

**BJMHR**British Journal of Medical and Health Research
Journal home page: www.bjmhr.com

Qualitative and Quantitative Comparison of the Remineralisation Potential of Three Different Natural Extracts: An In Vitro SMH and Sem-Edx Study

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ABSTRACT

Minimally invasive dentistry aims at restoring the early incipient caries thereby arresting the caries progression. Various remineralising agents boosts the calcium and phosphate deposition onto the incipient lesions restoring tooth architecture. This research aimed to evaluate the remineralisation potentiality of enamel surface lesion using different natural extracts quantitatively by surface microhardness and qualitatively by energy dispersive X-ray analysis. Forty single rooted teeth fitted in the criteria were collected and subjected to demineralization cycle for about of 7 days. Samples were allocated into 4 groups as : Group 1 – Artificial saliva, Group 2- Moringa leaf extract, Group 3 – Ashgaurd pulp extract, Group 4- Chicken egg shell powder based on the remineralisation agents employed and subjected to remineralization cycle for about of 14 days. Energy Dispersive X-ray Analysis (SEM- EDX) was used for Qualitative analysis and Vickers microhardness was used for Quantitative analysis. Qualitative EDS analysis revealed highest calcium and phosphorous content by Moringa leaf extract followed by Ashgaurd pulp extract. Quantitative SMH analysis revealed higher microhardness values for Moringa leaf extract. Herbal agents have a promising role in remineralization of early enamel carious lesions. Moringa leaf extract and Ashgaurd pulp extract shows higher remineralisation potential owing to its high calcium content

Keywords: Remineralisation, Energy Dispersive X-ray Analysis

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Received 29 November 2024, Accepted 25 December 2024

Please cite this article as: Rao SA *et al.*, Qualitative and Quantitative Comparison of the Remineralisation Potential of Three Different Natural Extracts: An In Vitro SMH and Sem-Edx Study . British Journal of Medical and Health Research 2024.

INTRODUCTION

Dental caries, a multifactorial disease always preceded by an array of distinct demineralization presents as opaque subsurface porosity of tooth surface owing to loss of mineral content caused by acidic pH which. This loss could be reverted back via deposition of mineral ions onto the tooth surface by remineralisation.¹ Demineralisation and remineralization plays a balanced ionic see- saw on the tooth where demineralization outrageous to produce dental caries.² Oral fluids flooded with Free fluoride drives calcium and phosphate ions into voids of crystal lattice of demineralised portion.^{3,4}

As traditional phytomedicines have paved their way towards regenerative dentistry as they are less toxic, possess bioactivity and anti- inflammatory properties.⁵ *Moringa oleifera* is one such phytomedicine which is leafy green legumes entombed with B-carotene, vitamin C and minerals including calcium, phosphorous, potassium and plant based oxalates. Though its beneficial effects against carious bacteria have been emphasized, there is an ongoing pioneer research halting to determine its remineralization potential of enamel lesions.^{6,7}

Ashgourd (*B. hispida*), a unique member of Cucurbitaceac family known for its medicinal and functional properties have prominent role in Ayurvedic medicine to treat gastro-intestinal problems. Peel, Pulp and Seeds of Ashgourd have proven to possess antioxidant, anti-inflammatory, detoxifying properties.⁸ The antacid properties of Ashgourd pulp are likely due to the presence of calcium, magnesium, iron, copper, zinc and selenium.^{8,9} Till date there is scarce of research and piqued the minds of researches to evaluate the remineralising potentiality of *B. hispida* against sub surface enamel lesions.

Chicken egg shell powder was reported as a medicinal and pharmaceutical agent used as bone substitute for treatment of maxillofacial defects and alternative to bone graft for regeneration of bone defects.¹⁰ It was registered as a rich source of calcium containing 94% of calcium carbonate, 1 % of calcium phosphate, 1% magnesium carbonate and 4% of organic matter.(10). It also contains fluoride and strontium.^{11,12} Due to its high calcium content it has the ability to promote remineralisation.

The remineralisation potential of normal saliva was described by Head in 1910.¹³ Artificial saliva has important substitutes that has caries preventive/ remineralising properties. It contains fluoride, calcium, phosphate ions have potential rehardening properties.

Hence, the objective of the present study is to evaluate the remineralisation potential of enamel surface lesion using different natural extracts quantitatively by surface microhardness and qualitatively by energy dispersive X-ray analysis.

MATERIALS AND METHOD

This in vitro study was approved by institutional review board of Mamata Dental College, Khammam, Telangana, India (EC/IRB NO. MDC – R – 088446). Forty freshly extracted human Maxillary Central Incisors were collected. The teeth were gently cleaned of residual debris and washed thoroughly under running water. The teeth were stored in sterile saline until use. Teeth with intact Enamel surface were included in the study. Teeth showing presence of caries, cracks, fractures, white spots, decalcification, fluorosis and developmental defects were excluded from the study.

Specimen Preparation

3mm×3mm wax pieces were cut from modeling wax sheet and placed on the labial surface to form a window. Except on the wax sheet all around the crown surface acid resistant nail varnish was coated in two layers. After the nail varnish dried red colored nail paint was used as third coat to differentiate the central window from adjacent area. Once the paint dried wax sheet was removed and the samples were ready with a exposed central isolated window of enamel.

Preparation of Demineralisation Solution

500 ml of demineralisation solution was prepared containing 2.2Mm of calcium chloride dehydrate , 2.2Mm potassium dihydrogen phosphate and 50mmol of acetic acid at pH of 4.01

Preparation of Remineralisation Solution (Figure 1)

PREPARATION OF *M.oleifera* LEAVES EXTRACT

200g of *M.oleifera* leaves powder were added to 1000ml of 60% ethanol and kept at room temperature for 48hr. After that the obtained mixture was magnetically stirred at 800rpm for 4hrs to achieve a homogenous mixture and then stored at 4⁰C for 24hrs. To allow the extraction of active ingredients the mixture was filtered through a muslin cloth, then filtered with whatman No.1 filter paper. The extract was incubated at 37⁰C for several days to allow for evaporation of the solvent to obtain final volume of 100ml.

PREPARATION OF *B. hispida* PULP EXTRACT

20g of pulp was grated and homogenized with 100ml of distilled water it was then filtered and remnant was discarded. The filtered liquid was centrifuged at 5000rpm for 15minutes to remove suspended particles. The clear supernatant was used as pulp extract.

PREPARATION OF EGG SHELL POWDER SOLUTION

1g of chicken egg shell powder dissolved in 20ml of 4% acetic acid in a beaker. The clear fluid which is collected at the top was transferred to a beaker. The pH of the solution was tested using pH meter which was 11.7.

PREPARATION OF ARTIFICIAL SALIVA

It is synthetic saliva containing sodium chloride, potassium chloride, calcium chloride, magnesium chloride, potassium mono hydrogen phosphate at pH7.0.

Demineralisation Protocol

The early artificial demineralization of enamel subsurface was achieved by immersing the specimens individually into a glass beaker containing 100ml of demineralization solution for 7 days. The solution was not replaced and not stirred throughout the demineralisation period.

Grouping

Specimens were randomly divided into four groups of n=40

GROUP 1 – Artificial Saliva (control)

GROUP 2 – Moringa Leaf Extract

GROUP 3 – Ashgaurd Pulp Extract

GROUP 4 – Chicken egg shell powder

Remineralisation Protocol

The teeth in each group were immersed in their respective remineralisation solution for 14 days. Half of the samples from each group were subjected to Scanning electron microscopic and Energy dispersive X-ray analysis for surface morphology and elemental composition respectively. And the other half samples were assessed for microhardness using Vickers micro hardness test.



Figure 1: Specimens from each group were immersed in their respective remineralisation solution for 14 days

Statistical Analysis

The obtained data was analyzed using SPSS software version 21.0. ANOVA test and Post Hoc Tukey test was used for intra-group comparison. Paired wise sample t test was used for inter-group comparison. P value of < 0.05 was considered significant.

RESULTS AND DISCUSSION

Table 1 show the microhardness values for all the groups and

The SEM images revealed decrease in pore volume of enamel structure in Moringa followed by Ashgaurd, chicken egg shell powder compared to control group (Figure – 1 to 4).

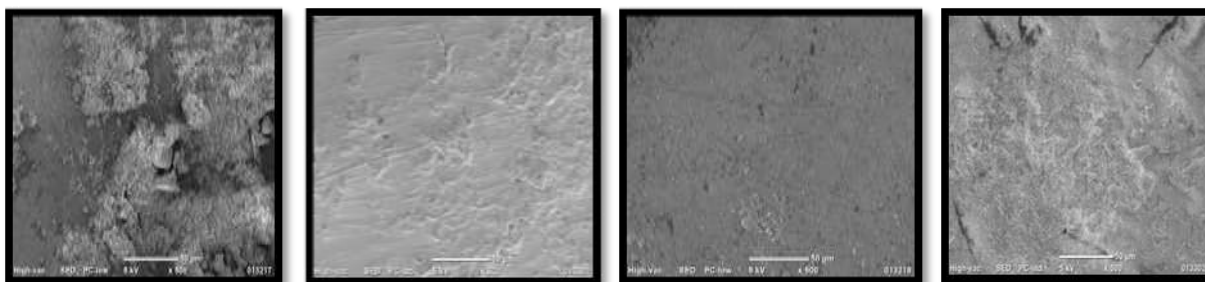


Figure 1: Moringa leaf extract

Figure 2 : Ashgaurd pulp Extract

Figure 3: Chicken egg shell powder

Figure 4 : Artificial Saliva

Table 1: Microhardness values

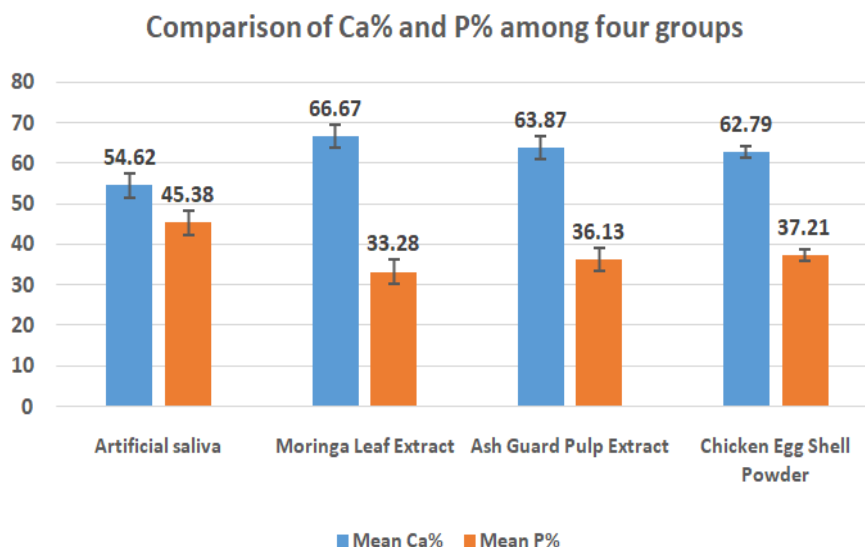
Group	Mean micro hardness	SD	F value	p-value
Artificial saliva	365.10 ^a	9.68	36.742	<0.001*
Moringa Leaf Extract	397.10 ^b	6.85		
Ash Guard Pulp Extract	386.70 ^c	9.17		
Chicken Egg Shell Powder	364.30 ^a	7.97		

Highest micro hardness value was seen in Moringa Leaf Extract group and least micro hardness was seen in Chicken Egg Shell Powder group. The overall difference in micro hardness values of the four groups was statistically significant. Pairwise comparison showed that micro hardness value in moringa leaf extract was significantly greater than that of the other three groups. Ash Guard Pulp Extract showed a significantly greater microhardness value as compared to the artificial saliva and chicken egg shell powder groups. There was a non-significant difference in the micro hardness values in between artificial saliva and chicken egg shell powder.

Least Ca% was found in artificial saliva and highest Ca% was seen in Moringa Leaf Extract. The overall difference in Ca% of the four groups was statistically significant. Pairwise comparison showed that Ca% in artificial saliva was significantly lower than that of the other three groups. There was a non-significant difference in the Ca% in between moringa leaf extract, ash guard pulp extract and chicken egg shell powder.

Highest P% was found in artificial saliva and least P% was seen in Moringa Leaf Extract. The overall difference in P% of the four groups was statistically significant.

Pairwise comparison showed that P% in artificial saliva was significantly greater than that of the other three groups. There was a non-significant difference in the P% in between moringa leaf extract, ash guard pulp extract and chicken egg shell powder.



DISCUSSION

Understanding the caries process has shifted the global approach against this progressive subsurface demineralization towards preservation of tooth structure by implementing either remineralisation or regeneration of lesion body structure.³ Different materials and methods are currently being evaluated to achieve new approaches for remineralisation of demineralised tooth structure and aid in treating dental caries.

Active white spot lesions are more likely to undergo remineralisation as compared to inactive lesion. This is because they have surface which is more porous and therefore allows better penetration of ions.¹⁴ Remineralisation therapy seeks to replenish the lost mineral content by introducing calcium and phosphate into demineralised pores.¹⁵ Calcium and phosphate ions must first penetrate the surface layer of enamel to bring about deposition of minerals through the body of the lesion and promote remineralisation.¹⁶

Fluorides are well known for their ability to prevent demineralization and promote remineralisation but excessive fluoride consumption leads to adverse effects and toxicity. Therefore instead of using fluorides plant extracts are known to have the effect on causative bacteria for tooth decay and it is proposed that these can be used to prevent and treat dental caries. This leads to search to find new remineralising agents that do not contain fluorides.¹⁷

In the present study experimental groups were Moringa leaf extract, Ashgaurd, Chicken Egg shell powder. Among which Moringa leaf extract shows higher remineralisation potential

The potentiality of Moringa leaf extract as remineralising agent may be due to high calcium content which interact via peptide guided remineralisation which is in accordance to the study

done by Sara H et al ³. Ethanolic extract of *M.oleifera* leaf extract was prepared according to the Alharbi et al. ¹⁸Moringa leaf extract has high levels of minerals and proteins provides a necessary alkaline environment for mineral deposition which leads to precipitation of calcium and phosphate.¹⁹

Ashgaurd has antibacterial and antifungal detoxification property and also has high nutritional values. ^{20 21} The higher content of minerals like Ca, Na, K, Fe, Zn in Ashgaurd have seeded a path as this is the first study to evaluate its remineralisation capability.⁸ In the present study ashgaurd shows highest remineralisation potential followed by Moringa leaf Extract.

In recent years chicken egg shell has attaining importance in numerous fields. Eman et al., returned the important role of chicken egg shell powder in remineralisation when applied topically to high concentration of bioavailable calcium.⁹

When compared with other natural calcium sources CESP has low levels of toxic metals like Pb, Al, Cd, Hg. The N-terminal sequence (Met-Ala-Val-Pro-Gln-Thr-Met-Val-Gln) of eggshell matrix proteins has been suggested in the increased calcium transport and considered as a potential significance of calcium when used as calcium supplements. The lesser remineralising ability of Chicken egg shell powder may be attributed to the poor anionic activity of phosphate and hydroxyl ions in lower concentrations which was in accordance to our study. ²²

Saliva has been chosen as our control group because it has calcium and phosphate concentrations maintained by various salivary proteins which may account for remineralisation.¹⁷ Saliva as well as remineralising solutions can remineralize early enamel lesions (23). In spite of enamel remineralisation potential of saliva, by itself it fails to initiate process of increasing levels of calcium and phosphate.¹⁶

Considering the importance of the surface layer in caries progression, the assessment of changes in this region is important. Surface microhardness provides a relatively simple, non-destructive and rapid technique in demineralization and remineralisation studies. ⁹

Various studies have used different methods to assess the process of enamel remineralisation. The commonly used micro hardness tests are Vickers hardness test and knop hardness test. In the present Vickers surface microhardness test was used.¹⁶

Moringa leaf extract shows highest surface micro hardness values due to its higher calcium content followed by Ashgaurd, Artificial saliva and Chicken Egg shell powder. According to the study done by Eman et al., Chicken egg shell powder yielded lower scores under VMHT which is in accordance to our study.

Various methods have been used to study remineralisation of carious lesion, such as microradiography, polarized light microscopy, microhardness, mineral analysis of calcium phosphate phases, transmission and scanning electron microscopy. One of the recent techniques that are used to quantitatively measures the small changes in the mineral content is Energy dispersive X-ray spectroscopy.⁹

Qualitative assessment was carried out SEM analysis. SEM images of sound enamel showed well organized enamel rods. The enamel crystals were homogenously arranged with clear outline. In our present study artificial saliva shows disorganized crystals with loss of structural characteristics. Remaining study groups demonstrated either amorphous crystals or particles scattered on the surface or lines of remineralisation along the prismatic borders.²

To the best of our knowledge this is the first study utilizing ashgaurd as a remineralising agent as it is cost effective and easily available moreover it can also extracted by simple real life method.

Limitations

As the study is limited to in vitro conditions. As in vitro conditions may be different when compared to the in vivo with dynamic complex biological system, further clinical trails are necessary to know the efficacy of potential of natural extracts. Though Moringa leaf extract shows higher remineralisation potential but the main drawback was discoloration of the specimens treated with its extract.

CONCLUSION

Within the limitations of the present study it can be concluded that Moringa leaf extract shows higher remineralisation potential followed by Ashgaurd, Chicken Egg shell powder and Artificial saliva. Moringa leaf extract shows highest SMH values due to its high calcium content.

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