The Synergistic Effect of Aqueous Extracts of Iraqi Propolis and CPP-ACP Paste on Enamel Microhardness after Demineralization Challenge.

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ABSTRACT

Aims: This study endeavors to estimate the synergistic effect of aqueous extracts of propolis on microhardness power of fluoridated CPP-ACP (MI plus) paste after demineralization challenge. Materials and methods: A total of (75) posterior wisdom teeth were used in the study. Enamel blocks were prepared and divided into five groups randomly, the teeth in all groups were subjected to demineralization cycle and then treated with: Sinjar's aqueous extract of propolis (AEP) -MI paste plus cream n. (15), Sulaymaniah's AEP-MI paste plus cream n. (15), Duhok's AEP-MI paste plus cream group n. (15), control positive group of MI paste plus alone n. (15), and control negative group of artificial saliva alone n. (15). Microhardness of enamel blocks was measured using Vickers microhardness tester machine at base line, after demineralization cycle and finally after treatment protocol. Results: Statistically, there were highly significant differences among study groups after demineralization cycle and there was a decrease in surface microhardness in all groups after demineralization, but the least reduction in surface microhardness belonged to mixture of Sulaymaniah's aqueous extract of propolis with MI paste plus followed by MI paste plus alone group after treatment protocol. Conclusions: Mixture of Sulaymaniah's aqueous extract of propolis with MI paste plus was significantly better than MI paste plus alone in preserving enamel's hardness and resisting the demineralization challenge.

Keywords: Enamel demineralization, propolis, CPP-ACP paste, microhardness.

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INTRODUCTION

Deminerlization of enamel leads to the appearance of white spot lesions, also it leads to the dissolution of apatite crystals and the net loss of calcium, phosphate, and other ions from the tooth. The objective of contemporary dentistry is to manage non-cavitated caries lesions non-invasively through remineralization in an attempt to inhibit the progression of dental disease (1).

Fluoridated casein phosphopeptide - amorphous calcium phosphate (CPP-ACPF) (MI Paste Plus) is one of the frequently used remineralization materials (1). CPP–ACP is made by the complication of casein phosphopeptide (CPP) with amorphous calcium phosphate (ACP) through a phosphorylated peptide chain (2). Numerous studies have stated the effectiveness of the CPP-ACP technology in preventing demineralization and enhancing remineralization of enamel and dentin (3). It has been recommended that remineralizing agents have anticariogenic and anti-erosive properties (4). It has been stated that adding fluoride to CPP-ACP could give a synergistic effect on enamel remineralization (5). On the other hand, the combination of CPP-ACP with fluoride led to localization of calcium and phosphate ions with fluoride ions at the enamel surface (6).

A new alternative to dental caries prevention is the development of natural materials as propolis and propolis’ fluoride. The choice of natural-based medicines is based on the fact that the side effects of traditional medicine are lesser than chemical or synthetic drugs (7).

Propolis is a natural substance collected by honey bees from different plants (8). It has anti-microbial and anti-inflammatory properties owing to the presence of flavonoids (9). In dentistry, propolis has been used for the treatment of gingivitis, periodontitis, candidiasis, aphthous ulcers, and pulpitis (10) and for prevention of dental caries lesions (11).

The aim of the current study is to speculate the synergistic effect of aqueous extracts of propolis on the microhardness power of fluoridated CPP.ACP paste after demineralization challenge.

MATERIALS AND METHODS

The study was approved by Research Ethics Committee board (University of Mosul, College of Dentistry, REC reference No. POP/M.13/12/20).

Collection and Extraction of Propolis
The unrefined propolis (Apis mellifera) was obtained from three different regions in the North of Iraq. The first type was from Sinjar's mountains/Nineveh, the second type was from Duhok's mountains and the third one was from Sulaymaniah's mountains. For aqueous extract preparation, Krell method (12) was followed. The extract was clear from any impurities, dark and viscous and its odor is also distinguishable for each specific type (13).

Formulations of the Fluoridated CPP–ACP Propolis Extracts Tooth Coating Complex

Both ingredients were combined into a cream formulation made from a base of starch and other suitable binding material (glycerin). The fluoridated CPP-ACP paste and distilled water were adjusted at a specific ratio and mixed with the base solution, then propolis extracts 30% were added after complete dissolving in ethanol, shaken well via vortex tube stirrer (Dragon Lab) then placed in the lyophilizer (Labconco, USA) till complete evaporation of the solvent and homogeneous cream was obtained for the three types of propolis as shown in Figure (1).

Sample Collection

The samples in this study consisted of (75) human permanent third molars extracted for the impaction reason. After extraction, the teeth were cleaned with tap water and examined with 10 × magnifying lens, the selection of the teeth followed specific criteria; the teeth must be sound, free from enamel defects, decay, stain,
cracks, hypoplasia, and fluorosis and unaltered by extraction procedure. The teeth were stored in 0.1% thymol solution at 4°C (14) to avoid dehydration and prevent bacterial growth until their use within three months.

**Preparation of Enamel Blocks**

Sound extracted third molars were cleansed accurately before using, they were polished with non-fluoridated pumice and white rubber prophylactic cup using a low speed hand piece, wiped free of soft tissue debris and rinsed in tap water then the crowns separated from the roots via a diamond disc bur in the high speed hand piece cooled with water, followed by mounting the crowns in cylindrical plastic tubes (16 mm diameter×14 mm depth) with cold cure acrylic resin with the outer buccal enamel surface exposed. The buccal surface of all samples polished using 240, 400, 600, and 1200 grit silicon carbide abrasive papers under flooding water to obtain standardized flat enamel surface (15) then smoothed by using the universal polisher machine (Metaserv, England). Each specimen was then coated under the digital stereomicroscope (X 40) with two layers of acid resistant nail varnish, leaving 3×3 mm² window on the middle third of the enamel surface to define the experimental area (16,17) as shown in Figure (2).

![Figure 2](image_url)

**Figure (2):** Preparation of Enamel Blocks, (A) Cylindrical Plastic Tubes, (B) Separation of Crowns from the Roots, (C) Crowns After Cutting, (D) Cold Cure Acrylic Mold & Varnish Application

**Materials**

Commercially available topical cream with bioavailable calcium and phosphate (GC America, Recaldent, Alsip, USA), which contains 10 % by weight of CPP–ACP in addition to So-
dium fluoride 0.20 % (MI Paste Plus) was used in remineralization of one group of the sample in addition to previously formulated fluoridated CPP–ACP-propolis aqueous extracts (AEP-MI plus) tooth coating complex of three types (Sinjar, Sulaymaniah and Duhok) for remineralization of other groups in the study.

Design of Study and Methods of application

The total number of teeth samples in the study was (75) samples, randomly divided into five groups, (15) samples in each group as follows:

Group 1: control negative group (N. =15), after immersion of the teeth samples in demineralization solution, they were immersed in artificial saliva only that it is changed daily for 14 days.

Group 2: control positive group (N. =15), after immersion of the samples in demineralization solution, the enamel surfaces were coated by a fine brush with a thin layer of Sinjar's AEP-MI plus paste complex and left for 30 minutes then washed with deionized water and kept in artificial saliva that it is changed daily. This procedure was repeated twice daily for 14 days.

Group 3: Sinjar's AEP-MI plus paste complex group (N. =15), after immersion of the samples in demineralization solution, the enamel surfaces were coated by a fine brush with a thin layer of Sinjar's AEP-MI plus paste complex and left for 30 minutes then washed with deionized water and kept in artificial saliva that it is changed daily. This procedure was repeated twice daily for 14 days.

Group 4: Sulaymaniah's AEP-MI plus paste complex group (N. =15), after immersion of the samples in demineralization solution, the enamel surfaces were coated by a fine brush with a thin layer of Sulaymaniah's AEP-MI plus paste complex and left for 30 minutes then washed with deionized water and kept in artificial saliva that it is changed daily. This procedure was repeated twice daily for 14 days.

Group 5: Duhok's AEP-MI plus paste complex group (N. =15), after immersion of the samples in demineralization solution, the enamel surfaces were coated by a fine brush with a thin layer of Duhok's AEP-MI plus paste complex and left for 30 minute then washed with deionized water and kept in artificial saliva that it is changed.
daily. This procedure was repeated twice daily for 14 days.

**Demineralization Procedure**

Before application of treatment protocol, each group was individually suspended in demineralizing solution for 5 days at temperature of 37 °C to create artificial caries like lesions. The demineralizing solution contained 2.2 mmol/L CaCl$_2$, 2.2 mmol/L NaH$_2$PO$_4$ and 50 mmol/L acetic acid adjusted to pH 4.5 with NaOH at 37 °C. The pH values of the demineralization solution were checked every day using a pH meter and the solution was changed every day (16).

**Surface Microhardness Measurement**

The surface microhardness (SMH) of the specimens in all groups was determined using a Vickers microhardness testing machine as shown in Figure (3) with a Vickers diamond pyramid indenter, which has a square-based diamond indenter with a 136° angle and 600 x lens magnification of scaled microscope (18). Enamel microhardness was measured for sound enamel at baseline, after demineralization-cycling and after treatment regime in each tested group with constant load of 500 g and time (15 seconds) throughout the whole study.

**Figure (3):** Surface Microhardness Measurement, (A) Vickers Microhardness Testing Machine, (B) Optic Microscope, (C) An Image of a Tetra Pyramidal Indentation Under Microscope.

**RESULTS**

The data were analyzed using SPSS program (version 19). Table (1) delineated one way analysis of variance (ANOVA) test for comparison of mean values of VHN (Vickers Hardness Number) between the groups at baseline, after demineralization cycle and after treatment scheme. Results
showed that there was a highly significant difference at $p \leq 0.01$ of mean microhardness values among tested groups in the three stages of the study.

Table (1): Analysis of Variance (ANOVA) Test of Mean Microhardness Values for Comparison between Aqueous Extracts of Propolis Groups & Controls at Every Stage in the Study.

| Time                 | Source of variance | Sum of Squares | DF | Mean Square | F     | Sig. |
|----------------------|--------------------|----------------|----|-------------|-------|------|
| **Baseline data**    | Between Groups     | 18893.872      | 4  | 4723.468    | 200.582 | .000*|
|                      | Within Groups      | 1648.414       | 70 | 23.549      |       |      |
|                      | Total              | 20542.286      | 74 |             |       |      |
| **After demineralization** | Between Groups   | 3296.033       | 4  | 824.008     | 136.270 | .000*|
|                      | Within Groups      | 423.282        | 70 | 6.047       |       |      |
|                      | Total              | 3719.315       | 74 |             |       |      |
| **After treatment**  | Between Groups     | 252602.850     | 4  | 63150.713   | 2.978E3 | .000*|
|                      | Within Groups      | 1484.494       | 70 | 21.207      |       |      |
|                      | Total              | 254087.344     | 74 |             |       |      |

Table (2) formulated means, number, standard deviation and Duncan's multiple range tests of VHN mean values of the enamel blocks of the tested groups. The results of the mean microhardness values were, statistically, significantly different for the tested groups at all stages but after treatment, the highest VHN mean value was found in Sulaymaniah's AEP-MI plus paste complex group followed by MI plus paste (control positive) group, then Sinjar's AEP-MI plus paste complex group followed by Duhok's AEP-MI plus paste complex group while the lowest value was found in control negative group that was preserved in artificial saliva only. It is obvious that all of the remineralizing treatment pastes increased the VHN mean values above the baseline means except for artificial saliva.
### Table 2: Mean Microhardness Values, Standard Deviation And Duncan's Multiple Range test for comparison between aqueous extracts for Sinjar, Sulaymaniah & Duhok's Propolis Groups & Controls in the three stages of experiment.

| Groups          | Variables | Baseline data | After demineralization | After treatment |
|-----------------|-----------|---------------|-------------------------|-----------------|
|                 | Mean      | 306.480 d     | 203.468 d               | 257.526 e       |
| Control -       | N         | 15            | 15                      | 15              |
|                 | Std. Deviation | 4.21448     | 2.27920                 | 3.24304         |
| Sinjar aqueous  | Mean      | 342.868 b     | 219.424 b               | 394.154 b       |
|                 | N         | 15            | 15                      | 15              |
|                 | Std. Deviation | 4.98179     | 2.55137                 | 6.14601         |
|                 | Mean      | 349.558 a     | 212.824 c               | 386.146 c       |
|                 | N         | 15            | 15                      | 15              |
|                 | Std. Deviation | 5.13307     | 2.43798                 | 5.96085         |
| Duhok aqueous   | Mean      | 348.653 a     | 213.254 c               | 428.248 a       |
|                 | N         | 15            | 15                      | 15              |
|                 | Std. Deviation | 5.03693     | 2.40104                 | 3.50343         |
|                 | Mean      | 336.370 c     | 222.838 a               | 367.866 d       |
|                 | N         | 15            | 15                      | 15              |
|                 | Std. Deviation | 4.84197     | 2.61180                 | 3.15257         |

*Duncan's Multiple Range Tests: Means with different letters are statically significant vertically (within the same column).

### DISCUSSION

In this study microhardness test was selected because it is simple, economical and as well an effective method to evaluate and compare the remineralization and demineralization changes (19). Thus, the square shape of indent obtained in Vickers hardness testing is easy and more accurate to measure, for that reason Vickers hardness testing was employed. Even the tiny changes in the square shape indent obtained after the test can be easily detected (20). Enamel hardness differs depending on the local variations from enamel rods and tufts, degree of mineralization of enamel and increased porosity near the dentino-enamel junction (21).

In current study the use of presynthetic acid followed by the application of remineralizing agent was done to enhance the resistance to acid challenge produced by acidic drinks and
foods in the oral environment (22). Also, artificial saliva was used as a control negative group and as a storage medium after the erosion process to be similar to the oral environment (23).

The results of this study revealed that the surface microhardness values of enamel specimens in all groups were decreased compared with the baseline values after demineralization cycle, next, microhardness of all groups increased after treatment protocol compared to the surface microhardness values measured after demineralization. These results disagree with the results of Hongal et al. (7) which did not show any remarkable difference after application of acid among the test groups. The result of the current study in agreement with Featherstone et al. (24) who used microhardness profiles to compare the artificial caries-like lesions and concluded that loss or gain of mineral in dental enamel due to demineralization and remineralization can be measured as hardness change. It was stated that the enamel hardness differs depending on the degree of mineralization of the enamel.

The unstabilized amorphous calcium phosphate (ACP) systems provide calcium ions with phosphate ions that cause immediate precipitation of ACP or, in the presence of fluoride ions, amorphous calcium fluoride phosphate (ACFP). In the intra-oral environment, these phases (ACP and ACFP) are potentially very unstable and may quickly convert into a more thermodynamically stable, crystalline phase (e.g., hydroxyapatite [HA] and fluorohydroxyapatite) (25).

On the other hand, Soekanto et al. (26) concluded that, the formulation of the CPP–ACP and propolis supported remineralization process. In addition, the propolis can be used as an alternative to prevent dental caries. Furthermore, the enamel surface microstructure of the CPP-ACP complex plus propolis gel displayed a homogeneous and smooth film at the surface of the enamel. It is recommended that the resin in the propolis might have bound with the CPP-ACP complex. This result was similar to research done by Franca et al. in 2014 (27).

Ordinarily, because of the high content of impurities which must be removed raw propolis is not suitable for pharmaceutical or cosmetic industry applications and food technology (28). To this end, by using organic solvents bioactive ingredients of propolis are extracted (29).

The present study showed that the microhardness mean values for all groups are significantly different from
each other at \( p \leq 0.05 \). Also, there was a highly significant difference of the mean microhardness values among tested groups in the three stages at \( p \leq 0.01 \).

Propolis showed minimal ability to inhibit demineralization; however, they were better than other groups. This shows a release of some remineralizing agents from those natural products, nevertheless with minimal effect on inhibition of demineralization process \(^\text{(30)}\). Also, propolis reduced accumulation of dental plaque and its insoluble external polysaccharide content \(^\text{(31)}\). On the other hand, propolis is a non-toxic material and its antimicrobial activity is related to the presence of flavonoids and terpenoids \(^\text{(32)}\). It contains minerals such as magnesium, iodine, potassium, sodium, copper, zinc, manganese, iron \(^\text{(33)}\). Also calcium, phosphate and fluoride \(^\text{(34)}\) and other minerals which are important in remineralization of tooth enamel.

Various studies reported that the influence of the geographic origin on the chemical composition of propolis and its biological activities \(^\text{(35,39)}\) and floral origins \(^\text{(37)}\). Nevertheless, presence and percentage content of composed material in propolis varies and depends on their origin, the species of bees that produced it and the type of plant pollen \(^\text{(38)}\).

Furthermore, the method of extraction and solvent used for this extraction method can change the chemical composition of propolis extract \(^\text{(39)}\). The results found in this study confirm the effect of the origin and type of the raw material \(^\text{(40)}\), as well as the extraction method \(^\text{(41)}\), in the composition and characteristics of the extracts \(^\text{(42)}\) investigated fifteen propolis samples from different botanic and geographic origins, verifying significant differences in their contents of polyphenols, flavonoids and active components. Comparing the results presented in Davequi-Nunes \textit{et al}. in 2018 \(^\text{(43)}\) in relation to the extraction method, it is possible to show a significant difference \((p>0.05)\) between the values for the flavonoid, phenolic and antioxidant activity (DPPH), where the ethanolic propolis extraction offered the best results among the samples and in the samples of different types. These results revealed the importance of the extraction method in the composition of the extract.

There is little data on the extraction of water solutions of propolis. Biologically active substances commonly are low soluble in water and there is low amount of phenolic compounds in wa-
Moreover, the aqueous extract resulted in antibacterial activity. A previous study was observed by Garedew et al. (2004) that compared different types of propolis extracts and showed that the water-extracted propolis solution had the weakest antifungal and antibacterial action. The difference to our results may be related to the intrinsic chemical composition of the propolis which is variable depending on their geographical origin.

CONCLUSIONS

Mixture of Sulaymaniah's AEP with MI paste plus was significantly better than MI paste plus alone in preserving enamel's hardness and resisting the demineralization challenge. The other types are less effective than MI paste plus alone but still have good resistance to acid cycling.

Limitations of the study:

The major limitation of this study is that it is an in vitro study in which demineralization cycle was obtained by using chemical products, and did not happen due to the presence of Streptococcus mutans and its acid byproducts. Also, surface microhardness in vitro may be different when compared to the dynamic conditions in the oral cavity in vivo.

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