Original Article

Remineralization Potential of Varying Concentrations of Two Plant-Based Extracts of *Cocos nucifera* on White Spot Lesions Using SEM and EDAX Analysis: An *In Vitro* Study

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ABSTRACT

Aim: To evaluate the remineralization potential of varying concentrations of two plant-based extracts of *Cocos nucifera* on white spot lesions using SEM and EDAX *in vitro* testing methods. **Materials and Methods:** The pulp was freshly obtained from coconut and divided into two. Then, coconut milk was obtained by blending, while the next portion was freeze-dried and lyophilized. Third molar teeth were processed into tooth slabs ($N = 40$) and split equally into five groups by block randomization. After demineralization, one tooth slab was taken from each, and SEM analysis was done. Remineralization was then performed among the various groups that included Group 1, which acted as a control and consisted of the remineralization solution. Groups 2 and 3 comprised 1:1 and 2:1 concentrations of the coconut milk, whereas Groups 4 and 5 consisted of 1:1 and 2:1 concentrations of the lyophilized extract. SEM and EDAX testing were done post-remineralization. Ca and phosphate values were tabulated, and statistical significance was determined for the obtained values using ANOVA. **Results:**Among the control and treatment groups, surface remineralization was better observed in 1:1 coconut milk and 2:1 coconut milk than in the 2:1 lyophilized coconut, control, and 1:1 lyophilized coconut. Between the control and treatment groups, Ca and phosphate percentages ($P < 0.001$) showed statistical differences. The lowest value of 2.3% was noted in the 2:1 lyophilized coconut group. **Conclusion:** Coconut extracts exhibit remineralization potential on the artificial carious lesion. Coconut milk exhibited significant improvement in the surface properties than lyophilized coconut.

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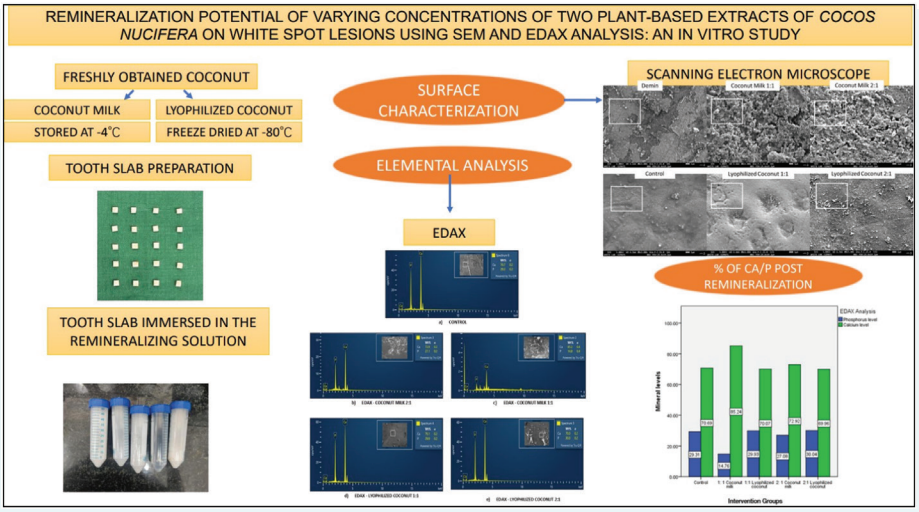
Quick Response Code:

Website: https://journals.lww.com/jpcd

DOI: 10.4103/jispcd.jispcd_146_23

How to cite this article: Balakrishnan N, Subramanian AK, Eswaramoorthy R, Angappan M. Remineralization potential of varying concentrations of two plant-based extracts of *Cocos nucifera* on white spot lesions using SEM and EDAX analysis: An *in vitro* study. J Int Soc Prev Community Dent 2024;14:129-35.



Received : 20-Sep-2023
Revised : 15-Feb-2024
Accepted : 20-Feb-2024
Published: 29-Apr-2024

KEYWORDS: *Coconut extract, demineralization, green synthesis, remineralization, white spot lesions*

INTRODUCTION

The hard tissues of teeth, especially the enamel and dentin, consist of inorganic component, which is biological apatite, or calcium-hydroxyapatite.[1] The biological apatite exists in the range of 1.67 in terms of the calcium-to-phosphate ratio.[2] Hence, obtaining ideal peak bone mass and maintaining bone mass depend mainly on calcium phosphate.[3] The dynamic processes of demineralization and remineralization are always at war within themselves.[4] These minerals are abundant in saliva, which aids in the preservation of this dynamic equilibrium. Moreover, during the process of demineralization, calcium is first released from the enamel, followed by phosphate.[5] Therefore, an increase in the bioavailability of calcium would be more effective than phosphate in stopping the demineralization process.[6]

Orthodontic fixed appliance therapy involves the bonding of orthodontic brackets over the dental enamel surface. The enamel surface is etched in this process where demineralization of the mineralized hard tissue surface occurs. With existing demineralized surfaces, the enamel with brackets is more prone to plaque and calculus accumulation, which in turn makes the enamel surface go devoid of calcium and phosphate ions from the saliva. Occasionally, dietary changes brought on by the expanding accessibility of carbonates in fizzy, sugary drinks can alter the structure of the hydroxyapatite crystal, thus making it more vulnerable to acid assault.[7] Additionally, studies have shown that bacterial ecology is also altered with higher acidogenic bacteria after the insertion of orthodontic fixed appliances.[8] Several other methods are also used for studying these changes on the enamel surface. They include analysis of surface microhardness, SEM EDAX, confocal microscopy,[9] etc.

Probiotic bacteria, fluoride treatment, and diet modification are all referred to as preventative measures. Wherein CCP-ACP application, resin infiltration, laser therapy, and many other methods are available for the management of such lesions.[10-12] Recently, different calcium phosphate (CaP) nanoparticle containing dental composites have been addressed as a potential treatment option for tooth demineralization.[13] Though there are several other materials existing, white spot lesions still remain more prevalent in orthodontically treated patients. These can be stopped from developing into carious lesions, causing concurrent loss of tooth structure, if treated at the right time. The plant-based extracts of Cocos nucifera were demonstrated to have good remineralization capacity in a prior investigation. Hence, the current study was made to evaluate the Remineralization Potential of Varying Concentrations of Two Plant-Based Extracts of *Cocos nucifera* on White Spot Lesions using SEM and EDAX *in vitro* testing methods.

MATERIALS AND METHODS

The study was done following CRIS guidelines (*in vitro* studies).[14,15] The null hypothesis was that the varying concentrations of two plant-based extracts prepared from *Cocos nucifera* had no difference in remineralization potential on artificially created white spot lesions.

Sample selection and randomization

G Power version 3.1 was used to calculate the sample size. (A-priori). The effect size was 0.8 and α error was 0.05 with a power (1-β error) of 0.95, two-tailed directionality of significance, and five treatment groups; the overall sample size was calculated as 35 (N). Hence, the number of samples included in each group was $n = 7$. Third molar teeth, that had been previously extracted for prophylactic or therapeutic purposes with intact enamel surfaces among the age groups 18–30 were collected. Block randomization was used to guarantee that the sample sizes in each group were equal. As a result, the principal investigator split the samples into blocks so that in each of the five treatment groups, seven samples were added. The investigator who performed the SEM and EDAX analysis and the one who computed the values were blinded throughout the entire experiment.

Sample preparation

Freshly purchased coconut was shredded, and the pulp was separated into two 200 g parts. To prevent material deterioration, half of the sample was used to extract the milk, and the other half was kept at −20°C. Freeze drying was at −80°C followed by 48 h of lyophilization.[16-18]

Demineralization solution preparation

Monobasic potassium phosphate, acetic acid, and calcium chloride were combined in recommended proportions. A pH of 4 was obtained by adding 1N hydrochloric acid and 2N sodium hydroxide.[16-18]

Remineralization solution preparation

Dipotassium hydrogen orthophosphate, potassium chloride, and calcium chloride ($CaCl_2$) were dissolved to create a remineralization solution. The solution's pH was adjusted to 7.[16-18] All of these were prepared and stored at room temperature. After that, the following control and treatment groups were created using the solution: Group 1 acted as a control and consisted of the remineralization solution. Groups 2 and 3 comprised 1:1 and 2:1 concentrations of the coconut milk, whereas Groups 4 and 5 consisted of 1:1 and 2:1 concentrations of the lyophilized extract.

Tooth-slab preparation

Third molar extractions were gathered, and 3 × 3 × 1.5 mm tooth slabs were created. 40 tooth slab samples were taken and divided among the five groups by block randomization.[16-18] After initial demineralization, due to the destructive nature of the investigation used, only one of the tooth slabs in each group was taken and analyzed for demineralization characteristics, while the others were all subjected to further remineralization procedure.

Remineralization procedure

The remineralization procedure was as follows: Demineralization: 3 days; washing with deionized water; remineralization: 14 days.[16-18]

Parameters assessed

SEM

Carbon tape was used to attach the sample to the brass stubs. The samples were then coated with platinum for 40 s in a vacuum chamber during sputtering. The photos were taken at various magnifications. FE-SEM IT800 model, JEOL company SEM was used. SEM values were recorded after demineralization of the samples and after remineralization in various groups.

EDAX

Energy dispersive X-ray analysis of the sample was done to identify the percentage of calcium and phosphorus deposition using the Oxford model. The working distance for EDX will be fixed at 10 mm. The sample was analyzed at a 20 kV voltage and EDX measurements were made for all post-treatment samples. The EDAX values were recorded at two points, and the intra-examiner's reliability was assessed. Kappa statistics were done, and they revealed a value of 0.8, indicating substantial agreement.

Results

SEM analysis of the demineralized enamel sample indicates an enamel surface that is irregular and filled with voids. In the post-remineralization groups, the control group containing the remineralizing, a line shows a regular pattern without any surface irregularities. Comparing the four experimental groups, the coconut milk group shows increased deposition on the surface of the enamel, with no demineralization zones visible on the demineralized enamel surface. The lyophilized coconut group at a concentration of 2:1 showed a better improvement of the surface characteristics, whereas at a lower concentration of 1:1, the surface irregularities were still visible, indicating a reduced amount of surface remineralization efficacy compared to the other three experimental groups [Figure 1].

Table 1 and Figure 2 indicate the Ca and phosphorus percentages among the various control and experimental groups analyzed post-remineralization. Of all the groups examined, the highest values of the percentage of calcium were noted in the 1:1 coconut milk group (85.2414%) and the lowest values of 69.9571% were noted in the 2:1 lyophilized coconut group. Among the groups comparing the phosphorus values, the highest was noted in the 2:1 lyophilized coconut group (30.0429%), and the lowest values were noted in the 1:1 coconut milk group (14.7586%). The

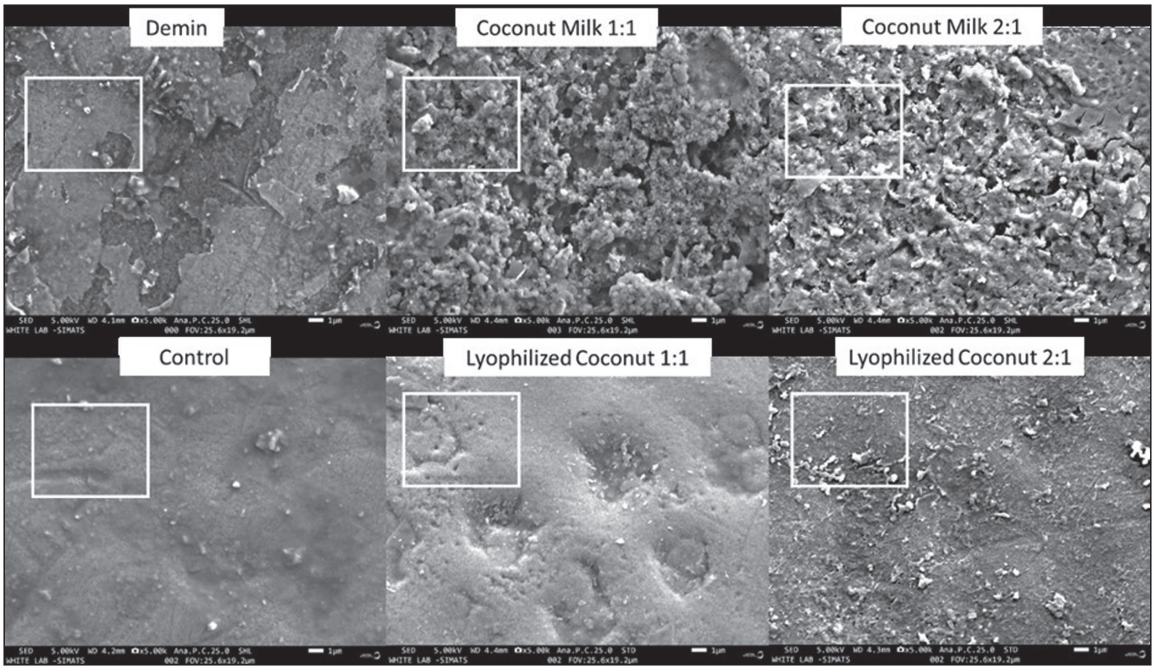


Figure 1: SEM images for demineralized enamel surfaces and various control and treatment groups in 1 µm magnification. Demineralized enamel surface shows visible enamel surface irregularities. Also, among the two concentrations of coconut milk and the lyophilized coconut extract, the higher concentration of the material shows increased remineralized surface changes compared to the low concentration

Table 1: Depicts the descriptive statistics for the amount of calcium and phosphorus obtained by EDAX analysis

		N	Mean (%)	Std. Deviation	Sig.
Control	Phosphorus	7	29.3071	1.66093	<0.001*
	Calcium	7	70.6929	1.66093	
1: 1 coconut milk	Phosphorus	7	14.7586	1.79769	
	Calcium	7	85.2414	1.79769	
1: 1 lyophilized coconut	Phosphorus	7	29.9286	1.98134	
	Calcium	7	70.0714	1.98134	
2: 1 coconut milk	Phosphorus	7	27.0757	2.38463	
	Calcium	7	72.9243	2.38463	
2: 1 lyophilized coconut	Phosphorus	7	30.0429	2.03458	
	Calcium	7	69.9571	2.03458	

*. The mean difference is significant at the 0.05 level

Ca and phosphate percentages ($P < 0.001$) among the various groups showed a significant difference [Tables 1–3]. The lowest Ca/P ratio of 2.3% was noted in the 2:1 lyophilized coconut group. A *post hoc* tukey test was done. Compared to the control group, the 1:1 coconut milk group shows statistically significant differences in the calcium phosphate percentage, whereas when the control was compared with other experimental groups, no statistically significant difference existed [Figure 3].

Discussion

Saliva's innate capacity to provide the tooth with bioavailable calcium and phosphate ions is linked to its capacity to remineralize enamel crystals. Normal saliva contains supersaturated calcium in the forms of hydroxyapatite, fluorapatite, phosphate, and bicarbonate at physiological pH.[18-22] Calcium and phosphate should be diffused to the subsoil by an ideal remineralizing agent. However, in any pH environment, it should have synergistic effects with saliva.[19]

SEM and EDAX analysis are quantitative tools effective in identifying the surface properties and deposition of calcium phosphate onto the surface.[20] Analytical methods like EDAX make it possible to identify the components contained in any kind of solid material. In our current evaluation, both SEM and EDAX were performed following the remineralization protocol among all five groups, and the results were as follows:

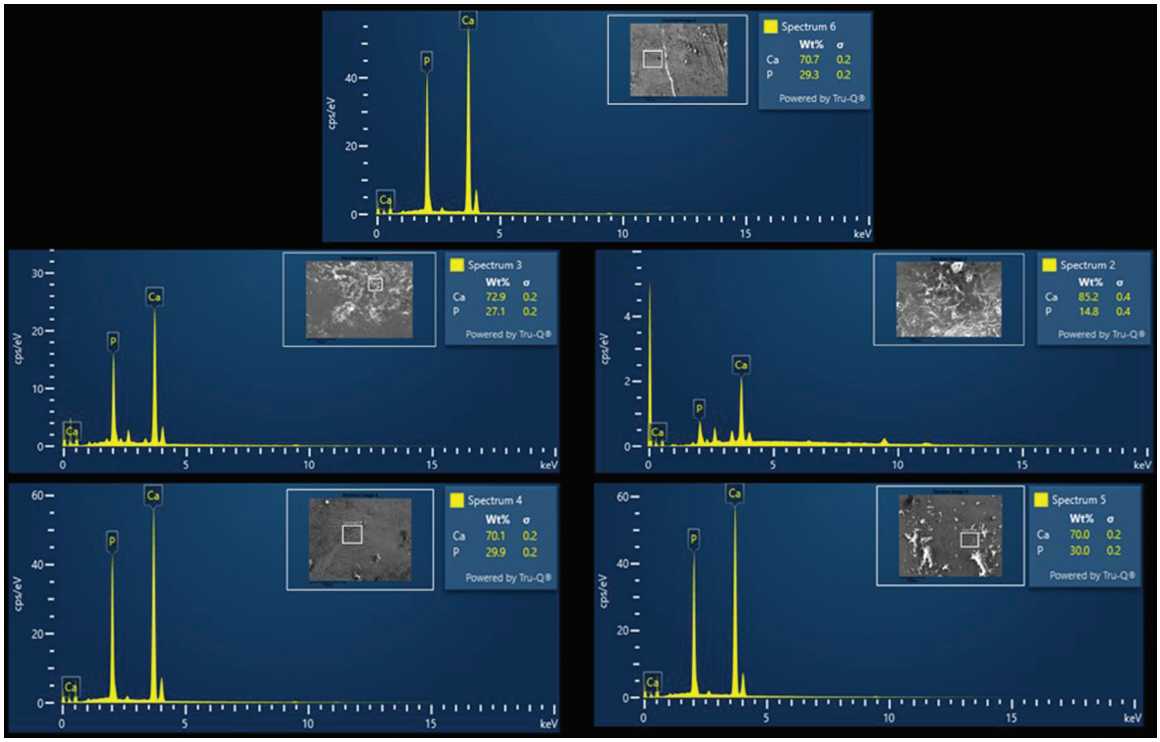


Figure 2: Results of the EDAX analysis for the estimation of the Ca and P ions on the remineralized enamel surfaces among the control and experimental groups

Table 2: Depicts the multivariate[a] statistics for the amount of calcium and phosphorus obtained by EDAX analysis

Effect	Value	F	Sig.
Intercept			
Pillai's trace	0.995	6095.311[b]	<0.001*
Wilks' Lambda	0.005	6095.311[b]	<0.001*
Hotelling's Trace	203.177	6095.311[b]	<0.001*
Roy's largest root	203.177	6095.311[b]	<0.001*
Groups			
Pillai's trace	0.909	75.344[b]	<0.001*
Wilks' lambda	0.091	75.344[b]	<0.001*
Hotelling's trace	10.046	75.344[b]	<0.001*
Roy's largest root	10.046	75.344[b]	<0.001*

[a]. Design: intercept + groups

[b]. Exact statistic

*. The mean difference is significant at the 0.05 level

- The demineralized enamel showed an irregular surface morphology with spaces and micropores, which is described to have a "honeycomb structure." Wherein the remineralized surface exhibits a mineral laydown and reduction in the surface voids, showing a good amount of mineral recovery.
- Except for the 1:1 lyophilized coconut group, almost all the other groups exhibited a Ca/P ratio of 2.2, which is closer to the ideal ratio.
- The remineralized surface in the 1:1 coconut milk group showed a more rugged surface morphology compared to that of the surface morphology of the 2:1 coconut milk group and the 2:1 lyophilized coconut group. Moreover, they did not display an even surface but a comparatively less rugged surface morphology than the 1:1 coconut milk group.

Previous studies on demineralized enamel showed an uneven surface morphology, which was characterized as a "honeycomb structure with voids and micropores"[21] with which our present findings are comparable. Also, in a study, Vitello *et al.*,[22] tested ACP functionalized with fluoride and carbonate-coated with citrate, nano-hydroxyapatite gel, 5% SF varnish, and CPP-ACP. Using a 200x magnification SEM study, it was

Table 3: Depicts the one-way MANOVA testing for the calcium and phosphorous values among the test groups

Source	Dependent variable	Type III sum of squares	Mean square	F	Sig.
Corrected model	Phosphorus level	1189.963[a]	297.491	75.344	<0.001*
	Calcium level	1189.963[b]	297.491	75.344	<0.001*
Intercept	Phosphorus level	24066.814	24066.814	6095.311	<0.001*
	Calcium level	190508.814	190508.814	48249.449	<0.001*
Groups	Phosphorus level	1189.963	297.491	75.344	<0.001*
	Calcium level	1189.963	297.491	75.344	<0.001*
Error	Phosphorus level	118.452	3.948		
	Calcium level	118.452	3.948		
Total	Phosphorus level	25375.230			
	Calcium level	191817.229			
Corrected Total	Phosphorus level	1308.416			
	Calcium level	1308.416			

[a]. R^2 = 0.909 (Adjusted R^2 = 0.897)

[b]. R^2 = 0.909 (Adjusted R^2 = 0.897)

*. The mean difference is significant at the 0.05 level

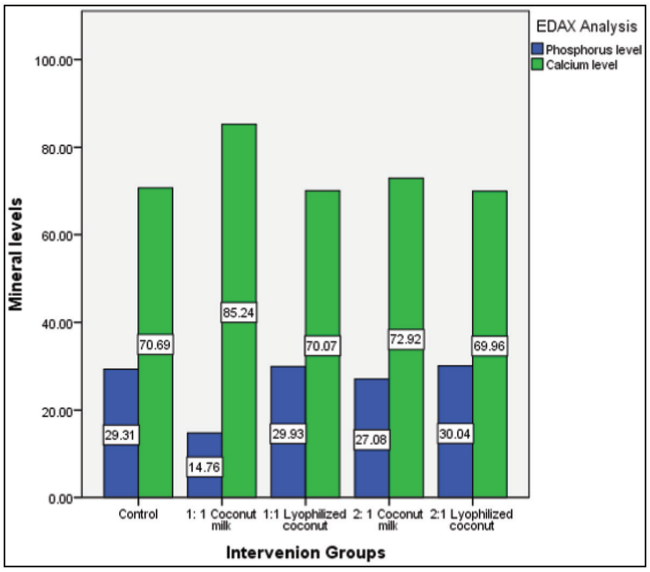


Figure 3: Mean calcium and phosphorus percentages on the tooth blocks among the various treatment groups. The highest percentage of Ca was noted in the 1:1 coconut milk group and 2:1 coconut milk group, whereas the lowest was noted in the 2:1 lyophilized coconut milk. The value shows a statistically significant difference in the amount of Ca and P deposition among the various groups (*P* value < 0.001*)

determined that the surface with the gaps had been remineralized, thus forming a more regular surface. In another study, SEM results showed that in the enamel substrate, CPP-ACP may restore a uniform and compact layer of disaggregated nanoclusters with a globular shape.[23]

In an earlier investigation, it was stated that these ideal values (1.67) were found on untreated tooth samples without subjecting them to any demineralization or remineralization. These values provide evidence of a closer-to-ideal biomimetic environment for remineralization.[24] Unquestionably, one unit cell of F-HA (Ca10(PO4)6F2) requires 10 Ca^{2+} and six PO_4^{3-} for every two F^-. Consequently, the oral environment's Ca^{2+} and PO_4^{3-} availability may play a significant role in the enamel remineralization process when F[25] is applied topically. Few studies have reported that the Ca/P ratios were 1.49–2.08% during the remineralization of dental enamel with various agents. The materials studied included CPP-ACP, CPP-ACP with fluoride, and fluoride varnish.[26] The test materials revealed a Ca/P ratio approximately close to 2. This shows the fact that the materials tested when used in higher concentrations promote a better remineralization potential used in the prevention and management of orthodontically induced white spot lesions.

The main limitations of the present study were the partial sample size and the *in vitro* simulated environment for white spot lesions. The properties of these materials *in vivo* need to be studied further.

Conclusion

Improvement in surface characteristics was noted with 1:1 coconut milk, 2:1 coconut milk, and 2:1 lyophilized coconut. Also, 2:1 coconut milk, 1:1 lyophilized coconut, and 2:1 lyophilized coconut showed an effective calcium/phosphate ratio, indicating better remineralization potential. Higher concentrations of both extracts showed better properties in comparison to their lower concentration counterparts.

Acknowledgements

We thank all the authors and research facilities for their valuable contributions.

Financial disclosure

Nil.

Conflicts of interest

Nil.

Author Contributions

NB and AKS: Conceptualized the research's goal and designed the methodology, validation, formal analysis, resources, and data curation; NB, RE, and MA: Performed investigation; NB and AKS: Initial Draft. All authors have approved the final version of the manuscript.

Ethical policy and Institutional Review board statement

The institutional review board granted the study ethical approval (SRB/SDC/PhD/ORTHO-2007/23/O55).

Patient Declaration of Consent

Not applicable.

Data Availability Statement

Not applicable.

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