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ANTIBACTERIAL AND MECHANICAL ASSAYS OF RESIN MODIFIED GLASS IONOMER CONTAINING PROPOLIS EXTRACT

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ABSTRACT

The antibacterial efficacy of restorative materials had an important role in preventing the recurrent caries. **The objective** of this study was to evaluate in-vitro the antibacterial and mechanical assays of Vitremer containing ethanolic extract of propolis (EEP). **Materials and methods**: **I-Antibacterial Assay**: The standard strain of Streptococcus *mutans* and Lactobacillus *acidophilus* were used for determination of minimum inhibitory concentration (MIC) of EEP/Vitremer. **II-Mechanical Assay: A-Shear bond strength (SBS)**: sixty half-crowns of non-carious extracted 2nd primary molars were placed at standard moulds containing Teflon disc that had 4mm x 3mm central hole and divided into 4 groups (n=15) according to the different EEP concentrations. Group I: 10%, group II: 25%, group III: 50% EEP-Vitremer mixture, and group IV (control): 0% EEP/ Vitremer and SBS was assessed using Instron machine. **B-Microhardness:** Sixty standard disc-shaped specimens were prepared from mixture 0%, 10%, 25%, and 50%, n=15 and nanoindentation value was recorded. Data were analyzed using one-way ANOVA and post-hoc test. **Results:** Only MIC of 10%, 25%, and 50% mixture showed growth inhibition against *S. mutans*, compared to 25% and 50% against L. *acidophilus*. SBS showed that 0% EEP recorded the highest value followed by10% mixture but the difference was not significant ($p>0.05$), while 25% and 50% reported the lowest values and the differences were significant ($p< 0.05$). 25% and 50% mixtures recorded the highest significant microhardness ($p<0.05$). 0% EEP and 10% mixtures displayed no significant differences between them ($P>0.05$). **Conclusions**: 25% EEP-Vitremer mixture was the most suitable concentration as it exhibits positive significant antibacterial and mechanical assays.

KEYWORDS: Propolis extract, fluoride-releasing restoratives, inhibition of recurrent caries, antibacterial and mechanical assays.

INTRODUCTION

One of the main contributing factors responsible for the failure of restoration is the recurrent caries. In deep caries, it is sometimes difficult to remove all carious dentin to avoid the incidence of pulp exposure. Also, the cariogenic micro-organisms can survive remaining under restorations causing recurrent caries and restoration failure [1-3]. One of the solutions to overcome this problem is the use

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of restorative dental materials containing fluoride as glass-ionomer cement (GIC). It inhibits the bacterial growth and metabolism, reducing their numbers [4], remineralizing the affected dentin and thus minimizing the risk of recurrent caries and pulp damage [5,6].

However, Takahashi et al.[5] showed that the amount of fluoride in GIC is insufficient for achieving the desired antimicrobial effects and did not verify whether the recurrent caries incidence can be reduced significantly [7]. Therefore, GICs may not prevent plaque proliferation and recurrent caries in some patients [8-10], and its fluoride concentration is too low to be effective, and so it is not considered the main factor in controlling biofilm formation [11].

Several studies [9,10] added antibacterial agents as chlorhexidine and antibiotics to restorative materials to improve its antibacterial effects. However, it was found that the addition of chlorhexidine or its derivatives might produce pulp toxicity when applied in deep cavities and jeopardize the basic properties of the material or both [12,13]. Despite the incorporation of antibiotics into the restorative materials had a significantly strong antibacterial effect, they may produce antibiotic resistance [14] and negatively affected the physicomechanical performance of the modified restoration [15,16]. Therefore, there is a need for alternative agents, which doesn't cause a change in physical properties of the restorative material. The particular antibacterial agent selected, its quality, quantity, and concentrations are important for incorporation into the restorative materials [16].

Recently, a worldwide trend increased to use biocompatible natural products for pharmacological purposes. Propolis is a natural resinous bee product, so, it is an easily available and cheap. It has been used by ancient Egyptians in folk medicine as a remedy for the treatment of many diseases [17] and in modern medicine due to a general back to nature trend. It is considered a promising agent due to its biological and pharmacological properties, such as bactericidal, antiviral, antifungal, analgesic, antioxidant, and anti-inflammatory effects [18,19]. In spite of these benefits, there are only limited reports about the addition of propolis to restorative materials as GICs.

Most of the studies [16,20-22] have focused on the antimicrobial effects of the restorative materials, but its physicomechanical properties have been overlooked. There are two forms of propolis: ethanolic and lyophilized. Ethanolic extract of propolis (EEP) is the most commonly used one, where ethanol acts as a solvent [21].

Most researches and cytotoxicity tests reported that propolis is safe and less toxic than other synthetic medicines [23-25].

Currently, new techniques, approaches, and materials have focused on dentistry on the minimally invasive procedures and maximum prevention [26]. According to this philosophy, resin-modified glass ionomer cement (RMGIC) is the material of choice, as it overcomes the drawback of GIC, such as poor handling characteristics, moisture sensitivity and the poor physicomechanical strength [14]. The biocompatibility and antimicrobial potential of RMGIC make the material more attractive [27,28].

Both *Streptococcus* mutans and *Lactobacillus* acidophilus are facultatively anaerobic, gram-positive bacteria found in the dental plaque and saliva of the human oral cavities. Mutans streptococci are responsible for dental caries initiation while the acidophilus lactobacilli are responsible for its progression. It is important to determine the minimal inhibitory concentration of propolis extract against both cariogenic bacteria without causing negative effects on the physicomechanical properties of incorporated RMGIC. No available data in the literature about the evaluation of antimicrobial assay against both cariogenic bacteria, and mechanical assay of EEP-RMGIC mixture. So, this study was undertaken to evaluate in-vitro the antibacterial and mechanical assays of RMGIC containing EEP.

MATERIALS AND METHODS

Preparation of Propolis Extract [23]

Twelve and a half gm of propolis powder (Royal Pharma, USA) was dissolved in 125 ml of ethanol 80% (vol/vol) using a magnetic stirrer for 24 h at room temperature. The solution was filtered, centrifuged at 8800 rpm for 30 min to produce EEP, and stored at 4ºC in dark bottle until use.

Preparation of EEP added RMGIC Vitremer [17]

Ethanolic extract of propolis was added to Vitremer Core Buildup/Restorative (*3M Dental Products, St Paul, MN, USA*) for further use in the antibacterial study (1gm powder=10 scoopful: 10 drops of each of 4 Vitremer liquid concentrations); EEP was added to the Vitremer liquid in 4 concentrations: 0%, 10%, 25%, and 50%: (i) 0% EEP (control group): Pure Vitremer [Powder (P): Liquid (L) ratio =1:1], (ii) 10% EEP-Vitremer mixture [$P^{Vitremer}$: $L^{Vitremer}$: L^{EEP} ratio=1:90:10], (iii) 25% EEP-Vitremer mixture [$P^{Vitremer}$: $L^{Vitremer}$, L^{EEP} ratio=1:0.75:0.25] and (iv) 50% EEP-Vitremer mixture [$P^{Vitremer}$: $L^{Vitremer}$, L^{EEP} ratio=1:0.5:0.5]. After mixing the powder and the liquid of Vitremer, EEP was added using a sterile micropipette.

I-Antibacterial Assay

Bacterial Strains and Inoculum Preparation

The antibacterial activity of EEP-Vitremer mixture was tested on standard strains of *Streptococcus* mutans (ATCC 25175) and *Lactobacillus* acidophilus (ATCC 4356) [KWIK-STIK, Microbiologics, USA] (fig 1). Blood agar plates were used for inoculation of both strains and then incubated at 37ºC for 24h to get pure colonies for further manipulation.

Determination of Minimum Inhibitory Concentration (MIC)

The MIC is the minimum concentration of the antimicrobial agent that inhibits the microbial growth. This parameter is used in vitro to determine the susceptibility or the resistance of microorganisms to the tested agent [29]. According to Weigand et al.[30] broth macro dilution method was used to test the antimicrobial activity of EEP-Vitremer mixture with the different prepared concentrations.

Four sets of 9 sterile glass test tubes 10ml containing 1ml of Mueller-Hinton broth (Oxoid) were used. The previously prepared mixture with the different concentrations was added to the first tube of each set to achieve dilution of 1000 mg/ml. Two serial dilutions of the mixture were performed up to the dilution of 3.9 mg/ml in the ninth tube in each set. The bacterial suspension was prepared by suspending colonies of tested control strain in a sterile saline to adjust turbidity as 0.5 McFarland. One ml of the bacterial suspension was added to each test tube in the 4 sets, and then the sets were incubated at 37ºC for 24h. The MIC value of the corresponding mixture was determined by the highest dilution in each set with no visible turbidity.

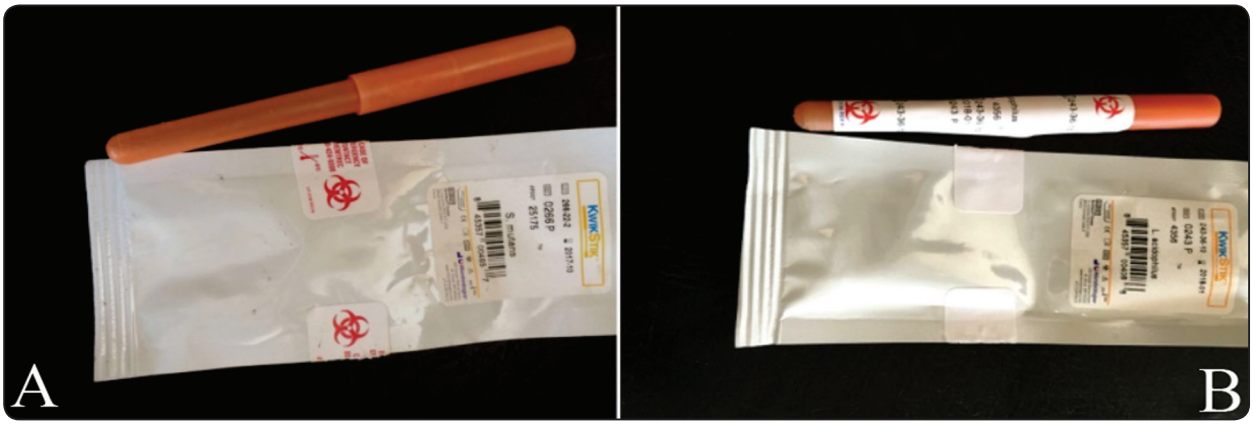

Fig. (1) Inoculating swabs of the standard strain for both *S*. mutans (A) and *L*. acidophilus (B).

II- Mechanical Assay

A. Shear bond strength testing

Thirty crowns of non-carious extracted second primary molars due to physiologic reasons were included in this study. Immediately after the extraction, the teeth were rinsed, stored, and sterilized in 2% formaldehyde solution at pH 7.0, at room temperature for 30 days. The teeth were cleaned, polished with slurry of pumice with a rubber cup for 15 sec, rinsed, and stored refrigerated at 4°C in deionized water until used [31].

Preparation of specimens

All 30 crowns were decoronated 1mm from the cemento-enamel junction and sectioned mesio-distally using a diamond disc under water cooling system to obtain 60 equal halves. Each specimen was placed at the center of a cylindrical aluminum mould using one-piece Teflon disc 3mm in thickness, with a central depression that fits the bottom of the mould. The mould was filled with self-cure acrylic resin, leaving only the buccal or lingual surface of the crown protruding well above the resin.

The buccal/lingual surfaces were ground on a water-cooled trimming abrasive wheel using a sequence of fresh 120-, 220- and 400-grit silicon carbide papers to expose flat dentinal surfaces. The specimens were ground to nearly the same depth midway between the dentino-enamel junction and the pulp, followed by manual polishing with wet 600-grit silicon carbide paper. The bonding area was demarcated with adhesive tape with a punch hole of 4mm in diameter in the center of prepared dentin surface. The specimens were randomly coded and divided into four equal groups (n=15), each group receiving equal numbers of buccal and lingual surfaces of primary second molars.

To build the restoration, another Teflon disc with a central hole of 4 mm in diameter and 3 mm in thickness was longitudinally split at the center. It was positioned over the specimens coinciding the central hole with the demarcated area on the prepared dentin (fig 2 A). Four groups were included in this study; Group I: 10% EPP-Vitremer mixture, Group II: 25% EPP-Vitremer mixture, Group III: 50% EPP-Vitremer mixture and Group IV (control): 0% EPP (pure Vitremer).

RMGIC build-up

The Vitremer primer was applied to the dentinal surface for 30 sec, dried for 15 sec and light-cured for 20 sec using Optilux curing light (Demetron/Kerr). All shades of Vitremer were equally distributed in all groups of study. The Vitremer mixture was syringed into the central hole of a properly oriented Teflon disc and bulk-cured from the top for 60 sec. The disc was separated gently using sharp scalpel blade and light-activated again for 60 sec. The finishing gloss was applied and light-activated for 20 sec. The specimen was removed from the mould and stored in a deionized water bath for 24 hours at 37°C (fig 2 B & C).

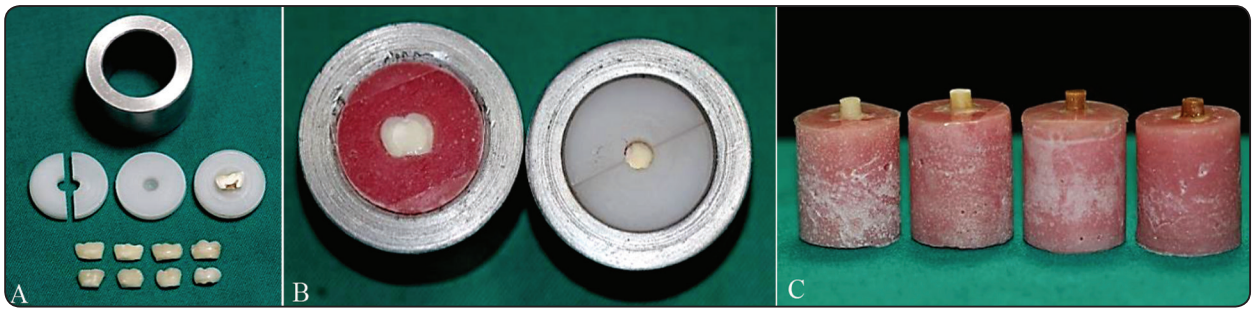

Fig. (2) Cylindrical aluminum mould, a disc longitudinally split at the central hole, Teflon disc with a circular central depression, another disc with a specimen attached to the central hole, and decoronated crown specimens of upper and lower second primary molars that were sectioned mesiodistally (A). Acrylic moulded specimen and adhesive tape with a circular hole within a metallic ring and a specimen before material application (B). Specimens representing 4 groups before SBS testing (C).

The specimens were thermocycled in deionized water for 100 cycles between 5°C and 55°C and for 30 sec in each dwelling temperature [31,32]. Every 100 thermocycles are equivalent to putting the specimen in the oral conditions for 10 days [33]. Any dislodged specimen was rejected and replaced.

Mounting of specimens

The shear bond strength (SBS) was assessed using an Instron machine (Bucks HP12 3SY, UK) at Dental Biomaterials Department, Faculty of Dentistry, Tanta University. A metallic tape 10 cm in length and 5 mm in width was used to apply the force at a crosshead speed of 0.5 mm/min until failure occurred (fig 3). The SBS was measured in Mega Pascal (MPa), which is derived by dividing the maximum load force (MPa) at the time of debonding by the bonded area (mm^2).

B-Microhardness

Nanoindentation testing

Sixty standardized disc-shaped specimens 6mm in diameter and 4mm in thickness using Teflon moulds were prepared from EEP-Vitremer (0%, 10%, 25%, and 50%) as mentioned before, 15 discs for each group. Both sides of all specimens were bulk-cured for 60 sec. To obtain a smooth and glossy surface, all specimens were ground with 400-2500 grit sandpaper and then polished by diamond pastes with mesh sizes of 1 and 0.5 microns (fig 4). All specimens were thermocycled as mentioned before, and then maintained at an ambient temperature of 24°C for 1h before applying the test using NanoTest Vantage Micromaterials Instrument, UK, at Institute of NanoScience and Nanotechnology, Kafrelsheikh University (fig 5).

An indenter prop was pressed into specific sites of the tested specimens by applying an increasing load of 750 μN with a constant rate of 15 $\mu N.s^{-1}$. Once the pre-set maximum value was reached, the normal load was decreased until partial or complete relaxation has taken place [34]. At least each specimen was subjected to 15-cycle nanoindentations located 25 μm away from each other and the mean microhardness values were recorded [35]. The results of the mechanical assay were subjected to one-way analysis of variance (ANOVA) and to Post-hoc test for pairwise comparison of subgroups when the ANOVA test is positive.

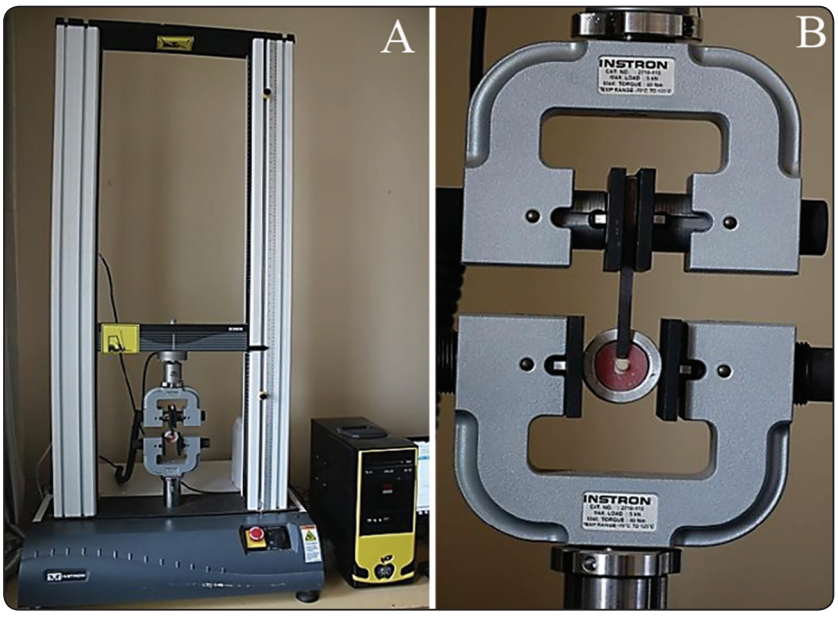

Fig. (3) Instron testing machine (A). The mounted specimen in Instron machine during SBS testing (B).

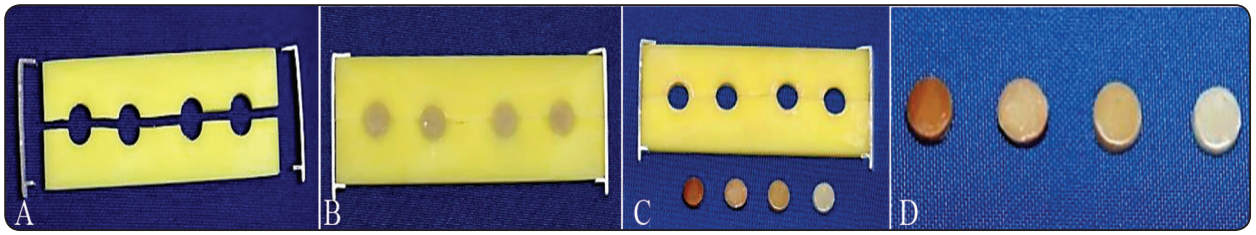

Fig. (4 Teflon mould with central holes 6mm in diameter and 4mm thickness (A), specimens within the mould and after light curing and removal from the mould (B&C), and the 4 discs representative specimens of the 4 groups (D).

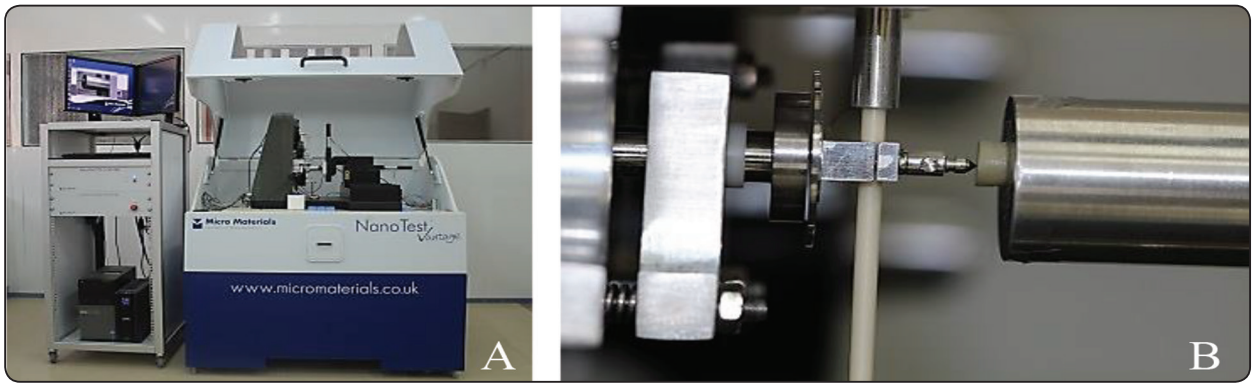

Fig. (5) NanoTest Vantage Micromaterials Instrument (A). An indenter prop is pressed into the tested specimen (B).

RESULTS

The results of broth macro dilution method used in this study showed that, the MIC values of 0%, 10%, 25% and 50% EEP-Vitremer mixture against the cariogenic bacteria were 1000 mg/ml, 500 mg/ml, 125 mg/ml, and 31.2 mg/ml for *S.* mutans, respectively, and >1000 mg/ml, 1000 mg/ml, 250 mg/ml, and 62.5 mg/ml for *L.* acidophilus, respectively.

In particular, the MIC value of 10%, 25%, and 50% mixture showed growth inhibition against *S.* mutans, compared to 25% and 50% mixture against *L.* acidophilus, MIC value of 0% EEP (control group) showed a negative effect on both cariogenic bacteria, while 10% mixture showed no efficacy for *L.* acidophilus.

The results of mean shear bond strength showed that 0% EEP (control group) recorded the highest values followed by 10% mixture, but there was no statistical significant difference between them ($P>0.05$). On the other hand, 25% and 50% mixtures recorded the lowest values and the difference was significant ($P< 0.05$). Table1.

The results of microhardness values obtained from the nanoindentation experiment of the specimens showed that 25% and 50% mixtures recorded the highest significant differences than 0% EEP and 10% mixtures ($p<0.05$). 0% EEP and 10% mixtures displayed no statistically significant differences between them ($P>0.05$). Table 2 and figure 6.

TABLE (1) The shear bond strength between experimental and control group (MPa).

Groups		Specimens No.	Shear bond strength			F-Test (p-value)	Post Hoc Test (P<0.05)
			Mean ±SD	Minimum	Maximum		
I	10% Mixture	15	7.82±0.30	7.10	8.6	305.386 (0.001)**	P_1**
II	25% Mixture		6.87±0.42	6.20	7.60		P_2**
III	50% Mixture		3.85±0.52	3.00	4.47		P_3
IV	0% Control		8.25±0.49	7.50	9.10		P4**
							P5**
							P6 **

#ANOVA Test *Significant difference **Highly significant difference

1-P_1: Comparison between 10% and 25% mixture. 2-P_2: Comparison between 10% and 50% mixture. 3-P3: Comparison between 10% mixture and control. 4-P4: Comparison between 25% and 50% mixture. 5- P5: Comparison between 25% mixture and control. 6-P6: Comparison between 50% mixture and control.

TABLE (2) Microhardness between experimental and control group (GPa) using Nano-scale measurement.

Groups		Specimens No.	Nano indentation micro hardness			F-Test (p-value)	Post Hoc Test (P<0.05)
			Mean ±SD	Minimum	Maximum		
I	10% Mixture	15	6.79±0.58	5.60	7.90	300.338 (0.001)**	P_1**
II	25% Mixture		11.51±0.44	10.80	12.20		P_2**
III	50% Mixture		17.00±0.93	16.000	19.90		P_3
IV	0% Control		5.41±0.53	4.26	6.30		P4**
							P5**
							P6 **

#ANOVA Test *Significant difference **Highly significant difference

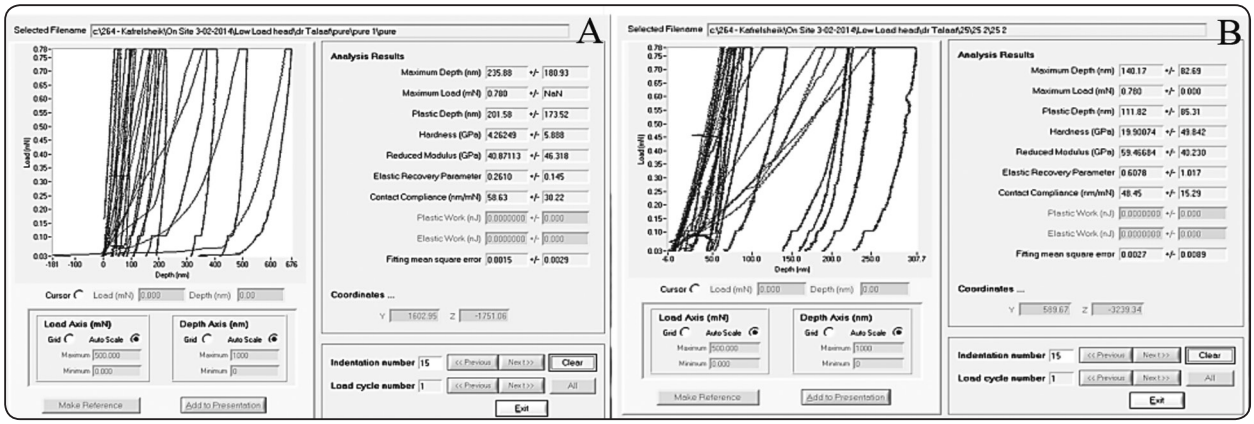

Fig. (6) Nano-scale measurement analysis illustrated with graphs showed the minimum value (A) and the maximum value (B).

DISCUSSION

Recently, utilization of antibacterial agents in restorative materials is recommended to aid in the prevention of recurrent caries [6], With the advanced technology in dentistry, new materials and approaches were developed [36,37]; propolis seems to be a great choice for dental treatment. It has been the topic of increasing the scientific interest and has the potential of use in the treatment of bacterial disease[38,39]. This encourages its addition in this study to the favorite modified GICs system in pedodontics restorative materials.

Resin-modified GICs have been developed by addition of 4.5%-6% resin-based composites to conventional GIC to overcome the drawback of physicomechanical properties of GICs, but maintaining their fluoride release and "recharging," and chemical adhesiveness to the tooth structure[40,41]. The significant anti-cariogenicity of GIC through long-term fluoride release makes the material more popular and widely used, especially in pedodontics. Therefore, the antibacterial modified RMGICs would provide an alternative approach to overcome this concept [26,42] and make it the material of choice to high caries-risk patients [43].

In this study, the ethanolic extract was used rather than lyophilized one as it was found that incorporation of lyophilized propolis leads to a hard and difficult manipulation with the color change of any GIC systems [14]. Additionally, there is a lack of reports on the use of the lyophilized form [44]. Often, EEP is directly used, resulting in effective antimicrobial potential [16,20].

This study focused on both antibacterial and mechanical assays of EEP-Vitremer mixture. Both *S*. mutans and *L*. acidophilus were used to determine antibacterial activity, as they are considered the main contributor to dental caries [23,45].

In this study, the buccal/lingual surfaces of specimens protruded above the resin mould to avoid any possible surface contamination by resin when the surfaces were ground flat. Also, the surfaces were ground to approximately the same depth midway between the dento-enamel junction and the pulp to represent a site nearly similar to the depth of a typical cavity preparation for testing the shear bond strength [46]. In the current study, to mimic the intra-oral environmental condition, all specimens were thermocycled 100 times; as it was reported that the thermocycling more than 100 cycles have been shown to be unnecessary [47].

Because the resin shades can influence the hardness [48] and bond strength [49], all shades of the Vitremer powder in this study were equally distributed in all groups. The lighter the shade, is the greater the hardness and bond strength [50].

In this study, the addition of EEP to Vitremer may show alterations, so, the antibacterial and mechanical assays were important to be investigated. The MIC, SBS, and Nanoindentation testing were applied.

The antimicrobial assay in this study reported that EEP has a positive impact on the antibacterial properties of Vitremer. The MIC value of 10%, 25%, and 50% mixture was 500 mg/ml, 125mg/ml, and 31.2 mg/ml respectively, showed growth inhibition against *S*. mutans, compared to 25% and 50% mixture against L. acidophilus which was 250mg/ml, and 62.5 mg/ml, respectively. Thus these only the MIC values of the mixture that have a significant activity against both cariogenic bacterial growth.

The results of MIC for *S* mutans are comparable to the findings of Hatunoğlu et al. [23], who found that MIC of both 0% and 10% EEP > 1000μg/ml, while 25% and 50% recorded 125 mg/ml and 31.2mg/ml, respectively. Kouidhi et al.[51] reported 8-32μg/ml, while Koo et al.[52] recorded 50-400 μg/ml. The change in MIC values may be due to differences in the botanical origin, geographical location and bee species, leading to change in chemical composition of propolis, and leading to a significant change of antibacterial activity of all specimens [53,54].

Also, this result is in agreement with the studies of Türkün et al.[13] and Deepalakshmi et al.[42] who reported that the antimicrobial efficacy was depended on the concentration of antibacterial added to restorative materials, but Jedrychowski et al.[55] showed no dose-response effects.

The mechanism of antibacterial effects of propolis is very complex. Some components such as high flavonoids concentration, caffeic[56], benzoic and cinnamic acids probably act on the bacterial cell wall or the cytoplasmic membrane, causing structural and functional damages [57]. Also, the antimicrobial effect may be due to the synergistic action of all components rather than an individual one[58] and/or inhibition of glucosyltransferase enzymes activity [38,39].

The increases of the antibacterial activity of the mixture in this study coincide with the study of Stuart [59] who reported that EEP added to GICs system has a greater positive antimicrobial effect, especially against *S. mutans,* most of the *Strept. Species* and *L. acidophilus* [60]. Waldner et al. [61] found that propolis may require high concentrations to develop into an antiseptic agent.

Also, the results of this study are in agreement with the in vitro study conducted by Herrera et al. [62] who revealed that Vitremer was the best material that showed bacterial inhibition against *Streptococcus spp, Lactobacillus spp*, and other bacterial species among the other glass ionomer filling materials as Ketac-Silver, Ketac-Fil, and Fuji II LC.

The variation in the antibacterial effects of GIC system might be due to their difference in compositions, such as presence or absence of oxides, type of acids, and fluoride release [63]. Therefore, in this study explaining the significant positive antibacterial activity of mixture may be related to the presence of polyacid, the antibacterial activity of EEP, and the high fluoride release that may results from addition of EEP [64], and the slowdown of acid-based reactions by the resin would lead to less mature ionic matrix and release of more fluoride[65,66].

The mean shear bond strength of 0% EEP, 10%, 25% and 50% mixture in this study was 8.25, 7.82, 6.87, and 3.85 MPa, respectively. This agrees with the study of Carrara et al. [67] and Di Nicoló et al. [68] who found that the shear bond strength of 0% EEP recorded 9.02 and 8.33 MPa, respectively; however, El-Kalla and Garcia-Godoy [69] reported 16.9 MPa.

This study revealed that the mixture had no significant deleterious effect on SBS at 10% and 25% mixture. This is in agreement with Troca et al. [14] who found that adding EEP has a negative effect on GIC system but disagree with the result of Hatunoğlu et al. [23] who reported that the increase of shear-peel band strength with the addition of EEP, but statistically the difference was not significant.

The significant inferior bond strength value of 50% mixture recorded 3.85 MPa may be attributed to the change in physical properties. The viscosity of Vitremer liquid decreases by adding EEP that prolongs the working time [23] and interferes with the network formation of GIC systems. The high percent of EEP would weaken the scaffold with unfavorable adhesion leading to a negative effect on the physical properties of the mixture [17].

Despite adding the antibacterial to GIC system enhanced its antimicrobial activity; Yesilyurt et al.[16] showed that the bond and compressive strengths of GIC system were negatively affected. However, de-Castilho et al.[70] stated that the negative results of compressive strength were not statistically significant.

Surface microhardness is one of the most important tests giving information about wear resistance and long-term durability of materials when exposed to the intra-oral environmental condition [71,72]. Nano-indentation measurement is a proper test for recording the mechanical properties of small-sized specimens and the surface properties of coatings.

In the present study, the microhardness of 25% and 50% mixture recorded the highest values than

10% mixture and control group and the difference was significant. A possible explanation for this finding is that EEP molecule contained highly active polyphenolic compounds and many aromatic fatty acids that have numerous favorable properties[73,74]; a chelation reaction was formed between the carboxylic group of Vitremer and phenolic hydroxyl of EEP [75]. The EEP act as a spacer for the dissociative carboxyl, provide highly active poly-salt bridging and increasing of cross-links. Following the addition of EEP, a greater amount of poly-salt bridges was formed that minimize the gaps existing among the crosslink networks with the increase of the molecules intensity on the surface[76-78].

One of the limitations of this study is that the mixture required accuracy of preparation to obtain adequate consistency without compromising the properties of the material. Another limitation is the color change of the mixture with the addition of a high EEP concentration that may compromise the restorative color match [14].

CONCLUSION

Based on the results of this in vitro study, despite some limitations; EEP-Vitremer mixture is promising; its incorporation into Vitremer in addition to the fluoride release will have a clinical interest due to their synergetic antibacterial action, significantly increase the microhardness, and had no significant deleterious effect on SBS except with 25% and 50% EEP concentration.

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