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Chitosan- (*Prunus avium*) Gum Nanocapsules Loaded with Orange Peel Extract

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Abstract

The Orange peel extract (OE) is an additive material that has been used widely as a natural antioxidant source and bioactive compound in the Pharmaceutical and food sectors. The poor stability and degradation of this extract were considered problems in the industry. New technologies have been introduced recently to prevent this degradation such as encapsulation. In this work, chitosan (CS) and Prunus avium gum (PG) were proposed as promising materials for the encapsulation of OE via the ionic gelation method. The effect of different ratios of CS: PG and CS: OE on encapsulation efficiency (EE %) of OE and the capsule size was investigated. The EE% of CS-PG ranged from 60.63 to 87.06 % with a size range of 40 to 95nm according to Atomic Force Microscope (AFM) images. The formulation with the highest EE% was chosen to be characterized by FTIR, scanning electron microscope (SEM), and in vitro release study. The FTIR spectra confirmed the cross-linking between the NH3+ group in CS and the negative functional group (-COO-) in PG. According to SEM micrographs, the capsules showed a spongy porous structure. The in vitro release study indicated that the release of OE from the CS-PG matrix in the acidic and neutral medium was 55.15 and 52.67% respectively after incubation for 240 minutes. This study found that CS-PG can be used as an effective wall matrix for encapsulating OE and delivering it into the gastrointestinal system.

Keywords: Orange peel extract, encapsulation, chitosan, *Prunus avium* gum.

كبسولات الكيتوزان -صمغ (*Prunus avium*) النانوية المحملة بمستخلص قشور البرتقال

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الخلاصة

يعد مستخلص قشر البرتقال (OE) مادة مضافة تم استعالها على نطاق واسع كمصدر طبيعي لمضادات الأكسدة والمركبات الفعالة حيوياً في المجالات الصيدلانية والغذائية. تعد عدم الثباتية وتدهور هذا المستخلص مشكلة في الصناعة. أدخلت تقنيات جديدة مؤخرًا- كالكبسلة- لمنع التدهور.هدف البحث إلى استعمال الكيتوزان وصمغ *Prunus avium* كمادة جدارية للكبسولات النانومترية لمستخلص قشور البرتقال (OE) وفق طريقة الهلام الأيوني. دُرس تأثير نسب إضافة مختلفة من الكيتوزان: الصمغ ونسب المستخلص : الكيتوزان في كفاءة الكبسلة، وحجم الكبسولات الناتجة. تراوحت كفاءة الكبسلة بين 60.63 و 87.06% وأبعاد الكبسولات بين 40 و95 نانومتر. تم اختيار المعاملة التي حققت أعلى كفاءة كبسلة ووصفت باستعمال تقنية FTIR والمجهر الإلكتروني الماسح (SEM) والقدرة على التحرر في الزجاج. . أكدت أطياف الـFTIR للكبسولات حدوث تجاذب كهربائي وارتباط بين مجموعات الأمين ($^{+}$NH3-) في الكيتوزان ومجموعات الهيدروكسيل ($^{-}$COO–) في الصمغ. ظهرت الكبسولات ببنى مسامية وفقاً لصور المجهر الإلكتروني الماسح. أوضحت دراسة القدرة على التحرر في الزجاج بأن النسبة المئوية لتحرر المستخلص الفينولي لقشور البرتقال في الأوساط الحامضية والقلوية وصل إلى 55.15 و52.67% على التوالي بعد التحضين لمدة 240 دقيقة. أظهرت الدراسة بأنه يمكن استعمال الكيتوزان وصمغ *Prunus avium* كمواد جدارية فعالة لتغليف مستخلص قشور البرتقال.

الكلمات المفتاحية: مستخلص قشور البرتقال، الكبسلة، الكيتوزان، صمغ *Prunus avium*

Introduction

The processing industries of fruits and vegetables generate a huge amount of by-products, such as peels, seeds, pulp, and certain other remnants that are discarded [(1)]. Scientists are interested in these wastes because they are sources of high-added value [(2)]. Orange peels, which remain after juice extraction, are an important source of bioactive compounds like phenols, pigments, and essential oil. These components, which have biological properties such as antioxidant, antibacterial, and anti-inflammatory, can be employed in the pharmaceutical and food industries [(3)]. Despite their importance, these compounds have limited bioavailability and low stability under processing conditions. The majority of phenolic compounds found in food are esters, polymers, or glycosides, and cannot be absorbed in this form [(4)]. Encapsulation in biodegradable polymeric nanoparticles is one approach to circumventing these constraints [(5)]. Hussein et al [(6)] reported that the nano-capsulation process increased the thermal stability of rosemary essential oil. Tavakoli et al [(7)] claimed that the encapsulation of the olive leaf extract rich in polyphenols masks its bitter taste and increases the nutritional value and stability of the extract in yogurt.

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Several methods for forming nanocapsules have been developed like emulsification, coacervation, ionic gelation, electrospinning, etc [8]. The ionic gelation approach was widely employed in this field due to the use of non-toxic and highly biocompatible polymers. This method is based on the interaction between oppositely charged polymers [9] like chitosan and gums. Chitosan (CS) is a naturally occurring cationic polysaccharide composed of glucosamine and N-acetyl-glucosamine produced by the alkaline deacetylation of crustacean chitin [10]. The amine groups of chitosan are ionized in acidic conditions. Plant gums are the high molecular hydrophilic polysaccharides produced from different parts of the plant (e.g. plant cell walls, tree exudates, seeds, tuber/roots) [11]. Prunus avium gum (PG) is a Rosaceae tree that exudates gum [12]. It is a by-product of the cherry tree in Syria as the result of the gummosis phenomenon. The physicochemical and functional properties of Prunus gums exudate showed promising properties for usage in a variety of sectors. [13, 14, 15], like nanoencapsulation of bioactive compounds. Gums can react with cationic polymers during ionic gelation by their negative functional groups [16]. Salarbashi et al [17], produced Prunus armeniaca gum nanoparticles loaded with curcumin and achieve high encapsulation efficiency (86.1%). A new nanoparticle delivery system for the encapsulation of quercetin was also created utilizing a mixture of almond gum (Amygdalus communis L.) and shellac as biopolymers [18]. Several studies indicated that the preparation parameters such as polymer concentration, chitosan–gum relative ratio, and the concentration of bioactive compounds can affect the encapsulation efficiency and particle sizes [19, 20, 21]. The aim of this study is to prepare and evaluate OE-loaded chitosan nanocapsules using Prunus avium gum (PG) as an ionic crosslinker. The impacts of the ratios of CS: gum and CS: OE, on encapsulation efficiency and capsule size were examined. Furthermore, the capsules with the highest encapsulation efficiency were characterized using a Scanning electron microscope (SEM), and Fourier transforms infrared (FTIR) and their release was investigated in acidic and neutral mediums.

Materials and methods

Materials

The peels of sweet orange (Citrus sinensis) were obtained -in 2018- from local juice shops in Damascus, Syria. A sample of cherry tree gum exudates (PG) was taken from the trunks and branches of cherry trees (Prunus avium) in Suwayda governorate, southern Syria. Chitosan (CS) low molecular weight (batch #STBH2613). All of the high-purity reagents and solvents utilized in this work were purchased from Sigma-Aldrich.

Preparation of orange peels extract (OE)

The peels of Valencia sweet orange (Citrus sinensis L.) were washed and dried at 40°C for 48 hours. The orange peel extract was prepared according to the optimal conditions studied previously. Grounded and dried orange peels were mixed with ethanol (50%) at the ratio of 1:10 (w/v) and put in a water bath at 36 °C for 2 hours and then centrifuged at 4500 rpm for 6 minutes. The supernatant was collected, filtrated through 0.45µm membranes, and kept in glass containers at −18 °C for further processing.

Determination of total phenolic compounds

Total phenolic compounds of OE were estimated using a Folin–Ciocalteu method. The extract samples (0.5 ml) were mixed with 2.5 ml of Folin-Ciocalteau reagent (0.2 N). After 5 minutes 2.0 ml of sodium carbonate (75 g/l) were added, and the mixture incubated at room temperature for 2 hours. The absorbance of the reaction was measured at 760 nm. Gallic acid at concentrations ranging from 25 to 250 mg/L was used in the preparation of a standard curve [22]

Purification of PG gum

The gum was purified using the modified method reported by Bhushette et al (23). The samples were powdered in an electric grinder (Starmix) and sieved (500µm sieve) to obtain a uniform particle size. The gum solution (5 %w/v) was heated to 50°C for 3 h, filtered to remove impurities, and concentrated twice at 50°C. The cherry gum exudate was precipitated with absolute ethanol (exudate solution: ethanol, 1:3 v/v). The precipitated gum was separated by centrifugation at 4500 rpm for 3 minutes, dried in the oven at 50°C for 48 hours to achieve a consistent weight, and then stored in an airtight container for future use (Figure1.).

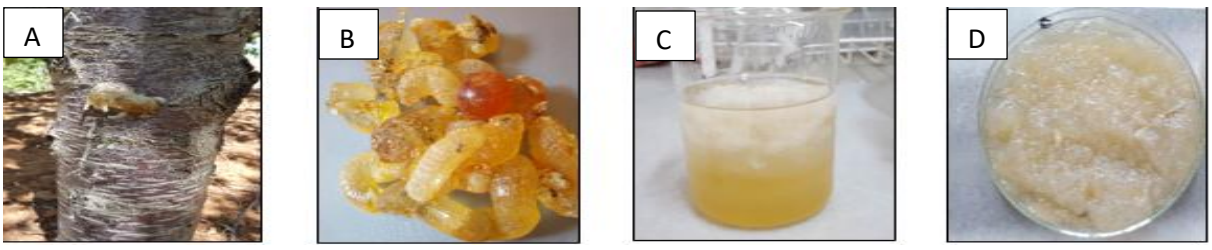

Figure 1. A and B: The sample of (*Prunus avium*) gum exudates, C: Gum precipitated with ethanol, D: Purified gum after separated by centrifugation) .

Preparation of CS-PG nanocapsule

Nanocapsules loaded with OE were produced according to the method described by Akinluwade et al [24] with some modifications. CS (0.5%) was dissolved in an acetic acid solution (1 %) then, OE was added to CS with different OE: CS ratios (0.5:1, 1:1, and 1.5:1 v/v) and stirred at 1300 rpm for 1 hour using a magnetic stirrer. After that, and during stirring the PG (2%w/v) was added to the CS-OE solution by drop-wise method at different PG: CS volume ratios (1:1, 2:1, and 3:1 v/v), and the mixture was stirred at room temperature for 2 hours. The formed capsules were separated by centrifuging at 13,000 rpm for 30 minutes at 4°C, and the supernatant was used to determine the encapsulation efficiency.

Encapsulation efficiency (EE)

The free phenolic compounds were determined in the supernatant after the separation of capsules by the Folin-Ciocalteau method which was described by Ebrahimzadeh et al **[22]** using a spectrophotometer at 765 nm and the EE% was calculated according to the following equation (1):

$$EE\% = \frac{\text{total amount of phenols} - \text{free phenols}}{\text{total amount of phenols}} \times 100 \quad (1)$$

Capsules sizes

The size, the particle size distribution, and surface morphology were examined using Atomic Force Microscopy measurements (AFM, Nanosurf easyScan2, PGitzerland: tapping mode (Tap190 Al-G), NanoSensors™, Neuchatel, Switzerland).

Characterizations of nanocapsules

The freeze-dried nanocapsules with the maximum EE% were characterized (Alpha1-2LDplus-Germany) by Scanning Electron Microscopy (SEM), Fourier transform infrared spectroscopy (FTIR), and in *vitro* release profile.

Scanning electron microscopy (SEM)

SEM (SEM, XMU II-VEGA) was used to study the morphology of prepared nanocapsules using a secondary electron detector at an acceleration voltage of 20 kV.

In vitro release study

In vitro release study was conducted using a modified method of Yadav et al [25]**.** The release of OE from the capsules was performed in phosphate buffer solution (0.2 M, pH=7.2) and HCl (0.1N, pH=1.2). The nanocapsules (0.03g) were placed in an Eppendorf tube with 1 ml of the release solution and incubated at 37°C in a shaking water bath for 240 min. At regular intervals (0,15, 30, 60, 90, 120, 150, 180, 210, and 240 minutes), the sample was withdrawn and centrifuged at 3500 rpm for 10 minutes to separate the released OE from simulated fluids. Then, the quantity of releasing OE was measured spectrophotometrically using the Folin–Ciocalteu method as described by Ebrahimzadeh et al [22].

Infrared spectrum analysis

FT-IR spectra for CS, PG, and CS-PG capsules were carried out using an FTIR-4200 Jasco spectrometer in the wavelength range of 4000–400 cm^{-1} at a resolution of 4 cm^{-1} using the KBr disc method.

Statistical analysis

The results of experiments were expressed as a mean± standard deviation of three replications of the experiment. Analysis of variance (ANOVA) two-way as a statistical model was performed, followed by Fisher's Least Significant Difference (LSD) test to evaluate the significant difference between treatments at the 5% level using Genstat software 12.

Results and Discussion

Encapsulation Efficiency (EE %)

The EE % of OE ranged from 60.63 to 87.06% for all formed nanocapsules with different ratios of CS: PG and CS: OE (Table 1). The results of EE% were lower than those reported by Mahmoud et al [26]**,** the difference in results could be attributed to the differences in methods of preparation nanocapsule and the difference in the physicochemical properties of wall materials. Mahdavi et al [27] reported that the type of coating material and the ratio of core material to coating material significantly impact the EE%.

Table 1. EE% of OE -loaded nanocapsules created via different treatments.

CS: OE ratio	CS: PG ratio		
	1:1	**1:2**	**1:3**
1:0.5	**87.06±0.39^{a}**	NF	NF
1:1	72.47±0.78^{bc}	73.85±0.36^{b}	79.62±0.79^{ab}
1:1.5	60.63±5.84^{d}	65.03±5.3^{cd}	76.05±1.67^{b}

***Mean values ±SD values. Different letters indicate a significant difference (*P*<0.05) between the results. NF: not formed.**

According to the statistical analysis in Table 2, the OE ratio had a significant influence on the EE% (P<0.05). Our results showed that as the ratio of OE increased the EE % decreased, this may be explained by the fact that when the ratio of OE increased the solution density increased, and the cross-linking between the matrix and OE was lost. This finding was in agreement with a previous study for preparing Viola odorata Linn. extract microcapsules [28]. On the other hand, the statistical analysis revealed that the EE% was dependent on the ratio of CS: PG, and the maximum EE% was achieved at the high ratio of CS: PG. Several previous studies have found that increasing the ratio of encapsulating material improves EE% [19, 16]. This increase in the EE% with an increasing CS: PG ratio could be explained by insufficient encapsulation materials to form a strong structural matrix and a protective layer at a low ratio of CS: PG. While a denser and stronger wall layer with more active sites is formed around nanocapsules at the higher ratios of CS: PG, leading to a higher EE % [29].

Particle morphology and size distribution

AFM imaging is an -efficient approach for providing surface morphology as well as more precise size and size distribution [30]. The results in Table 2 and AFM images confirmed that the capsules of CS-PG prepared via the ionic gelation method were in nanometre dimensions (<100 nm). The AFM microscopy in Figure. 2 showed that the produced nanocapsules had a uniform spherical shape. One of the most important aspects of particulate system formulation is the particle size distribution [31]. The histograms (Figure 2, C) revealed the capsules created by CS and PG are with the same mean size.

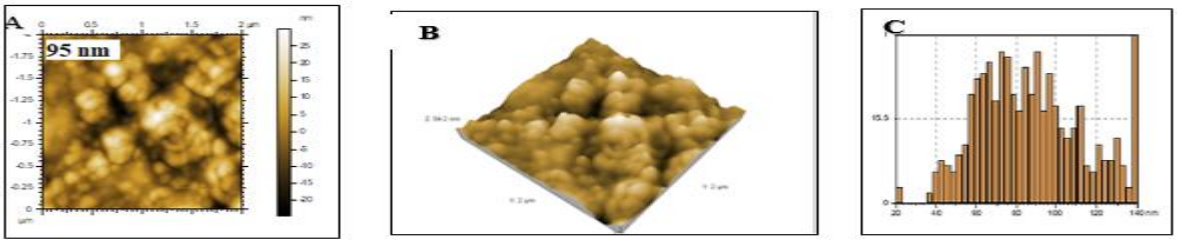

Figure 2. 2 × 2μm^2 AFM images (A: 2Dand B: 3D images) and capsules size distribution (C) of capsules entrapping OE: (1:1 of CS-PG and 1:0.5 of CS: OE)

The results in Table 2 showed that the capsules produced in this study have a mean size in the range of 40 to 90 nm. The results also showed when the CS: PG ratios were, 1:2 and 1:3 and CS: OE ratio was 1:0.5 no capsules were formed. This result may be due to the viscosity of PG at high ratios resulting in a limited reduction in the functional groups [32] and limiting the interaction between PG hydroxyl groups and CS amine group

Table 2. Size of CS-PG capsules loaded with OE (nm).

CS: OE ratio	CS: PG ratio		
	1:1	**1:2**	**1:3**
1:0.5	95±7.07^{d}	NF	NF
1:1	70±1.36^{c}	40±1.71^{d}	41±1.41^{d}
1:1.5	60±3.4^{bc}	60±1.42^{a}	52.5±3.53^{b}

*** Mean ± SD values of triplicate samples. Different letters in the results indicate a significant difference (*P*<0.05).NF: not formed.**

As shown in table 3 the individual variables and their interactions had a significant effect on capsule size (P<0.05). A significant decrease in size was observed when the PG concentration was increased. This is due to a stronger interaction between the negative charge of the PG and the positive charge of the CS, which is reflected in the small size [33]. These results are consistent with those reported by Vahidmoghadam et al [34].On the other hand, the size of the nanocapsules was impacted significantly by the ratio of CS: OE added in the preparation, and as the CS: OE ratio increased the size of the capsules increased. Rajabi et al [19] claimed that the nanocapsule's sizes were increased when the core increased because of a rise in the surface of the carriers as a result of their increased load, resulting in a thicker layer of complexes chitosan-gum around them. Based on the findings of Akdeniz et al [35], when core material ratios increase, the coating material is insufficient to encapsulate it, resulting in the coalescence of capsules and larger particle size. A previous study had found similar results [36].

Table 3. Analysis of variance of CS-PG capsules sizes.

Source of variation	d.f	M.S.	F
Gum ratio	2	417.3333	**<.001**
Phenolic extract ratio*	1	675	**0.005**
gum×phenolic extract	2	217	**<.001**

***The ratio of phenolic extract (1:0.5) which did not form any capsules was excluded from the statistical analysis of CS-CG nanocapsules.**

Nanocapsules structure

Figure 3 illustrates the SEM microscopy of the nanocapsules with the highest EE% (1:1 CS-PG and 1:0.5 CS: OE). The nanocapsule particles seemed to be in an irregular form with noticeably sharp edges (Figure. 3). Also, the porous structure was observed in freeze-dried powder. Tavares et al [37] reported similar results for encapsulation of garlic extract. Kuck et al [38] observed the irregular structure and a broken glass shape for freeze-drying particles of grape skin phenolic extract. The spongy porosity structure of the particles is a result of the freeze-drying process and the development of ice crystals in the substance [39].

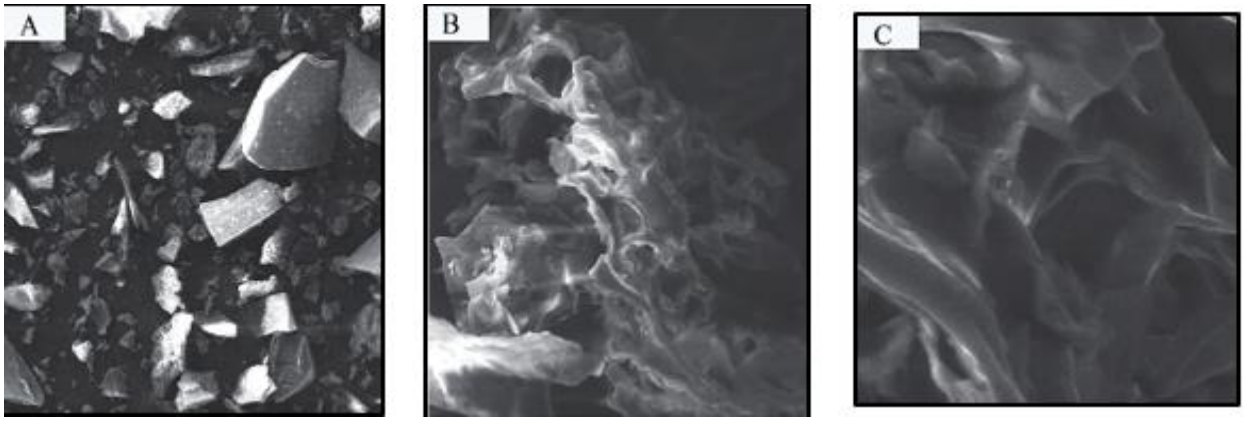

Figure 3. SEM images of capsules entrapping OE at different magnification scales; A:200µm, B: 20 µm, and C: 5 µm.

The release profiles of OE from nanocapsules

Encapsulated structures should not only preserve the bioactive compounds entrapped in them, but they should also be able to release them so that they may perform their biological functions [40]. The release profile for the best formulation, based on the highest EE%, was studied in simulated gastric fluid (pH =1.2) and in simulated intestinal fluid (pH= 7.2). It is nutritionally preferable for the capsules to release slowly in simulated gastric media and quickly in simulated intestinal media [41]. In acidic conditions, the solubility of CS increases and the polymer matrix swells, resulting in an increase in the target material's release rate [19]. As shown in Figure 4, phenolic compounds were released from capsules during the incubation periods, and 55.15% of OE was released from the CS-PG matrix after 240 min. At pH> 6.5 the porosity in the structure of nanoparticles observes due to the swell in the gum chain, resulting in the release of bioactive compounds [16]. As illustrated in Figure 4, OE release in neutral media was significantly lower than in acidic media, with OE release reaching 52.67 after 4 hours. The time required to release 50% of the encapsulated OE (t50%) is about 207.51 and 236.01 min under acidic and neutral media respectively. The amount of phenolic extract released in the literature is higher than that reported in this study; Rajabi et al [19] reported that about 80% and 70% of the saffron extract was released from chitosan-Arabic gum nanocapsule after 240 minutes in acidic and neutral media, respectively. This difference can be explained by differences in the chemical compositions of the two environments, as well as differences in CS solubility. On the other hand, the chemical structure of saffron extract and OE extract differs in terms of molecular weight and size of molecules, as well as negative charge potential, resulting in differing release behavior of the two substances. Our results indicate that the nanocapsules created by CS-PG could be used as an efficient strategy for retaining OE bioactive components and delivering them safely into the gastrointestinal tract.

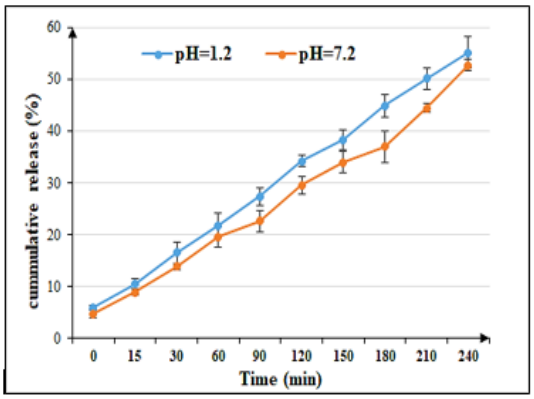

Figure 4. In *vitro* cumulative release of OE from CS-PG nanocapsules in various pH media.

Fourier transform infrared analyses (FTIR)

The interactions between the wall components (chitosan and gum) and between wall components and extract within the nanocapsules were studied using FTIR measurements (Figure. 5). The CS spectra showed peaks at 3362.28 (OH and NH2 stretch), 2880.17 (CH stretch), 1652.7 (C=O

stretching in the amide I), 1423.21 (C-N stretch in amine I), and 1068.37 cm−1 (C- O- C stretch). PG powder show peaks at 3320.82 (O-H stretching), 2924.52 (CH stretching), 1617.98, 1429.96 (stretching vibration of carboxyl), and 1039.77 cm−1 (stretching of the C-O bond).

An increase in the wavenumber of the hydroxyl group from 3362.28 in CS to 3399.89 cm−1 in nanocapsules, indicated the improvement of hydrogen bonding between OE and CS [37]. The peak of amide I in CS and the peak of carbonyl in PG shifted from 1617.98 and 1652.7 cm-1 to 1621.84 cm-1. Also, the peak at 1429.96 and 1423.21 cm-1 in PG and CS shifted to 1449.24 cm-1 in nanocapsules. These changes indicate the complex formation via electrostatic interaction between the negative functional groups of the gum and the positive ones of chitosan within the nanoparticles [19, 43].

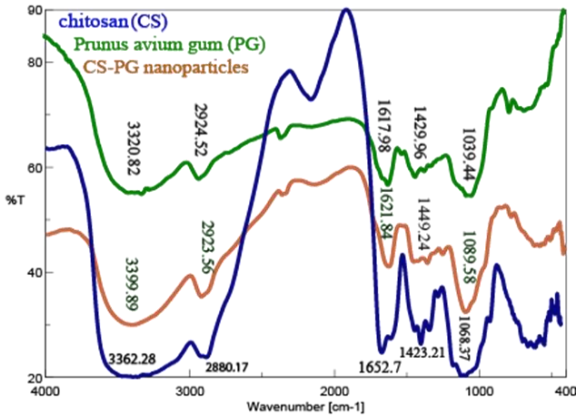

Figure 5. FTIR spectra: (A) CS, PG, and their nanocapsules

Conclusion

In this work, OE-loaded CS-PG nanocapsules were successfully prepared via the ionic gelation method. The ratio of CS: PG and CS: OE significantly affects both the capsules size and EE%. The highest EE% (87.06%) with nanometre dimensions (95 nm) was obtained when the CS: PG and CS: OE were added at the ratios 1:1 and 1:0.5 respectively. The release experiments demonstrated that the produced nanocapsules can release OE over time (55.15 and 52.67 % were released after 240 min in acidic and neutral media respectively). According to SEM images, the nanocapsules had a spongy porous structure.

The findings revealed that CS-PG may be utilized as wall materials for bioactive compound delivery in food sectors and that PG could be used as a natural and safe polymer. Future research will focus on a study the effect of fortification of various food products with OE encapsulated with CS-PG on their quality parameters.

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