THE REMINERALIZING POTENTIAL OF NATURAL DRUG LOADED NIOSOME VERSUS ITS EXTRACT ON DEMINERALIZED ENAMEL

Ahmed Adel Abdelaziz*1, Heba fathy Elemam**1 and Ahmed Hamdy Abdu***1

ABSTRACT

Objectives: This in-vitro study was designed to evaluate the remineralizing potential of (Peganum harmala) as a natural plant, niosome loaded harmala extract versus sodium fluoride on demineralized enamel surface.

Materials and methods: A 90 caries-free upper first premolar teeth were collected. The specimens were cut 2 mm in a horizontal plane, sectioning of the coronal part into two halves resulting in 180 specimens. Then, embedded in acrylic blocks. Specimens were then immersed in demineralizing solution of 0.3% citric acid for 8 hours to produce artificial caries-like lesions. The specimens randomly divided into five groups: group (A) Peganum harmala extract , group (B) niosome loaded harmala, group (C) sodium fluoride, group (D) positive control, group (E) a negative control. Two concentration groups (5% and 10%), two different time (20 days and 40 days). Specimens of each group were examined for microhardness measurement, data was then recorded, statistically analyzed using analysis of variance (ANOVA). Also, specimens were examined using scanning electron microscopy (SEM). Energy Dispersive X-ray analysis (EDX).

Results: 10% concentrations appeared more effective than the 5% in all tested groups. The median hardness for 10% harmala was 176.4.6 in 40 days compared to 151.6 for 5% harmala. Also, niosome 10% showed the highest median microhardness overall (243.6 in 40 days). Also, there was an increase in microhardness values from 20 to 40 days for all preparations. SEM results and EDXA analysis confirmed these observations.

Conclusions: P. harmala plant extract and niosome loaded harmala enhanced the remineralization potential of demineralized enamel surface.

KEYWORDS Peganum harmala, niosome, remineralization, demineralized enamel, microhardness
INTRODUCTION

Enamel is the hard calcified dental tissue, it is made up of a high percentage of minerals (96%) and a complex arrangement of hydroxyapatite crystals. It has been subjected to dynamic processes of de- and remineralization during the whole life span of the tooth. Additionally, any defect in the enamel will result in the morphological changes in tooth structure and later on dentin exposure and hypersensitivity (1).

Demineralization of enamel is defined as the removal of mineral ions from hydroxyapatite HA crystals of calcified enamel. This happens at acidic pH ≤ 5.5 when hydroxyapatite reacts with hydrogen ions. Accordingly, signs of demineralization are appearing in the form of loss of tooth surface texture as it becomes rough with white chalky spot lesions (2). While dental erosion is a chemical process not involving acids of bacterial origin. Clinically, it has a destructive effect on the tooth surface which appeared as white spot lesions with progressive loss of tooth structure (3).

Furthermore, hard dental tissue remineralization is considered a natural repair process in which the calcium and phosphate ions are deposited into crystal voids in demineralized enamel. A variety of remineralizing agents have been used. The early enamel carious lesion management like fluoride, hydroxyapatite, Casein Phosphopeptide–Amorphous Calcium Phosphate (CPP-ACP), antibacterial bonding agents, laser, Xylitol, some herbal medicaments, and Ozone (4).

Fluorides act to enhance the exchanging of the hydroxyl group in hydroxyapatite crystals of enamel to form fluorapatite which results in an increase in acidic resistance and the remineralization process (5). Fluoride has injurious effects in high doses where it can pass through the cell membrane resulting in fluorosis with injurious effects on the tooth structure (6). Additionally, topical application of fluorides in the form of solutions gels, or varnishes results in the deposition of calcium fluoride on the enamel surface and exposed dentinal tubules, which is more efficient in the treatment of dentin hypersensitivity (7).

Harmala (Peganum harmala) is a flowering plant of the family Zygophyllaceae which is found in the Mediterranean region, central Asia, North Africa and Iran (8). P. harmala has different properties some of them are antibacterial, antifungal, antipyretic, anti-inflammatory, vasorelaxant, anti-tumor activity, anti-protozoal besides monoamine oxidase inhibition, and high antioxidant activities (9). Alkaloids can be produced in different parts of the plant that correspond to their pharmacological properties where beta-carbonyl derivatives such as harmalin, harmalol, pegane, isopegane, deoxyisopegane; as well as quinazoline derivatives such as vasicinone, vasicine, and deoxyvasicinone were considered from the most important alkaloids that derived from seeds and roots of this plant (10).

Therefore, this plant has been implicated in diabetes mellitus, jaundice, bronchial asthma, dermatitis, and other medical diseases (11). It has been found that the ethanolic extract of P. harmala can inhibit the growth of Streptococcus pyogenes, Staphylococcus aureus, and Staphylococcus epidermidis. Also, can inhibit other microorganisms found in oral cavity like lactobacillus and Candida albicans (12).

Nanotechnology is the study of the materials’ properties and their applications in the range of 1–100nm, where unique phenomena exist. It depends on properties of volume effect, surface effect, quantum size, and quantum tunnel from the materials at nanoscale level. Also, it has a higher ability to release ions than microparticles. Nowadays, nanomaterials are often added to resin composite restorations to release different ions that aids in the remineralization of dental substrates (13). Different nanoparticles including nanorings, nanopores, nanotubes, nanocapsules, nanospheres,
and dendrimers are being used nowadays in different fields. The smaller the diameter of nanoparticles, the larger the specific surface area, the more unique phenomena enabled and the more novel applications could be achieved (14).

Niosomes are nanostructures, possessing both hydrophilic and lipophilic properties, comprised mainly of nonionic surfactants and cholestrol and prepared by self assembly of nonionic surfactants in an aqueous medium forming bilayer closed nanovesicles. It is characterized by stability when stored at 4°C up to 6 months in compared to other nanovesicles (16), ease of preparation, shaping, low toxicity and it has ability to deliver many bioactive compounds, nutraceuticals including therapeutic agents. Consequently, it has a niosomal carrier preparation (17). Also, it was applied in the cosmetic industry because of being osmotically active, non-toxic, non-immunogenic, biocompatible and biodegradable.

Niosome is considered to be a promising drug delivery platform for different administration routes such as oral, parenteral, dermal/transdermal, ocular, and pulmonary (18). Also, it improves the pharmacokinetic and pharmacodynamic properties of herbal bioactive agents due to its ability to encapsulate either hydrophilic or hydrophobic compounds and resist its degradation (19). Hence, many trials and studies adopted aimed to improve the bioavailability and anticancer activities of different drugs by enclosing them into niosomes such as topotecan, letrozole, curcumin, and melittin (20).

There are two types of lipids nanoparticles: solid lipid nanoparticles (SLNs) and nanostructured lipid carriers (NLCs). The compounds of lipid nanoparticles are generally safe components, have a low production cost, less toxic. SLNs are prepared only with solid lipids, whereas the combination of solid and liquid lipids yields NLCs which results in providing a better drug entrapment ability and greater physicochemical stability (21).

Many researches have been adopted to measure antibacterial effect of P. harmala but non of them have been conducted before to test the remineralization capabilities of such a plant and when loaded as Niosome on dental substrate whether enamel or dentin.

The aim of this in-vitro study was to evaluate the remineralizing potential of harmala (Peganum harmala) as a natural plant, niosomes loaded harmala extract versus sodium fluoride on demineralized enamel surface. The null hypothesis claims that all of the agents used will have a potential remineralization effect on the demineralized enamel surface.

MATERIALS AND METHODS

Ethical Committee Consent:

The current study was granted approval from Badr University in Cairo BUC Institutional Ethical Committee with approval number: BUC-IACUC-230306-18.

Sample Size Calculation:

Sample size calculation was based on a continuous response variable derived from matched pairs in a prior study by Magalhães et al, 2021 (22). The investigation sought to evaluate how different suspensions impact the Knoop hardness values (KHN) of human teeth. This involved employing a dependent t-test or an equivalent non-parametric test to compare two distinct composites with results ranged from 70.6±6.8 to 80.5±3.2.

For determining Specimen sizes, G Power statistical analytic software (version 3.1.9.6) was utilized. It was established that having 10 samples in each group would be sufficient to detect an effect size (d = 1.86), achieving an actual power (1-β error) of 0.99 (99%) and a significance level (α error) of 0.05 (5%) for a two-sided hypothesis test.
Selection and preparation of teeth

A total of 60 freshly intact, extracted human upper first premolars were collected from patients requiring therapeutic extraction for orthodontic treatment. Calculus and soft debris were removed using a hand scaler and cleaned using distilled water. After that, teeth were cut 2 mm below the cementoenamel junction in a horizontal plane using a water-cooled diamond disc to separate the crown and root of the teeth from each other, the coronal part was sectioned longitudinally in mesiodistal direction into two halves resulting in 120 specimens using high-speed diamond disc under the copious amount of coolant. The buccal and palatal surfaces of the crowns were then embedded in acrylic blocks with their buccal and palatal surfaces exposed and facing up then a window of 3*3 mm was created on the enamel surfaces of all specimens by painting the remaining sample surface with nail varnish. \(^{(24)}\)

Sample grouping:

For Microhardness Assessment:

A total of 120 specimens have been used in this study for microhardness assessment. They were then divided into five main groups as shown in (Table 1)

- Negative control group (baseline group) (group E): 10 specimens of sound teeth were placed in distilled water throughout the study.

- Positive control group (demineralization group) (group D): 10 specimens were placed into 100 mL of 0.3% citric acid (pH 3.5) as a demineralization solution for 8 hours every day for 5 days.

The remaining 100 specimens were demineralized as specified in the demineralization solution (citric acid) as follows: A demineralization solution 0.3% citric acid (pH 3.5) was prepared by the Faculty of Pharmacy Cairo University. The pH of the demineralization solution was adjusted by the gradual addition of NaOH to the citric acid using a pipette. pH was measured and kept constant using a pH meter. Then, teeth were immersed in the demineralizing solution (100 ml) for 8 hours every day for consecutive 5 days to produce artificial caries-like lesions. The demineralized solution was changed daily. Subsequently, the specimens were rinsed thoroughly with deionized water to remove any residual acid \(^{(23, 24)}\).

Then, specimens were subdivided into 3 groups according to the different remineralizing agents used as follows:

- **Group A**: 40 specimens were placed in the Peganum harmala extract plant and were further divided into two equal subgroups of 20 specimens each according to plant extract concentration prepared:
  - **Group A1**: 5% concentration.
  - **Group A2**: 10% concentration.

Then each subgroup was further subdivided over two time intervals:

  - 10 specimens for 20 days
  - 10 specimens for 40 days

- **Group B**: 40 specimens were placed in niosome loaded harmala and were further divided into two equal subgroups of 20 specimens each according to plant extract concentration prepared:
  - **Group B1**: 5% concentration.
  - **Group B2**: 10% concentration.

Then each subgroup was further subdivided over two time intervals:

  - 10 specimens for 20 days
  - 10 specimens for 40 days

- **Group C**: 20 specimens were placed in sodium fluoride NaF and were further divided into two equal subgroups of 10 each according to the two-time intervals:
  - 10 specimens for 20 days
  - 10 specimens for 40 days
For EDXA & SEM Assessment:

A total of 30 extracted maxillary premolar teeth resulting in 60 specimens after the sectioning of crowns have been used in this study for EDXA and SEM assessment. They were then divided into five main groups as applied for the microhardness group. Five specimens for each subgroup were taken as representatives for Energy Dispersive X-Ray Analysis (EDXA) and Scanning Electron Microscope Unit (SEM) analysis.

TABLE (1) Variable groups used in this Study

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Immersion Period</th>
<th>5%</th>
<th>10%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>20 days</td>
<td>40 days</td>
</tr>
<tr>
<td>Group B</td>
<td>Niosome loaded harmala</td>
<td>15 (B1)</td>
<td>15 (B1)</td>
</tr>
<tr>
<td>Group C</td>
<td>Sodium fluoride NaF</td>
<td>15 (C1)</td>
<td>15 (C2)</td>
</tr>
<tr>
<td>Positive control group (D)</td>
<td>Demineralized enamel</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Negative control group (E)</td>
<td>Sound teeth (Baseline group)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Storage of the specimens:

Treated groups were placed in distilled water at room temperature and it was replaced daily for the whole period of this study.

Preparation of Peganum harmala extracts:

Peganum harmala dried mature seeds were collected. The former director of El-Orman Botanic Garden consultant at the Ministry of Agriculture, Mrs. Therese Labib, confirmed the authenticity, purity, and identity of such plant. A voucher specimen (18.1.17) was placed at the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Cairo University (Cairo, Egypt).

Extraction of harmala alkaloids: (preparation *)

P. harmala seeds (2.5 Kg) were ground to fine powder and extracted by maceration with 70% ethanol (3\times5 L). The ethanol extract was concentrated under reduced pressure to yield 520 g of a dark reddish-brown residue. The concentrated ethanol extract (450 g) was subsequently dissolved in HCl (5%, 2 L), filtered, and partitioned with dichloromethane (4 \times 300 mL). The aqueous acid layer was made alkaline to pH 9 with \(\text{NH}_4\text{OH}\) and extracted with methylene chloride (4\times500 mL) to yield 60 g of reddish-brown dichloromethane dry extract (25).

Preparation of drug-loaded niosome: (preparation **)

HAF-loaded SLNs were fabricated employing oil/water (O/W) emulsion approach using sonication as described elsewhere with some modifications (26). In brief, the oil phase, formed of HAF stearic acid (SA), and phosphatidylcholine (PC) in a molar ratio (2:1), was heated at 80\(^\circ\). Then, it was added to a preheated aqueous solution containing polymer 188 (1% w/v) while homogenized at 15,000 rpm in an ice bath, forming HAF/SLNs. Finally, the SLNs were ultrasonicated for 15 minutes in a bath sonicator (Elma Hans Schmidbauer, Singen, Germany).

** This drug loaded noisome was prepared by Dr. Sherif A. Fahmy: Department of Chemistry, School of Life and Medical Science, Hertfordshire University hosted by Global Academic Foundation. New Administrative Capital, Cairo, Egypt.

* These extract was prepared by Dr. Marwa Y. Issa: Department of pharmacognosy, Faculty of pharmacy, Cairo university, Egypt.
Characterization

Characterization of the prepared HAF/SLNs

Zetasizer Nano (Malvern Instruments, UK) was used to measure mean particle sizes, polydispersity index (PDI), and zeta-potential, where all measurements were carried out at 25 °C.

A transmission electron microscope (JEOL-JEM 2150, Musashino, Akishima, Tokyo, Japan), operating at 160 Kv, was used to study the morphological features of the developed niosomal nanovesicles.

Determination of entrapment efficiency % (EE)

The percent of HAF entrapped (EE %) into HAF/SLNs was determined using the indirect method. Briefly, an aliquot of each specimens (2 mL) was ultracentrifuged at 14,000 rpm for 120 min at 4°C then the supernatant of each specimens was separated, ultrafiltrated, afterward the UV-Vis spectrophotometry (Peak instruments T-9200, USA) at 270 nm was used to determine unentrapped HAF. The EE (%) was calculated employing equation (1) (Bhardwaj P, et al., 2020) (27).

\[
EE \% = \frac{\text{Initial amount of drug-the amount of unentrapped drug}}{\text{Initial amount of drug}} \times 15
\]

In-Vitro Release Study

The release rates of HAF were studied by exploiting the dialysis bag method at pH values (pH 7.4). Concisely, a dialysis bag (14KD cut off) containing 1 mL of each preparation was immersed in 25 mL of PBS (pH 7.4) as the release medium. Sodium dodecyl sulfate (SDS, 0.5%) was added to the release medium to increase the solubility of the hydrophobic drugs and to decrease the release resistance. Then, the system was located in a shaking incubator (Jeio tech SI-300, SEOUL, KOREA) at 37±0.5°C and a shaking rate of 130 rpm. At definite time intervals, 1 mL of each specimen was retrieved for estimation by either HPLC or UV-vis spectrophotometry (as previously described) and immediately replenished with an equal volume of fresh medium to maintain sink conditions. The release % of Y-oil and Ox was estimated using equation (2).

\[
\text{Release} \% = \frac{\text{Amount of released drug}}{\text{Initial amount of loaded drug}} \times 15
\]

Preparation of sodium fluoride NaF 5%:

A sensitive balance was used to measure the sodium fluoride powder (0.5 gm by weight) that was then mixed with 1 liter of distilled water, 8 gm propylene glycol (surfactant and co-surfactant respectively), and 8 gm glycerol, then transferred into a clean bottle. All of these solutions were freshly prepared at the Drug Manufacturing Unit (DMU), Faculty of Pharmacy, Cairo University, Egypt.

Preparation of 5% and 10% concentrations of the plants:

For both Peganum harmala and niosome loaded harmala concentrations, a stock solution (100 mL) of the Peganum harmala alkaloid rich fraction (PARF) in saline of concentration 1 mg/1 mL (100%) was prepared. The diluted concentrations were prepared by dilution with saline to get two different concentrations 5 and 10 % solutions of PARF.

Application of different remineralizing agents:

The buccal enamel surface for all prepared groups was immersed in the prepared concentrations of 5% and 10% of remineralizing agents for 15 minutes every day for 20 days and 40 days based on their subgroups. Then microhardness, SEM, and EDXA were investigated after each time interval (28), and tested solutions were refreshed daily.

Surface microhardness Assessment:

The specimens’ surface microhardness values were evaluated, after 20 and 40 days, using Vickers
hardness tester (Willson Microhardness tester HMV-2 Series, USA). A digital microhardness tester was used at baseline. Three indentations were made with a rate of 15 seconds and a load L of 150gf (HV 0.1) at a distance d of 500 μm from the edge, and three readings were recorded for each specimen. The indentations length was measured and Vickers values were converted into micro-hardness values. The Vickers hardness (HV) is calculated using this equation:

\[ HV = 1854.4 \frac{L}{d^2} \]

**Scanning electron microscope analysis:**

Then, all specimens of all subgroups were mounted on stubs and examined using a Scanning Electron Microscope Unit (SEM) at the Egyptian National Research Center (NRC) for determination of the morphological changes occurring on the tooth surface. Specimens were mounted on scanning electron microscope plate and the surface enamel was examined at magnification of 1500x and 2000x.

**Energy Dispersive X-Ray Analysis (EDX) analysis**

The quantitative composition of the studied surfaces was determined by Energy Dispersive X-ray Analysis (EDXA) which measured the number of emitted X-rays versus their energy. This analysis system works as integrated features of SEM (JXA-840 electron probe microanalyzer, JEDL, Japan). Data was then recorded and tabulated. Data was displayed as wt% for Calcium, Phosphorus, and Carbon.

**Statistical Analysis**

The statistical analysis of the provided data was conducted utilizing IBM SPSS software package version 24.0 (Armonk, NY: IBM Corp) and GraphPad Prism 18. Descriptive statistics, including means and standard deviations, were generated for the groups. Group comparisons were carried out using the Two-Way Analysis of Variance (Two Way ANOVA) followed by Tukey’s post hoc test for multiple comparisons, preceded by an assessment of normality through the Kolmogorov–Smirnov test and the Shapiro–Wilk test. The results of these tests indicated a normal distribution for the parametric data under consideration.

**RESULTS**

**Microhardness Results**

Table (2) presents Follow-up of Vicker’s Hardness of Human teeth after Different Remineralizing Preparations using Paired t-test and Two Way ANOVA. Five treatments were tested - 10% and 5% concentrations of harmala and niosome, and 5% NaF. Vickers hardness was measured at 20 days and 40 days after application.

The table shows that all treatments significantly increased tooth hardness from baseline after 20 days, with the 10% niosome showing the greatest increase of 69.7 Vicker’s units. After 40 days, hardness further increased substantially for the 10% and 5% harmala treatments, with the 10% concentration now conferring the greatest hardness improvement of 46.6 units.

Table (3) presents the descriptive statistics on the Vickers Hardness values of human teeth treated with different remineralizing preparations. It compares the effects of three different preparations (Harmala, niosome, sodium fluoride) at two different concentrations (5%, 10%) over the 40 day-period. The table also provides statistics for demineralized and sound enamel as controls.

Several notable results emerge from the data. First, all the remineralizing preparations increased the hardness relative to the etched enamel control, indicating they helped in remineralization and strengthening of the teeth. Second, the 10% solutions appeared somewhat more effective than the 5% solutions in all cases. The median hardness for 10% harmala was 176.4 in 40 days compared to 151.6 for 5% harmala. Third, niosome 10% led to the highest median hardness overall
suggesting that it may be the most effective preparation. Fourth, there was an increase in hardness from 20 days to 40 days for all preparations, implying the remineralization continued over the 40 days period. Fifth, the range and standard deviations are quite wide, indicating substantial variability among the teeth in response to the treatments.

These results demonstrate that the natural remineralizers harmala and niosome can effectively harden softened enamel, potentially reversing the early stages of decay. The 10% niosome led to the greatest overall hardening after 40 days. The study shows the feasibility of using these natural compounds as an alternative remineralization therapy to fluoride. Further research could explore if combinations of harmala and niosome have an additive effect, and perform clinical trials on decay prevention and arresting capabilities. The potential to remineralize enamel using non-toxic natural products is promising for improved dental health.

Table (4) presents the results of a study comparing the Vickers hardness of human teeth following treatment with different remineralizing agents. The study utilized a two-way ANOVA and Tukey’s post-hoc test to analyze the data.

The post-hoc analysis reveals some notable findings. harmala 10% and niosome 10% resulted in significantly higher hardness than other groups in 20 days. In 40 days, niosome 10% remained highest, while harmala 10% dropped to levels comparable to other treatments. This suggests the early benefits of harmala 10% diminish over time compared to niosome 10%. NaF 5% also showed significantly higher hardness than some groups by 40 days, indicating a delayed but meaningful remineralizing effect.

Overall, the data indicates that 10% concentrations of both harmala and niosome provide early remineralization benefits, but niosome appears more durable over time. NaF also imparts benefits but with a slower onset. The sound and etched enamel groups represent positive and negative controls, with expected hardness levels.

In summary, this table presents a well-designed study comparing remineralizing agents using appropriate statistical tests. The post-hoc analysis reveals interesting trends over time, with niosome 10% appearing most effective for durable remineralization. The data could help dentists to select optimal treatments to strengthen enamel and prevent tooth decay. Further studies on larger specimens would help confirm these preliminary findings.
TABLE (3) Descriptive statistics of Vicker’s Hardness of Human Teeth after Different Remineralizing Preparations:

<table>
<thead>
<tr>
<th></th>
<th>Harmala 10%</th>
<th>Harmala 5%</th>
<th>Niosome 10%</th>
<th>Niosome 5%</th>
<th>NaF 5%</th>
<th>Etched Enamel</th>
<th>Sound Enamel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20 days</td>
<td>40 days</td>
<td>20 days</td>
<td>40 days</td>
<td>20 days</td>
<td>40 days</td>
<td></td>
</tr>
<tr>
<td>Min</td>
<td>128.6</td>
<td>175.2</td>
<td>109.1</td>
<td>150.3</td>
<td>171.2</td>
<td>228.1</td>
<td>137.8</td>
</tr>
<tr>
<td>25% Percentile</td>
<td>128.8</td>
<td>175.2</td>
<td>109.1</td>
<td>150.5</td>
<td>171.2</td>
<td>230.8</td>
<td>137.8</td>
</tr>
<tr>
<td>Median</td>
<td>129.4</td>
<td>176.4</td>
<td>117.9</td>
<td>151.6</td>
<td>171.7</td>
<td>243.6</td>
<td>138.6</td>
</tr>
<tr>
<td>75% Percentile</td>
<td>130.5</td>
<td>177.8</td>
<td>118.1</td>
<td>152.5</td>
<td>172.3</td>
<td>249.4</td>
<td>140.6</td>
</tr>
<tr>
<td>Max</td>
<td>131.4</td>
<td>177.8</td>
<td>118.1</td>
<td>152.6</td>
<td>172.4</td>
<td>253.2</td>
<td>140.6</td>
</tr>
<tr>
<td>Range</td>
<td>2.8</td>
<td>2.6</td>
<td>9</td>
<td>2.3</td>
<td>1.4</td>
<td>25.1</td>
<td>2.8</td>
</tr>
<tr>
<td>M</td>
<td>129.6</td>
<td>176.5</td>
<td>115</td>
<td>151.5</td>
<td>171.7</td>
<td>241.4</td>
<td>139</td>
</tr>
<tr>
<td>SD</td>
<td>1.039</td>
<td>1.301</td>
<td>5.139</td>
<td>9.347</td>
<td>0.5601</td>
<td>9.642</td>
<td>1.442</td>
</tr>
<tr>
<td>SEM</td>
<td>0.424</td>
<td>0.7513</td>
<td>2.967</td>
<td>3.936</td>
<td>0.2286</td>
<td>3.936</td>
<td>1.45</td>
</tr>
<tr>
<td>Lower 95% CI</td>
<td>128.5</td>
<td>173.2</td>
<td>102.3</td>
<td>150.5</td>
<td>171.1</td>
<td>231.3</td>
<td>135.4</td>
</tr>
<tr>
<td>Upper 95% CI</td>
<td>130.7</td>
<td>179.7</td>
<td>127.8</td>
<td>152.5</td>
<td>172.3</td>
<td>251.5</td>
<td>142.6</td>
</tr>
</tbody>
</table>

*M; Mean, SED; Standard Error of Difference   *Significant difference a P<0.05.

TABLE (4) Multiple Comparisons of Vicker’s Hardness of Human Teeth following using Different Remineralizing Preparations using Two Way ANOVA and Tukey’s Post Hoc Test:

<table>
<thead>
<tr>
<th></th>
<th>Harmala 10%</th>
<th>Harmala 5%</th>
<th>Niosome 10%</th>
<th>Niosome 5%</th>
<th>NaF 5%</th>
<th>Etched Enamel</th>
<th>Sound Enamel</th>
<th>P-value (Two Way ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20 days</td>
<td>40 days</td>
<td>20 days</td>
<td>40 days</td>
<td>20 days</td>
<td>40 days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
<td>E</td>
<td>F</td>
<td>G</td>
<td>H</td>
<td>I</td>
</tr>
<tr>
<td>Tukey’s Post Hoc</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td></td>
</tr>
</tbody>
</table>

*M: mean           SD: standard deviation   *Significant difference a P<0.05.

Means with the same superscript letters in the same row were insignificantly different as P > 0.05.

Means with different superscript letters in the same row were significantly different as P <0.05.
Scanning Electron Microscope Results

**Negative control (group E)**

Smooth surface of the enamel structure has been observed with no porosity with minute few depressions, with relatively smooth enamel surface at the cervical third, with evident perikymata, and the occlusal third showed shallow pits corresponding to Tomes’ processes of ameloblasts were occasionally seen.

**Positive control (group D)**

Teeth in the demineralization group revealed uneven and rough enamel surface with marked increased porosities. Rough surface at the cervical third with numerous pits occupying distinctly surface area were observed. Cervical region with higher magnification revealed pits with variable depths and widths, few of them are wide and others are deep. At the middle, occlusal thirds showed apparent defects and enamel rod ends with a honeycomb pattern of demineralization.

**Harmala 5% (group A1)**

Teeth in this group revealed enamel surface with partial occlusion of some rod cores with obviously thickened interprismatic substance.

**Niosome 5% (group B1)**

Teeth in this group revealed an enamel surface with occlusion of more rod cores with obviously thickened interprismatic substance with increasing areas of remineralization and shallow depressions with variable sizes can be detected.

**Harmala 10% (group A2)**

Teeth in this group revealed that harmala 10% remineralization showed mineral particles deposition on the enamel surface and smooth surface that was surrounded by a layer of less remineralized crystals.

**Niosome 10% (group B2)**

The teeth in this group revealed intact enamel surface at the cervical third with no detectable defects in many areas with regular surface. Less regular surface has been observed at the middle and occlusal thirds than that of the cervical third with non-pitted defective areas. With high magnifications, shallow pits on the enamel surface were occasionally seen.

![Fig. (1) Scanning electron micrographs of negative control group (E): E1 middle third showing smooth surface with few depressions (arrow)- E2 cervical third showing smooth enamel surface with perikymata (arrow) -E3 Occlusal third showing smooth enamel surface with shallow pits (arrow) and positive control group (D): D1 cervical third showing rough enamel surface with numerous pits (arrow) (D2) higher magnification of cervical area reveals the depth and width of numerous pits (arrow) (D3) middle, occlusal thirds showing apparent defects of enamel surface with a honeycomb pattern of demineralization.](image-url)
Sodium fluoride (group C)

Teeth in this group revealed that remineralization with Sodium fluoride resulted in the deposition of mineral particles on the enamel surface with less regular enamel surface and some areas of pitted defective areas that was surrounded by a layer of remineralized crystals.

Energy Dispersive X-Ray Analysis (EDXA) results

Energy Dispersive X-Ray Analyzer (EDXA) is an analytical technique used to identify the elemental composition of materials in this study in conjunction with SEM analysis to provide elemental identification and quantitative compositional information for the deposited mineral particles. Surface Ca, PO4, and F weight percent (wt%) were also estimated on the enamel surface.

![Fig. (2) Scanning electron microscope of different remineralizing agents: group A1: (A1-a) showing enamel surface with partial occlusion of some rod cores (arrows), (A1-b) Higher magnification showing noticeable areas of remineralization with thickened interprismatic substances (arrows). Group B1:(B1-a) showing enamel surface with occlusion of more enamel rods, (B1-b) higher magnification showing more areas of remineralization and shallow depressions (arrows). Group A2: (A2-a) showing deposition of mineral particles on enamel surface at cervical third, (A2-b) higher magnifications of enamel rods with mineral particles on the enamel surface. Group B2: (B2-a) cervical third showing intact enamel surface with no detectable defects, (B2-b) middle third showing less regular surface with areas of non-pitted defective areas (arrows) (B2-c) higher magnification showing shallow pits on enamel surface. Group C: (C1) deposition of mineral particles on enamel surface with less regular enamel surface and some areas of pitted defective areas (C2) higher magnifications of enamel rods with mineral particles deposited.](image-url)
The changes in the concentration of the total mineral of specimens in the different groups are summarized in Table (5) and presented in Figure 3. As for all groups, the positive control group (D) had the lowest Ca/P ratio (59.2/65.48) and the highest Carbon content (15.3). On the other hand, niosome 10% (group B2) had the highest Ca/P ratio (332.12/207.79).

**TABLE (5) Representing different EDXA spectra for the selected specimens (wt%) for different minerals**

<table>
<thead>
<tr>
<th>Group</th>
<th>C</th>
<th>O</th>
<th>P</th>
<th>Ca</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control group (E)</td>
<td>6.56</td>
<td>115.35</td>
<td>383.37</td>
<td>526.38</td>
</tr>
<tr>
<td>Positive control group (D) (demineralized)</td>
<td>15.3</td>
<td>79.35</td>
<td>65.48</td>
<td>59.2</td>
</tr>
<tr>
<td>Sodium fluoride group (C)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Harmala 5% group (A1)</td>
<td>3.26</td>
<td>11.88</td>
<td>69.74</td>
<td>136.13</td>
</tr>
<tr>
<td>5% group (B1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Harmala 10% group (A2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Niosome 10% group (B2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. (3): Representative figures for the elemental analysis by EDX spectra of enamel surface of specimens (E, D, A1, B1, A2, B2, C) illustrate different EDX spectra for the selected to initiate from all examined groups.
DISCUSSION

Natural products are considered the center of attraction of many studies, to be used as new therapeutic agents. Remineralization can be defined as the process whereby ions from the external source as calcium and phosphate where deposited into crystal voids of demineralized enamel, resulting in the rebuild of the crystalline structure with minerals and preserving the remaining tooth structure. (Jawale et al, 2017) (28).

The study aimed to evaluate the remineralization effect of harmala (Peganum harmala) as a natural plant, niosome-loaded harmala extract and sodium fluoride on artificially demineralized enamel using different testing methods which are microhardness test, Energy Dispersive X-ray Analysis (EDXA) and scanning electron microscope. The null hypothesis tested in our present study was accepted since all of the evaluated materials studied showed variable remineralization potential effects on the demineralized enamel surface of premolar teeth.

Surface enamel has enamel rods, rod sheaths and interrod substance, since these features and the abrupt changes in crystal orientation from one rod to another make it difficult to investigate, so examined using scanning electron microscope SEM is an advantage technique to render enamel surface more visible. (Sabel, 2012) (29). Where it has a large field depth, and much higher resolution allowing more specimens to be focused at one time with higher magnification of closely spaced specimens as stated by Mohanty, et al, in 2014 (30). The use of EDXA in this study is considered a confirmatory and precise test for mineral content evaluation percentage on the enamel surface before and after remineralizing agents were applied where it demonstrates the percentage of calcium and phosphorous since those minerals are found in a high concentration on the enamel surface (Sathe et al, 2014) (31).

Enamel surface was selected in this study rather than dentin because it is hard structure as it has local differences in porosity and acid solubility; where acids penetrate deeply into enamel results in dissolving tooth minerals locally. Hence, remineralization of early enamel lesions is more valuable to protect underlying dentin as mentioned by Talwar et al, in 2019 (32). While dentine remineralization is more difficult and ineffective than in enamel because enamel lesions have remaining seed mineral crystals, that not occurred in dentine lesions Cao et al, 2015 (33).

In this study, non-carious human premolars stored in distilled water to initiate the artificial subsurface lesion which was examined by using a magnifying lens (TH-600600, China.) of ×7 to exclude any tooth with cracks or other structural defects (Abdelaziz et al, 2009) (34). Demineralization of enamel has been achieved through the using of citric acid in-vitro at pH 5 or higher. Where this acid has effectiveness in producing apparent demineralized enamel lesion and the PH cycling models have been used to mimic PH periodic alteration in-vivo as stated by Abdelaziz et al, in 2019 (35).

Moreover, the specimens that had been exposed to the acidic demineralizing solution showed regions of uneven and rough enamel surface with marked increased porosities and regions of enamel surface dissolution. This finding agreed with El-Haddad, in 2018 (36). Additionally, we demonstrated apparent defects and enamel rod ends with a honeycomb pattern at the occlusal thirds denotes demineralization in our findings which were in accordance with Karlinsky et al, in 2012 (37) who revealed that using acids dissolve calcium and phosphate ions, causing gaps between crystals that result in enamel porosity. This occurs when hydroxyapatite reacts with hydrogen ions at acidic pH ≤ 5.5 leading to the creation of gaps among crystals that produce a rough surface and the appearance of white chalky spot lesions, which is reversible by remineralization at the enamel surface.

In this study, we used EDXA to determine the amount of the calcium and phosphorus on the tooth
surface. Also, it has been confirmed the SEM findings in our results because the demineralization group had a lower Ca/P ratio, which indicated a chemical demineralization process. This could be illustrated by a decrease in PH below a certain point, which causes the dissolution of enamel hydroxyapatite and subsequent demineralization as explained by Yu et al, in 2017 (38). These results agreed with Seow et al, in 2005 (39), who examined the etching effects of the acidic beverage on the enamel surface, and in accordance with Colombo et al, in 2016 (40), who revealed the diffuse demineralization involved the rod core compared with that in the interprismatic areas gave the enamel a “honeycomb pattern” or “keyhole pattern” of demineralization.

In concern with group of drug-loaded niosome harmala plant, it showed higher microhardness values than group of harmala extract. These findings were somehow relevant to Wang, et al., in 2011 (41) who explained the smaller size of nanoparticles with high effectiveness in form of better ionic exchange with hard nanoparticles crystal on the enamel surface and higher remineralization.

At our present study, 10% concentration used for both harmala plant extract and niosome loaded harmala recorded the highest mean microhardness value than using 5% concentration. Also, there was an increase in the mean microhardness value recorded for harmala plant extract and niosome loaded harmala after 40 days of immersion comparable to the period of 20 days which showed a lower mean value of microhardness. The highest mean microhardness value recorded was seen in niosome group 10% concentration after 40 days (228.1, 253.2). Also, the lowest mean microhardness value was seen in harmala group 5% after 20 days (109.1, 118.1).

It was shown that sodium fluoride 5% concentration showed high mean microhardness values for both periods 20 days and 40 days in comparison to 5% concentration in both harmala and niosome loaded harmala.

Our findings of treated groups with harmala as a natural extract showed improvement to varying degrees in terms of deposition of mineral particles on the enamel surface and smooth surface that agreed with Mohamed, et al, in 2023 (41) who demonstrated the effect of solution forms of theobromine and silver diamine fluoride in the remineralization potential of the demineralized enamel surface. Also, this agreed with the results of Bilgin et al, in 2016 (42) who explained that two herbal mixtures of chocolate, ginger, and honey have a positive effect on the remineralization of early enamel lesions.

On the other hand, treated groups with harmala promoted enamel remineralization as P. harmala was found to be rich alkaloid sources especially in seeds and roots of the plant, which has a role in protecting the plant against microorganisms, herbivores, and insects in the statement of Kalesinskas et al, in 2014 (43). This notion is supported by scientific documentation of Motamedifar et al, in 2018 (44) who mentioned that the growth of S.mutans can be restrained when using ethanolic extract of P. harmala.

Surface Microhardness (SMH) of the specimens in this study was done to estimate demineralization and remineralization process of the enamel surface as it is considered a simple, popular technique because it permits determination of hardness in the same specimens before and after the treatments with minimal experimental failure Kamh, et al, 2018 (45).

The results of the current study demonstrated that the mean microhardness values of all samples have been decreased after immersion in the demineralizing agent which was in accordance with other studies that reported that initial enamel lesions with intact surfaces recorded low mineral content due to loss of calcium and phosphate ions when compared to sound enamel; thus, demonstrating a lower microhardness value at the surface than for sound enamel tissue as mentioned by Soares, et al, in 2017 (46).
Niosomes are vesicles similar to liposomes with non-ionic surfactants. Additionally, they are capable of encapsulating either hydrophilic or hydrophobic compounds providing shields for degradation, and upgrading their bioavailability and therapeutic effect. Moreover, it enhances the pharmacokinetic and pharmacodynamic properties of herbal bioactive decreasing its toxicity. Therefore, niosomes can be used as carriers to deliver different drugs, hormones, and antigens as stated by Machado, et al, in 2021 (47).

Many literatures state that fluoride application remains the best remineralizing agent for the treatment of early enamel carious lesions. Unfortunately, it lacks the power to guide the mineral crystal formation, and is unable to form mineral crystals on the enamel surface (Fan, Sun and Moradian-Oldak, 2009) (49) beside exposure to high fluoride doses can lead to dental fluorosis or even skeletal fluorosis in severe cases as mentioned by Aoun, et al, in 2018 (50).

In our study, the sodium fluoride group with concentration of 5% showed the highest microhardness values than other groups with the same concentration, this was attributed to that Fluoride strengthens enamel by preventing, preventing dissolution of hydroxyapatite crystals. Also fluorides acting by exchanging the hydroxyl group in hydroxyapatite of enamel to form fluorapatite that was found to be more resistance to acidic attacks. For this purpose, generally, fluorides have been used in toothpaste formulations. Whereas during the process of partially dissolved minerals, fluoride ions will be more valuable when they are included into the crystalline network, and the remineralization process occurs as stated by Kim et al, in 2021 (53).

The current invitro study was done away from the patient’s mouth as the saturation degree of oral fluids (saliva and plaque) controls the remineralization process, and when exceeding the remineralization this leads to healing process. An increase in calcium or fluoride concentrations in the oral fluids would appear promising to improve the remineralization process of different remineralizing agents as in a statement by Sharma, et al, 2017 (54).

The enamel surface of the specimens treated with harmala plant extract and loaded niosome showed a remineralization effect on the demineralized enamel surface with regular enamel surface at the cervical area with non-pitted defective areas at the middle and occlusal thirds comparable to sodium fluoride group that showed deposition of mineral particles on the enamel surface with less regular enamel surface and some areas of pitted defective areas that was surrounded by a layer of remineralized crystals.

Histological examination using SEM and mineral content measurement by EDXA have been used at this study for comparison with the results obtained by microhardness test for more relevant and accurate results and it has been showed that the results were almost similar, correspondent and highly matching.
CONCLUSION

It was concluded that time and concentration increase the remineralization potential for different plant materials used and also for sodium fluoride. Harmala plant extract and niosome-loaded harmala enhanced the remineralization potential of demineralized enamel and hence could be considered effective natural therapeutic remineralizing agent.

This data provides evidence that all three remineralizing preparations help strengthen the etched enamel, with niosome 10% appearing the most effective overall. The strengths of the study include testing multiple preparations at two concentrations over two-time points and the inclusion of etched and sound enamel controls. Limitations are the small sample size per group and the lack of statistical tests comparing the differences between groups. Further studies could analyze these data statistically and investigate the mechanisms behind the different remineralization efficacy. But overall this provides useful pilot data on the remineralizing potential of these preparations.

CLINICAL RECOMMENDATIONS

Corresponding studies of this herb in vivo are suggested to investigate the cytotoxicity on oral epithelial cells for more accurate results, to use precise preparations and concentrations of this plant as mouthwash for enamel remineralization.

Acknowledgment

The Authors would like to give their thanks and gratitude to Dr. Marwa Y. Issa and Dr. Sherif A. Fahmy for the preparation and supplying the plant extract and niosome-loaded used in this study.

REFERENCES


