therefore, a study on quantitative analysis of the mechanical recovery and mineral content change of enamel and dentin after remineralization is highly needed.

The present study aimed to evaluate further the effect of nano HA on biomechanical aspects and mineral content change. Furthermore, the biocompatibility of nano HA as an oral care ingredient was assessed using a cytotoxicity and inflammation study to be used as an oral care product.

2. MATERIAL AND METHODS

Nanohydroxyapatite (Nano HA)-fortified toothpaste was formulated using nano HA (produced by Dept. of Physics, IPB University) and C. *Aeruginosa* oil (extracted by Tropical Biopharmaca Research Center) as an active agent following previous study [20]. Twenty toothpaste formulas containing 0.2 – 2%wt nano HA and 0 – 0.2%wt C. *Aeruginosa* oil (Table 1) were tested in human premolar teeth through a demineralization-remineralization study and biocompatibility study as an oral care product.

The in vitro study was approved by the Ethics Committee. Following a previous study [20], twenty extracted human premolar teeth were prepared by cleaning under water, then scaling and soaking in H_2O_2 (Merck) for deproteination. Following deproteination, the prepared teeth were dried at room temperature and followed by cutting with micro motor into eighty specimens for demineralization and remineralization study. The demineralization was conducted to obtain the artificial incipient caries lesion with a modified method from the previous study [21]. A demineralization study was carried out with constant stirring at 150 rpm at room temperature for 24 hours by maintaining the pH at 4.3. Moreover, the demineralization process was also applied for the remineralization study by changing the demineralization solution with toothpaste slurry. In this study, toothpaste slurry was obtained from the mixture of each toothpaste formula with distilled water (1:1). The tooth specimens after demineralization and remineralization were characterized using the microhardness Vickers test (LM 800AT Vicker microhardness tester) to measure the mechanical recovery of enamel and dentin after remineralization. In addition, energy dispersive x-ray (EDX, SEM FEI Quanta 650) was used to observe the chemical elements for analyzing the mineral content change relative to baseline after demineralization and remineralization.

Prior to being used as an oral care product, safety assessment was also conducted through cytotoxicity and inflammation study. The cytotoxicity study was tested using the lactate dehydrogenase (LDH) assay by measuring LDH activity as an index of cell death. In this assay, human embryonic kidney (HEK293T) cells were seeded in 96 well tissue culture plates at the density of 1000 cells/well and incubated in a culture medium for 24 hours in a humidified 37^{o}C incubator equilibrated with 5% CO_2. Each toothpaste formula sample was added with 100 µL/well and incubated for 48 hours in a favorable environment (37^{o}C, 5% CO_2, 90% humidity). As a control requirement, control cells without treatment (untreated cell) were also observed. LDH activity was determined by using a spectrophotometer at 450 nm with the addition of 50 µL lysis buffer, and the results are presented as the percentage of cytotoxicity. The percentage of cytotoxicity was calculated as [22]:

$$\%\text{Cytotoxicity} = \frac{\text{Mean OD of treated cell - Mean OD of untreated cell}}{\text{Mean OD of standard blank - Mean OD of untreated cell}} \times 100 \quad (1)$$

Table 1 Twenty toothpaste formulas

Run	Sample Code	% Weight						
		Nano HA	C. *Aeruginosa* Oil	Na-CMC	Hydrated Silica	SCG	Peppermint	Water
1	F1	0.2	0.2	0.3	4.0	1.2	0.1	17.8
2	F2	0.2	0.0	0.3	4.0	1.2	0.1	18.0
3	F3	2.0	0.2	0.3	4.0	1.2	0.1	16.0
4	F4	2.0	0.0	0.3	4.0	1.2	1.3	15.0
5	F5	1.5	0.2	0.3	4.0	1.2	1.6	15.0
6	F6	0.2	0.0	0.3	4.0	1.2	3.1	15.0
7	F7	1.9	0.0	0.3	4.0	2.6	0.1	15.0
8	F8	0.2	0.2	0.3	4.0	4.0	0.1	15.0
9	F9	0.2	0.0	0.3	4.0	4.2	0.1	15.0
10	F10	2.0	0.0	0.3	5.2	1.2	0.1	15.0
11	F11	0.2	0.2	0.3	6.8	1.2	0.1	15.0
12	F12	0.2	0.0	0.3	7.0	1.2	0.1	15.0
13	F13	0.2	0.0	0.4	4.0	1.2	0.1	17.9
14	F14	2.0	0.0	0.4	4.0	1.2	0.1	16.1
15	F15	0.2	0.2	0.4	4.0	1.2	2.7	15.0

Run	Sample Code	% Weight						
		Nano HA	C. *Aeruginosa* Oil	Na-CMC	Hydrated Silica	SCG	Peppermint	Water
16	F16	0.2	0.0	0.4	4.0	1.2	2.9	15.0
17	F17	0.2	0.2	0.4	4.0	1.2	0.1	17.7
18	F18	2.0	0.2	0.4	4.0	2.2	0.1	15.0
19	F19	1.6	0.0	0.4	5.5	1.2	0.1	15.0
20	F20	0.2	0.2	0.4	6.6	1.2	0.1	15.0

For the inflammation study, the determination of interleukin-1alpha (IL-1 alpha) was analyzed using the commercially available Human IL-1 alpha ELISA Kit. This study was run to measure the release of IL-1 alpha as an indicator of acute irritation. The results were expressed in picogram (pg) of IL-1alpha per ml of culture media for each toothpaste formula. Moreover, control cells without treatment (untreated cells) and commercial products were also observed.

3. RESULTS AND DISCUSSION

A comprehensive study of nano HA-fortified toothpaste on tooth remineralization should be carried out, reflecting the integration of important parameters comprising microstructure, mineral content, and mechanical behaviour. The previous study [20] evaluated 20 toothpaste formulas containing nano HA and C. *Aeruginosa* oil, focusing on the microstructure aspect. The results showed the tooth remineralization indicated by the disappearance of porous surface and the changes in the gray image levels [20]. The current study corroborates the previous study on the efficacy of nano HA-fortified toothpaste in tooth remineralization from mechanical recovery and mineral content changes.

Figure 1 presents the mechanical recovery of the tooth after remineralization, shown by the changes in Vickers Hardness Number (HVN) of samples relative to baseline. The demineralization process by creating artificial incipient caries lesion reduced microhardness of tooth specimen shown by negative value for most samples (except F17). The negative value of VHN change relative to baseline indicates the weakened tooth due to the enamel erosion along the demineralization process. Interestingly, the changes of VHN after remineralization increased for most toothpaste formulas with a positive value. The increase in the percentage changes of VHN quantifies the efficacy of each toothpaste in inducing tooth remineralization. The maximum remineralization occurred on samples F10, F12, F16, F17, and F20, in which sample F10 had a high content of nano HA (2%weight) while the other four samples had low content (0.2%). This result informs that tooth remineralization was not solely influenced by the nano HA content but also related to the interaction between components in toothpaste. These microhardness results follow the previous study confirming the influence of the interaction between components in higher changes in the gray image levels [20].

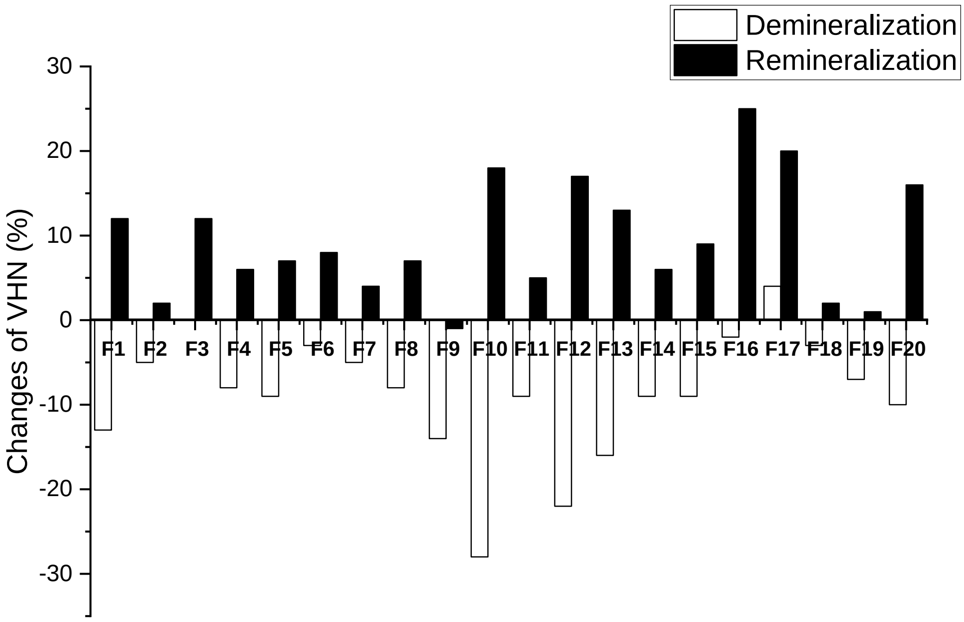
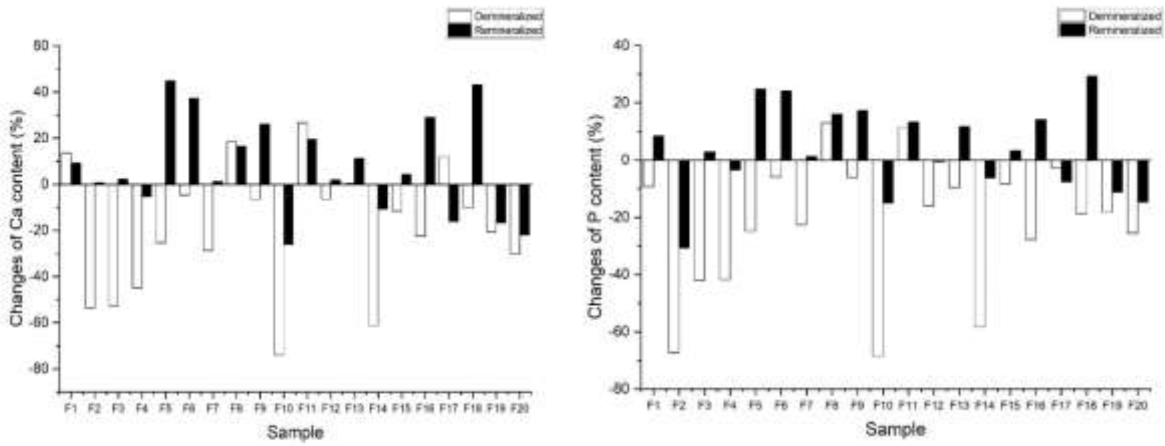


Figure 1. The percentage changes of Vicker's Hardness Number after demineralized and remineralized, relative to baseline.



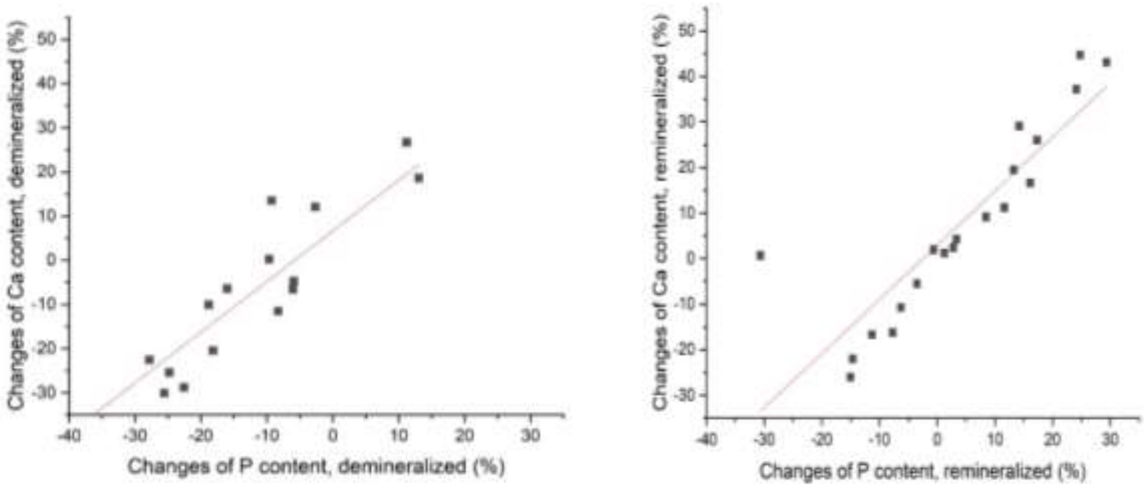
(a) (b)

Figure 2. The percentage changes of (a) Ca and (b) P contents after demineralized and remineralized, relative to baseline.

Another parameter to evaluate the effectivity of nano HA as remineralizing agent is the mineral content of remineralized tissue. Elemental analysis using EDX on the demineralized and mineralized samples showed a decreased trend of calcium (Ca) and phosphorous (P) contents following demineralization. It was shown by the changes in Ca and P contents in demineralized teeth compared with baseline (Figure 2a and 2b). The Ca and P content of the teeth samples following demineralization has a negative percentage change in Ca and P content. This value indicates that demineralized samples had lower Ca content than untreated samples. In addition, the application of nano HA-fortified toothpaste performed the ability to induce remineralization, shown by increased changes in Ca and P content. The positive percentage change of Ca and P content confirms mineral content recovery of remineralized tissue. The maximum remineralization occurred on samples F5, F6, F9, F10, F16, and F18 in the content of nano HA ranging from 0.2 – 2%. Similar results occurred from mechanical and mineral content aspects where the interaction between components in toothpaste matters in tooth remineralization. In general, most tested formulas show efficacy in tooth remineralization. Thus, being fortified into toothpaste, nano HA plays a major role in helping smoothen the surface [14] either through attachment to the enamel surface by a deposit in small depressions or biochemically induced formation of a protective layer of the outer enamel [15].

The fitting of percentage changes in Ca and P content in demineralized samples (Figure 3a) showed a linear correlation (Pearson's R = 0.96) with a gradient of 1.15. The value indicated that treatment of teeth samples in demineralizing solution at pH 4.3 resulted in both Ca and P ions dissolution. The gradient of 1.15 showed that the dissolution of Ca ions was slightly higher than that of P ions. A similar pattern was also observed with the percentage changes of P content in remineralized teeth samples. The fitting of percentage changes in Ca and P content in remineralized samples (Figure 3b) showed a linear correlation (Pearson's R = 0.88). The value indicates that toothpaste formulae tested in this study can improve both teeth' Ca and P content. Interestingly, the gradient of this fitting is 1.19, which is higher than the gradient of demineralization. Thus, being the reservoir of Ca and P ions [13], nano HA provides greater uptake of Ca during remineralization compared to the dissolution of Ca during demineralization.

Table 2 indicates the Ca/P ratio of samples. The Ca/P ratio of the untreated teeth was 1.26 – 2.30, with an average of 1.88. The Ca/P of the demineralized and remineralized samples also showed similar values.



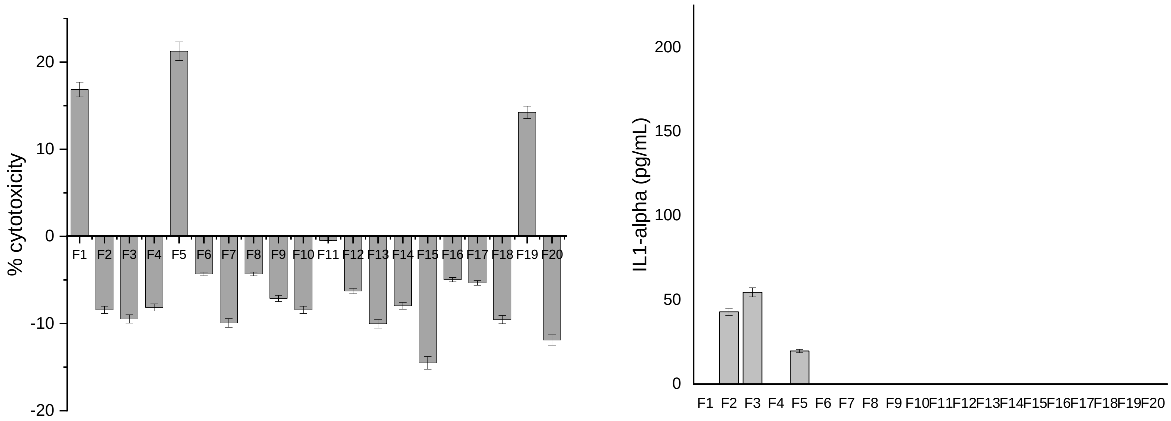
(a) (b)

Figure 3. The fitting of percentage changes of Ca and P contents in a) demineralized and b) remineralized samples.

Table 2 Ca/P ratio of baseline, demineralized and remineralized teeth samples

Sample	Ca/P (Standard Deviation)		
	Baseline	Demineralized	Remineralized
F1	1.90 (0.03)	2.38 (0.02)	1.91 (0.06)
F2	1.26 (0.10)	1.80 (0.09)	1.83 (0.05)
F3	2.30 (0.07)	1.87 (0.03)	2.29 (0.06)
F4	1.84 (0.04)	1.73 (0.08)	1.80 (0.03)
F5	1.80 (0.09)	1.78 (0.03)	2.08 (0.02)
F6	1.77 (0.01)	1.79 (0.04)	1.95 (0.06)

F7	1.99 (0.02)	1.83 (0.02)	1.99 (0.03)
F8	1.81 (0.01)	1.90 0.01)	1.82 (0.02)
F9	1.81 (0.03)	1.80 (0.04)	1.94 (0.04)
F10	2.10 (0.07)	1.70 (0.09)	1.83 (0.07)
F11	1.89 (0.02)	2.15 (0.09)	1.99 (0.02)
F12	1.77 (0.03)	1.98 (0.06)	1.82 (0.05)
F13	1.84 (0.03)	2.04 (0.02)	1.83 (0.04)
F14	1.94 (0.02)	1.79 (0.09)	1.85 (0.10)
F15	1.99 (0.06)	1.92 (0.01)	2.00 (0.03)
F16	1.78 (0.01)	1.91 (0.09)	2.02 (0.08)
F17	2.09 (0.05)	2.41 (0.13)	1.90 (0.03)
F18	1.75 (0.05)	1.94 (0.08)	1.94 (0.03)
F19	1.95 (0.05)	1.90 (0.04)	1.83 (0.06)
F20	2.04 (0.10)	1.92 (0.10)	1.87 (0.09)



(a) (b)

Figure 4. Biocompatibility of nano hydroxyapatite-fortified toothpaste with HEK293 cell line comprising (a) percentage of cytotoxicity based on lactate dehydrogenase (LDH) activity and (b) IL1-alpha, an indicator of acute irritation.

As an oral care ingredient, a safety assessment of nano HA-fortified toothpaste was carried out in a biocompatibility study comprising cytotoxicity and inflammation study. The cytotoxicity of all formulas was tested by detecting the lactate dehydrogenase (LDH) activity. The release of LDH indicates the mechanism of necrosis, which is one of the cells death types [22]. LDH is secreted from damaged plasma membrane cells. Therefore, the elevation of LDH activity indicates increased cytotoxicity. The LDH activity of cell control was used as a reference for converting LDH activity to the percentage cytotoxicity [22]. We obtained this calculation that most of the formulas did not exhibit high cytotoxicity. Most of the formulas had a negative value of cytotoxicity (Figure 4a), indicating that the LDH activity of the assay containing the cell control with formulated toothpaste was lower than the assay containing only cell control. Some toothpaste formulas (F1, F5, and F19) showed a positive value but a low degree of cytotoxicity. Therefore, in the perspective of cytotoxicity, all tested formulae are considered to be safe. Moreover, the sensitivity of toothpaste samples was also conducted through IL-1alpha determination as an indicator of acute irritation. This assay measured the release of IL-1alpha in a culture medium due to the role of IL1 cytokines as one of the primary initiators of the dermal pro-inflammatory response. Figure 4b shows the release of IL-1alpha level as an indicator of the acute irritation of nano HA-fortified toothpaste. As it can be observed, the study revealed zero values for most toothpaste formulas indicating the absence of acute irritation. Nevertheless, some formulas, such as F2, F3, and F5, showed a positive value of IL-1alpha but low concentration (less than 55 pg/ml). In short, nano-hydroxyapatite are safe to be fortified in toothpaste for oral care product.

4. CONCLUSION

In this study, the demineralization-remineralization study reveals the ability of nano hydroxyapatite-fortified toothpaste in tooth remineralization, reflecting the mechanical and mineral content recovery of remineralized tissue. The results evidence an effectivity of nano hydroxyapatite in the tooth remineralization indicated by the increase of Vicker's Hardness Number relative to the baseline. Nevertheless, the concentration of nano hydroxyapatite fortified into the toothpaste does not ensure the remineralization rate but the interaction between components in toothpaste matters. Pre-clinical study shows safety results comprising no cytotoxicity effect and acute irritation for most toothpaste formulas. These results confirm the safety of toothpaste fortified with nano hydroxyapatite in combination with C. *Aeruginosa* oil for oral care products.

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