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# Novel Ca/Mg/ Chitosan Layered Double Hydroxide Modified with Hydroxyapatite as New Dental Nanocomposites; Mechanical and Antibacterial Study

**Mohammadmehdi Moshafia**
Comenius University Bratislava
**Mehdi Ranjbar**
Mehdi.Ranjbar@kmu.ac.ir
Kerman University of Medical Sciences
**Azadeh Aminzadeh**
Kerman University of Medical Sciences
**Iman Mohammadzadeh**
Kerman University of Medical Sciences

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# Abstract

In this study we demonstrate an efficient and cost effective synthesis of novel Ca/Mg/Chitosan layered double hydroxides (LDHs) modified with hydroxyapatite. The chitosan as a biotemplate was extracted from whiteleg shrimp (Litopenaeus vannamei (shell. Modification and functionalization of the LDHs with chitosan structures can lead to new biotechnological and biomedical applications. Ca $(NO_3)_2.4H_2O$ in this approach was applied as simultaneous precursor for synthesis of Ca/Mg/Chitosan LDHs and also $Ca_{10}$ $(PO_4)_6(OH)_2$. The final nanocomposites were characterized extensively with scanning electron microscopy (SEM), Fourier Transform Infrared Spectroscopy (FTIR), Dynamic light scattering (DLS), Thermo-gravimetric analysis (TGA) and differential thermal analysis (DTA). The experimental results showed for the new nanocomposites mechanical properties such as the flexural strength, modulus and compressive strength at 0.1 mg $H_2PO_4$ can be promoted to 198.0 MPa, 28.5 GPa and 198.0 MPa and also at 2 mg chitosan evaluated 210.0 MPa, 21.2 GPa and 211.0 MPa respectively. The antibacterial test demonstrated that this resin nanocomposite has minimal inhibitory concentration (MIC) about $2 < MIC \leq 4$ for gram-positive and gram-negative bacteria.


# 1. Introduction

In recent years the use of composite resins with a strength close to the tooth has received much attention(1, 2). The use of composite resins is moving in the direction of being able to completely replace fillers such as amalgam(3, 4). The main problem of these composites is volumetric contractions, which occur during the polymerization process (5, 6). In addition to causing tension in the teeth, these volumetric contractions(7, 8), Empty spaces are created between the tooth and the filling, which causes food particles and fluids to accumulate in it and intensifies tooth decay(9, 10). With the help of LDH nanostructures(11, 12), the percentage of volumetric contractions can be reduced and the strength of the composite can be significantly increased(13, 14). Double-layered hydroxides are positively charged nanostructures that can form nanobio-hybrids with negatively charged biomolecules such as vitamins, drugs, and DNA strands (15, 16). Layered hydroxide compounds are a special type of compound whose crystal structure is made up of two-dimensional units placed by weak forces(17, 18). The LDHs are unique materials which can be added to a polymer composition in small amounts(19) and can produce polymer nanocomposites (without much difference in plastic appearance) that have very prominent properties such as lighter weight, properties anti-scratch and impermeable properties against bactrim side effects(20, 21). Mechanical behavior of the LDHs such as their strength and adhesion have a significant contribution to performance in various dental cases(22, 23). The LDH structures due to the unique properties of the layer and the high surface-to-volume ratio, are able to trap the chitosan polymer structures in their structural layers, thereby creating a synergistic effect on mechanical properties(24, 25). Ordinary fillings may break down gradually and under various stresses, or decay may develop over time at the site where the filling is attached to the tooth(26). Nanotechnology has the potential to reduce these damages by producing dental restoratives that are both more durable and durable than today's fillers and more resistant to secondary caries(27, 28). The problem with anti-corrosion composite fillers

is due to an additive in the powder to which calcium and phosphate ions are added for stable release(29, 30). These ions are essential for the longevity of the fillers (31, 32). The high surface area relative to the volume of the LDH structures makes them much more efficient at releasing ions, and thus a much smaller amount of these materials is required to produce the same effect(33, 34). Calcium phosphate nanocomposite filler in a tooth can intelligently release caries agents that protect the tooth from the acid produced by the kettles(35, 36). In addition, it can regenerate minerals from tooth loss by releasing ions into the inefficient mineral area of the tooth(37). Many properties of hydroxyapatite, including mechanical properties such as Fracture strength and fracture toughness, surface properties, biocompatibility and solubility, are affected by the shape and dimensions of the nanomaterial crystals of this material(38). Hydroxyapatite nanoparticles homogeneously and uniformly with a high degree of purity have a spherical structure similar to living tissue, in this structure the bonding reaction as well as the stability on the surface of natural or artificial bone is significantly improved(39). In recent years, much research has been done on hydroxyapatite(40) and chitosan(41) structures due to the unique and improved properties widely used in dentistry. In this study, after preparation of Ca/Mg/Chitosan LDHs modified with HA nanocomposites, these structures were evaluated for the mechanical properties such as flexural strength, modulus. The antimicrobial effects on seven microbial strains such as *E.coli*, *K.pneumoniae, S.marcescens*, *M.luteus, B.sabtilis, S.aureus* and *P.aeruginosa* have been investigated. The final products were characterized with SEM, FT-IR, DLS and TGA.

## 2. Experimental

# 2.1. Materials

Shrimp and vannamei source chitosan was prepared from a local market in Kerman. Commercial materials such as Ca $(NO_3)_2.4H_2O$ (MW:236.15 g/mol, CAS Number: 13477-34-4), $Mg(NO_3)_2.\ 6H_2O$ (Magnesium nitrate hexahydrate, ACS reagent, 99%), Hydrochloric acid (37% Merck KGaA, Darmstadt, Germany) and cetyltrimethylammonium bromide [$(CH_3(CH_2)_{15}N(Br)(CH_3)_3$, ≥ 98%, micellar avg mol wt 62,000] were purchased from Sigma-Aldrich through their representative in Iran Kimia Exir, N, N Dimethylmethanamide, $HCON(CH_3)_2$, MW: 73.09, 99.8% and $H_2PO_4$ powder (96.986 g/mol, reagent-grade) were purchased from Merck company through their representative in Iran Kimia Exir. All the aqueous solutions were prepared by using Deionized water (DW) which was purchased from the ABSAN laboratory.

# 2.2. Preparation of Chitosan from Litopenaeus vannamei

In this research, chitosan was prepared from *Litopenaeus vannamei* shrimp shell according to the method stated in the literature(42). Afterwards, to remove and modified the loose tissue the shells of these species were placed in temperature conditions 50 ˚C for 24 hours. In the next step for the demineralization, the shells washed with 5mL HCl 0.1M solution at room temperature (25 ◦C). Finally, the white precipitate obtained was dried at 60 ˚C for 4 days.

# 2.3. Ca/Mg/Chitosan Layered Double Hydroxide

0.392g $Ca(NO_3)_2$ and 0.065g $Mg(NO_3)_2$ were dissolved in 20 mL of deionized water at reflux system under nitrogen gas atmosphere and then after 45 minutes 0.01g CTAB were added to this mixed solution drop by drop under vigorous stirring, followed by 45 minutes. The precipitates were separated by filter paper, washed with ratio 2:1 distilled water: ethanol for several times and dried in an oven at 60 ◦C for 48 h. To the synthesis of Ca/Mg/chitosan LDHs 0.02 g of chitosan was dissolved in 10 mL ratio 2:1 distilled water: dimethylformamide as capping agent, complexing and supplement agent under reflux system with 400rpm at 50 ◦C for 2h.

## 2.4. Ca/Mg/Chitosan LDHs modified with Hydroxyapatite (HA)

In the next step to prepare the Ca/Mg/Chitosan LDHs modified with hydroxyapatite, the solution obtained from the previous step (Ca/Mg/ chitosan-LDHs) 0.1 g of $H_2PO_4$ powder (dissolved in 5 ml of distilled water) was added drop by drop under vigorous stirring to the reaction mixture, to adjustment the pH of the mixed precursor about 7.1–7.9 NaOH 1M solution was used. Then above solution was placed in microwave device under the irradiation at 300watt, 600watt and 900watt for 10 minutes and 5 minutes with alternating on (30 seconds) and off (60 seconds) pulses. The final products were dried at a 50◦C for 48h. Experimental different conditions for the preparation of Ca/Mg/Chitosan LDHs modified with HA are summarized in Table 1.

Table 1
Laboratory experiments for the preparation of Ca/Mg/chitosan LDHs modified with HA dental nanocomposites

| Sample No. | CTAB (g) | pH | Microwave power (watt) | Time (min) | Figure No. |
|---|---|---|---|---|---|
| S1 | 0.01 | 7.1–7.9 | 300 | 5 | 3a |
| S2 | 0.01 | 7.1–7.9 | 300 | 10 | 3b |
| S3 | 0.01 | 7.1–7.9 | 600 | 5 | 3c |
| S4 | 0.01 | 7.1–7.9 | 600 | 10 | 3d |
| S5 | 0.01 | 7.1–7.9 | 900 | 5 | 3e |
| S6 | 0.01 | 7.1–7.9 | 900 | 10 | 3f |

[Here insert Table 1]

## 2.5. Mechanical Assessment

To assessment the mechanical properties of the Ca/Mg/Chitosan LDHs modified with HA nanostructures, different concentrations of the chitosan and $H_2PO_4$ powder were added to Heliomolar Flow (Ivoclar Vivodent AG, FL-9494) resin composites. The universal testing machine according to ISO 9917(43), in the stainless steel cylindrical template with 6mm ×4mm dimensions were measured.

## 2.6. Antibacterial assay

In this present study, the antimicrobial activity of the as synthesized Ca/Mg/Chitosan LDHs modified with hydroxyapatite was investigated based on the agar dilution method. In the solid-state dilution method, successive dilutions are prepared from the tested antimicrobial agent and transferred to sterile plates. Then a certain amount of bacterial suspension is inoculated using a sampler on the surface of the plates. The minimum concentration of antimicrobial agent that inhibits the growth of microorganisms is considered as MIC (minimum inhibitory concentration). To prepare the microbial suspension, the loop was first sterilized on a flame and used to remove microbial colonies and transferred to the Müller-Hinton broth medium. 1 cc of the microbial suspension in a 10 cc flask was diluted to 10 times. The final dilution in the plates reached 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125 µg/mL. Positive and negative control plates will also be considered.

# 3. Results and Discussion

Fourier transform infrared spectroscopy (FTIR) used as a powerful and developed method based on the absorption of radiation and the study of vibrational mutations of polyatomic molecules and ions for determining the structure and measurement of chemical species. FT-IR spectra of Ca/Mg/chitosan - LDHs and Ca/Mg/Chitosan LDHs modified with HA are shown in Fig. 1a and 1b. The results clearly show that a series of weak bands of metal-oxygen (M-O) stretching vibrations and oxygen-metal-oxygen (O-M-O) bending between 400 $cm^{-1}$ and 800 $cm^{-1}$. The bands at about 900 $cm^{-1}$, 1300 $cm^{-1}$ and 1400 $cm^{-1}$ can be attributed to the stretching modes and C-O-C, C = O or C-N and CHO or C–H bending vibrations of $–CH_2$ in chitosan structures and Ca/Mg –LDHs. The band in the range 3500 $cm^{-1}$ which is also widespread is related to O–H stretching in Ca/Mg/chitosan-LDHs nanostructures. After modified Ca/Mg/chitosan LDHs modified with HA the bands in the frequency range 500 $cm^{-1}$ and 980 $cm^{-1}$ can be related to $PO_4^{3-}$ group. Also $CO_3^{2-}$ group at 1480 $cm^{-1}$ indicates the presence of $Ca_5 (PO_4)_3OH)_2$ structures in the final products.

[Here insert Fig. 1a and 1b]

Thermo-gravimetric analysis (TGA) and differential thermal analysis (DTA) were conducted via METTLER TOLEDO TGA/DTA instrument at a heating rate of 10 ºC/ min under nitrogen ($N_2$) atmosphere and a cooling rate of 58 C/min in the range of 50–400 ºC. The TGA/DTA curve of the Ca/Mg/chitosan-LDHs modified with HA is shown in Fig. 2a. As the results show the final products have a good thermal stability as only one weight loss step in the temperature range of 250◦C. The weight loss evaluated be ~ 30% which can be related to loss of water molecules and weakening of intermolecular hydrogen bonds at Ca/Mg/Chitosan LDHs structures. After 250◦C up to 400◦C with the loss of all covalent bonds complementary thermal degradation occurs. According to DTA curve two endothermic stage related to the loss of water molecules from the structure around 100◦C and the thermal decomposition of Ca/Mg/Chitosan LDHs modified with HA nanostructures can be viewed at about 250◦C up to 400◦C. To investigate the particle size and the normal scattering distribution we used dynamic light scattering (DLS) technique. Figure 2b shows the DLS diageram of the Ca/Mg/Chitosan LDHs Modified with HA

about Dn 10%:7.72 nm, Dn 50%:10.19 nm and Dn 90%:15.44 nm and also mode: 9.73 nm, mean: 10.95 nm, Dev: 28.05% intensity: 100% were estimated. The existence of a sharp and uniform peak indicates the accuracy in the construction of nanostructures and the desired method.

[Here insert Fig. 2a and 2b]

Scanning electron microscopy (SEM) was used as an analysis to investigate the morphology of surface and and cross-section properties. The SEM images of the samples show which the power and time as the most effective device independent variables have the greatest effect on the particle size and morphological properties. The effect of irradiation time and power on the morphology of products are shown in Fig. 3a-3f. SEM images in Fig. 3a and 3b show that with increasing, the irradiation time from 5 minutes to 10 minutes at 300watt, sufficient time is created for the formation of nanoparticles with uniform dispersion. By increasing the microwave power to 600watt at 5 minutes, particles have inhomogeneous adhesions and separate in some areas. This can be related to increase the surface-to-volume ratio in the particles, which increases in 10 minutes. The influence of microwave power and irradiation time on the morphology of the Ca/Mg/Chitosan LDHs modified with HA at 900watt for 5 minutes indicated which the provided energy is exorbitance hence, the particles are formed as agglomerated and heterogeneous structures. The ability of microwave power to create high energy led to an increase in size and heterogeneous uniformity. Due to the high surface area to volume and the nature of being layered of the Ca/Mg/Chitosan LDHs modified with HA increase in microwave power and irradiation time can cause packed structures.

[Here insert Fig. 3a and 3b]

Flexural strength, compressive strength and modulus tests are the most effective dependent variables in the study of mechanical properties of resins. To assess the physical performance of the as-synthesized Ca/Mg/Chitosan LDHs modified with HA dental nanocomposites. We evaluated Ca/Mg/Chitosan LDHs modified with HA nanocomposites in different concentrations of chitosan and $H_2PO_4$ powder to achieve the best results for the flexural strength, compressive strength and modulus tests. Figure 4 indicates the evaluation of the effectiveness of increasing chitosan polymer in different concentrations on mechanical properties such as the flexural strength, compressive strength and modulus. The results indicate that with increasing concentration of chitosan solution mechanical properties such as the flexural strength, modulus and compressive strength up to 2 mg shows an upward trend. This can be due to the stabilizing properties of the chitosan through cross-linked band to increase mechanical properties. The most mechanical properties related to 2 mg chitosan for the flexural strength, compressive strength and modulus tests evaluated 210.0 MPa, 21.2 GPa and 211.0 MPa respectively.

[Here insert Fig. 4]

Figure 5 shows the evaluation of mechanical properties such as flexural strength, modulus and compressive strength related to the increase of $H_2PO_4$ powder as the precursor for the formation of calcium hydroxyapatite in the final products. The results show that increasing the concentration of the

$H_2PO_4$ powder causes more calcium hydroxyapatite nanoparticles and then agglomeration happens in the dental nanocomposite. Due to the increase in the surface to volume ratio of nanoparticles, aggregates are formed which at high concentrations reduce the mechanical properties of the final products. The most mechanical properties such as the flexural strength, modulus and compressive strength tests can be viewed at 0.1 mg $H_2PO_4$, 198.0 MPa, 28.5 GPa and 198.0 MPa respectively.

[Here insert Fig. 5]

The results of antimicrobial effects of the as synthesized Ca/Mg/chitosan LDHs modified with HA dental nanocomposites are shown in Table 2. In this MIC assay, a positive sign means bacterial growth and a negative sign means no bacterial growth. The synthesized nanocomposites have shown antimicrobial effect on *Escherichia coli*, *Klebsiella pneumonia, Pseudomonas aerogios* and *Serratia marcesceus* which are gram-negative bacteria $2 < MIC \leq 4$ and also the final products indicate antimicrobial effect on *Bacillus subtillis, Micrococuus luteus* and *Staphylococcus aureus* which are gram-positive bacteria $2 < MIC \leq 4$. Figure 6 shows antimicrobial effects.

Table 2
Results of antimicrobial effects of nanoparticles based on agar dilution method.

| **Concentration** | **64** | **32** | **16** | **8** | **4** | **2** |
|---|---|---|---|---|---|---|
| **Bacteria** | **µg/ml** | **µg/ml** | **µg/ml** | **µg/ml** | **µg/ml** | **µg/ml** |
| *E.coli* PTCC1330 | - | - | - | - | - | + |
| *K.pneumoniae* PTCC1053 | - | - | - | - | - | + |
| *S.marcescens* PTCC1621 | - | - | - | - | - | + |
| *M.luteus* PTCC1110 | - | - | - | - | - | + |
| *B.sabtilis* PTCC 1023 | - | - | - | - | - | + |
| *S.aureus* PTCC1112 | - | - | - | - | - | + |
| *P.aeruginosa* PTCC1074 | - | - | - | - | - | + |

[Here insert Table 2]

[Here insert Fig. 6]

# 4. Conclusion

The current study aimed at investigating the physicochemical properties of Ca/Mg/Chitosan LDHs Modified with HA nanocomposites. In this study chitosan source extracted from shrimp shell. This polysaccharide is usually discarded and left in the environment leading to the environmental pollution. The great amount of chitosan can be found in shrimp shells compared with the commercial chitosan to the preparation of Ca/Mg/Chitosan LDHs Modified with HA nanocomposites. According to the results

the optimization of Ca/Mg/Chitosan LDHs Modified with HA as dental nanocomposites show the most mechanical properties for 2 mg chitosan about 210.0 MPa, 21.2 GPa and 211.0 MPa and at 0.1 mg $H_2PO_4$198.0 MPa, 28.5 GPa and 198.0 MPa for the flexural strength, compressive strength and modulus tests respectively. The Minimum inhibitory concentration of antimicrobial effect of the final nanocomposites was evaluated about $2 < MIC \leq 4$ gram-negative and gram-positive bacteria.

# Declarations


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Institute of Neuropharmacology, Kerman University of Medical Sciences, also Laboratory of Nanostructures and Nanodrugs Materials, Kerman,Iran. This study received ethical approval 400000180 from the local ethical committee of the Kerman University of Medical Sciences.


### Declaration Conflict of Interest

The authors declare that they have no conflict of interest.

Compliance with Ethics Requirements

This article does not contain any studies with human subjects.

### Author Contribution

Mehdi Ranjbar conceived of the presented idea and supervised the findings of this work.

Mohammad Hasan Moshafi developed the theory and performed the computations.

All authors discussed the results and contributed to the final manuscript.


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# Figures

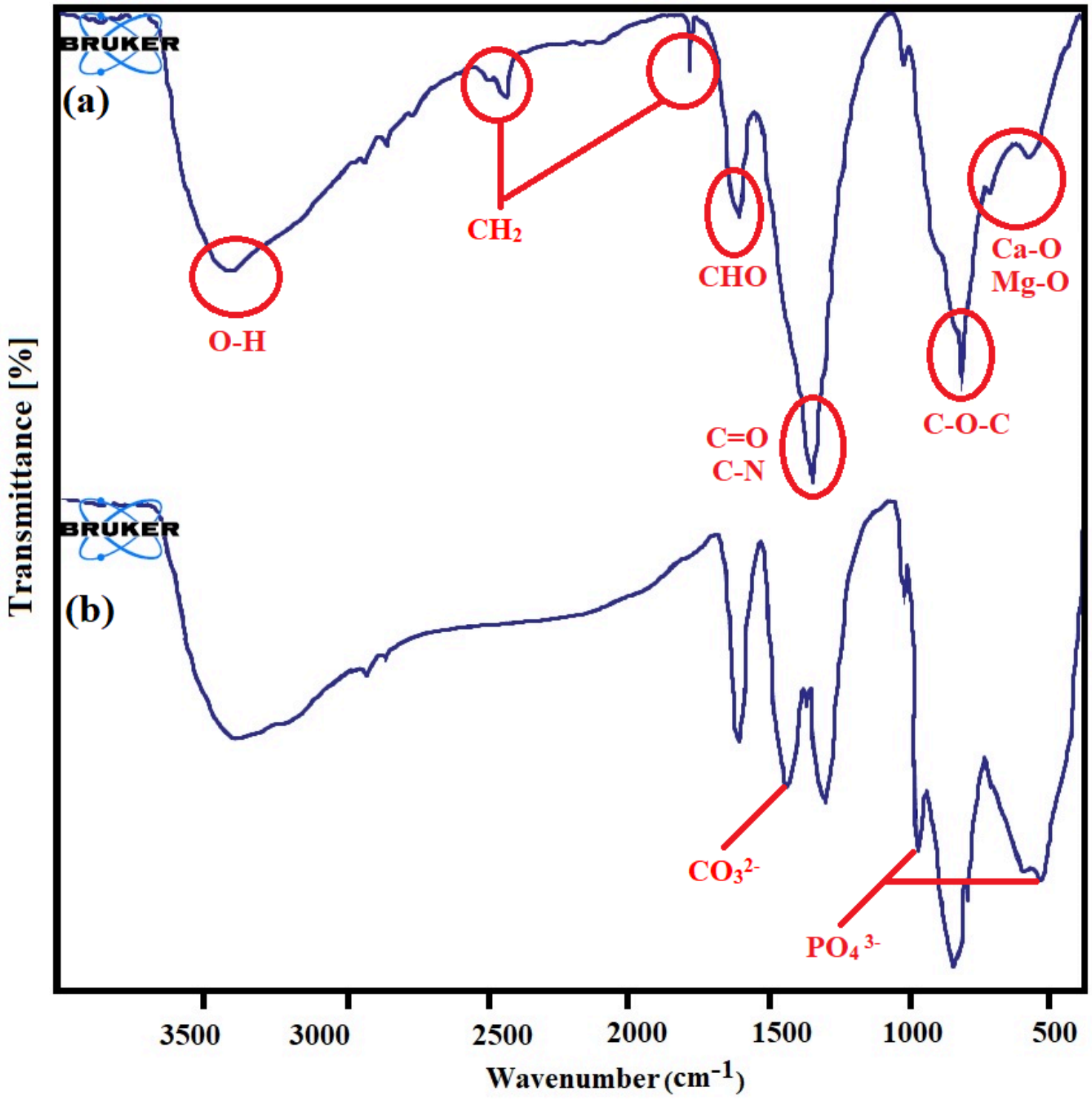



**Figure 1**

The FT-IR spectroscopy of the Ca/Mg/chitosan-LDHs nanostructures (a) and Ca/Mg/ chitosan LDHs modified with HA in the range of 500-3500 $cm^{-1}$ (b).

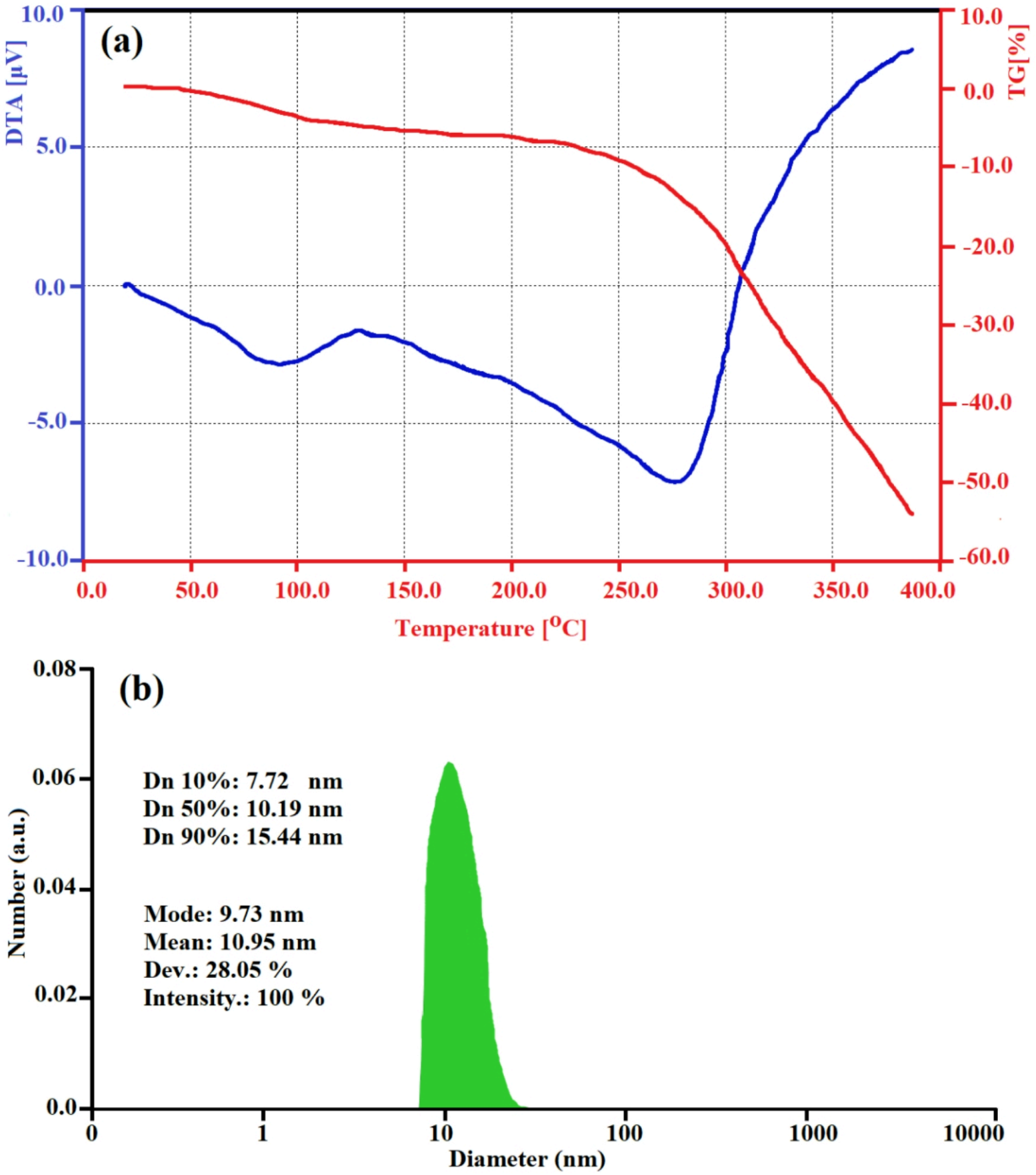
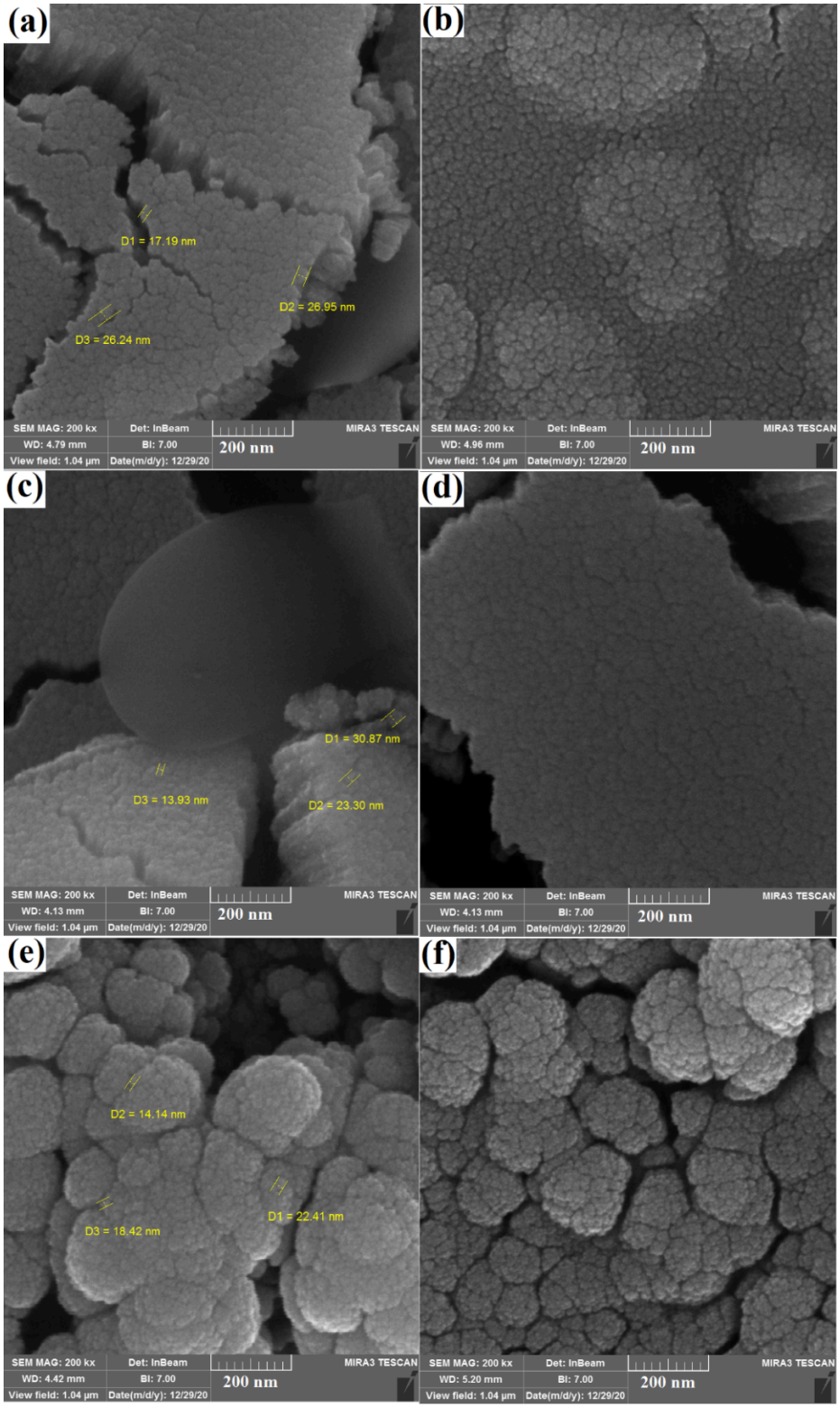



**Figure 2**

The TGA/DTA curve of the Ca/Mg/ chitosan LDHs modified with HA nanocomposites under nitrogen ($N_2$) atmosphere in the range of 50–400 °C (a) and the DLS diagram of the final product (b).




## Figure 3

The SEM images of Ca/Mg/Chitosan LDHs modified related to Sample1 (a), Sample2 (b), Sample3(c), Sample4 (d), Sample5 (e), Sample6 (f).

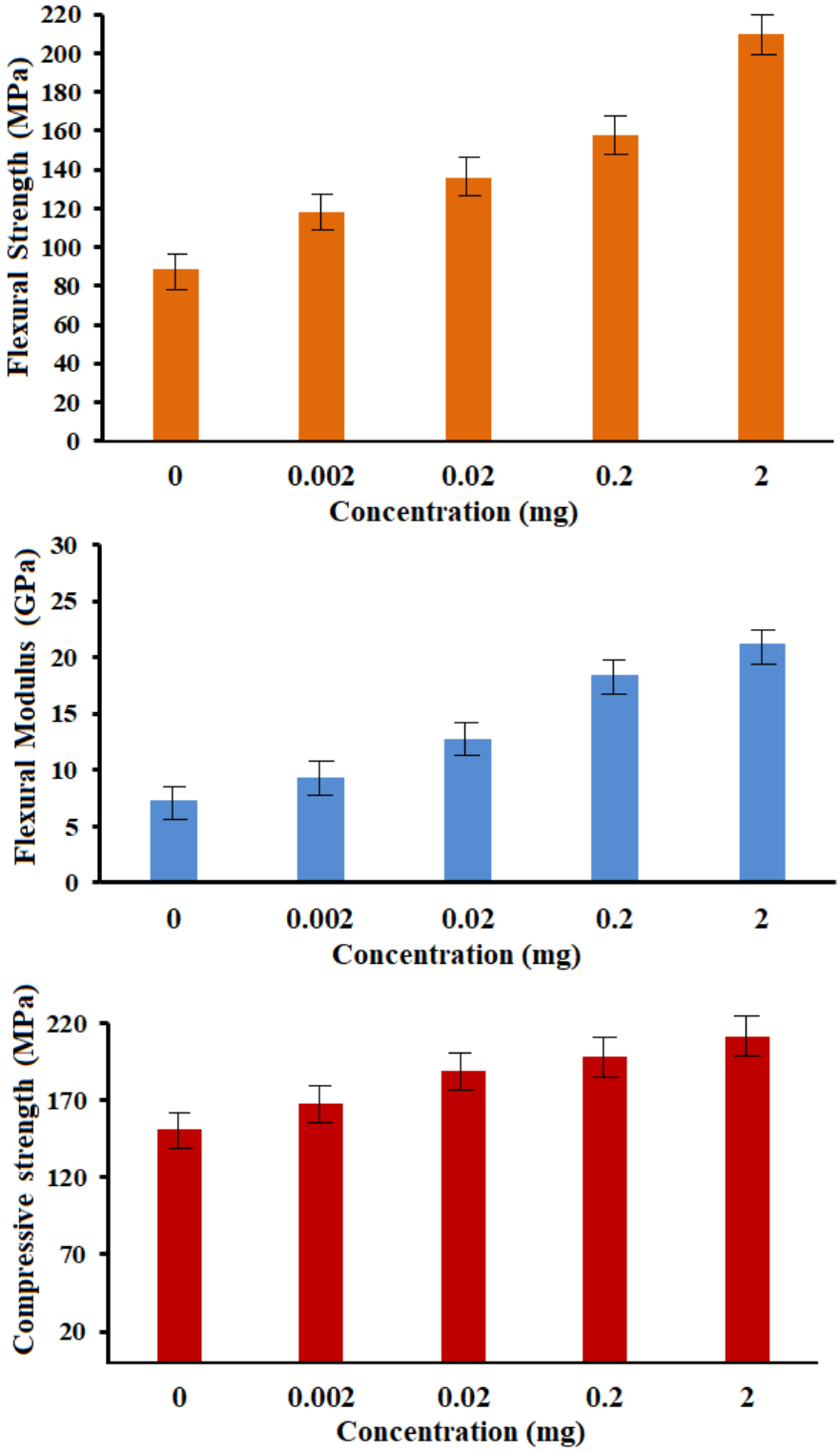



**Figure 4**

Mechanical properties of the Ca/Mg/chitosan LDHs modified with HA resin nanocomposites with different concentration of chitosan on flexural strength (MPa), flexural modulus (GPa) and compressive strength (MPa).

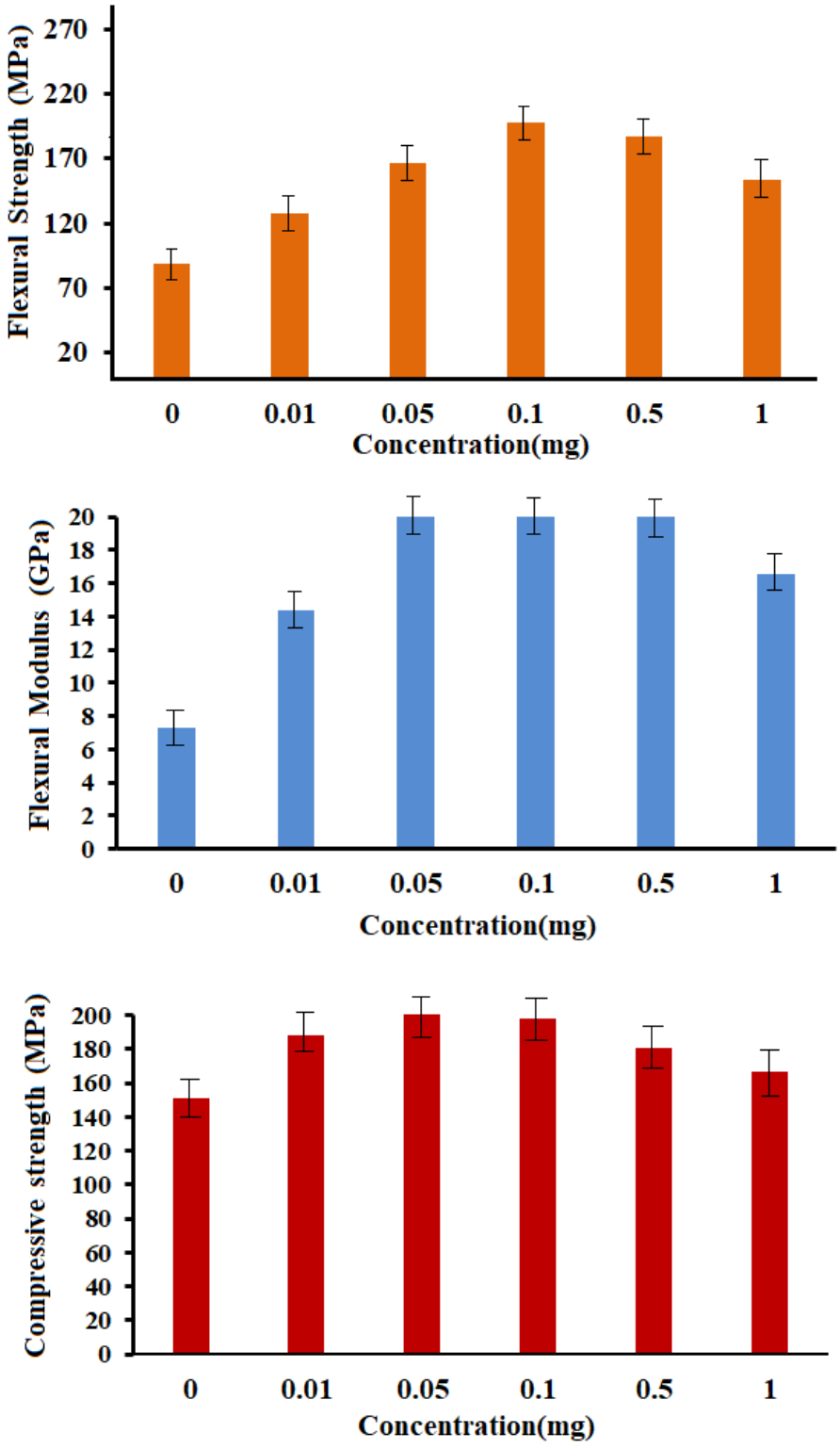



**Figure 5**

The evaluation of mechanical properties such as flexural strength (MPa), modulus (GPa) and compressive strength (MPa) for $H_2PO_4$ powder with different concentration.

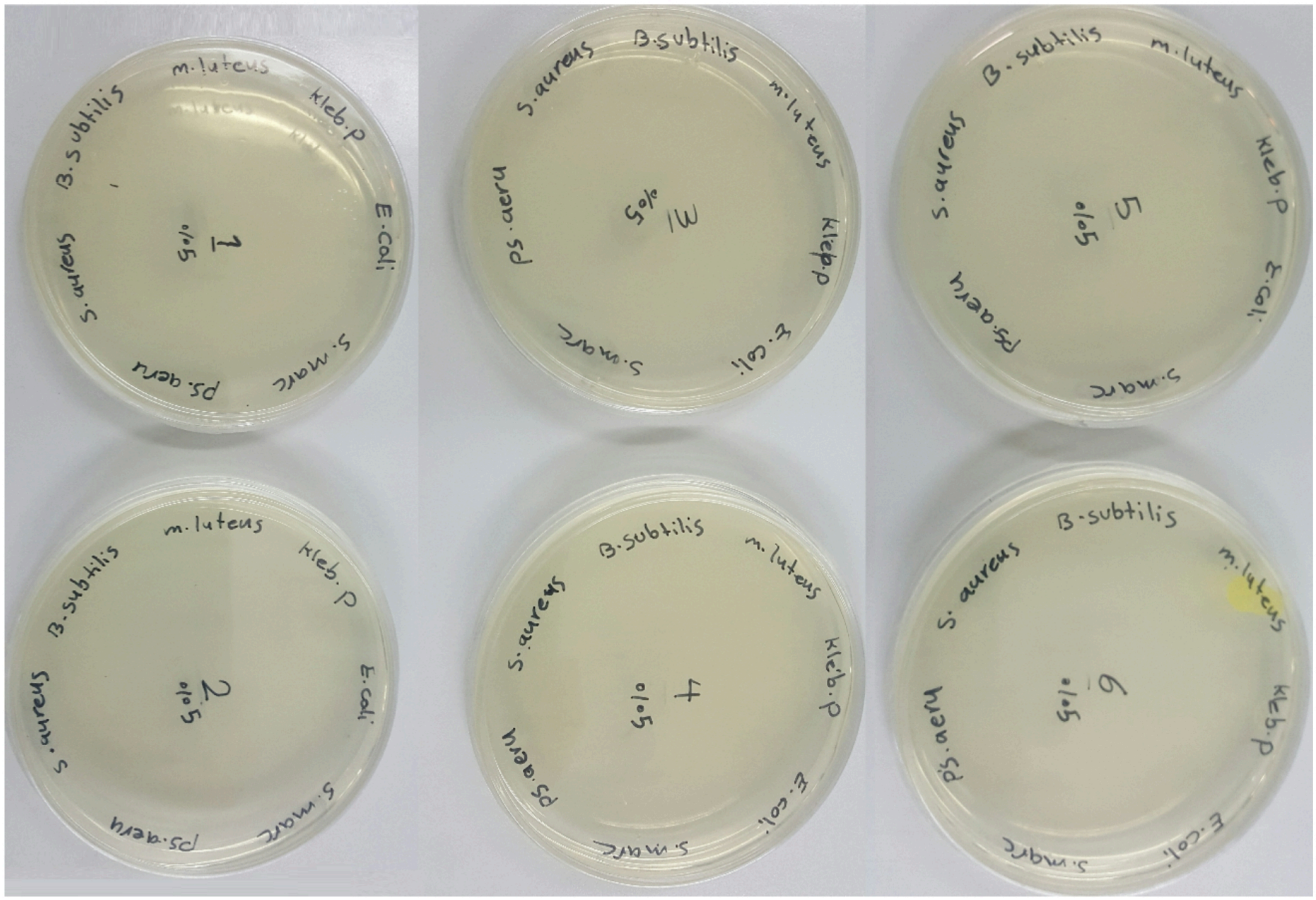



**Figure 6**

The final concentrations for plates were achieved from respectively 2 µg/mL, 4 µg/mL, 8 µg/mL, 16 µg/mL, 32 µg/mL, 64 µg/mL for the as synthesized Ca/Mg/chitosan LDHs modified with HA dental nanocomposites.