Nanomed Res J 3(2):109-116, Spring 2018

RESEARCH ARTICLE

Characterization of active nanochitosan film containing natural preservative agents

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ARTICLE INFO

Article History:
Received 27 February 2018
Accepted 09 April 2018
Published 01 May 2018

Keywords:
Film
Grape Seed
Mentha Spicata
Essential Oil
Nanochitosan
Pomegranate Peel

ABSTRACT

Objective(s): The aim of this study was to improve different characteristics including antibacterial, antioxidant and physical properties of nanochitosan film by incorporating *Mentha spicata* essential oil (EO) and methanolic pomegranate peel and grape seed extracts.

Methods: The determination of the chemical composition of *M. spicata* EO was conducted by means of gas chromatography with mass spectrometry (GC-MS). Thickness, color, *in-vitro* antioxidant and antimicrobial properties of prepared films were evaluated.

Results: The most abundant constituents of *M. spicata* EO were carvone (78.76%), limonene (11.50%) and *β*-bourbonene (11.23%). The thickness of designated films and straight chitosan film were similar, however, the increasing value was observed for films containing *M. spicata* EO, pomegranate peel and grape seed extracts ($P > 0.05$). The lower lightness, higher redness and consequently a darker color was found in film incorporated with methanolic pomegranate peel and grape seed extracts. The highest antioxidant activity was found in nanochitosan enriched with the EO and grape seed extract. The antibacterial activities of all extracts and the EO were in the order: *M. spicata* EO > grape extract > pomegranate peel extract.

Conclusions: The nanochitosan film incorporated with EO and extracts have shown good antibacterial and antioxidant activities which can be barrier against microbial and chemical contamination in food industries.

How to cite this article
Shahbazi Y, Shavisi N. Characterization of active nanochitosan film containing natural preservative agents. Nanomed Res J, 2018; 3(2): 109-116. DOI: 10.22034/nmrj.2018.02.008

INTRODUCTION

In recent decades, increasing studies have been concentrated on the development and application of biodegradable and biocompatible polymer materials because of concerning in term of the negative environmental effects resulted in the application of plastic compounds [1]. Chitosan has been examined extensively for the production of active pure/composite films and coatings owing to appropriate characteristics including intrinsic antimicrobial effectiveness as well as good physico-mechanical characteristics [2]. Chitosan, a linear compound, consists of β-(1–4)-2-acetamido-D-glucose and β-(1–4)-2-amino-D-glucose units [3]. It has good properties especially biocompatibility and biodegradability[4]. Hence, it is considered as one of the most important potential compounds for packaging of different types of foods [5-7]. Enrichment of biodegradable films and/or coatings using essential oils (EOs) and extracts in order to enhancement of antimicrobial, antioxidant, physical and mechanical properties were examined by several researchers [2, 3, 6, 8-10].

Since ancient times, the aerial parts of *Mentha spicata* genus specially its leaves and its EO has been approved for treatment of common important

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disorders like malaria, infections and cold. It is also frequently applied in making of traditional foods and as a spice in dairy products such as Dough (Iranian yoghourt based drink) and yoghourt [11]. Application of other antimicrobial constituents such as extracts of by-products obtained from pomegranate peel and grape seed could reduce the used concentrations of EOs and subsequently decrease their unsatisfactory effects on the sensorial properties of food stuffs [12, 13].

Pomegranate is widely cultivated throughout the world particularly Asia, North Africa, Americas and Mediterranean region [14]. In Iranian folk medicine, this fruit was applied for treatment of diarrhea and healing wounds [15]. Previous studies indicated that high phenolic compounds of pomegranate's peal and fruits are the most important reason of their antimicrobial, antioxidant and anti-cancer properties [14, 16, 17]. In addition, grape seed as a by-product derived from grape has high contents of flavanols, procyanidins and phenolic acid. These compounds have critical roles in antimicrobial and antioxidant properties of grape seed extract [18]. Iran is considered as one of the most important exported countries of pomegranate and grape fruits and large contents of peel and seed as waste compounds were produced here [14]. Moreover, enrichment of biodegradable films with aforementioned compounds probably can improve their antimicrobial, antioxidant and physic-mechanical properties [19]. Hereto, the current study aimed to enhance some properties of active nanochitosan film including antibacterial, antioxidant and physical properties using addition of *M. spicata* EO, methanolic pomegranate peel and grape seed extracts.

MATERIALS AND METHODS

Plant material, isolation of essential oil and its GC-MS analysis

The collection of the *Mentha* plant, EO isolation and gas chromatography–mass spectrometry (GC–MS) analysis of the obtained EO were previously described in our study [20].

Preparation of fruit extracts

Pomegranate (Saveh variety) was purchased from a local garden in Saveh city (Tehran, Iran). The peel of pomegranate fruit was cut in small size, dried, powdered and dissolved in methanol (1:20 g/ml) and finally isolated on a shaker at ambient condition (25 ± 1 °C) for 1-2 days. The obtained extract was filtered and dried using a rotary evaporator at 40 ± 1 °C approximately after 2 days. Commercial grape seed extract was purchased from Gol Adonis Daru (Tehran, Iran). Both extracts and also EO was stored at chilled condition (4 ± 1 °C) for using in our experiment [14].

Preparation of nanochitosan film

For preparation of nanochitosan film, amount of 2 g of nanochitosan powder (75-85% deacetylated and medium molecular weight: 250 kDa) was poured in a beaker containing 1% (v/v) glacial acetic acid and shake on a magnetic stirrer/hot plate at 37 °C for 8 h. Glycerol as a plasticizer and Tween 80 as an emulsifier were added at levels of 0.75 ml/g nanochitosan and 0.2% of EO or extracts, respectively. Soon after of stirring on a magnetic stirrer/hot plate at 37 °C, the EO (0 and 2%), methanolic pomegranate peel (0 and 2%) and grape seed (0 and 2%) extracts were incorporated to the nanochitosan mixture. Finally, 50 ml of active chitosan film solution was placed on the glass petri dish and dried for 48 h at room temperature conditions [21].

Film properties

Thickness

Film thickness was assessed using digital micrometer (Mitutoyo, Mitutoyo Corporation, Japan). Determinations were conducted in triplicate.

Fourier Transform Infrared Spectroscopy

The interaction between the components in designated chitosan films was evaluated using Fourier Transform Infrared Spectroscopy (FTIR; Bruker, model ALPHA, Germany) within the wave number range of 400–4000 cm^{-1}.

Surface morphology

Surface structure of designated chitosan films was observed using a TeScan MIRA3 SEM; during the measurement samples were sputtered with a layer of gold to prevent the charging effect.

Color measurement

Lightness (L), redness (a) and yellowness (b) values of designated films were evaluated using Minolta Chroma Meter Model CR-400 (Minolta Co Ltd, Osaka, Japan). The corresponding values of the standard white plate were L^* = 93.49, a^* = -0.25 and b^* = -0.09. Color differences (DE) were determined using the following equation [21]:

Color differences (DE) =

$$\sqrt{(L*-L)2(a*-a)2(b*-b)2}$$

Antioxidant activity

The antioxidant property of designated active nanochitosan films was evaluated by 2, 2-diphenyl-1-picrylhydrazyl hydrate (DPPH) free radical scavenging activity according to Moradi, *et al.* [19].

Antimicrobial activity

The *in-vitro* antibacterial effect of designated active nanochitosan films was evaluated against growth of *Staphylococcus aureus* (ATCC 6538), *Bacillus subtilis* (ATCC 6633), *Bacillus cereus* (ATCC 11774), *Listeria monocytogenes* (ATCC 19118), *Salmonella typhimurium* (ATCC 14028) and *Escherichia coli* O157:H7 (ATCC 10536). The mentioned pathogen microorganisms obtained from the Iranian Research Organization for Science and Technology (Tehran, Iran). Bacterial strains were sub-cultured at 37 °C for 24 h in Brain Heart Infusion broth (BHI) and adjusted to a final density (10^8 CFU/ml) and used a as inoculum dose [22].

The antibacterial effects of designated active nanochitosan films were examined using agar disk diffusion assay based on the procedure published by Ojagh *et al.* [21]. After preparing sterile molted BHI agar, 15 ml of the medium was poured into microbiological petri dishes (diameter = 90 mm). after that, 0.1 ml of the aforementioned bacterial suspensions were cultured on the surface of the medium. Then, the designated active nanochitosan films were put on the surface of inoculated BHI agar. The cultured media were incubated at 37 ± 1 °C for 24-48. The diameter of the inhibition zones were calculated. The antimicrobial examinations were conducted in triplicate.

Statistical analysis

The analysis was performed using SPSS 16.0 for Windows (SPSS, Chicago, IL, USA) software package. Significance level was considered $P<0.05$ in all experimental data.

RESULTS AND DISCUSSION

Chemical compositions of M. spicata essential oil

The chemical composition of *M. spicata* EO were completely reported in our previous studies [20]. The *M. spicata* EO contained mainly carvone (78.76%), limonene (11.50%) and β-bourbonene (11.23%).

Film properties

Thickness

The effects of incorporating *M. spicata* EO, pomegranate peel and grape seed extracts in the thickness chitosan films on the performance of the nanocomposite is shown in Fig. 1. The thickness of designated films and straight chitosan film were similar, however, the increasing values was observed for films containing *M. spicata* EO, pomegranate peel and grape seed extracts ($P > 0.05$). Similarly, Shahbazi [23], Moradi *et al.* [19], Jouki *et al.*, [24] and Peng *et al.*, [2] reported that incorporation of red grape seed extract + *Ziziphora clinopodioides* EO, *Zataria multiflora* Boiss EO + grape seed extract, thyme + oregano EOs and lemon + thyme + cinnamon EOs had increasing effects on thickness property of tested films, respectively.

Fourier Transform Infrared Spectroscopy

The FTIR spectra of chitosan, chitosan + *M. spicata* EO, chitosan + pomegranate peel extract,

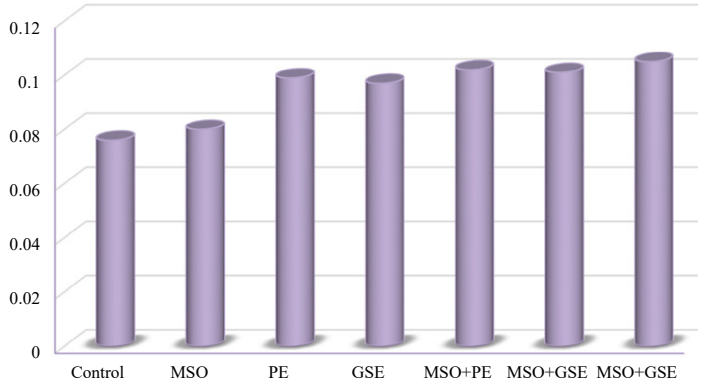

Fig. 1. Thickness properties of nanochitosan film incorporated with *M. spicata* essential oil (MSO), pomegranate peel extract (PE) and grape seed extract (GSE)

and chitosan + grape seed extract films are given in Fig. 2. The major peaks of chitosan film were as follow: 3428.59 cm^{-1} (stretching vibrations of O-H and N-H), 2927.26 cm^{-1} (aliphatic C-H stretching vibration), 3068.88 cm^{-1} (CH_2 stretching vibration), 3027.81 cm^{-1} (CH_3 stretching vibration), 1702.25 cm^{-1} (C=O stretching of amide I), 1664.02 cm^{-1} (NH of amide II stretching vibration), 1110.29 cm^{-1} (C3-OH stretching vibration) and 1039.48 cm^{-1} (C6-OH stretching vibration) [25]. For film containing pomegranate peel extract, *M. spicata* EO and grape seed extract, peaks around 1000-1800 cm^{-1} might be attributed to the stretching of C=O, -C=C-C=O, -C=C- [(in-ring) aromatic] and -C-C- [(in-ring) aromatic] found in the phenolic components [19, 26].

Surface morphology

Surface structures of chitosan, chitosan + *M. spicata* EO, chitosan + pomegranate peel extract, and chitosan + grape seed extract films are shown in Fig. 3a-d. Based on our findings, straight chitosan film had compact and uniform structure without pores (Fig. 3a). Comparison of the films containing pomegranate peel and grape seed extracts did not present any obvious structural differences (Fig. 3b-c). In these films, roughness of surface structure were observed. In chitosan + *M. spicata* EO obvious pores and cavities were found (Fig. 3d) which is probably occurred during evaporation of the EO at casting method [27].

Color

Color values (lightness (*L*), redness (*a*) and yellowness (*b*)) of prepared nanochitosan based films using incorporation of *M. spicata* EO, pomegranate peel and grape seed extracts are shown in Fig. 4. Lower L^* value (lightness), higher b* value (yellowness) and DE* (color difference) were found for films enriched with *M. spicata* EO, pomegranate peel and grape seed extracts compared to pure nanochitosan film ($P < 0.05$). The straight nanochitosan film had the higher L^* value and lower b* value by $L = 84.1$, $a = -1.76$ and $b = 29.11$ as compared to other groups. As it can be observed in Fig. 4, moderate color changes were observed in prepared films containing *M. spicata* EO. It can be concluded that for all designated films,

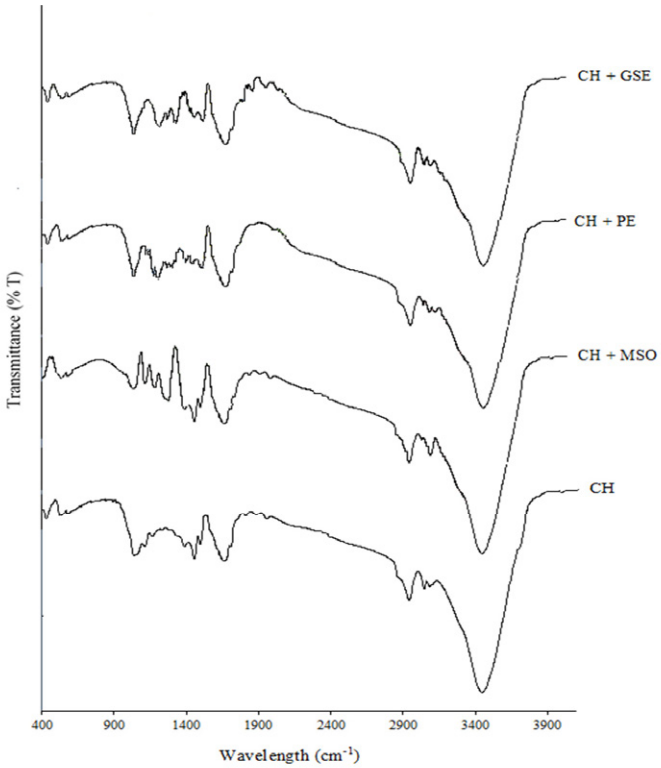

Fig. 2. FTIR spectra of CH (chitosan), CH + *M. spicata* essential oil (MSO), CH + pomegranate peel extract (PE) and CH + grape seed extract (GSE) films

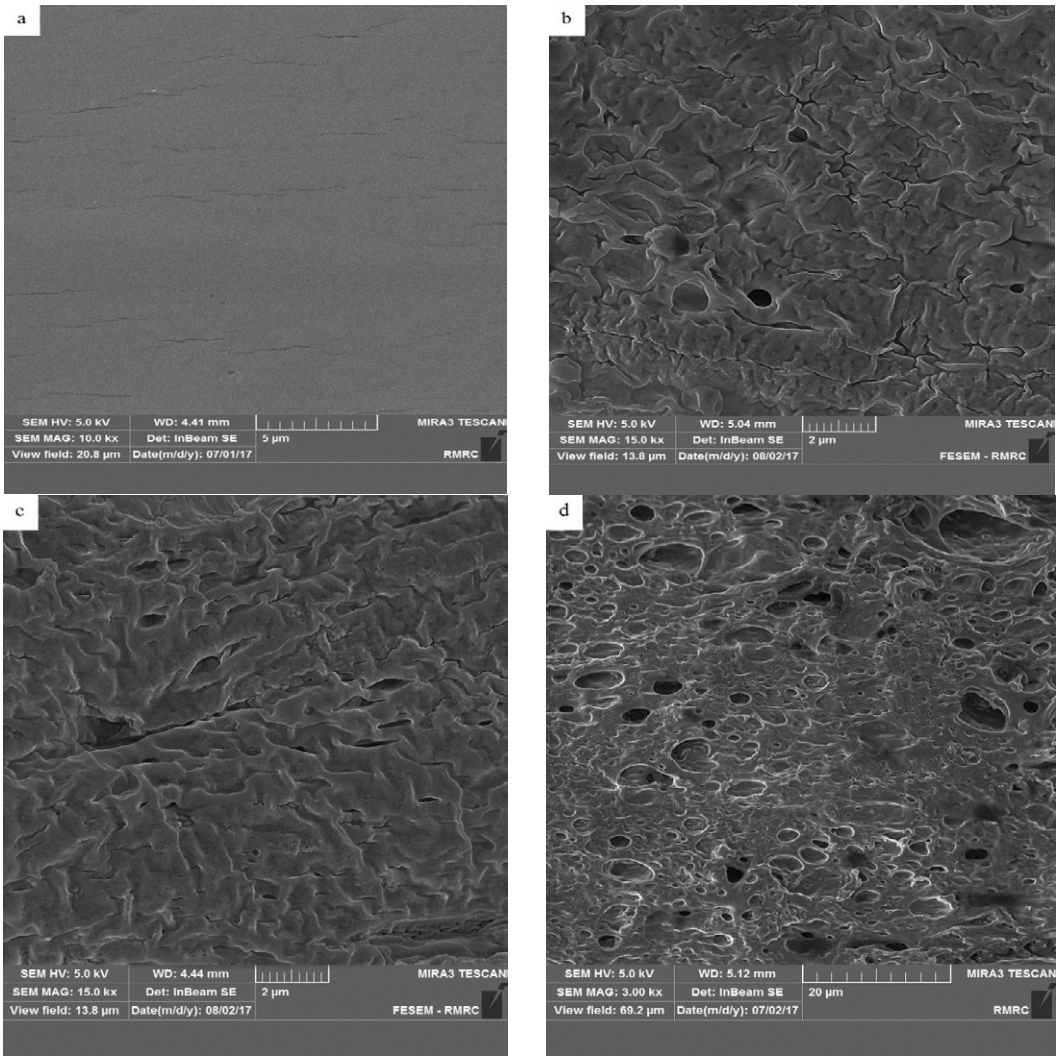

Fig. 3. SEM of chitosan (a), chitosan + pomegranate peel extract (b), chitosan + grape seed extract extract (c) and chitosan + *M. spicata* essential oil (d) (MSO)

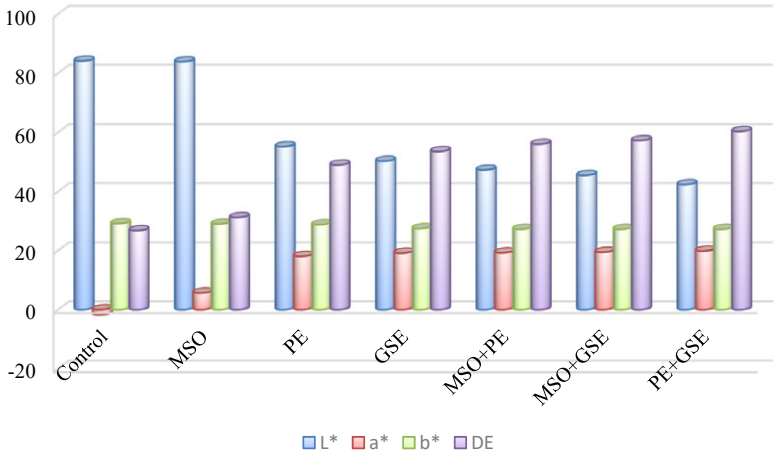

Fig. 4. Color of nanochitosan film incorporated with *M. spicata* essential oil (MSO), pomegranate peel extract (PE) and grape seed extract (GSE)

incorporation of *M. spicata* EO, pomegranate peel and grape seed extracts lead to the greater changes in color and subsequently resulting in darker films. The most likely reason of color changes of tested films is the presence of phenolic compounds in *M. spicata* EO, pomegranate peel and grape seed extracts and the internal structure developed during chitosan film drying [2, 28]. Changes in color of gelatin film incorporated with cinnamon, clove and star anise extracts [29], chitosan + tea polyphenols [30], soy protein film + grape seed extract + nisin [31] and pea starch film + grape seed extract were also reported by other researchers.

Antioxidant activity

Based on the results presented in Fig. 5, the free radical scavenging activity of all active nanochitosan films was much higher than that of chitosan film without *M. spicata* EO, pomegranate peel and grape seed extracts. The maximum scavenging activity was found for film containing *M. spicata* EO + grape seed extract; in this group free radical scavenging activity was more than four folds compared with chitosan film without natural compounds. Moreover, pure film had slight antioxidant activity, which might be related to the reaction among the free radicals and the free residual amino groups to form ammonium groups [19]. It could be noted that the highest scavenging activity of nanochitosan film containing *M. spicata* EO, pomegranate peel and grape seed extracts strongly could be attributed to the high extent of phenolic compounds of pomegranate peel and grape seed extracts as well as high amount of monoterpene hydrocarbons of *M. spicata* EO [4]. Numbers of researches indicated that there was positive relation between total phenolic content of EOs/extracts incorporated to the biodegradable films and their antioxidant activities [2, 4, 19, 32].

Antibacterial activity

The results of antibacterial effects of nanochitosan films containing *M. spicata* EO and methanolic pomegranate peel and grape seed extracts are shown in Fig. 6. Based on our findings, pure nanochitosan film showed antibacterial inhibitory effects against all used microorganism pathogens at the contact area. It might be attributed to the presence of positively charged amino groups of chitosan which are strongly interacted with negatively charged groups on the cell membranes and walls of Gram-positive and Gram-negative bacterial pathogens, and finally cause to the leakage of essential constituents of the bacteria which critical role in survival and growth of the microorganisms [21, 33]. The lack of clear inhibition zone in pure film of nanochitosan is related to this fact that chitosan is not capable to diffuse through agar medium when it is in a solid form. Hence, only the growths of bacteria that directly contact with the chitosan are suppressed [10]. The results presented in Fig. 6 showed that the antibacterial activities of all extracts and the EO were in the order: *M. spicata* EO > grape seed extract > pomegranate peel extract. The hypothesis is that the most important compounds of *M. spicata* EO including carvone, limonene and β-bourbonene as well as high phenolic compounds of grape seed

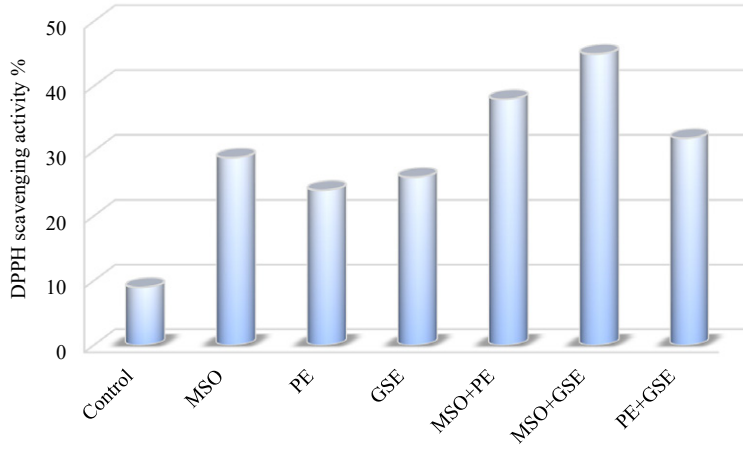

Fig. 5. DPPH scavenging activity of nanochitosan film incorporated with *M. spicata* essential oil (MSO), pomegranate peel extract (PE) and grape seed extract (GSE)

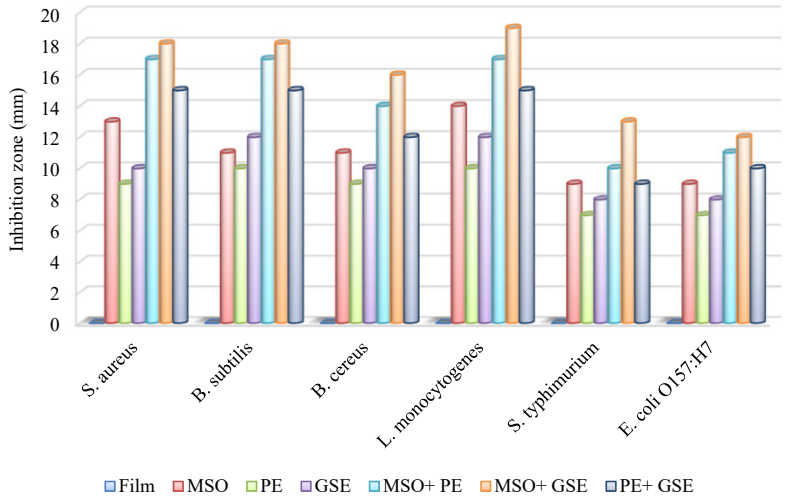

Fig. 6. The antibacterial activities of nanochitosan film incorporated with *M. spicata* essential oil (MSO), pomegranate peel extract (PE) and grape seed extract (GSE)

and pomegranate peel extracts especially flavanols, procyanidins and phenolic acid could can easily enter through the cell walls and membranes of the microorganisms, disrupt their structures and cause the leakage of cellular components [34, 35]. In consistent with our findings, previous studies indicated good antibacterial effectiveness of pomegranate peel extract against some microorganism pathogens including *E. coli*, *P. aeruginosa* and *S. aureus* [16, 36, 37]. Khan and Hanee [38] demonstrated that the major phenolic compounds of pomegranate peel extract which have good antibacterial effect were polyphenols, tannins, flavonoids and anthocyanins. Based on our findings in Fig. 6, the effects of designated films against Gram-positive microorganisms were more than Gram-negative microorganisms. These results could be likely due to the hydrophobic nature of lipopolysaccharide outer membrane structure of Gram-negative microorganisms which reduce the release of antimicrobial compounds into the internal area of microorganisms [13].

CONCLUSION

It may be suggested that nanochitosan films containing *M. spicata* EO, methanolic pomegranate peel and grape seed extracts exhibit antimicrobial and antioxidant properties against several food-borne pathogenic bacteria. According to the results of the current study, thickness and color properties of prepared nanochitosan films were affected by incorporating *M. spicata* EO, methanolic pomegranate peel and grape seed extracts. Scanning electron microscope study and FTIR showed good interaction of investigated compounds with nanochitosan films. In addition, further study is necessary to determine the phytochemical constituents of *M. spicata* EO, methanolic pomegranate peel and grape seed extracts responsible for the antibacterial effects against the bacterial pathogens and also antioxidant property.

CONFLICT OF INTERESTS

The author claims that there is no conflict of interest.

ACKNOWLEDGEMENTS

The author acknowledges Razi University for providing instrumentations.

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