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Preparation, Characterization of Two Modified Irradiated Pulp Capping Materials and Evaluation of Anti-Bacterial Activity and Compressive Strength

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ORIGINAL STUDY

Preparation, Characterization of Two Modified Irradiated Pulp Capping Materials, and Evaluation of Their Antibacterial Activity and Compressive Strength

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Abstract

Purpose: The present study aimed to prepare and characterize an irradiated cross-linked chitosan/calcium hydroxide/Urbical copolymer combined with propolis powder as a modified pulp capping material. Evaluation of the antibacterial activity and compressive strength was also studied. **Materials and methods:** The cross-linked chitosan/calcium hydroxide copolymer was prepared by mixing 5 ml of calcium hydroxide (1 %) and 6 ml of chitosan 1 % (1 g of chitosan was wetted with 3.5 ml lactic acid for 48 h which improves the solubility of chitosan in water), then 100 ml distilled water was added, and 2.5 ml of HEMA under 25 kg irradiation dose and the resultant was mixed with Urbical (0.03 g base and 0.03 g catalyst). Propolis powder (irradiated and nonirradiated) was mixed with calcium hydroxide powder at a ratio of 1 : 1.5. Propolis was irradiated with a dose of 20 kg. Characterization of the structure of the prepared samples was done using Fourier transform infrared spectroscopy. Compressive strength and antibacterial activity were then studied. **Results:** Regarding compressive strength, the significantly highest compressive strength mean value was recorded in the calcium hydroxide group, followed by the nonirradiated propolis/calcium hydroxide group; however, the cross-linked chitosan/calcium hydroxide copolymer recorded the lowest mean value. Results showed that the highest antibacterial activity against *Streptococcus mutans* and *Escherichia coli* was recorded in the cross-linked chitosan−calcium hydroxide/Urbical copolymer group. However, the antibacterial activity against *Staphylococcus aureus* showed no statistically significance difference between tested groups ($P = 0.734$). **Conclusion:** Gamma radiation enhanced the antibacterial activity of the two modified irradiated pulp capping materials tested, but it negatively affected their compressive strength.

Keywords: Chitosan, Compressive strength, Irradiation cross-linking

1. Introduction

Biocompatibility and osteoconductivity are important criteria in restoring dental and osseous defects. Therefore, researchers attempt to produce new pulp capping materials with good biocompatibility and osteoconductivity [1]. Dental caries is defined as an infectious, transmissible disease that is caused by interactions between acidogenic bacteria, substrate, and environmental factors along with the host's characteristics [2].

Pulp necrosis may occur as a result of pulp exposure due to the spreading of caries which leads to pain and then pulpitis (reversible, irreversible). Irreversible pulpitis may lead to teeth extraction or root canal treatment. Direct pulp capping is the treatment of small noninfected pulp exposure to preserve pulp vitality [3]. Stimulation of the tertiary dentine is one of the causes of using calcium hydroxide as a pulp capping material. Bactericidal effect and fibroblast activation are caused by having a high pH [4].

Building and repairing honeycombs can be done by propolis, as a natural resin substance obtained by honeybees from various plants. Propolis has an anti-inflammatory, antibacterial activity with no toxicity, and therefore aids in pulp regeneration

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and dentinal tubule formation. A mixture of calcium hydroxide and propolis can increase the biocompatibility and antibacterial activity of calcium hydroxide [5].

Chitosan, a natural compound, has several characteristic features such as excellent biocompatibility, biodegradability, reactivity of acetylated amino groups, permeability to selective elements, polyelectrolyte action, antibacterial activity, anti-inflammatory properties, and the ability to accelerate wound healing [6].

Pulp capping material should have enough compressive strength to resist fractures from masticatory forces and placement pressure [7]. Retaining the biocompatibility, modification, and sterilization of the biopolymer can be achieved by irradiation cross-linking. The absence of any chemical additives and single-step application makes it a low-cost effective process to change biopolymers [8].

The present study aimed to prepare and characterize irradiated cross-linked chitosan/calcium hydroxide/Urbical copolymer and propolis powder as a modified pulp capping material. Also, evaluation of the antibacterial activity and compressive strength had been studied.

2. Materials and methods

2.1. Materials used in the present study

(1) Self-cure calcium hydroxide cavity liner (Sigma–Aldrich Co., 3050 Spruce, St Louis, USA).
(2) Chitosan (deacetylated chitin, poly-D-glucosamine) medium molecular weight (Sigma–Aldrich Co.).
(3) HEMA: 2-Hydroxyethyl methacrylat, 1, 2 Ethanediol mono (Sigma–Aldrich Co.).
(4) Urbical: self-curing radiopaque calcium hydroxide paste (Promedica).
(5) Propolis powder (raw material).

2.2. Materials preparation

2.2.1. Preparation of propolis

Propolis powder (irradiated and nonirradiated) was mixed with calcium hydroxide powder at a ratio of 1: 1.5 [9]. Propolis was irradiated with 20 kg (according to a pilot study).

2.2.2. Preparation of cross-linked chitosan/calcium hydroxide/Urbical copolymer

Several trials were made to achieve our goal of obtaining cross-linked chitosan/calcium hydroxide to be used as a pulp capping material.

According to the pilot study, a mixture of 5 ml of calcium hydroxide 1 % and 6 ml of chitosan dissolved in lactic acid was prepared. Different ratios of HEMA (0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5 ml) were used. Many of these preparations gave satisfactory results on consistency. At this stage, another problem arises, the long setting time of the hydrogels. To overcome this problem, calcium hydroxide paste (Urbical) was added to the preparation using 0.03 base and 0.03 catalyst to shorten the setting time.

Finally, 5 ml of calcium hydroxide (1 %) was mixed with 6 ml of chitosan 1 % (1 g of chitosan was wetted with 3.5 ml lactic acid for 48 h which improves the solubility of chitosan in water), then 100 ml of distilled water was added, and 2.5 ml of HEMA under 25 kg irradiation dose and the resultant was mixed with Urbical (0.03 g base and 0.03 g catalyst) [10]. The samples were irradiated at the National Center of Radiation Research and Technology, Atomic Energy Authority, Cairo, Egypt.

This in-vitro study has received approval from the Research Ethics Committee at the Faculty of Dental Medicine for Girls, Al-Azhar University (REC-CL-23-08), and each participant signed an informed consent form with approval.

2.3. Samples' grouping

The experimental study was divided into four groups according to the type of material. Regarding the antibacterial assay, three subgroups were developed from the main groups by the type of tested bacteria.

Group 1: calcium hydroxide powder.
Group 2: cross-liked chitosan/calcium hydroxide copolymer.
Group 3: nonirradiated propolis/calcium hydroxide.
Group 4: irradiated propolis/calcium hydroxide.

2.4. Sample size calculation

Based on the sample size calculation from a previous study [11], six samples were deemed sufficient to detect an effect size of 0.4, with 90 % power and a significance level of 0.05.

2.5. Chemical characterization by Fourier transform infrared spectroscopy

The samples of each group were ground, and then structurally characterized by Fourier transformation

infrared spectroscopy (FTIR) for identifying the chemical structure and the functional groups of the prepared materials.

2.6. Testing procedures

2.6.1. Antibacterial assay

The antibacterial activity of the tested groups was assessed using the "agar diffusion test". Standardized strains of *Staphylococcus aureus* (ATCC: 13,565), *Streptococcus mutans* (ATCC: 25,175), and *Escherichia coli* (ATCC: 10,536) were tested on the medium of nutrient agar. A DMSO solvent was used as the (negative) control. Concentrations of 15 mg/ml were used to test the compounds against bacterial strains. The sterilized media was poured into sterilized Petri dishes (20–25 ml), each Petridish and was allowed to solidify at room temperature. A microbial suspension was prepared in sterilized saline equivalent to McFarland 0.5 standard solution (1.5×10^5 CFU/ml) and its turbidity was adjusted to OD = 0.13 using a spectrophotometer at 625 nm. Optimally, within 15 min after adjusting the turbidity of the inoculum suspension, a sterile cotton swab was dipped into the adjusted suspension and was flooded on the dried agar surface and then allowed to dry for 15 min with the lid in place. Wells of 6 mm diameter were made in the solidified media with the help of a sterile borer. A measure of 100 μl of the solution of the tested compound was added to each well with the help of a micropipette. The plates were incubated at 37 °C for 24 h in case of antibacterial activity. This experiment was carried out in triplicate, and zones of inhibition were measured in mm scale [12]. The materials were seeded in the form of disks (the antibacterial assay was done at the Micro Analytical Center, Faculty of Science, Cairo University).

2.7. Compressive strength

According to ISO 9917–1: 2007 Standard [13], the samples were made using 4 × 6 mm diameter molds. Samples were seated between two compression plates of a universal testing machine (Instron model 3345/England). Compressive mode of failure was applied at a cross-head speed of 1 mm/min up to specimen failure. Data were calculated and recorded in MPa using computer software (Blue Hill Universal Instron/England).

2.8. Statistical analysis

Statistical Package for the Social Sciences (SPSS Statistical package for social science IBM USA), version 20 was used for managing the results and statistical analysis. Data normality was explored by checking the distribution of the data using Shapiro–Wilk and Kolmogorov–Smirnov tests. Normally distributed numeric variables using the analysis of variance test were done for comparisons between groups. For pairwise comparisons, the Bonferroni post hoc test was performed.

Two-sided *P* values were used. Significant *P* values were less than or equal to 0.05.

3. Results

3.1. Chemical characterization by Fourier transform infrared spectroscopy

IR spectra of the cross-linked chitosan/calcium hydroxide copolymer showed characteristic absorption bands. The characteristic absorption bands appeared at 1709 cm^{-1}. A strong band occurred at 1562 cm^{-1} and a modest band at 1450 cm^{-1} (Fig. 1).

The IR spectra of the nonirradiated propolis show a peak at 3550–3200 cm^{-1}, peaks at 2972 and 2853 cm^{-1}, stretching vibration at 1608 cm^{-1}, peaks at 1508–1515 cm^{-1}, and stretching vibration at 883–880 cm^{-1}. The irradiated propolis approximately has the same FTIR peaks (Figs. 1 and 2).

3.2. Antibacterial assay

Table 1 and Fig. 3 show the inhibition zone (mm) of *E. coli* (ATCC: 10,536). There was a statistically

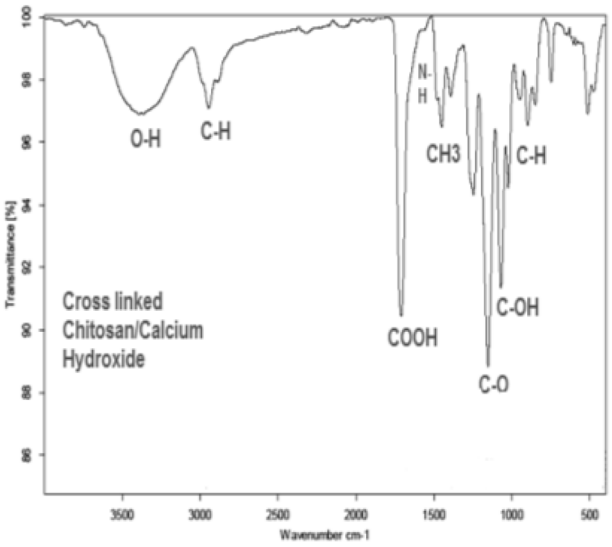

Fig. 1. FTIR spectrum of cross-linked chitosan/calcium hydroxide/HEMA. FTIR, Fourier transform infrared spectroscopy.

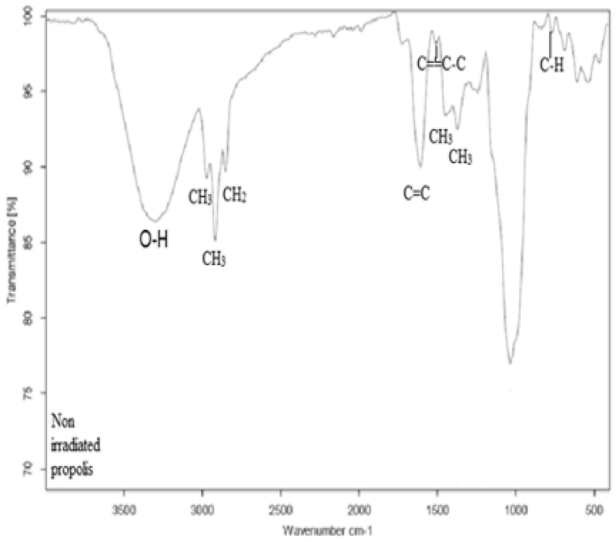

Fig. 2. FTIR spectrum of nonirradiated propolis. FTIR, Fourier transform infrared spectroscopy.

significant difference between all tested groups. Results revealed that the highest mean value was recorded in the cross-linked chitosan-calcium hydroxide/Urbical copolymer group (16 ± 1.74), followed by the irradiated Propolis/calcium hydroxide group (14.68 ± 1.49), then by nonirradiated propolis/calcium hydroxide group (13.27 ± 1.22), while no activity was recorded in the calcium hydroxide group.

Table 2 and Fig. 4 show the inhibition zone (mm) of *S. aureus* (ATCC: 13,565). There was no statistically significant difference between tested groups ($P = 0.734$).

Table 3 and Fig. 5 show the inhibition zone (mm) of *S. mutans* (ATCC: 25,175). There was a statistically significant difference between all tested groups. The highest mean value was recorded for the irradiated propolis/calcium hydroxide group (19 ± 1.1), followed by the cross-linked chitosan−calcium hydroxide/Urbical copolymer group (18.33 ± 0.61), and the then the nonirradiated propolis/calcium hydroxide group (16.33 ± 1.51). However, the calcium hydroxide group showed no activity.

3.3. Compressive strength

Table 4 and Fig. 6 show the mean compressive strength values of the tested groups. The significantly highest compressive strength mean value was recorded in the calcium hydroxide group (4.10 ± 0.640), followed by the nonirradiated propolis/calcium hydroxide group (3.68 ± 0.83). Results revealed that the cross-linked chitosan−calcium hydroxide/Urbical copolymer recorded the lowest significant compressive strength value compared with all tested groups ($P = 0.000$). However, the difference between the calcium hydroxide group and the nonirradiated propolis/calcium hydroxide group was insignificant.

4. Discussion

Direct pulp capping is considered a well-established method of treatment where the exposed pulp is covered by the capping material. In this study, calcium hydroxide was used as a direct pulp capping material as calcium hydroxide has been considered as the "gold standard" cement for pulp protection. Chitosan was used as a direct pulp capping material based on its promising properties in dentistry [5].

Chitosan mixed with calcium hydroxide as a direct pulp capping material was used in the present study. However, most of the studies use chitosan as a drug-loaded scaffold in direct pulp capping. The present study was an attempt to mix chitosan with calcium hydroxide. As chitosan is easily soluble in acidic solutions, the reaction of acid and base forms precipitated salt and water, hence no homogeneous mix occurs. Therefore, cross-linking of chitosan to form a network is the only way to prepare chitosan hydrogels. Satisfied results were obtained by

Table 1. Descriptive statistics of the inhibition zone (mm) of Escherichia coli *and comparison between tested groups (analysis of variance test).*

Groups	Mean	SD	95% confidence interval for mean		Minimum	Maximum	*F* value	*P* value
			Lower bound	Upper bound				
Calcium hydroxide	0.00[c]	0.00	0.00	0.00	0.00	0.00		
Cross-linked chitosan−calcium hydroxide/Urbical copolymer	16.00[a]	±1.74	14.17	17.83	13.30	18.00	195.60	0.000*
Nonirradiated propolis/calcium hydroxide	13.27[b]	±1.22	11.99	14.55	11.10	14.50		
Irradiated propolis/calcium hydroxide	14.68[ab]	±1.49	13.12	16.24	14.00	17.70		

*Significance level *P* value less than or equal to 0.05.
a,b,c indicate the significant differences between the groups.

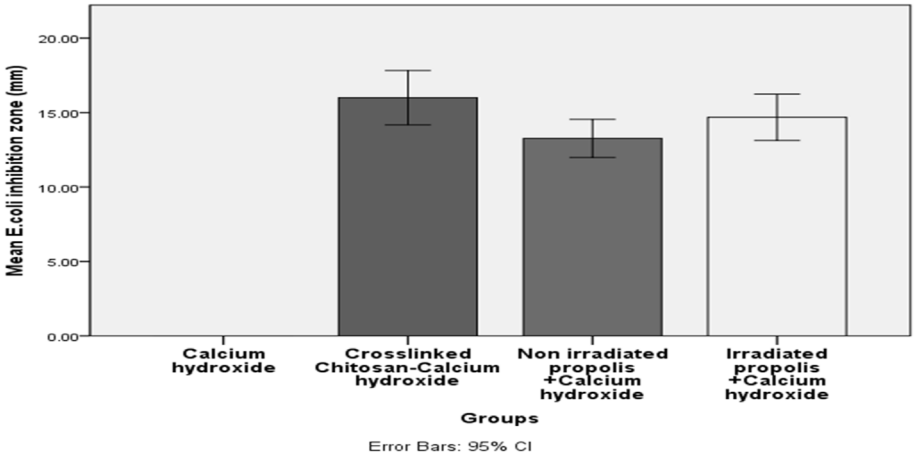

Fig. 3. Bar chart showing mean value of the inhibition zone (mm) of Escherichia coli *of different tested groups.*

Table 2. Descriptive statistics of the inhibition zone (mm) of Staphylococcus aureus *and comparison between tested groups (analysis of variance test).*

Groups	Mean	SD	95% confidence interval for mean		Minimum	Maximum	*F* value	*P* value
			Lower bound	Upper bound				
Calcium hydroxide	16.733[b]	±0.589	16.115	17.351	16.000	17.400		
Cross-linked chitosan−calcium hydroxide/Urbical copolymer	16.733[b]	±1.506	15.153	18.313	14.400	19.000		
Nonirradiated propolis/calcium hydroxide	15.733[b]	±2.100	13.529	17.937	13.000	19.200	0.430	0.734 ns
Irradiated propolis/calcium hydroxide	16.0[b]	±2.757	13.107	18.893	13.000	20.000		

NS, nonsignificant.
Significance level *P* value less than or equal to 0.05.
a,b,c indicate the significant differences between the groups.

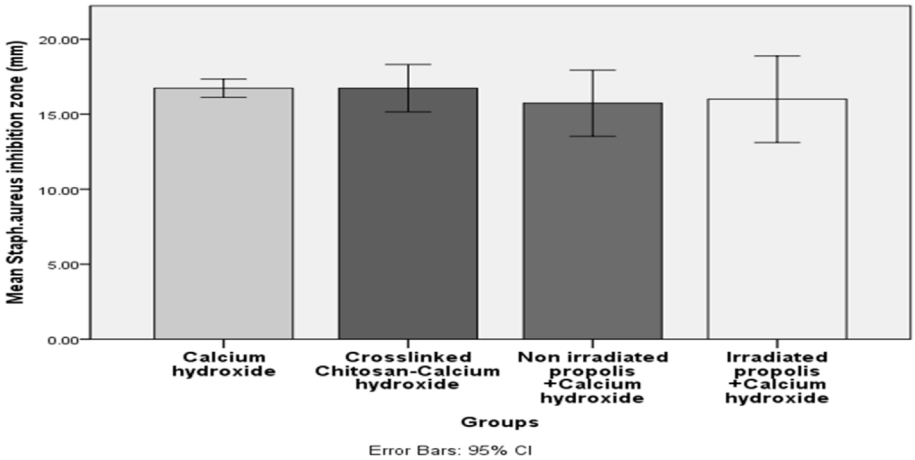

Fig. 4. Bar chart showing mean value of the inhibition zone (mm) of Staphylococcus aureus *of different tested groups.*

combining a chemical cross-linker hydroxy ethyl metha acrylate (HEMA) and irradiation technique, which produce a lot of active sites and facilitate the combination of HEMA. HEMA as a chemical cross-linker was chosen for its wide uses as a constituent in many bonding systems and glass ionomer cements. It has many biomedical advantages for being biocompatible, highly permeable, hydrophilic material, and compatible with blood proteins and cells [14].

Table 3. Descriptive statistics of zone of inhibition (mm) of Gram-positive bacteria Streptococcus mutant *and comparison between tested groups (analysis of variance test).*

Groups	Mean	SD	95% confidence interval for mean		Minimum	Maximum	*F* value	*P* value
			Lower bound	Upper bound				
Calcium hydroxide	0.00[c]	0.00	0.00	0.00	0.00	0.00		
Cross-linked chitosan–calcium hydroxide/Urbical copolymer	18.33[a]	±0.61	17.70	18.97	17.50	19.00	508.93	0.000[a]
Nonirradiated propolis/calcium hydroxide	16.33[b]	±1.51	14.75	17.91	15.00	18.00		
Irradiated propolis/calcium hydroxide	19.0[a]	±1.10	17.85	20.15	18.00	20.00		

a,b,c indicate the significant differences between the groups.
[a] Significance level *P* value less than or equal to 0.05.

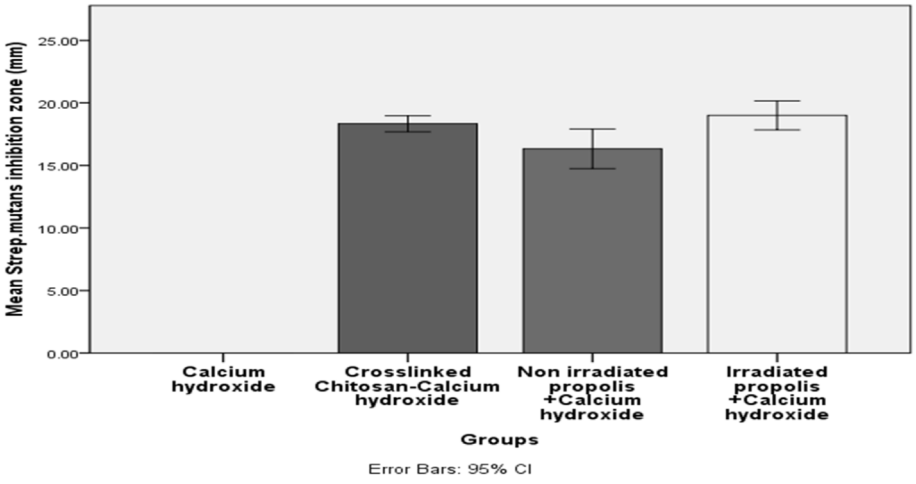

Fig. 5. Bar chart showing the mean value of zone of inhibition (mm) of Gram-positive bacteria Streptococcus mutants of different tested groups.

Table 4. Descriptive statistics of compressive strength (MPa) and comparison between tested groups (analysis of variance test).

Groups	Mean	SD	95% confidence interval for mean		Minimum	Maximum	*F* value	*P* value
			Lower bound	Upper bound				
Calcium hydroxide	4.10[a]	±0.64	3.43	4.77	3.59	4.92		
Cross-linked chitosan–calcium hydroxide	0.07[c]	±0.01	0.06	0.08	0.06	0.08	40.36	0.000[a]
Nonirradiated propolis/calcium hydroxide	3.68[a]	±0.83	2.81	4.55	2.62	4.32		
Irradiated propolis/calcium hydroxide	2.28[b]	±0.93	1.30	3.26	1.37	3.42		

a,b,c indicate the significant differences between the groups.
[a] Significance level *P* value less than or equal to 0.05.

In the current study, irradiation cross-linking was used, as a professional technique for cross-linking action. Ionizing radiation as a method of preparing hydrogels has many advantages, including simultaneous sterilization, cross-linking through free radical polymerization on exposure to gamma radiation, without the use of a cross-linking agent or chemical initiator, and being safe for humans and the environment [7]. The irradiation dose used in the current study was 25 kg which was within the acceptable range and gives the optimum consistency as the pulp capping material.

The longer setting time of the prepared cross-linked chitosan/calcium hydroxide/HEMA hydrogel was another problem. Therefore, calcium hydroxide paste (Urbical) was added to the preparation to

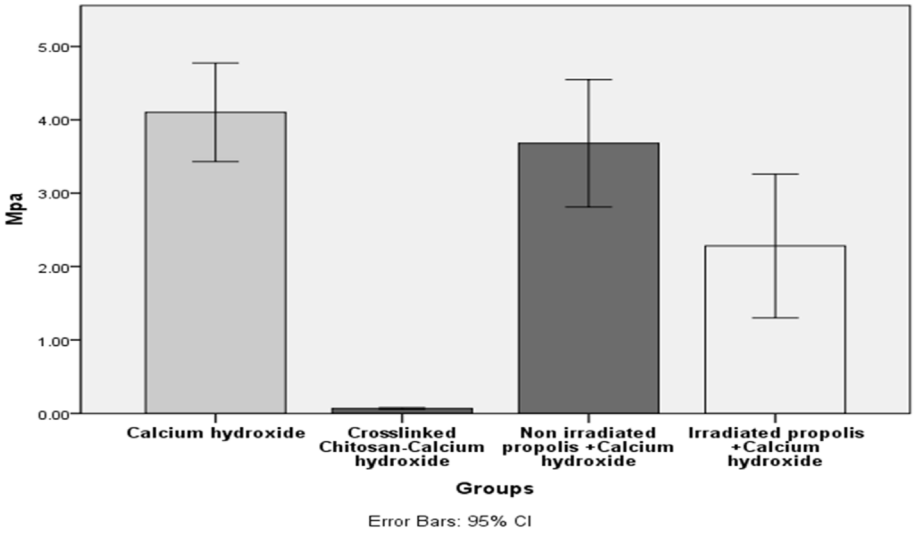

Fig. 6. Bar chart showing the mean value of compressive strength (MPa) of different tested groups.

shorten the setting time. The partial solubility of calcium hydroxide dispersed in the cement, as well as its dissociation to Ca2+ and OH− ions, increases the number of OH− ions, which, when combined with the OH− ions produced by irradiation cross-linking, leads to more hydrogen abstraction, resulting in more free radicals and increasing the rate of the setting reaction. As a result, the time required for setting is reduced [15].

The molecular structure and the functional groups associated with cross-linked chitosan/calcium hydroxide/HEMA, nonirradiated propolis were studied by FTIR. The FTIR spectra of the tested materials were in agreement with the spectra found in the literature [16]. The interaction of chitosan's amine (-NH_2) groups with the hydroxyl (-OH) groups in calcium hydroxide caused chitosan to bind to the calcium hydroxide carrier. Consequently, the O−H band at 3392 cm^{-1} was notably broader in the cross-linked chitosan/calcium hydroxide/HEMA copolymer, and the C−N peak (1070 cm^{-1}) became deeper with the inclusion of chitosan. On comparing the FTIR spectra of the parent chitosan with that of cross-linked chitosan/calcium hydroxide/HEMA, additional peaks appeared in the spectra which indicate structural changes.

The occurrence of an intense band at 1562 cm^{-1} and a moderate band at 1450 cm^{-1} were attributed to the symmetric COO− (which overlaps with the N−H band) and asymmetric axial deformations of COO−, respectively, confirms the introduction of chitosan groups. The characteristic peaks of both chitosan and calcium hydroxide were seen in the FTIR spectra of the chitosan/calcium hydroxide formulations as shown in Fig. 1 [16].

Phenolic compounds or their esters were shown to be present by the spectra pattern (aromatic C−H at 3100−3000 cm^{-1}, (O−H) at 3307−3368 cm^{-1} and C=C aromatics at 1500−1600 cm^{-1}). The flavonoids were indicated (C=O at 2972, 2933, 2879 cm^{-1}). The tested material contains unsaturated C=C bonds as the band at 1605 cm^{-1} was present. The weak absorbance of C=C at 1651−1659 cm^{-1}, and C−O at 1320−1000 cm^{-1} assures the presence of terpenes. The amino acids and amino acids aromatic were shown by the absorbance of symmetric N−O and C−N at 1334−1290 cm^{-1} and N−H at 3400−3250 cm^{-1}. Fatty acids stilbenes, steroids, and carboxylic acids exhibit absorbance at C−O and O−H at 1200−1000 cm^{-1} [17]. The irradiated propolis approximately has the same FTIR peaks, which means that radiation does not affect the structure of propolis.

In the current study, the species of microorganisms were chosen to represent the Gram-positive and Gram-negative bacteria. *S. mutans*, *S. aureus*, and *E. coli* were used in the antibacterial assay of the present study. Dental caries are primarily due to *S. mutans* [15], and *S. aureus* and *E. coli* were frequently isolated from dental infections. The agar diffusion test was used in the current study to assess the antibacterial activity of the tested materials because of its simplicity. It compares the antibacterial activity of materials by inhibition zone size [18].

Calcium hydroxide was chosen in the present study because of its antibacterial activity and mineralization capability; however, its activity against certain bacteria was not sufficient [19]. Therefore, it was essential to add chitosan to $Ca(OH)_2$ to improve its antibacterial activity, dissociation, and biocompatibility [19].

Many organisms can be inhibited by chitosan through different mechanisms. Interactions between the chitosan NH^{3+} sites and the negative cell membranes of microbes are considered the simplest mechanism, causing permeability alteration of the microbial cell and intracellular material release. Chitosan binding to microbial enzymes and nucleotides may cause cell structure disruption in *E. coli* and *S. aureus*. The polycationic form of chitosan reveals a broad spectrum of antibacterial activity. Chitosan interacts with surface anionic structures in Gram-negative bacteria, such as proteins and lip polysaccharides. However, the negatively charged cell wall components, consisting of peptidoglycan and teichoic acids, interact with chitosan, in Gram-positive bacteria [20].

In the current study, results of the antibacterial activity revealed that the irradiated cross-linked chitosan–calcium hydroxide/Urbical copolymer recorded the highest mean value against *E. coli* among the tested groups. This could be attributed to the fact that reducing chitosan polymer length and molecular weight improves its biological activity. It has been proven that γ irradiation is the most effective method for modifying the physical and chemical properties of chitosan polymers to improve their solubility, antimicrobial and antioxidant properties, and plant-growth-promoting properties [21].

The inhibition of growth of all tested bacteria against (nonirradiated–irradiated) propolis was noted, but zones of inhibition varied according to the type of bacteria. Irradiated propolis exhibited the highest antibacterial activity against *S. mutans* when compared with the other groups and the highest antibacterial activity against all tested bacteria as compared with nonirradiated propolis. This is due to the cumulative effect between the antibacterial effect of radiation and propolis. The high content of phenolic and flavonoids might be the reason for the antibacterial effect of propolis [22].

Furthermore, results showed that calcium hydroxide had no effect on *E. coli* and *S. mutans*. This finding could be explained by the slow release of hydroxyl ions during the period of contact. The absence of an antibacterial impact on some strains of bacteria suggests that the production of hydroxyl ions from calcium hydroxide was insufficient to inhibit the growth pH of these microorganisms [23].

Pulp capping material can be empowered to resist compressive force from the restorations by adequate compressive strength [6]. In the current study, the results revealed that the calcium hydroxide group recorded the highest mean compressive strength value, followed by the nonirradiated propolis/calcium hydroxide group. Such a decrease could be attributed to an increase in the number of weak hydrogen bonds that form between hydrogen atoms (in aromatic compounds from propolis) and oxygen atoms in water, resulting in a reduced compressive strength for the material. This finding was consistent with a prior study [13], which found that the compressive strength of calcium hydroxide/propolis was significantly lower than calcium hydroxide alone. The weak structure may be attributable to the fact that propolis includes phenolic acid, which is a weak acid that can react with a strong base (calcium hydroxide) to produce salt and water. Higher water content materials yield weaker structures and, as a result, lower compressive strength.

The irradiated propolis/calcium hydroxide group had lower compressive strength values than the nonirradiated propolis group because of the scission of the molecules done by radiation; gamma irradiation affects the basic structure of polymers through the production of free radicals, and accordingly, molecular weights were reduced [7]. However, the irradiated cross-linked chitosan/calcium hydroxide/Urbical copolymer was significantly lower than all tested groups, because chitosan is a naturally modified polysaccharide with hydrophilic properties after treatment with lactic acid, so it degrades on exposure to radiation and the main chains of glycosidic bonds was broken down [7]. Moreover, the solubility of chitosan can also be increased by applying gamma radiation, as gamma irradiation affects the intermolecular and intramolecular hydrogen bonds and weakens the van der Waals force [24].

4.1. Conclusion

Within the limitation of the current study, gamma radiation aided in the enhancement of the antibacterial activity of the two modified irradiated pulp capping materials tested and negatively affected their compressive strength.

4.2. Recommendation

Further investigations should be performed to understand the structure of the newly prepared materials.

Conflict of interest

There are no conflicts of interest.

Acknowledgments

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