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Application of Taguchi method in the optimization of synthesis of cellulose-MgO bionanocomposite as antibacterial agent

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In this study, optimal conditions to form cellulose-MgO nanocomposite with antibacterial properties were evaluated. Applying the Taguchi method, 9 experiments were designed and the effects of different concentrations of biopolymers cellulose (0.5, 1 and 2 mg/ml), MgO nanoparticles (2, 4 and 8 mg/ml) and stirring times (30, 60 and 90 min) on antibacterial activity of synthesized nanocomposites were assessed. The characterizations of products were investigated by dynamic light scattering (DLS), raman spectroscopy, scanning electron microscope (SEM), thermogravimetric analysis (TGA) and differential thermal analysis (DTA). The results showed that the nanocomposite produced in the conditions of experiment 9 (MgO 8 mg/ml, cellulose 2 mg/ml and stirring time of 60 min) has the strongest antibacterial activity. The outcomes of both methods of colony forming units (CFU) and disc diffusion indicated that the antibacterial activity of cellulose-MgO nanocomposite was significantly higher than its components ($P < 0.05$). Thermal analysis indicated improvement in the thermal stability of the cellulose biopolymer after the formation of the nanocomposite. Due to the improvement of the antibacterial properties of cellulose-MgO nanocomposite compared to its components, we can use it as a new antibacterial agent in the fields of pharmaceutical, medicine and dentistry.

Keywords: cellulose biopolymer, magnesium oxide nanoparticles, nanocomposite, antibacterial activity, Taguchi method.

INTRODUCTION

Despite the therapeutic advances in recent years, no effective solutions have been so far provided to cure some diseases such as cancer[1–3], autoimmune diseases[4, 5], chronic pains[6, 7] and microbial infections[8]. Bacterial infections are still known as one of the major causes of morbidity and mortality in the world[9]. *Escherichia coli* and *Staphylococcus aureus* bacteria cause some of the most important common infections in different situations in the society or transmission through the food chain[10]. According to the World Health Organization, *Staphylococcus aureus* and *Escherichia coli* bacteria have shown resistance to some commonly used antibiotics in more than 50% of cases. Nowadays, despite increased bacterial resistance to antibiotics and adapting to them, finding new solutions to eliminate bacteria has become necessary[8, 9].

The use of nanotechnology and producing nanoparticles have developed new hopes for solving the humans today's problems. In nanoparticles, due to increased surface to volume ratio, all physical and chemical properties are changed, and as a result, their reactivity will increase[11]. Recently, the metal oxides have drawn the attention due to strength in different circumstances, being safe for humans and the environment. Metal oxide nanoparticles show many capabilities and features with their unique structural features such as fine granularity, more solubility, easier passing through the cellular barrier and more reactivity[12]. Some of metal oxide nanoparticles, such as silver oxide, titanium oxide, zinc oxide, copper oxide, iron oxide and magnesium oxide have shown favorable antimicrobial properties[13, 14]. One of the widely used nanoparticles in industry and medicine are MgO NPs. Having features such as biodegradability, affordable cost and environmental adaptability, these nanoparticles have numerous applications in various industries. MgO NPs can be used alone or in combination with other antimicrobial groups as a potential effective antibacterial agent[14, 15]. One of the most limitations of using metal nanoparticles is their instability due to strong tendency to accumulation. The use of stabilizing agents or surfactants prevents the unwanted accumulation of nanoparticles by maintaining their size and controlling their shape[16]. In recent years, the design of nanocomposites has been studied extensively for various applications, and various studies have been done on biodegradable polymer-based nanocomposites containing metal nanoparticles. The use of biodegradable polymers, according to their desired characteristics and effects in improving the properties of metal nanoparticles, has covered a major part of the research[11, 17, 18].

Due to having unique features such as biodegradability, availability, renewability and nontoxicity, cellulose has drawn much of attention[19, 20]. Cellulose production by microbial methods is an alternative to protect natural resources. Bacterial cellulose is a microbial polysaccharide with higher purity compare to plant cellulose. The polymer is composed of β 1→4 units of D–glucose form that its similar forms can found in plants. Bacterial

cellulose is mainly produced by bacteria of the genus *Gluconacetobacter* that are nonpathogenic bacteria, among which, *Gluconacetobacter xylinum* produces the greatest amount of cellulose[21, 22]. The use of polymer-based nanocomposites containing metal nanoparticles has several advantages over metal nanoparticles, including trapping of nanoparticles within the polymer matrix to prevent their accumulation, better surface distribution of nanoparticles, greater contact of nanoparticles with the bacteria and faster removal of bacteria[23]. Previous studies have shown that the ratio of nanoparticles to the biopolymer as well as their stirring time are among the factors affecting the antimicrobial activity of nanocomposites[18, 24].

The aim of this study was to produce microbial cellulose for the synthesis of novel cellulose-MgO nanocomposite and evaluating its antibacterial activity. The characterizations of products were investigated by dynamic light scattering (DLS), raman spectroscopy, scanning electron microscope (SEM), thermogravimetric analysis (TGA) and differential thermal analysis (DTA). The antibacterial activity of nanocomposite on *Staphylococcus aureus* and *Escherichia coli* was studied using the colony forming unit (CFU) and disc diffusion methods.

EXPERIMENTAL

Synthesis of Cellulose Biopolymer

The cellulose biopolymer was obtained from the 7-day culture of *Gluconacetobacter xylinus* in Hestrin-Schramm medium under static conditions at 30°C. The resulting cellulose was placed for 1 h in boiling 0.5 M sodium hydroxide for treatment. It was then washed with distilled water for removal of possible remaining residues, and finally, was dried at 40°C[25].

Synthesis of Magnesium Oxide Nanoparticle

The MgO NPs were synthesized using the co-precipitation method. In this method, the solutions of 0.1 M Mg $(NO_3)_2$ and 1 M NaOH were prepared with a volume of 50 ml. Each solution were separately stirred for 1 h at room temperature to become perfectly uniform. Then, the magnesium nitrate solution was added to the sodium hydroxide solution drop by drop under constant stirring condition. After combining the two solutions, the resulting solution was continuously stirred for 1 h at 40°C until a milky sediment was obtained. The resulting precipitate was isolated by centrifugation and rinsed with double-distilled water for three times to remove other impurities. The resulting precipitate was placed in oven at 60°C to obtain the powder compound of Mg $(OH)_2$. The resulting Mg $(OH)_2$ powder was calcined in the electric furnace for 2 h at 450°C to obtain MgO NPs.

Synthesis of Cellulose-Mgo Nanocomposites

To provide optimum conditions for the synthesis of antimicrobial nanocomposites of cellulose-MgO, 9 experiments were designed by using the Taguchi method and Qulitek-4 software (Table 1). To this end, different levels of cellulose biopolymer powder added to 50 ml of twice-distilled water in separate containers to form solutions containing 0.5, 1 and 2 mg/ml. Also, solutions containing 2, 4 and 8 mg/ml MgO NPs was obtained with added NPs powder to three separate containers containing 50 ml twice-distilled water. The all solutions were sonicated for 10 min to be completely homogenized. Then, the MgO NPs solutions were added drop by drop to solutions containing cellulose biopolymers with continuous shaking. The resulting mixtures were stirred continuously for 30, 60 and 90 min at temperature of 40°C until a uniform solution was obtained. Each of the resulting solutions were divided into two parts. A part was stored at a temperature of 4°C to investigate their antibacterial properties, and the other part was put in a 40°C oven to obtain the nanocomposites powder for analysis their properties[26, 27].

Antibacterial Activity

To investigate the antibacterial activity effect of the synthesized nanocomposites on gram-positive bacteria (*Staphylococcus aureus*) and gram-negative bacteria (*Escherichia coli*), two different estimation methods of colony forming units (CFU) and disc diffusion were used. The nutrient agar medium was used to prepare the studied bacterial suspension. Once the culture media were prepared, *Staphylococcus aureus* and *Escherichia coli* bacteria were cultured on them and incubated for 48 h at a temperature of 37°C. After isolating the bacteria and preparing the suspension, some of the bacteria colonies were removed and dissolved in distilled water. To achieve the desired concentration of bacteria, the solutions were diluted with distilled water to reach an approximate concentration of 10^8 CFU/ml. A volume of 5 ml of solution containing bacteria and 5 ml of solutions containing different concentrations of synthesized nanocomposites were transferred to the falcons. The falcons were vortexed to make them well homogeneous. Then, they were shaken for 6 h in a shaking incubator at 37°C at 140 rpm.

Table 1. Taguchi design of experiments and effects of synthesized nanocomposites on the survival rate of *Staphylococcus aureus* and *Escherichia coli* bacteria

Experiment	MgO (mg/ml)				Cellulose (mg/ml)				Stirring time (min)				Bacterial survival (Log_{10} CFU/ml)	
	2	4	8		0.5	1	2		30	60	90		Gram positive	Gram negative
1		2				0.5				30			4.42	5.02
2		2				1				60			3.28	4.32
3		2				2				90			4.72	5.59
4		4				0.5				60			3.67	3.96
5		4				1				90			0.49	1.04
6		4				2				30			3.32	3.89
7		8				0.5				90			3.76	4.11
8		8				1				30			0.98	0.66
9		8				2				60			0.00	0.00

For testing the colony forming unit, the bacteria suspensions were diluted using a 10-fold serial dilution. A volume of 0.9 ml sterilized distilled water were poured into 1.5 ml microtubes. Then, 0.1 ml of different concentrations of bacterial suspensions and synthesized nanocomposites were added to each microtube. The resulting solutions for each dilution were then cultured onto nutrient agar medium, and the plates were incubated at 37°C for 24 h. After incubation, the rate of grown colonies on each plate were counted, and their averages for each of the 9 experimental units designed with the Taguchi method were calculated. All experiments at this stage were performed with three times repeat.

After determining the best ratio of biopolymer to nanoparticle and the stirring time with the highest antibacterial activity, the antibacterial activity rate of the synthesized nanocomposite with its constituents were evaluated by using colony forming units (CFU) and disc diffusion methods. In the disc diffusion method, a homogeneous suspension of *Staphylococcus aureus* and *Escherichia coli* bacteria nearly concentration of 10^8 CFU/ml was prepared and transferred onto the nutrient agar medium. The medium was fully cultured using cotton swabs. Then, we placed there 3 paper discs containing nanocomposite, cellulose and MgO NPs on the medium in each plate and incubated them for 24 h at 37°C. Finally, the plates were examined under the light, and the diameter of inhibition zone for each disc was measured using a ruler.

Characterizations

The dynamic light scattering (DLS), ZEN3600 model (Malvern, UK), was used to confirm the synthesis and examine the size of MgO nanoparticles. A Raman spectrum was prepared from cellulose biopolymer using a spectrometer (Nicolet Almega Thermo, USA) in the range of 200–3500 cm^{-1}. The SEM images of cellulose-MgO nanocomposite were obtained using a scanning electron microscope (TESCAN, Czech Republic) at the magnifications of 100 nm and 200 nm. The thermogravimetric analysis and differential thermal analysis of samples were measured using a thermal analysis device (Linseis STA PT1000, Germany). For this purpose, an empty aluminum pan as the reference and the nitrogen atmosphere were used. The samples weighing approximately 0.3 gr with the speed of 10°C/min at the temperature range of 0–800°C were scanned.

RESULTS AND DISCUSSION

Antibacterial Activities Analysis

To determine the optimal conditions for the synthesis of cellulose-MgO nanocomposite with the highest antibacterial activity, 9 experiments were designed based on the Taguchi method, and the effects of synthesized nanocomposites under various conditions on the survival rate of *Staphylococcus aureus* and *Escherichia coli* bacteria were assessed (Table 1). The results showed that the nanocomposites produced under the conditions of experiment 9 (MgO 8 mg/ml, cellulose 2 mg/ml and stirring time of 60 min) has the strongest antibacterial activity against gram-positive and gram-negative bacteria, and no bacteria grew in their presence.

Examining the antibacterial activity of cellulose-MgO nanocomposites by colony forming unit (CFU) method indicated that the antibacterial activity of the synthesized nanocomposite against the studied bacteria were significantly higher than its components (Table 2). Evaluation of antibacterial activity of cellulose-MgO nanocomposite by using the disc diffusion method revealed similar results, as the growth inhibition zone of cellulose-MgO based nanocomposites against *Staphylococcus aureus* and *Escherichia coli* bacteria were as 20 mm and 19 mm, respectively, which were significantly higher than cellulose (0 mm, 0 mm) and magnesium (15 mm, 15 mm) (Table 2, Fig. 1).

The study results suggested that the incorporation of cellulose biopolymer and MgO NPs leads to the formation of cellulose-MgO nanocomposite, which not only

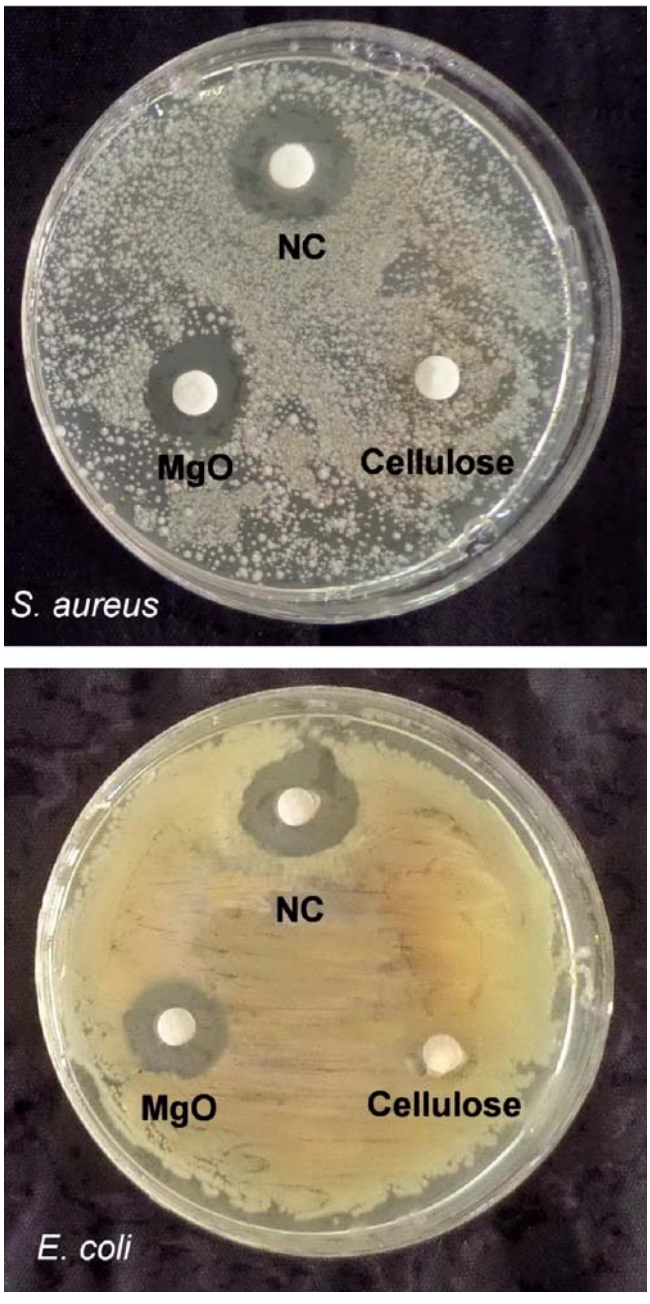

Figure 1. Comparison of inhibition zone of cellulose-MgO nanocomposite (NC), MgO NPs and cellulose biopolymer against *Staphylococcus aureus* and *Escherichia coli* bacteria

Table 2. Antibacterial activity of cellulose-MgO nanocomposite, cellulose biopolymer and MgO NPs against *Staphylococcus aureus* and *Escherichia coli*

Factors	Gram positive bacteria (*Staphylococcus aureus*)		Gram negative bacteria (*Escherichia coli*)	
	Bacterial survival (Log_{10} CFU/ml)	zone of inhibition (mm)	Bacterial survival (Log_{10} CFU/ml)	zone of inhibition (mm)
MgO	3.23^{b}	15.00^{b}	3.87^{b}	15.33^{b}
Cellulose	7.88^{a}	0.00^{c}	7.69^{a}	0.00^{c}
Cellulose- MgO	0.00^{c}	20.33^{a}	0.00^{c}	19.66^{a}
SEM	0.03	0.59	0.04	0.47

Means within columns with different superscripts show significant difference according to LSD test ($P < 0.05$); SEM, standard error of the mean.

retains the characteristics of its components, but also, the interaction effect of the constituents on each other leads to the formation of new properties and improved properties of the synthesized nanocomposites. This increasing effect can be due to improved structural properties of nanocomposite, including its increased surface area, enhanced reactive sites and optimized oxidative capacity, which improve the biological activities of the nanocomposite compared to its components.

In agreement with these results, the previous studies reported the antibacterial activity of MgO NPs alone or in combination with other materials as the nanocomposites[14, 28]. Furthermore, it has been reported that increased concentration and reduced size of MgO NPs improve the antibacterial activity of these nanoparticles[29]. The exact action mechanism of MgO NPs, causing bacterial death, is not quite clear yet; however, several mechanisms have been proposed. The most important of these mechanisms may include oxidative stress induction, releasing toxic metal ions and damaging cell membranes and disrupting its activities[30]. Tang and Lv[31] indicated that MgO NPs have a better performance against gram-positive bacteria than the gram-negative ones. Leung and coworker[30] reported that the mechanism of cell death in the gram-negative *Escherichia coli* bacterium in the presence of MgO NPs is not related to oxidative stress and lipid peroxidation, and the damage to the cell membrane is the leading cause of death in these cells. This difference can arise due to different structure of the membranes of gram positive and negative bacteria. Gram-negative bacteria have a complex outer membrane structure that reduces the ROS penetration into the cell as a primary barrier. Various studies reported improvement in the antibacterial activities of different metal nanoparticles in combination with cellulose and nanocomposite formation with this polymer[26, 32]. By trapping nanoparticles within its matrix, the cellulose biopolymer prevent their aggregation, causing better surface distribution of nanoparticles and their close contact with the bacteria, and thus, improve the antibacterial activity of nanoparticles.

Table 3 indicates the impact of factors of MgO, cellulose and stirring time on the survival rate of *Staphylococcus aureus* and *Escherichia coli* bacteria. The results suggest that the MgO NPs at level 3, and cellulose biopolymer and stirring time at level 2 have the greatest effect on reduced survival rate in both gram-positive and gram-negative bacteria groups. The interaction effects of factors on the survival rate of gram-positive and gram-negative bacteria are displayed in Table 4. Cellulose biopolymer at level 3 and the stirring time at level 2 showed the highest interaction influence on each other and on reducing the growth rate of *Staphylococcus aureus* as 32.30 and *Escherichia coli* as 42.21. MgO NPs and cellulose biopolymer at level 3 showed significant interaction effect on reducing the growth of gram-positive bacteria (21.61) and gram-negative bacteria (19.85). The lowest percentage of interaction influence index belonged to MgO NPs at level 3 and the stirring time at level 2 as 15.78 and 6.88 for *Staphylococcus aureus* and *Escherichia coli* bacteria, respectively.

Analysis of variance of parameters affecting the growth reduction of gram-positive and gram-negative bacteria are shown in Table 5. The greatest impact on reducing the growth of *Staphylococcus aureus* and *Escherichia coli* bacteria related to MgO NPs as 17.96% and 31.77%, respectively. The cellulose biopolymer effect size on *Staphylococcus aureus* and *Escherichia coli* bacteria was as 11.21 and 4.68, respectively. The factor of stirring time showed no effect on the studied bacteria.

Taguchi method, after data analysis and evaluating the effect of each of the factors and their interaction, predicted the optimum conditions for the synthesis of cellulose-MgO nanocomposite with the maximum antibacterial activity (Table 6). Based on the results, the MgO NPs and the stirring time had the highest and the lowest contributions in reducing the survival rate of

Table 3. Main effects of MgO, cellulose and stirring time on the survival rate of gram positive and gram negative bacteria

Factors	Gram positive bacteria (*Staphylococcus aureus*)			Gram negative bacteria (*Escherichia coli*)		
	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
MgO	4.14	2.49	1.58	4.98	2.96	1.59
Cellulose	3.95	1.58	2.68	4.36	2.01	3.16
Stirring time	2.91	2.32	2.99	3.19	2.76	3.58

Table 4. Effects of interacting factor pairs on the survival rate of *Staphylococcus aureus* and *Escherichia coli bacteria*

Interacting factor pairs	Gram positive bacteria (*Staphylococcus aureus*)				Gram negative bacteria (*Escherichia coli*)			
	Column	Severity Index [%]	Reserved column	Optimum conditions	Column	Severity Index [%]	Reserved column	Optimum conditions
Cellulose × Stirring time	2×3	32.30	1	[3,2]	2×3	42.21	1	[3,2]
MgO × Cellulose	1×2	21.61	3	[3,3]	1×2	19.85	3	[3,3]
MgO × Stirring time	1×3	15.78	2	[3,2]	1×3	6.88	2	[3,2]

Table 5. ANOVA test for factors affecting the growth reduction of gram positive and gram negative bacteria

Factors	Gram positive bacteria (*Staphylococcus aureus*)						Gram negative bacteria (*Escherichia coli*)					
	DOF	Sum of Squares	Variance	F-Ratio (F)	Pure Sum	Percent (%)	DOF	Sum of Squares	Variance	F-Ratio (F)	Pure Sum	Percent (%)
MgO	2	10.10	5.05	1.80	4.48	17.96	2	17.41	8.70	2.57	10.65	31.77
Cellulose	2	8.42	4.21	1.50	2.80	11.21	2	8.33	4.17	1.23	1.57	4.68
Stirring time	2	0.81	0.40	0.14	0.00	0.00	2	1.01	0.50	0.15	0.00	0.00

Table 6. Predicted the optimum conditions of cellulose-MgO nanocomposite synthesis with the maximum antibacterial activity

Factors	Gram positive bacteria (*Staphylococcus aureus*)		Gram negative bacteria (*Escherichia coli*)	
	Level	Contribution	Level	Contribution
MgO	3	1.16	3	1.59
Cellulose	2	1.15	2	1.17
Stirring time	2	0.42	2	0.42
Total contribution from all factors		–2.73		–3.18
Current grand average of performance		2.73		3.18
Bacterial survival at optimum condition		0.00		0.00

studied bacteria, respectively, while cellulose biopolymer revealed a size effect between these two factors closer to the MgO. The level 3 for MgO and level 2 for two factors of cellulose biopolymer and stirring time were introduced as the most appropriate levels. Based on the results, it was estimated that the synthesized nanocomposite in optimal condition inhibit bacterial growth nearly as 100%, and no bacteria grow in presence of this compound. To the best of our knowledge, this research firstly introduced cellulose-MgO nanocomposite and evaluated its antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*, which led to desired results.

Characterizations

The results of the DLS analysis of MgO nanoparticles are shown in Figure 2. The average size of synthesized MgO nanoparticles in this study was about 29 nm. Figure 3 shows the Raman spectrum of cellulose biopolymer within the wavelength range of 200–3500 cm^{-1}. The sharp peak observed in the 380 cm^{-1} range refers to the CCH, COH methane bending in the cellulose biopolymer spectrum. The broadband observed at 1095 cm^{-1} and 1120 cm^{-1} is attributed to the symmetric COC stretching. The sharp band observed in the 1380 cm^{-1} range is caused by HCH, HCC, HOC, COH bending. The band seen in the 2900 cm^{-1} range is due to C-H stretching[33]. The observed bands in the prepared spectrum confirmed the cellulose biopolymer synthesis.

The structures of MgO nanoparticles and the cellulose--MgO nanocomposite were evaluated using the FESEM (Fig. 4). The MgO nanoparticles were almost spherical and showed aggregation in some parts. The SEM image

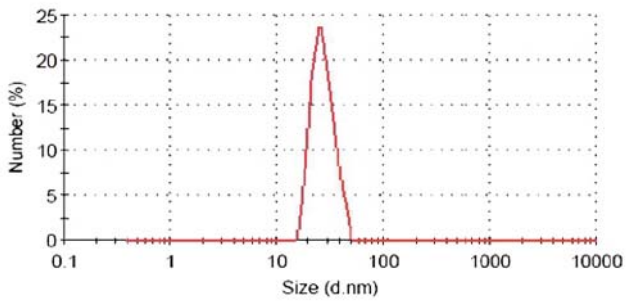

Figure 2. DLS graph of MgO nanoparticles

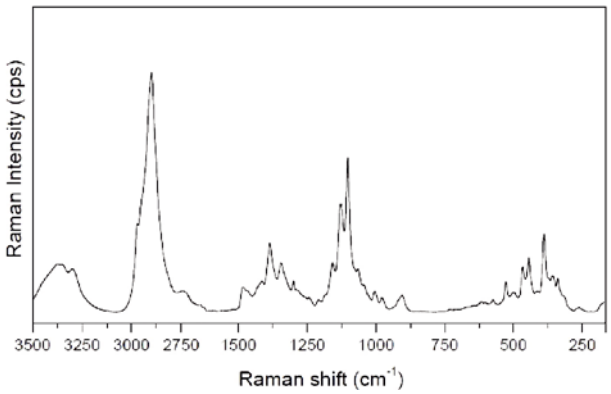

Figure 3. Raman spectrum of cellulose biopolymer

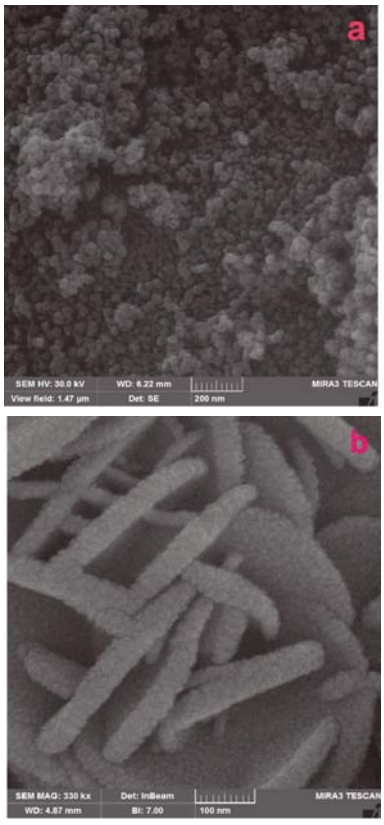

Figure 4. SEM images of MgO nanoparticle (a); cellulose-MgO bionanocomposite (b)

of the nanocomposite revealed the coating of the MgO nanoparticles on the cellulose biopolymer and confirmed

the formation of the cellulose-MgO nanocomposite. Previous studies have demonstrated that the formation of nanocomposites increases the contact surface and improves the performance of the nanoparticles[24, 27].

Thermal analysis

The thermal behavior of cellulose biopolymer and cellulose-MgO nanocomposite was evaluated with thermogravimetric analysis and differential thermal analysis (TGA/DTA) (Fig. 5). The thermogravimetric analysis curve for cellulose in the heat range of 250 to 350°C indicated a reduction volume as 85.4. The differential thermal analysis of cellulose biopolymer showed an endothermic peak. The onset and offset points of this peak were respectively at 312°C, 355°C and its peak point was at 338°C. Reviewing the results of two peaks (TGA/DTA) suggests that the endothermic peak at 338°C in DTA is in accordance with the cellulose degradation in the range of 250 to 350°C in the TGA curve. Change in the structure of cellulose biopolymer due to heat mainly occurs through dehydration, depolymerization and the formation of Glucosan. Cellulose dehydration occurs in a temperature range of 180–280°C, leading to the formation of 1,4 and 1,6 anhydroglucopyranoside. The beginning of depolymerization occurs at about 310°C due to cutting the chain at 1,4 glycosidic bond. The cellulose pyrolysis process continues at higher temperatures to other compounds[34].The cellulose-MgO nanocomposite in the temperature range of 100 to 450°C showed changes in the volume of about 36.06 at TGA curve. The nanocomposite DTA curve analysis represented an endothermic peak with an onset point, a peak point and an offset point with degrees as 355°C, 377°C and 399°C respectively. By comparing the curves (TGA/DTA) of cellulose biopolymer and cellulose-MgO nanocomposite, one can conclude that the stability rate and thermal resistance have improved by nanocomposite formation. Stability improvement of the synthesized nanocomposite will facilitate its long-term storage and usability. This economically reduces the costs and make the commercial production of nanocomposites economic.

CONCLUSIONS

Synthesis of MgO nanoparticles, cellulose biopolymer and cellulose-MgO nanocomposite were confirmed by DLS, Raman spectroscopy and SEM analysis, respectively. The nanocomposite produced by using 8 mg/ml magnesium oxide nanoparticles, 2 mg/ml cellulose biopolymer and the stirring time of 60 min (experiment 9) had the highest antibacterial activity against gram-positive and gram-negative bacteria. The antibacterial activity of cellulose-MgO nanocomposite was significantly better than its components in both colony forming units (CFU) and disc diffusion methods. Moreover, thermal analysis demonstrated improvement in the thermal stability of the cellulose biopolymer after the formation of the nanocomposite. Due to favorable antibacterial properties of synthesized cellulose-MgO nanocomposite, it can be used as a green alternative to replace antimicrobial compounds available in various fields, including food industry, pharmaceutical industry, medical and dentistry.

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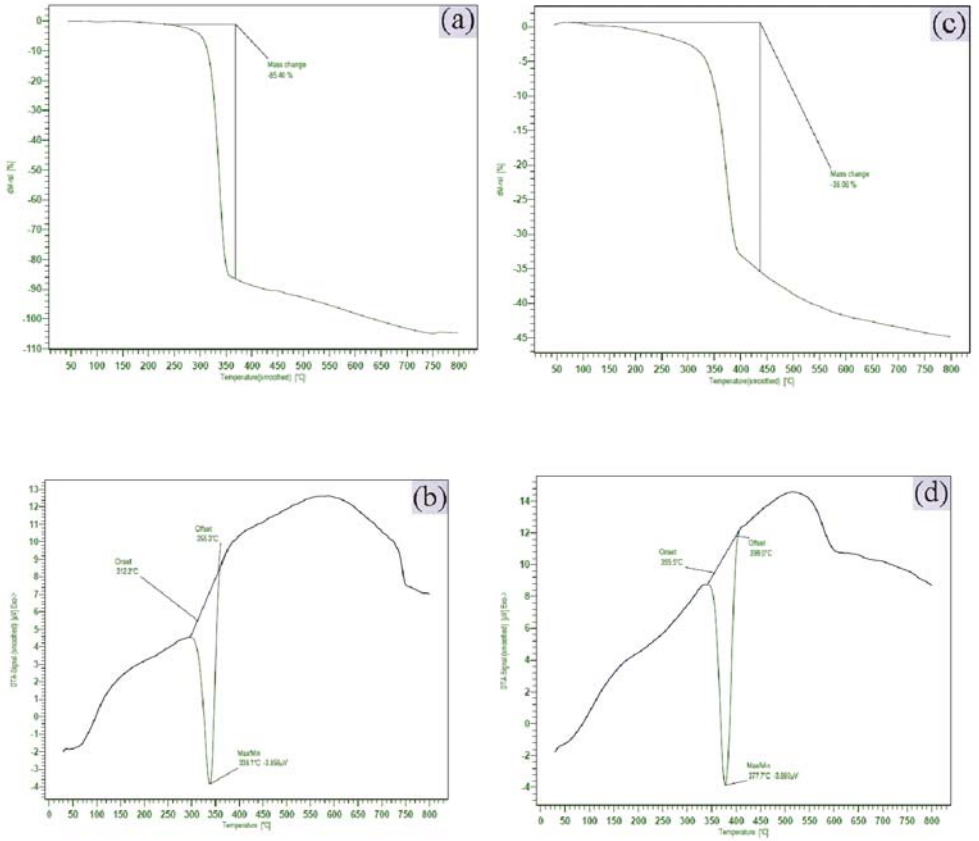

Figure 5. Thermogravimetric analysis of cellulose biopolymer (a) and cellulose-MgO nanocomposite (b); differential thermal analysis of cellulose biopolymer (c) and cellulose-MgO nanocomposite (d)

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