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Preparation and Characterization of Nano-Antioxidant Via Ionically Cross-Linked Propolis Nanoparticles with Chitosan Polymer as A Carrier

Article Info.

Author

Abrar S. Abdul-Razak [1,2] Rashad Fadhil Ghadhban[1]

1-Department of Physiology, Pharmacology and Biochemistry, College of Veterinary Medicine, University of Basrah, Basrah, Iraq

2-Department of Physiology, Pharmacology and Toxicology, College of Pharmacy, University of Basrah, Basrah, Iraq

Corresponding Author Email Address: rashad.ghadhban@uobasrah.edu.iq

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Abstract

This study aimed to prepare and characterization of nano propolis by encapsulating propolis extract with Chitosan (CS) using the ionic gelation method to produce Chitosan-propolis Nanoparticles (Cs-Pr NPS). Propolis is well known as an antioxidant, anti-inflammatory, but its clinical efficacy is limited due to poor water solubility. To overcome these challenges, nanoencapsulation technology has been used to enhance its therapeutic efficacy. Ultraviolet–visible Spectroscopy (UV-VIS), Fourier Transform Infrared (FTIR), Field Emission Scanning Electron Microscopy (FESEM), X-ray diffractometer (XRD) and Atomic Force Microscopy (AFM) analysis were applied to confirm the success of nano-formulation and structural integrity of nanoparticles. UV-Vis spectroscopy confirms the preservation of biologically active components, as absorption peaks corresponding to flavonoid and phenolic compounds. FTIR showed the presence of hydroxyl (-OH) and carbonyl (C=O) functional groups of propolis and nanopropolis. FESEM of nanopropolis show smooth spherical particles with a size between (35-51nm), XRD confirmed the crystalline nature of the Cs-Pr NPs. In addition to AFM, confirm well-dispersed nanoparticles with spherical morphology and smooth surface.

Keywords: Nano propolis, propolis, Antioxidant, Ionic Gelation, polyphenols and flavonoids.

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Introduction

Propolis is a resinous product made by worker bees by mixing collected tree buds and resin with secretions from their glands (1). It has a complex chemical composition, with 70% of its bioactive components consisting mainly of flavonoids, terpenes and phenols, in addition to a variety of amino acids, enzymes, vitamins and many trace elements (2). The biological properties of propolis are due to its richness in polyphenols and flavonoids (3). Propolis is a potent antioxidant that acts by scavenging free radicals, inhibiting the formation of reactive oxygen species (ROS), chelating metal ions and exerting synergistic roles with other antioxidants (4). Its clinical efficacy is limited due to poor water solubility, rapid and intense metabolism, and low bioavailability. Moreover, the absorption of bioactive components of propolis, especially polyphenols and flavonoids, is greatly affected by changes in pH of the gastrointestinal tract, causing degradation, alteration or structural transformation of them (3). On the other hand, gut microbes also affect the flavonoid metabolites and bioavailability, causing 20% only to be absorbed in the small intestine and enter the systemic circulation, while the majority passes into the colon (5). Encapsulating propolis can improve its bioavailability by protecting the bioactive substances in propolis from metabolic degradation in the digestive tract, improving their solubility and stability, permeability and achieving slow release and targeted delivery of the active substances (6). Propolis is coated with chitosan by different methods, including emulsion-based cross-linking, complex coacervation, droplet coalescence, reverse micellar, solvent diffusion/evaporation and ionotropic gelation methods (7). Among these methods, the ionotropic gelation method is often preferred due to its simplicity, non-toxicity and low cost (8). Therefore, this study investigates the preparation and characterization of nanoencapsulation of propolis using ionic cross-linking between propolis and chitosan polymer to produce chitosan propolis Nanoparticles (Cs-Pr NPS).

Materials and Methods

Preparation of Cs-Pr NPS

Chitosan (Sigma-Aldrich, Germany) and ethanolic propolis extract 70% (Taian Biotechnology CO., LTD, Xi'an, China) were used to prepare Cs-Pr NPS by the ionic gelation method. (9) demonstrated a method to prepare Chitosan- propolis extract adduct. Cs-Pr adduct synthesized by mixing equal amounts of these materials and using the Dean–Stark (Clevenger, OEM, India) apparatus in the presence of xylene for condensation reaction until the water had been separated. Filtration, washing several times with various solvents (methanol, hot distilled water and ethanol respectively) was utilize to separate chitosan amide product, then dried at 50 $^{\circ}$C via electric oven and weighted. Cs-Pr NPS were obtained by use of an ionic-gelation method by adding Tripolyphosphate (TPP) with Cs-Pr adduct as follows: First, 1% w/v of acetic acid solution was used as a solvent for dissociation of 25 mg/ ml of Cs- Pr adduct to obtain a clear solution. Second, the TPP solution was mixed with Cs- Pr solution with ratios 1: 2.5 (w/ w) % with continuous stirring at ambient temperature for six hours. TPP initiated ionic gelation mechanism and produced

Cs- Pr /TPP nanoparticles. Precipitate of these nanoparticles was formed by several processes, including separation, washing, and drying. Finally, it was re-suspended in water and dried (10).

Characterization of S-CSNPs

Characterization tests of Nanoparticles were performed in the material research laboratories at the Ministry of Sciences and Technology, Environment and Water Research and Technology Director (EWRTD) in Baghdad.

Ultraviolet–visible Spectroscopy (UV-VIS)

It is used to confirm the formation of nanoparticles. The absorbance spectra of propolis extract and CS-Pr NPs solution were scanned in the range of 200 to 800 nm by using UV-VIS double-beam spectrophotometers. According to the method described by (11).

Fourier Transform Infrared (FTIR)

FTIR analysis (Shimadzu, Japan) was used to identify the functional groups on the surface of propolis extract and Cs-Pr nanoparticles. The spectra were scanned at a resolution of 4 cm^{-1} in the (400–4000 cm^{-1}) range. According to the method described by (12).

Field Emission Scanning Electron Microscopy (Fe- SEM)

It was utilized to examine the morphology and particle size of the NPs' surface that was prepared. The Brucker Scanning Electron Microscope was used to characterize morphology of samples. According to the method described by (13).

X-ray diffractometer (XR)

was employed to determine the crystalline nature of the prepared NPs. XRD scanning was recorded by using an X-ray diffractometer at 2θ angle scanning mode (operational voltage 40 kV and current 30 mA, Cu K (a) radiation λ = 1.540). The particle size of Cs-Pr NPs was determined by using the Debye–Scherrer equation as follows: $D=0.9\boldsymbol{\lambda}/\beta \cos\theta$, where D diameter of the NPs, $\boldsymbol{\lambda}$ is the wavelength of the x-ray, θ is the diffraction angle (Bragg's angle) in radians and β is the full width at half maximum (FWHM) of the peak in radians (14).

Atomic Force Microscopy (AFM)

It offers the ability to image 2D and 3D objects, as well as qualitative and quantitative details, many physical properties, including size, shape, surface texture, and roughness. According to the method described by (15).

Results

Synthesis of Cs-Pr NPs:

Chitosan nanoparticles loaded with propolis (Cs-Pr NPs) were prepared successfully by the ionic gelation method. TPP (tripolyphosphate) had been added as a cross-linker, which induce to form cross linking between protonated amine groups (NH3+) of chitosan and the negatively charged phosphate group of TPP, forming spherical nanoparticles. The ionotropic gelation process modifies the molecular structure of chitosan, resulting in a change in its solubility and transforming it into a liquid form. The loading of propolis extract into nano-chitosan was confirmed firstly by the appearance of the solution, which was clear, thus confirming the success of the encapsulation process.

Characterization of synthesized Cs-Pr NPs

UV-Vis Spectroscope:

Ultraviolet analysis of nanopropolis and propolis extract was the first step to confirm successful loading of propolis into the nanoparticles and verify the spectral properties of them by scanning the ready solution with UV- visible spectroscopy wavelengths (200-800 nm). As shown in Figure (1) and Table (1), the absorption peaks of propolis extract were (3.436,3.763 and 3.913) at a wavelength 294, 288 and 281nm which is usually related to flavonoids and phenolic compounds, while the peaks of nanopropolis show slight shifting to right in absorbance intensity (0.437,0.38 and 0.736) at wavelength (289, 269 and 222 nm) As shown in Figure (2) and Table (2)

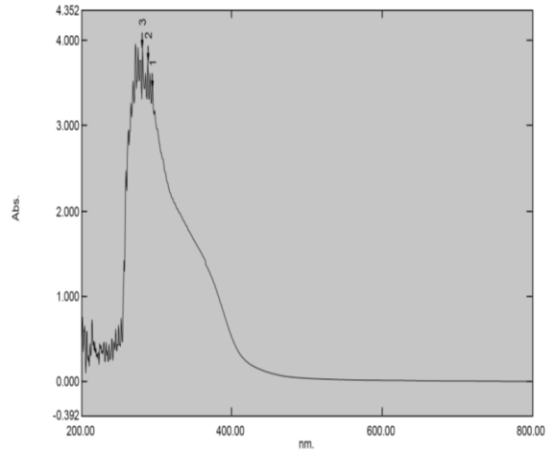

Figure (1) UV-visible spectroscope in Propolis extract

Peak No.	wavelength	Absorbance
1	294.00	3.436
2	288.00	3.763
3	281.00	3.913

Table (1): UV- Visible spectral analysis result of propolis extract

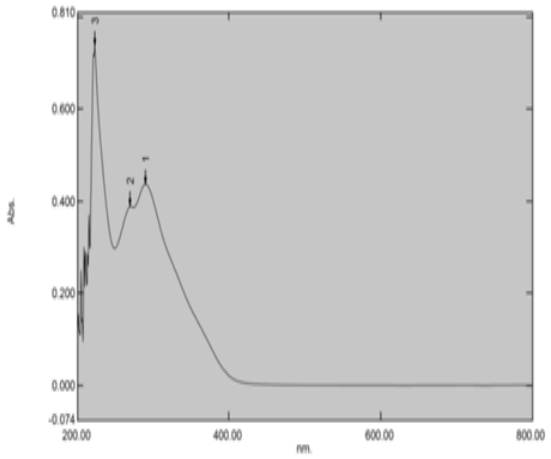

Figure (2) UV-visible spectroscope Nanopropolis

Table (2): UV- Visible spectral analysis result of propolis

Peak No.	wavelength	Absorbance
1	289.00	0.437
2	269.00	0.388
3	222.00	0.736

Fourier Transforms Infrared Spectroscopy Analysis (FTIR):

To determine function groups and detect any shifting that represents molecular interaction or encapsulation, Fourier transform infrared (FTIR) analysis was conducted on both proplis extract and Nanopropoplis using wavelength ranges of (400-4000 cm^{-1}). As show in figure (3) and (4), absorption peaks of propolis extract were around (3356 cm^{-1}) which ascribed to the hydroxyl group (O-H) stretching vibration in phenolic groups in propolis While the absorption peak of the nanopropolis showed a right shift and was 3223 cm^{-1} which ascribed to the hydroxyl group (O-H) stretching vibration in phenolic groups in propolis and chitosan, also assign to amino (-NH) groups of chitosan. A bands at 3070, 2924 and 2854 cm^{-1} of propolis extract are assigned to stretching vibration of C-H of aromatic ring, asymmetric and symmetric C-H in alkanes (aliphatic saturated chains) respectively, compared to the shift in absorption peak of nanopropolis, which was 2914 cm^{-1} and 2841 cm^{-1} which representing asymmetric and symmetric C-H in alkanes of propolis and chitosan. Stretching vibrations shown by the FTIR spectra band of propolis extract at 1639 cm^{-1} are attributed to C=C and C=O groups, typically associated with lipids, flavonoids, phenolic compounds and aromatic ring deformations that well known for antibacterial and antioxidant properties.

Also, the band was shown at 1712 cm^{-1}, assigned to the C=O stretching vibration of carboxylic acid, which is associated with carboxylic acid derivative from ferulic acid or caffeic acid. In nanopropolis, more intense absorption and a shift to 1691 cm^{-1} are likely associated with the stretching vibrations of the amide (C=O stretching) groups, which are common functional groups in propolis and chitosan. In propolis extract, the absorption bands at 1508 cm^{-1} and 1452 cm^{-1} correspond to the C=C stretch of the aromatic ring. But this band's move to 1571 cm^{-1} and 1435 cm^{-1} in FTIR nanopropolis. The band 1571 cm^{-1} represents the amide II vibration that is created from N-H bending and C-N stretching, while the peak at 1435 cm^{-1} is assigned to the C=C stretch of the aromatic ring.

FTIR spectra for propolis extract revealed a band at 1359 cm-1, which was assigned to C-H stretch vibration; on the other hand, nanopropolis showed a band at 1325 cm^{-1} attributed to C-N stretch vibration. An absorption band appear at 1159 cm^{-1} and 1099 cm^{-1} corresponding to the C=O group of carboxylic acid, ascribed to flavonoids and lipids in propolis. This band shifts to a wider and more intense band at 1136 cm^{-1}. Absorption bands at 815 cm^{-1}, 736 cm-1 and 694 cm^{-1} in propolis extract and 630cm^{-1} and 545 cm^{-1} in nanopropolis demonstrate stretching and bending vibration of C–H groups, especially from aromatic rings, associated with phenolic compounds, whereas the peak near 640 cm^{-1} and below were associated with ring distortion of phenolic compounds of both propolis and nanopropolis.

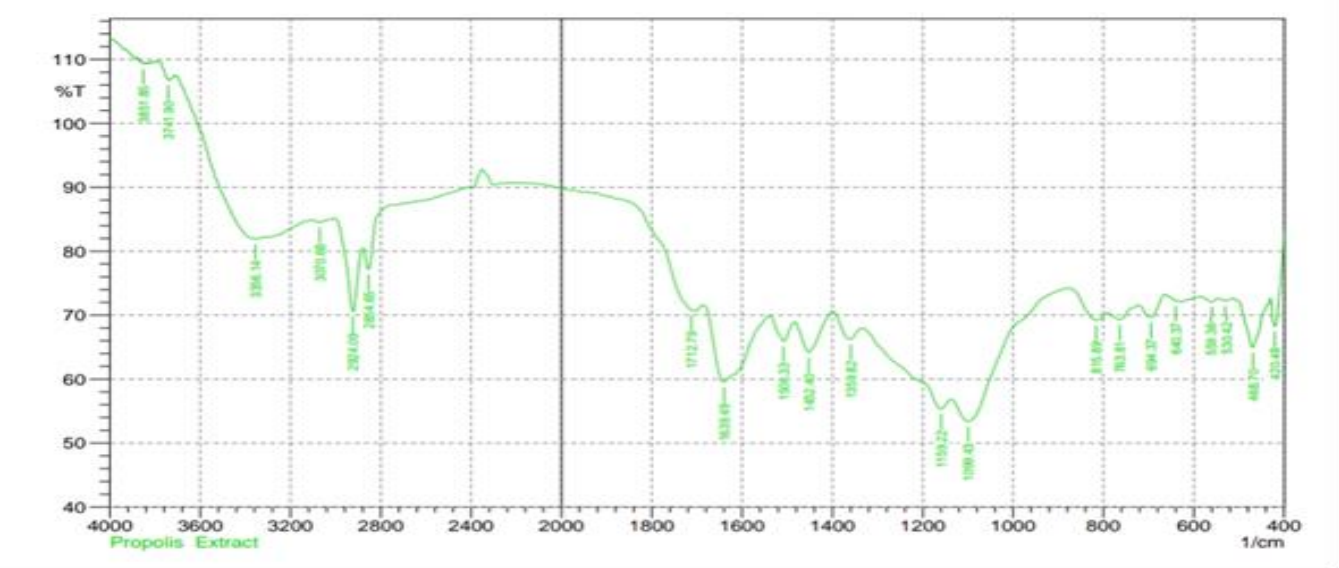

Figure (3) FTIR spectrum showing functional groups of Propolis extract

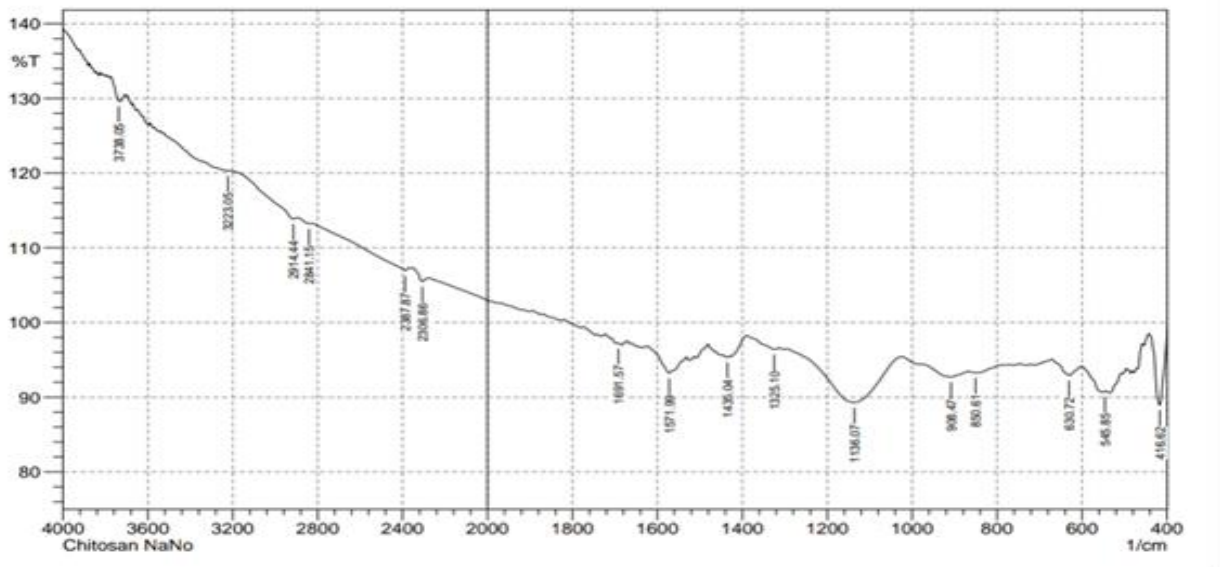

Figure (4) FTIR spectrum showing functional groups of Nanopropolis

Field Emission Scanning Electron Microscope (FESEM):

It was used to assess surface morphology and size of nanoparticles. The image of FESEM nanopropolis show smooth spherical particles, with a diameter ranging between (35-51nm), which is consistent with the required range of pharmaceutical applications**.** Figure (5)

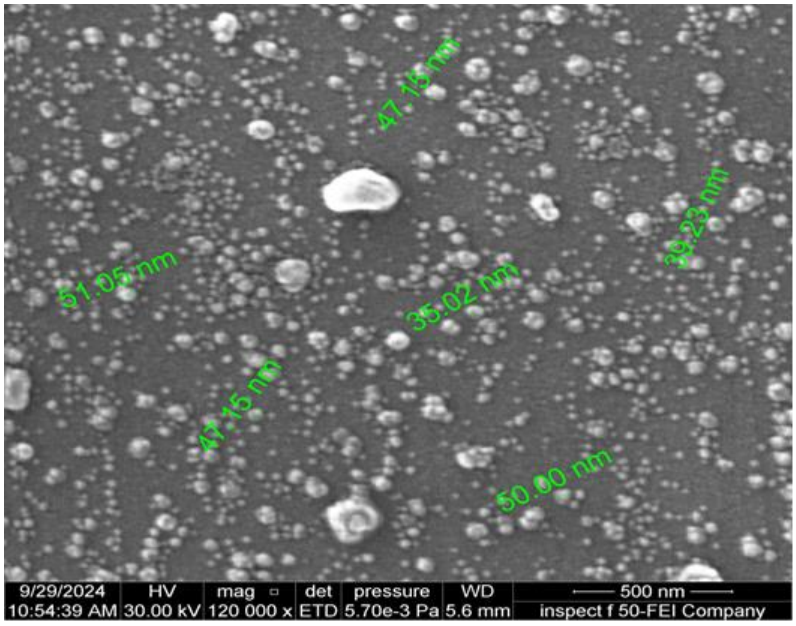

Figure (5) Field Emission Scanning Electron Microscopy (FESEM) image of Nanopropolis

X-Ray Diffraction Analysis (XRD)

X-ray diffraction was employed to investigate crystalline structure and size of prepared Cs-Pr NPs to confirm SEM results, as shown in Figure (6). The diffractogram showed several characteristic peaks at 2θ values 8.73°, 11.9°, 22.4°, 23.2494°, 23.2°, 24.0°, 30.7°, 31.7°, 33.4°, 35.8°, 37.4°, 45.9°, 47.4°, 52.4°,58.2°, 63.8°.

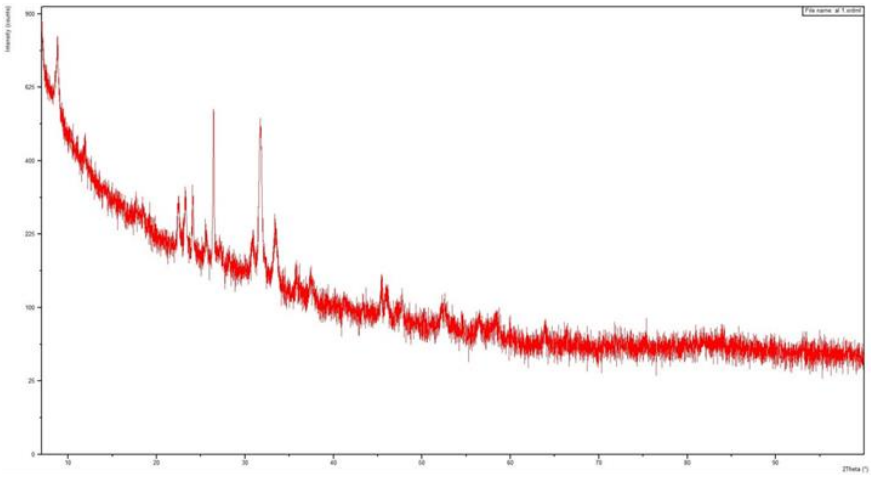

Figure (6): XRD of biosynthesized Propolis Nanoparticles

Atomic Force Microscopy (AFM)

AFM was utilized to investigate the surface topography and roughness of encapsulated nanoparticles through 2D and 3D images and a table containing the diameters of particles. It has the ability to give a micrograph and analyze surface of the Nano formulation to give very precise statistical values of average size, distribution, surface roughness and confirm the result of SEM. The AFM results shown in figures (7A, B and C) reveal that the particles of Nano propolis have an average size of 32.56 nm with a 10.76 nm roughness of the surface.

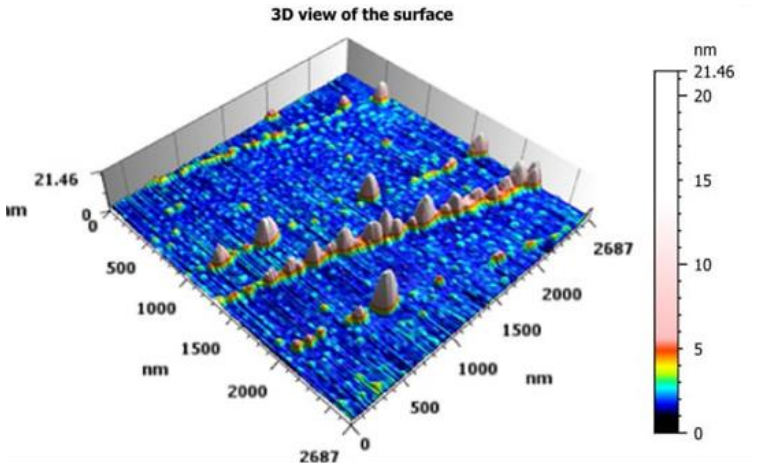

Figure 7A: Atomic Force Microscopy (AFM) showed a three-dimensional view of propolis Nanoparticles

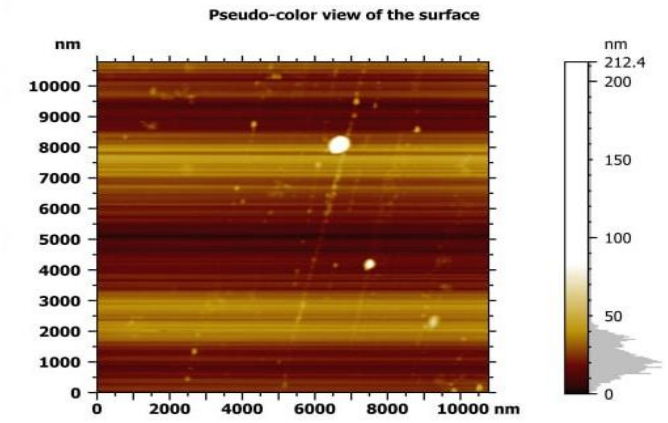

Figure7B: Atomic Force Microscopy (AFM) showed a two-dimensional view of propolis Nanoparticles.

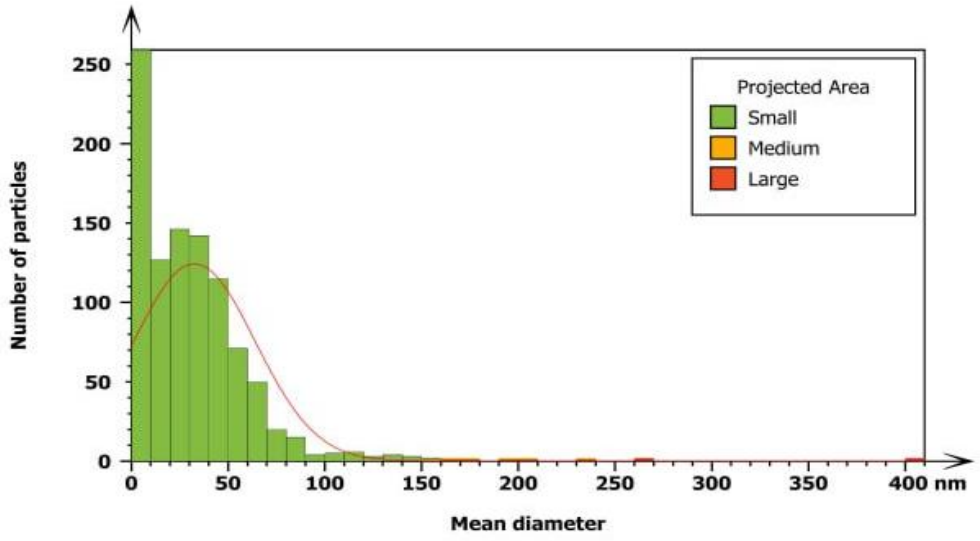

Figure 7 C: Atomic Force Microscopy (AFM) Diagram Shows the Size Range of Nanopropolis

Discussion

Drug delivery encapsulation technology plays an important role in protecting the biologically active ingredients in encapsulated propolis and increasing therapeutic efficacy through biocompatibility, biodegradability, targeted delivery, and controlled release. In the current study, the ionic gelation method was able to provide these properties by fabricating nanoparticles depending on the electrostatic interconnection between positively charged chitosan amino groups and negatively charged TPP phosphate groups, on condition that one of the components is polymeric (chitosan), propolis can be trapped inside the polymer chains, ultimately becoming encapsulated within the nanoparticle structure. The cross-linking between chitosan and TPP led to a change in both the molecular structure of chitosan and its solubility in acidic environments, forming a hydrogel or liquid solution (16).

The primary analysis used to confirm the occurrence of encapsulation and nanoparticle formation is UV spectroscopy analysis. However, it has been found that the main absorption peaks of propolis are between 250 and 400 nanometers with a very intense peak at 290 nanometers; these peaks belong to phenol and flavonoid compounds in propolis (17). In the current study, UV spectroscopy peaks for propolis extract were 294.00 nm, 288.00 nm and 281.00 nm, while Cs-Pr NPS exhibited 289.00nm, 269.00nm and 222.00 nm. These slight shift in peaks of nanopropolis indicate successful formation of nanoparticles, polymeric interaction between propolis and polymers as well as the encapsulation of the propolis with still contain the same bioactive compound (phenols and flavonoids), This result is consistent with the findings of (18), whose results showed minor shift in UV wavelength for both propolis and chitosan/Arabic gum particles encapsulating propolis, with maximum absorption shown at 290 nm.

Structural characterization of polymeric nanoparticles entrapping propolis was performed by FTIR spectroscopy. FTIR of our study exhibited numerous spectra that indicated the presence of

numerous bioactive compounds, including flavonoids, phenolic compounds, aromatic compounds, hydrocarbons, primary and secondary alcohols, and amino acids in both propolis and nanopropolis. However, FTIR of propolis displays a band 3356 cm^{-1} corresponding to the O-H functional group, which confirm the presence of alcohol or phenolic compounds such as flavonoids this result a line with (19) while nanopropolis showed change in band shape and right shift (3223 cm^{-1}) which belong to hydrogen bond between OH of propolis and NH of chitosan, indicating of encapsulation of polyphenol within chitosan polymers. This result is consistent with the result of (20). Propolis show three peaks between (3200 – 2800 cm^{-1}) assigned to stretching vibration of C-H of flavonoids, aromatic ring and aliphatic saturated chains consistent with the result of (21), while nanopropolis showed two merged peaks to a single peak (2914 cm^{-1}) with a shift of the third peak (2841 cm^{-1}) consistent with the result of (22). Propolis show two peaks in spectra between (1800-1600cm^{-1}), which are attributed to the C=O group typically associated with lipids, flavonoids, and phenolic compounds that are well known for antibacterial and antioxidant properties. In nanopropolis, the merged two peaks into one peak with more intense absorption and a shift to 1691 cm^{-1} suggested the occurrence of a hydrogen bond interaction between the phenolic OH of propolis and chitosan amide because chitosan has C=O stretching of amide I at 1645 cm^{-1}, this indicate successful of encapsulation. Propolis exhibited two peaks in spectra between (1600-1400cm^{-1}) which attribute to C=C and C-H, CH2 and CH3 group association with flavonoids and aromatic rings, shifting to 1571 cm^{-1} and 1435 cm^{-1} in FTIR nanopropolis indicating occurrence interaction between chitosan and propolis, in addition to another interaction between chitosan amine group and TPP phosphate group because chitosan has amide II at 1550cm^{-1}, indication successful of ionic interaction and encapsulation (23).

FTIR spectra for propolis extract revealed a single band in the spectra fingerprint region (1400 - 1200 cm^{-1}). On the other hand,, nanopropolis showed a band at 1325 cm^{-1} attributed to cross-linking between chitosan and TPP because chitosan has amide II and C-N at (1310-1350cm^{-1}). An absorption band of propolis appear two peaks at (1200 -1000 cm^{-1}) corresponding to the C=O group of carboxylic acid, ascribed to flavonoids and lipids in propolis. This band shifts to a wider and more intense band at 1136 cm^{-1}, a clear indicator of successful cross-linking between chitosan and TPP because chitosan has a C-O-C bridge at 1153 $cm^{-1,}$ and the shift occurs due to ionic interaction. Absorption bands between (1000-600 cm^{-1}) propolis extract and nanopropolis demonstrate stretching and bending vibration of C–H groups, especially from aromatic rings, associated with phenolic compounds. FTIR for our study of propolis and nanopropolis is consistent with the results of previous studies (18, 20, 21). Depending on the results, the presence of hydroxyl in propolis and hydroxyl and carbonyl groups in Cs-Pr NPs indicates chemical interaction between the propolis and chitosan. Also, forming new compounds is related to changes in the functional groups. These results a line with the findings of (24), who exhibited that the shifted peaks refer to the formation of a new compound.

The image of FESEM revealed that the Cs-Pr NPs have spherical morphology and are well distributed with a homogenous size of nanometer dimensions (35-51nm), which is consistent with the results of AFM. However, previous studies demonstrate that use of the ionic gelation method

produces a smaller particle size of nanoparticles, which play a crucial role in increasing therapeutic efficacy, solubility and bioavailability of bioactive ingredients, which is in line with the findings of (25). XRD diffraction pattern of Cs-Pr NPs shows broad peaks with low intensity, indicating interaction of chitosan with propolis. Previous study mentions XRD peaks of standard chitosan nanoparticles were at 2θ 11.7, 16.4, 18.41, 20.53 and 22.3 (26-27). In our study, the shifting of these peaks to 8.73°, 11.9°, 22.4°, 23.2494°, 23.2°, 24.0, 30.7 and 31.7° confirms interaction of chitosan-loaded propolis to produce Cs-Pr NPs. While other peaks from 33.4° to 63.8° belong to Cs-Pr NPs, the X-ray diffraction peaks are formed as a result of X-ray reflections from crystal planes. However, in our result, the broad peaks in X-ray diffraction are attributed to the inverse relationship between particle size and peak width, as small crystals have a restricted number of reflection planes with low intensity, while large crystals have a large number of reflection planes with high intensity (28). In addition, the bioactive compounds, such as flavonoids, phenolic acids and terpenes found in propolis extract, play an important role in facilitating interaction between propolis and chitosan and stabilizing crystal size and uniform size distribution in nanopropolis (29). This confirms the formation of stable, crystalline particles with small nanoscale sizes as a result of the interaction between propolis and chitosan. These results agreed with the findings of (30). AFM revealed the Cs-Pr NPs have small spherical morphology particles with a smooth surface, where the smooth surface confirms homogeneity and success of encapsulation of propolis by the ionic gelation method, agreeing with the findings of (31).

Conclusion

The current study confirms successful synthesis of nano propolis with preserved bioactive compounds and improves physiochemical properties of nanoparticles, thus enhancing bioavailability and therapeutic efficacy.

Conflicts of interest

The authors declare that there is no conflict of interest.

Ethical Clearance

This work is approved by The Research Ethical Committee.

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تحضير وتوصيف مضاد أكسدة نانوي عبر الربط الأيوني بين البروبوليس وبوليمر الكيتوزان كوسط ناقل

أبرار سلمان عبد الرزاق [1,2] , رشاد فاضل غضبان[1].

1-فرع الفسلجة والادوية والكيمياء الحياتية، كلية الطب البيطري، جامعة البصرة، البصرة، العراق.

2- فرع الفسلجة والادوية والسموم، كلية الصيدلة، جامعة البصرة، البصرة، العراق

الخلاصة

هدفت هذه الدراسة إلى تحضير وتوصيف العكبر النانوي عن طريق تغليف مستخلص العكبري بالكيتوزان باستخدام طريقة التجليد الأيوني لإنتاج جسيمات نانوية من العكبر المغلف بالكيتوزان. ان العكبر معروف جيدا بخصائصه المضادة للأكسدة والالتهابات، إلا أن فعاليته السريرية محدودة بسبب ضعف ذوبانه في الماء. وللتغلب على هذه المشكلة، استخدمت تقنية التغليف النانوي لتعزيز فعاليته العلاجية. تم استخدام التحليل الطيفي للأشعة فوق البنفسجية والمرئية، وتحليل الأشعة تحت الحمراء بتحويل فورييه، وتحليل المجهر الإلكتروني الماسح ذي الانبعاث الميداني، وتحليل حيود الأشعة السينية، وتحليل المجهر الذري الماسح لتأكيد نجاح التركيبة النانوية والسلامة الهيكلية للجسيمات النانوية. اكد التحليل الطيفي للأشعة فوق البنفسجية والمرئية الحفاظ على المكونات النشطة بيولوجيًا، حيث تتوافق قمم الامتصاص مع مركبات الفلافونويد والفينول, أظهرت تقنية تحليل الأشعة تحت الحمراء بتحويل فورييه وجود مجموعات وظيفية من الهيدروكسيل و الكاربونيل في العكبرونانوعكبر, تُظهر صور المجهر الإلكتروني الماسح للنانوعكبر أشكالًا كروية ناعمة يتراوح حجمها بين (35-51 نانومتر), أكدت تقنية حيود الأشعة السينية الطبيعة البلورية لجسيمات الكيتوزان-عكبر النانوية. بالإضافة إلى ذلك، تؤكد تقنية المجهر الذري الماسح وجود جسيمات نانوية متفرقة بشكل جيد ذات شكل كروي وسطح أملس.

الكلمات المفتاحية: العكبر النانوي، العكبر، مضادات الأكسدة، التجليد الأيوني، البوليفينولات والفلافونويدات.