ISSN: 2538-5887; Pathobiology Research.

Synergistic Antibacterial Activity of synthesized Graphene Oxide/Chitosan nanocomposite with Rosmarinus officinalis L. Essential Oil against *Multidrug-Resistant* Acinetobacter baumannii, Pseudomonas *aeruginosa* and Escherichia coli isolates

ARTICLE INFO

Article Type
Original Research

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ABSTRACT

Treatment of infections caused by multidrug-resistant bacteria has become a global challenge. The combined therapies involve the simultaneous use of two or more biological agents with different mechanisms of action, which are more effective than traditional treatments for diseases that act only in one way. The aim of this study was synergistic antibacterial activity of synthesized graphene oxide/chitosan (GO/CS) nanocomposite with *Rosmarinus officinalis* L. essential oil against multidrug-resistant (MDR) bacteria. *R. officinalis* essential oil was extracted and its chemical compounds were analyzed by gas chromatography and mass spectrometry. The GO/CS nanocomposite was synthesized. The size and structure of the synthesized nanoparticles were evaluated by EDS, XRD, FE-SEM, and FTIR analysis. Antibacterial activity of chitosan, graphene oxide, GO/CS nanocomposite and *R. officinalis* essential oil was studied by broth microdilution method against 5 MDR isolates of *A. baumannii, P. aeruginosa* and *E. coli.* The antimicrobial interaction of the essential oil and GO/CS composite was studied by checkerboard titration method. The results showed that chitosan, graphene oxide and GO/CS had no antimicrobial activity in the studied concentrations. The MIC of *R. officinalis* essential oil was obtained between 0.12-256 µl/ml. *R. officinalis* essential oil in combination with GO/CS nanocomposite had a synergistic effect against 5 isolates of *P. aeruginosa* and 2 isolates of *A. baumannii*, and caused an additive effect against two isolates of *E. coli.* Based on the findings of this study, this combination can be effective against some MDR isolates and could be used to treat infections caused by these isolates.

Keywords: Antibacterial Agent, Nanocomposite, Essential oil, Multidrug Resistant, Drug Synergism.

Introduction

Today's Infectious diseases remain one of the leading causes of morbidity and mortality worldwide. The World Health Organization (WHO) and Centers for Disease Control (CDC) have expressed serious concern regarding the continued increase in the development of multidrug resistance among bacteria that causes many infection diseases. The numbers of infections produced by such resistant isolates are increasing globally. This acquired resistance of pathogens presents a key challenge for many antimicrobial drugs (1). There is greatest concern when antibiotic resistance occurs with *Enterococcus* spp., *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp., together given the acronym ESKAPE, which highlights the ability of these microorganisms to escape the action of antimicrobial agents (2). Nanotechnology applied to the synthesis of new antibiotics is an important approach, since the use of nanometric size materials can result in greater contact between the compound and the bacteria, with improved bioavailability, increased absorption, faster passage of the drug into the cell, and enhanced muco adhesion (3).

Chitosan is composed of β-(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine randomly distributed within the polymer. The cationic nature of chitosan is rather special, as the majority of polysaccharides is usually either neutral or negatively charged in an acidic environment. This property allows it to form electrostatic complexes or multilayer structures with other negatively charged synthetic or natural polymers (4). Besides, chitosan is reported to have other biological properties, such as antitumor, antimicrobial, and antioxidant activities (5). Graphene oxide (GO), a well-known monolayers of carbon atoms that form dense honeycomb structures containing hydroxyl and epoxide functional groups on the two accessible sides and carboxylic groups at the edges (6). Moreover, it has unique properties including large surface area, low cytotoxicity, and good water stability. Hence, it can act as the platform for growing metal nanoparticles and stabilizing them. Graphene oxide exhibits remarkable biological properties such as antibacterial activity (7) and anticancer activity resulting from its photothermal performance (8). For this reason, GO has great potential in diverse biomedical fields including drug delivery, tissue engineering, biosensing, and bioimaging (9). However, plenty of studies have indicated that GO did not exhibit obvious toxicity only at low doses, which undoubtedly limits its applications in humans. Fortunately, the toxicity of GO can be eliminated by the addition of biocompatible polymer CS. CS and GO can readily form composites through covalent bonding, hydrogen bonding, or electrostatic interactions (10). Recent studies have shown that the synergistic effect between CS and GO has excellent in vitro and in vivo biocompatibility (11), angiogenic and cell growth effect (12) and antimicrobial properties (13).

On the other hand the traditional use of plants as medicines provides the basis for indicating which essential oils and plant extracts may be useful for specific medical conditions (14). Various herb species have been used worldwide in prevention and treatment of diseases for many centuries. Today, several drugs have been developed from plants that are active against many common diseases (15). *Rosmarinus officinalis*, commonly known as Rosemary, is a flavoured herb. The *Rosmarinus officinalis*, essential oils (ROEO) is a mixture of several major and minor compounds that have been demonstrated its antimicrobial, antiproliferative, anti-inflammatory and antioxidant activities (16, 17).

The aim of study was synthesis of Graphene oxide-Chitosan (GO/CS) nanocomposite and evaluation of its synergistic antibacterial affects with essential oil of *Rosmarinus officinalis* against multiple drug resistant bacteria.

Material and methods

Bacterial Strains

This study was conducted on the bacteria *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Escherichia coli* isolated from 50 clinical samples including blood, wounds, feces and urine of patients referred to Milad Hospital in Tehran. The samples were cultured on blood agar and McConkey agar and incubated at at 37◦C for 24-48 hours. Differential biochemical tests were performed in the laboratory to identify and isolate bacteria.

Antibiotic Susceptibility and Determination of MDR isolates

Sensitivity of *Acinetobacter baumannii*, *Escherichia coli and Pseudomonas aeruginosa* isolates to antibiotics was determined using disk diffusion method (Bauer-Kirby) according to CLSI instructions. The susceptibilities of Ciprofloxacin (5µg), Ceftazidim (30µg), Cefotaxime (30µg), Gentamycin (10µg), Piperacillin (100µg), Piperacillin-tazobactam (100µg), Trimethoprim-sulfamethoxazole (1.25/23.75µg), Tetracyclin (30µg), Imipenem (10µg), Meropenem (10µg), Amikacin (30µg) antibiotics (Padtan-Teb Co., Iran) were characterized by agar diffusion, according to CLSI, 2021 (Table 1). Bacterial concentration based on 0.5 McFarland standard was 1.5×10^8 CFU/mL. The results were reported as resistant, intermediate, and susceptible. *Escherichia coli* ATCC 25922 were used as quality control isolates. Finally, isolates showing resistance to more than 2 antibiotics were recognized as MDR isolates (18).

Table1. Antibiotics and their growth inhibition zone based on CLSI 2021.

Antimicrobial agent	Sensitive	Intermediate	Resistance
Acinetobacter baumannii			
Ciprofloxacin (Fluoroquinolones)	>21	16-20	<15
Ceftazidim (Cefem)	>18	15-17	<14
Cefotaxime (Cefem)	>18	15-17	<14
Gentamycin (Aminoglycosides)	>15	13-14	<12
Piperacillin (penicillin)	>21	18-20	<17
Piperacillin-tazobactam (Beta lactam)	>21	18-20	<17
Trimethoprim- sulfamethoxazole	>16	11-15	<10
Tetracyclin (Tetracycline)	>15	12-14	<11
Meropenem (Carbapenem)	>18	15-17	<14
Imipenem (Carbapenem)	>22	19-21	<18
Amikacin (Aminoglycosides)	>17	15-16	<14
Pseudomonas aeruginosa			
Ciprofloxacin (Fluoroquinolones)	>25	19-24	<18
Ceftazidim (Cefem)	>18	15-17	<14
Gentamycin (Aminoglycosides)	>15	13-14	<12
Piperacillin (penicillin)	>21	15-20	<14
Piperacillin-tazobactam (Beta lactam)	>21	15-20	<14
Imipenem (Carbapenem)	>19	16-18	<15

Meropenem (Carbapenem)	>19	16-18	<15
Amikacin (Aminoglycosides)	>15	13-16	<12
Escherichia coli			
Ciprofloxacin (Fluoroquinolones)	>26	22-25	<21
Ceftazidim (Cefem)	>21	18-20	<17
Cefotaxime (Cefem)	>26	23-25	<22
Gentamycin (Aminoglycosides)	>15	13-14	<12
Piperacillin (penicillin)	>21	18-20	<17
Piperacillin-tazobactam (Beta lactam)	>21	18-20	<17
Trimethoprim- sulfamethoxazole	>16	11-15	<10
Tetracyclin (Tetracycline)	>15	12-14	<11
Imipenem (Carbapenem)	>23	20-22	<19
Meropenem (Carbapenem)	>23	20-22	<19
Amikacin (Aminoglycosides)	>17	15-16	<14

Rosmarinus officinalis essential oil preparation

The branches of *R. officinalis* plant were prepared from Research Institute of Forests and Rangelands, (Tehran, Iran) and the taxonomic identification of plant materials was confirmed by a botanist. 200 g of dried aerial parts were extracted for 3 h by Clevenger's apparatus and dehydrated by anhydrous sodium sulfate. The sample stored in a dark closed container, away from light and at 4°C.

Analysis of _Rosmarinus officinalis_ essential oil (ROEO)

The analysis of *R. officinalis* essential oil compounds were performed using an Agilent Technologies mass spectrometer Agilent technologies 7890A gas chromatograph equipped with an HP5MScapillary column (30 m, 0.25 mm, 0.25µm), and a mass detector MS 5975C VL MSD was operated in the EI mode. The identification of components was based on the comparison of their mass spectra with those of WILEY and NIST Libraries as well as on the comparison of their retention indices of the authentic standard with the literature (19).

Synthesis of Graphene Oxide (GO)

GO was prepared according to the modified Hummer method [In detail, 5g of graphite and 2.5g of $NaNO_3$ were mixed with 108mL H_2SO4 and 12mL H_3PO_4 and stirred in an ice bath for 10min. Next, 15g of $KMnO_4$ were slowly added so that the temperature of the mixture remained below 5°C. The suspension was then reacted for 2h in an ice bath and stirred for 60min before again being stirred in a 40°C water bath for 60min. The temperature of the mixture was adjusted to a constant 98°C for 60 min while water was added continuously. Deionized water was further added. So, that the volume of the suspension was 400mL. 15mL of H_2O_2 was added after 5min.The reaction product was centrifuged and washed with deionized water and 5% HCl solution repeatedly. Finally, the product was dried at 60°C (20).

Synthesis of GO/CS Nanocomposite

First, 2% v/v solution of acetic acid was prepared. Then 0.4 g of chitosan powder was gradually added to 20 mL of 2% V/V solution of acetic acid and dissolved to obtain a 2% W/V solution of chitosan. Then the solution was stirred for 24 hours at room temperature by placing a magnet inside the container. Then, the solution was placed in an ultrasonic bath with a temperature of 25°C for one hour, and 3 mL of glutaraldehyde (GLA) was slowly added to the chitosan solution as a cross-linking agent, and stirring was continued for half an hour. 0.3 g of graphene oxide was added to the mixture and stirred continuously for 90 min in a water bath with a temperature of 50°C by placing a magnet inside the reaction container. 0.1 M NaOH solution was slowly added to it until the pH was about 9.5 and placed in a water bath for 60 minutes at a

temperature of 80◦C and the resulting solution was poured into the funnel. First, it was washed with ethanol and then several times with deionized water. The final product was dried at 40◦C. After drying, CS/GO was completely ground in a mortar (21).

The structures of materials were measured by Fourier-trans form infrared spectra analyzer (FT-IR; Thermo Nicolet inc., NEXUS 870, USA) and X-ray diffraction (XDR; Philips PW 1800, USA. The microscopic morphologies of samples were characterized by Field-emission scanning electron microscopy (FE-SEM; Sigma inc., Zeiss, Germany).

Determination of Minimum Inhibitory Concentrations:

The minimum inhibitory concentration (MIC) values of Chitosan, Geraphene Oxide, GO/CS nanocomposite and *R. officinalis* essential oil were determined by 96-well polystyrene microtiter plates with Mueller Hinton broth (MHB). The GO/CS was serially diluted twofold with deionized water in concentrations ranging from 0.39-400 µg/mL. The essential oil was serially diluted two-fold with 4% DMSO containing 0.12-256 µl/mL of essential oil. After shaking, 100 mL of diluted GO/CS or essential oil was added to each well of 96-well microtiter plates. Muller-Hinton broth (Merck, Germany) was used as the broth medium. Microbial suspensions were adjusted to 0.5 MacFarland and diluted to 1×10^6 cfu/ml, and then 100mL of the suspension was added to each well. The final volume of all wells was 200 microliters. Finally, the microplates were placed on a shaker (250 rpm) for 1 minute until the mixture was completely uniform and incubated at 37◦C for 24 hours. Only *R. officinalis* essential oil dissolved in MHB (oil control), only bacterial suspension in MHB (bacterial control), and 4% DMSO with bacterial suspension (diluent control) were also included. MIC values were determined as the lowest concentration of compound that inhibited bacteria after 24 hours. MIC of CS and GO was performed according to the above method.

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Determination of synergistic Activity of GO/CS and ROEO by Checkerboard Method

Eight serial, two-fold dilutions of GO/CS nanocomposite and *R. officinalis* essential oil were prepared and used in the MIC tests. 50µL of each dilution of oil essential was vertically added to the wells of the 96-well microtiter plates, and 50µL of GO/CS dilution was added horizontally to the wells. 100µL of microbial suspension (1×10^6 cfu/ml) was added to each well and incubated at 37◦C for 24 hours. Fractional inhibitory concentrations (FICs) were calculated using the MIC of the combination of GO/CS and essential oil by the MIC of GO/CS or essential oil alone. The interaction between the two antimicrobial agents was estimated by calculating the fractional inhibitory concentration (FIC) indices (FICI) using the following formula 1, 2:

$$\text{(1) FIC} = \frac{\text{MIC of the essential oil or nanocomposite in combination}}{\text{MIC of the essential oil or nanocomposite alone}}$$

(2) FICI = FIC of the essential oil + FIC of anocomposite

The interaction was interpreted by using the following criteria: FICI ≤ 0.5, synergy; 0.5 < FICI ≤ 1, additive; 1 < FICI ≤ 4, indifference (no effect); and FICI > 4, antagonism (22).

Statistical analysis:

Statistical significance was determined by Duncan's multiple range test and a Student-t test procedure of SPSS 21.0 (SPSS Inc., Chicago, IL, USA). The level of statistical significance was $p < 0.05$.

Results:

Results of the analysis of the constituents of *Rosmarinus officinalis* essential oil

In *R. officinalis* essential oil, the composition of Eucalyptol with 21.301%, camphor with 18.200% and alpha-pinene with 15.358% had the highest amount. The chemical compositions of the ROEO and GC-MS chromatograms were shown in Tables 2 and Figures 1

Table 2. Compositions and percentages in *R. officinalis* essential oil

Rate Time	Compound Number	Mol Weight (amu)	CAS Number	%
7.871	.ALPHA.-PINENE	136.125	000080-56-8	15.358
8.118	.alpha.-Thujene	136.125	002867-05-2	0.877
9.868	.gamma.-Terpinene	136.125	000099-85-4	4.63
10.428	.ALPHA.-TERPINOLENE	136.125	000586-62-9	3.843
10.955	Camphene	136.125	000079-92-5	5.326
11.465	Sabinene	136.125	003387-41-5	2.203
11.915	Myrcene	136.125	000123-35-3	7.913
12.026	4(10)-Thujene	136.125	003387-41-5	0.927
13.529	p-Cimene	134.11	000099-87-6	2.869
18.771	Eucalyptol	154.136	000470-82-6	21.301
19.425	LINALOOL L	154.136	000078-70-6	4.156
19.527	2,3,4-Trimethylpyrrole	109.089	003855-78-5	0.18
21.914	TERPINENE 1-OL	154.136	000586-82-3	2.43
23.741	Camphor	152.12	000076-22-2	18.2
24.055	Isoborneol	154.136	000124-76-5	2.175
24.582	BORNEOL L	154.136	000464-45-9	3.203
25.151	Terpene-4-ol	154.136	000562-74-3	0.169
25.627	p-Mentha-2,4(8)-diene	136.125	000586-63-0	0.149
27.614	Verbenone	150.104	000080-57-9	1.217
29.263	Caryophyllene	204.188	000087-44-5	2.385
30.851	.alpha.-Caryophyllene	204.188	006753-98-6	0.489
				100

Results of GO/CS nanocomposit synthesis

The chemical structure of the adsorbent and the type of its functional groups were identified using the Fourier transform infrared (FTIR) spectrum. Figure 2(a), (b), (c), respectively, shows the IR spectra of GO, GO/CS, and chitosan in the region of 400-4000 cm-1. In the spectrum of GO/Cs composite (Figure 2(b)), the intensity of peaks appearing at 3400 and 1075 has increased compared to GO (spectrum (a)). The presence of these peaks is caused by the vibrations of the ─OH groups of chitosan, which are adsorbed on the GO surface. The peak located at 1743 cm-1 (related to the carboxylic groups of GO) has disappeared in the spectrum of GO/CS. This confirms that the carboxylic groups of GO have reacted with chitosan for the synthesis of GO/CS composite. By examining the FTIR spectrum of GO/CS composite, it can be concluded that carboxyl groups of GO interact with NH2─chitosan groups rather than amides (band located at 1660 cm-1) and form the carboxyl band (11400-cm). The nucleophilic attack of amine on GO epoxy groups also causes the formation of amine. The increase in the intensity of the band at 1608 cm–1 in the GO/CS spectrum confirms this. The crystal structure of the absorbent was evaluated by X-ray diffraction (XRD) technique, the XRD patterns of graphite, GO, GO/CS, and CS are shown in Figures 3(a), (b), (c) and (d) respectively, are observed. The XRD patterns of chitosan, GO/CS in Figures 3(c) and (d), both have a strong peak at θ2=19.91, which is attributed to the amorphous state of chitosan. This indicates that the amorphous structure like CS remains preserved in GO/CS nanocomposite. Figures 4(a), (b), (c) show the FE-SEM images of graphite, GO and GO/CS, respectively. In Figure 4(b), the layered structure of GO with a uniform surface and relatively sharp and much wrinkled edges can be seen well. The surface of GO/CS (Figure 4(c)) becomes more uneven. This confirms the placement of high amounts of chitosan on GO layers.

Antibacterial activity CS, GO, GO/CS nanocomposite and *R. officinalis* essential oil

The antimicrobial effect of chitosan, graphene oxide, GO/CS nanocomposite and *R. officinalis* essential oil was investigated by broth microdilution method against 5 MDR isolates of *Acinetobacter baumannii, Pseudomonas aeruginosa* and *Escherichia coli*. The studied concentrations of CS, GO and GO/CS did not show any effect against MDR isolates and bacteria grew in all wells of the 96-well microplate. MIC of *R. officinalis* essential oil for MDR isolates was obtained in the range of 0.12-256 µl/ml. *Acinetobacter* isolates were the most sensitive and *Pseudomonas aeruginosa* isolates showed the most resistance. The MIC values of all agents against all isolates are shown in Table 3.

Synergistic activities of GO/CS nanocomposite combined with *R. officinalis* essential oil

The results of the antimicrobial interaction showed that *R. officinalis* essential oil in combination with GO/CS nanocomposite had a synergistic effect against 5 isolates of *P. aeruginosa* and 2 isolates of *A. baumannii*, as defined by FICI values of 0.0703-0.2578, and caused an additive effect against two isolates of *E. coli,* as specified by FICI value of 0.5078. This combination had no effect against other isolates (Table 4)

Table 3. MIC of Chitosan, Geraphene Oxide, GO/CS nanocomposite and *R. officinalis* essential oil (ROEO).

	CS µg/ml	GO µg/ml	GO/CS µg/ml	ROEO µl/ml
Acinetobacter baumannii				
Isolate No. 15	400 >	400 >	200>	8
Isolate No. 16	400 >	400 >	200>	0.12
Isolate No. 17	400 >	400 >	200>	0.12
Isolate No. 18	400 >	400 >	200>	1
Isolate No. 19	400 >	400 >	200>	1
Pseudomonas aeruginosa				
Isolate No. 6	400 >	400 >	200>	256
Isolate No. 7	400 >	400 >	200>	256
Isolate No. 8	400 >	400 >	200>	256
Isolate No. 9	400 >	400 >	200>	256
Isolate No.10	400 >	400 >	200>	256
Escherichia coli				
Isolate No. 24	400 >	400 >	200>	1
Isolate No. 25	400 >	400 >	200>	4
Isolate No. 26	400 >	400 >	200>	4
Isolate No. 27	400 >	400 >	200>	8
Isolate No. 28	400 >	400 >	200>	8

Table 4. Synergistic Activity GO/CS nanocomposite and *R. officinalis* essential oil (ROEO).

	FIC GO/CS	FIC ROEO	FICI	Interaction
Acinetobacter baumannii				
Isolate No. 15	0.0078	1	1.0078	indifference
Isolate No. 16	0.0078	1	1.0078	indifference
Isolate No. 17	0.0078	0.25	0.2578	synergy
Isolate No. 18	0.0078	0.25	0.2578	synergy
Isolate No. 19	0.0078	1	1.0078	indifference
Pseudomonas aeruginosa				
Isolate No. 6	0.0078	0.25	0.2578	synergy
Isolate No. 7	0.0078	0.25	0.2578	synergy
Isolate No. 8	0.0078	0.125	0.1328	synergy

Isolate No. 9	0.0078	0.125	0.1328	synergy
Isolate No.10	0.0078	0.0625	0.0703	synergy
Escherichia coli				
Isolate No. 24	0.0078	1	1.0078	indifference
Isolate No. 25	0.0078	1	1.0078	indifference
Isolate No. 26	0.0078	0.5	0.5078	additive
Isolate No. 27	0.0078	1	1.0078	indifference
Isolate No. 28	0.0078	0.5	0.5078	additive

synergy; 0.5 < FICI ≤ 1, additive; 1 < FICI ≤ 4, indifference (no effect); and FICI > 4, antagonism

Discussion

Multiple drug resistance (MDR) is a common form of clinical resistance and is defined as the ability of a disease-causing organism to survive in lethal doses of various drugs or chemicals (Mostofi et al., 2011). The high prevalence of these resistant bacteria has exposed patients to a serious threat in hospital infections and has caused the death of thousands of people. The treatment of infectious agents resistant to this antibiotic requires new antimicrobial substances. Among these Methods can be referred to combined treatment. The combination of bioactive essential oil with nanoparticles is a novel applied method and could be beneficial, as a synergistic or additive interaction or deleterious, as an antagonistic or toxic outcome) (23).

In this research, graphene oxide/chitosan nanocomposite was synthesized and characterized by FTIR, XRD and FE-SEM analyses. Antimicrobial effect of chitosan, graphene oxide, graphene oxide/chitosan nanocomposite against MDR isolates of *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Escherichia coli* was investigated. The results showed that chitosan, graphene oxide and GO/CS composite had no effect against MDR isolates in the studied concentration. Although the antimicrobial effect of these compounds has been confirmed in some studies (13, 24), but these studies were conducted on standard isolates of bacteria and so far no study has been conducted against drug-resistant isolates. Among these studies, we can refer to the study of Chowdhur et al that confirmed the antimicrobial effect of graphene oxide nanocomposite modified with chitosan decorated with ZnO nanoparticles against *S. aureus* and *E. coli* (25). Jiang et al., investigated the antimicrobial activities of chitosan/graphene oxide magnetic composite against *E. coli* bacteria (26). In relation to the mechanism of the effect of GO on bacteria, many studies have been conducted, which show that damage to the bacterial cell membrane caused by the direct contact of the bacteria with the very sharp edge of the GO nanoparticle is an effective mechanism in the inactivation of bacteria (27). Chitosan is soluble in water and has a positive charge, and this feature is very important from a technical point of view because it enables the polymer to interact with large molecules and polyanions in the aqueous environment. After the interaction of chitosan with anionic groups on the surface of the cell, the destruction of the membrane begins.

Therefore, GO/CS nanocomposite is also applied to wound healing Due to be focused on improving the injectability, water-absorption and water-retention capability, mechanical property, and antibacterial activity. Besides, GO/CS composite with high antibacterial activity against gram-negative and gram-positive bacteria occupies a key position among wound dressings (28).

One of the promising solutions to eliminate drug-resistant microorganisms is the use of effective plant compounds. Herbal compounds can significantly increase the success of treatment and prevent the spread of new diseases. In this study, the antibacterial effects of *R. officinalis* essential oil against the MDR isolates were evaluated by determining the minimum inhibitory concentration. Based on the obtained results, the MIC of *R. officinalis* essential oil for MDR isolates was between 0.12-256 µl/ml and had the greatest effect on *P. aeruginosa* isolates. In the analysis of *R. officinalis* essential oil, the composition of eucalyptol with 21.301%,

camphene with 18.200% and alpha-pinene with 15.358% had the highest amount. These results are consistent with other (16). These phenolic compounds in essential oils can lead to cases such as disruption of the bacterial cytoplasmic membrane, inhibition of the proton motive force and electric current, and coagulation of cell contents. On the other hand, the chemical structure and the presence of hydroxyl group in the structure of these compounds is one of the most important reasons for their antimicrobial effects (29).

The results of the antimicrobial interaction showed that *R. officinalis* essential oil in combination with GO/CS nanocomposite had a synergistic effect against 5 isolates of *P. aeruginosa* and 2 isolates of *A. baumannii*, and caused an additive effect against two isolates of *E. coli.* As noted, the combining drugs with each other can lead to a greater inhibitory effect than the sum of the effects of each of these substances alone, and reduce the dosage of one or both substances (30) and Our results confirm that these compounds exerted synergistic and additive effects against some MDR stain when GO/CS nanocomposite was combined with essential oil. These results suggest that combination of GO/CS nanocomposite with *R. officinalis* essential oil can be used as an antimicrobial compound to treat infections caused by multi-drug resistant bacteria.

In this study, a search was made to find alternative medicines from different sources. Chitosan (CS) has biodegradability, non-toxic properties and antimicrobial properties. On the other hand, graphene oxide (GO) is also known as a biocompatible material with excellent chemical and mechanical properties (31). Both chitosan and graphene oxide have antibacterial activity, but individually they cannot act as strong antibacterial agents. GO/CS nanocomposites have a strong competitive edge in wound healing (32). Current studies mainly focus on improving their antibacterial effect, generally achieved through chemically modifying GO or CS alone or simultaneously with some drugs Considering the physical characteristics also influencing its antibacterial effect, GO/CS nanocomposites are a convenient and advantageous research topic (28).

Therefore, in order to expand the use of these two substances, it seems that the synthesis of Go/CS nanocomposites can increase their antimicrobial effects and the combination of these two factors with *R. officinalis* essential oil can increase their effect.

Bacterial infection in the wound area leads to delayed healing. Therefore, wound dressings containing antibacterial substances have attracted the attention of many researchers. Wound dressings with excellent antibacterial ability can accelerate the wound healing process by killing pathogenic bacteria on the wound and maintaining a sterile environment for wound healing. GO/CS are both good antibacterial materials, also GO/CS nanocomposite has several advantages including sufficient mechanical strength, anti-inflammatory and tissue adhesive properties (28). Therefore, GO/CS nanocomposites impregnated with *R. officinalis* essential oil can be used for dressing wounds and treating infections caused by multi-drug resistant bacteria in the form of hydrogel.

Conclusion

In this research, a simple method was used to synthesize graphene oxide/chitosan nanocomposite. The results of the present study showed the effectiveness of *R. officinalis* essential oil against multi-drug resistant isolates. Also, the synergistic and additive effect between the synthesized nanocomposite and *R. officinalis* essential oil was confirmed. Therefore, the combination of these two antimicrobial agents can be used in the treatment of infections caused by drug-resistant bacteria.

Conflict of interest

There is no conflict of interest between the researchers in this study.

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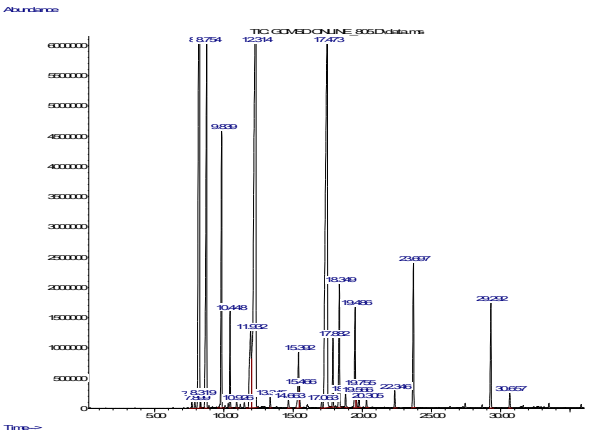


Figure 1. GC-MS chromatogram of the *R. officinalis* essential oil

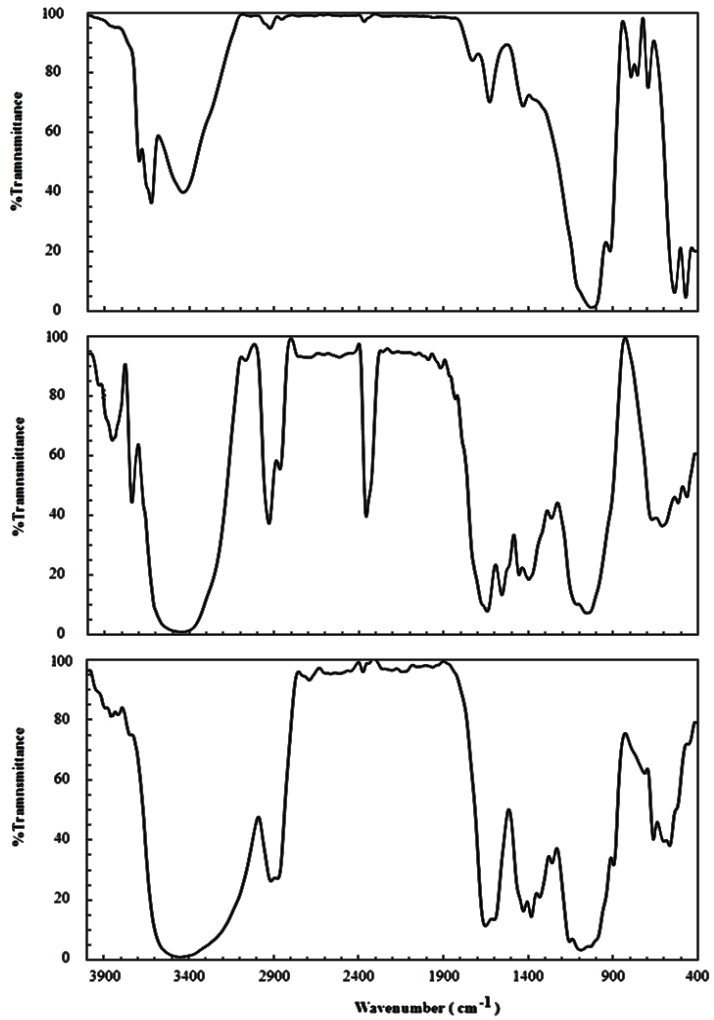


Figure 2. FTIR analysis results (a) GO, (b) GO/CS, (c) chitosan.

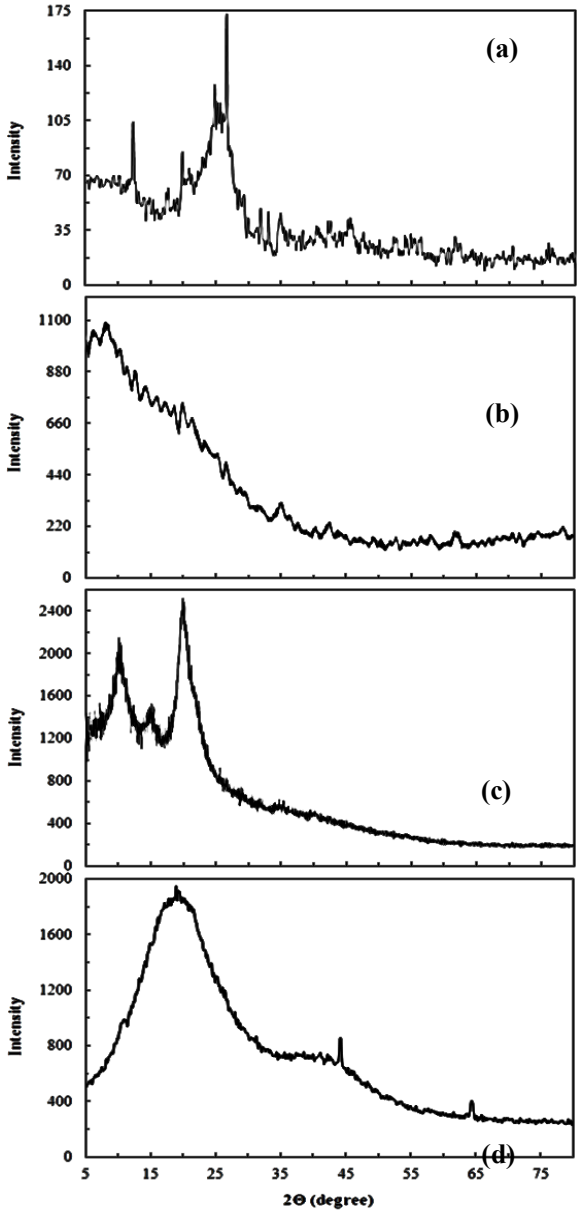
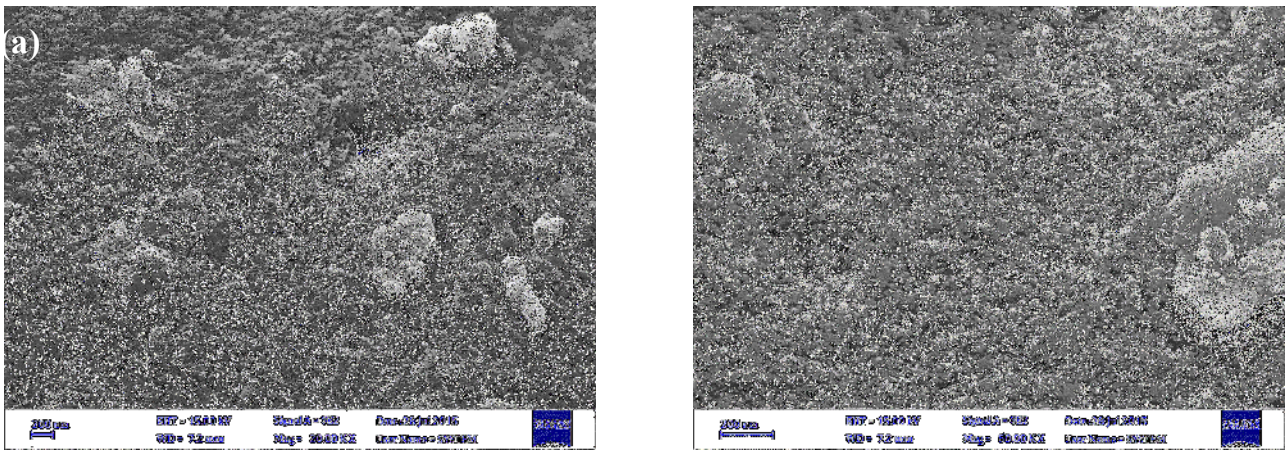


Figure 3. X-ray diffraction (XRD) pattern (a) graphite (b) GO, (c) GO/CS, (d) chitosan.



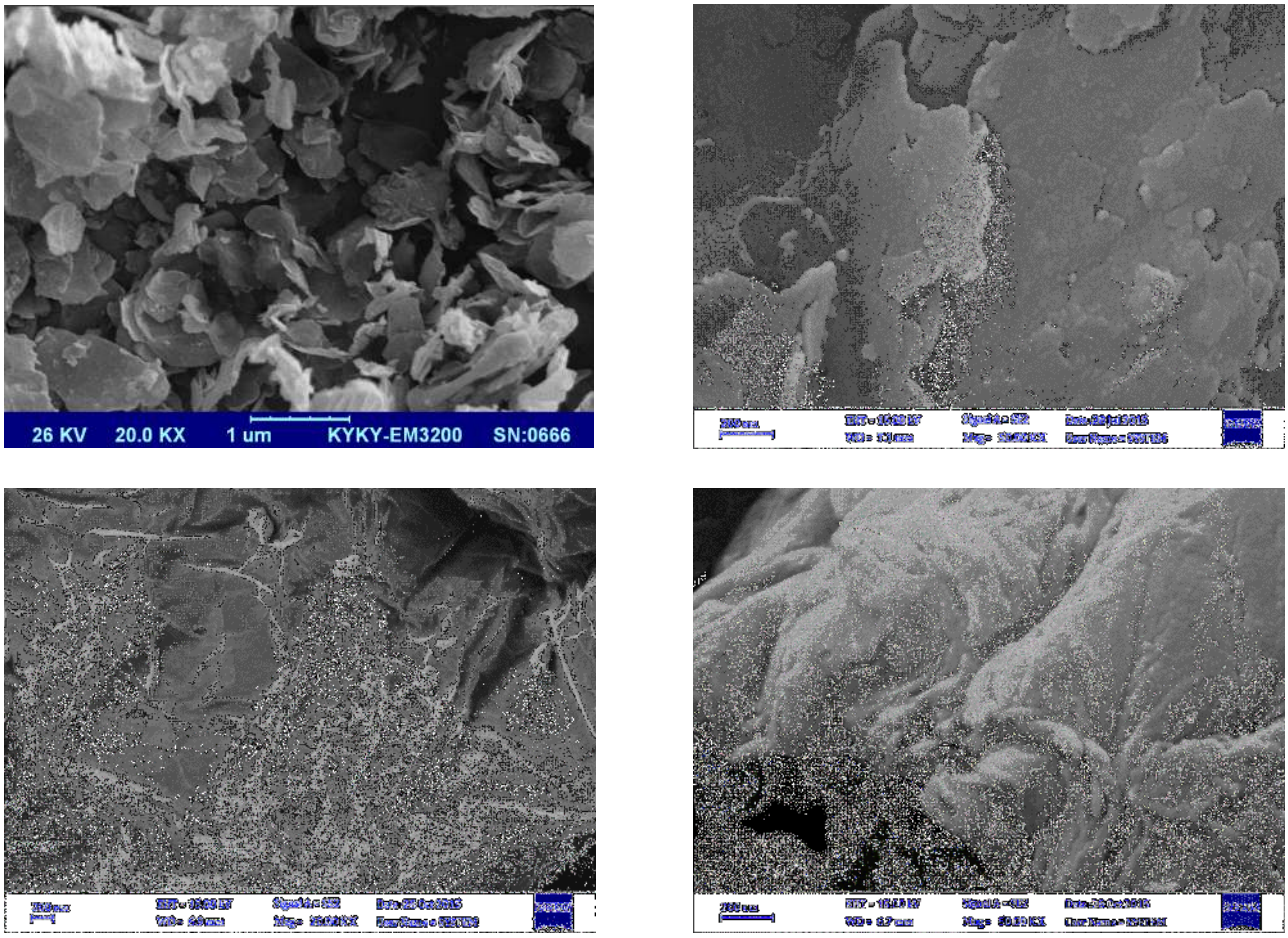


Figure 4. FESEM images of (a) graphite, (b) GO, (c) GO/CS. The magnification of the images on the left is 20KX and the images on the right are 50KX times