

Article

Evaluation of Antioxidant and Antibacterial Activity of Gelatin Nanoparticles with Bitter Orange Peel Extract for Food Applications

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Abstract: Bitter orange is a citrus fruit rich in bioactive compounds, but its waste is currently underutilized. One potential solution is to encapsulate these bioactive compounds. This research aims to synthesize gelatin nanoparticles loaded with an ethanolic extract of bitter orange peel and to evaluate their in vitro antioxidant and antibacterial activities. Coaxial electrospray was used to encapsulate the ethanolic extract of bitter orange with bovine gelatin as wall material, considering a voltage of 15 kV, a wall solution flow rate of 0.1 mL/h, and a core solution flow rate of 0.08 mL/h. Characterization of the nanoparticles was performed using scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FT-IR). Antioxidant activity was evaluated by the total phenolic content, flavonoids, and antioxidant capacity by the DPPH•, ABTS•+, and FRAP assays. Antibacterial activity was assessed by the well diffusion technique on Mueller–Hinton agar against *Listeria monocytogenes* and *Escherichia coli* O157:H7 bacteria. SEM images confirmed that the nanoparticles were spherical in shape, while FT-IR analysis indicated that the incorporation of the extract did not alter the amide bonds of the gelatin protein. The nanoparticles containing the extract exhibited higher antioxidant activity and heightened inhibition against *E. coli* O157:H7, indicating their potential food applications.

Keywords: *Citrus aurantium*; nanotechnology; coaxial electrospray; bioactive compounds; foodborne pathogens

1. Introduction

The bitter orange (*Citrus aurantium*) is a fruit that grows on a tree belonging to the *Rutaceae* family. Native to East Africa, Arabia, and Syria, it is actively cultivated in regions such as Spain, Italy, and North America [1]. In Sonora, Mexico, *Citrus aurantium* is primarily used as an ornamental tree, and its fruits are typically discarded [2]. Bitter orange is a globose fruit with a slightly textured surface and an intense orange color when ripe (Figure 1). Due to its rich content of diverse bioactive compounds such as phenolics, flavonoids, essential oils, and vitamins, bitter orange is commonly employed for medicinal purposes (antibacterial, anti-inflammatory, anticancer, antioxidant, and cardiovascular

properties) and in the food industry (juices and essential oils) [3]. Bitter orange biowaste currently produces atmospheric and aquatic pollution contamination since it accumulates in the soil when it is not consumed or it is not fully used [4]. The predominant residue of the bitter orange is its peel, comprising approximately 40% of its fresh weight. In the peel, the documented phenolic compounds primarily consist of phenolic acids (74%) and flavonoids (23%). Among these, the most prevalent phenolic compounds identified are p-coumaric acid and ferulic acid [5].



Figure 1. Tree (a) and fruit (bitter orange) (b) of the *Citrus aurantium* species.

The previously mentioned compounds can help prevent food spoilage and cross-contamination, which pose significant health risks to consumers and can lead to temporary digestive issues such as gastroenteritis, diarrhea, fever, or even infections in other systems. Bacteria such as *Escherichia coli* and *Listeria monocytogenes* are often found in raw or poorly processed foods, including meat, dairy products, seafood, and fruits, making them two of the primary etiological agents of foodborne diseases [6]. There is currently a growing interest in obtaining bioactive compounds from biowaste due to their antioxidant and antimicrobial properties. These compounds also modulate the intestinal microbiota and promote the immune system [2]. Biowaste is rich in phenolic molecules, which contain aromatic rings with hydroxyl groups. These molecules are known to inhibit microorganisms by interacting with their cytoplasmic membranes, cell walls, and nucleic acids, thereby disrupting cellular functions, slowing growth, and causing cell death [7,8].

Nanoencapsulation is a process in which an active ingredient, an active molecule of a food (antioxidants, essential fatty acids, vitamins), or living cells (probiotics) are trapped within a wall material (polymers of carbohydrates, proteins, and lipids). Encapsulation serves not only to enhance the stability, bioavailability, and controlled release characteristics of biomolecules but also aids in concealing undesirable odor and taste [9]. Different nanoencapsulation techniques exist, notable among them are nanoprecipitation, emulsion–diffusion, double-emulsification, emulsion–coacervation, polymer coating, and electrospray techniques. However, electrospray is relatively simple, low cost, requires a low amount of solvents and the particles are obtained in a single step [10]. The principle of electrospray lies in the ability of an electric field to deform the droplet interface to produce nanometer-range droplets. The Rayleigh method serves to determine the rupture of the drop. This limit occurs when the surface tension of the drop is elevated by the electrostatic force [11]. The electrospray control variables, such as solution flow rate, electric potential, collector distance, environmental humidity, and polymer solution viscosity, conductivity, surface tension, and concentration, must be considered [12]. On the other hand, coaxial electrospray modifies the uniaxial electrospray process by using a coaxial capillary needle and syringes for two solutions [13]. It is an electrohydrodynamic process in which multilayer nanoparticles are formed by introducing coaxial electrified jets. Coaxial electro-

spray offers several potential benefits, including high efficiency, a simple and cost-effective process, the ability to preserve biological activity, and the production of particles with a uniform size distribution [12]. Therefore, the objective of this research is to produce by coaxial electrospray gelatin nanoparticles containing an ethanolic extract of bitter orange peel (*Citrus aurantium*) and to analyze their morphological, structural, antioxidant, and antibacterial properties as potential food applications.

2. Materials and Methods

2.1. Plant Material

Bitter orange fruits were harvested in the first quarter of 2022 at the University of Sonora, Hermosillo, Sonora, Mexico. A total of 900 g of fruit was washed with a neutral detergent, rinsed with potable water, and the peel was removed. The orange peel was cut into 3 cm by 3 cm squares and dried for 24 h at 60 °C in a convection oven (Blue M, New Columbia, PA, USA) until it reached a moisture content of 17%. Following drying, the material underwent grinding and sieving (size 60 sieve).

2.2. Chemical and Reagents

Absolute ethanol ($\geq 99.5\%$) and hydrochloric acid (HCl, 37.5%) were from Meyer Reagents (Ciudad de México, MEX); gallic acid, 2 N Folin solution, quercetin, 2,2-diphenyl-1-picrylhydrazyl (DPPH[•]) radical, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), tripyridyl-s-triazin (TPTZ), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS^{•+}) radical, glacial acetic acid, and bovine gelatin type B were obtained from Sigma-Aldrich (St. Louis, MO, USA); potassium persulfate ($K_2S_2O_8$, $\geq 99.0\%$) was from Jalmek (San Nicolás de los Garza, NL, MEX); anhydrous sodium carbonate (Na_2CO_3 , $\geq 99.5\%$), anhydrous sodium acetate ($C_2H_3NaO_2$, $\geq 99.0\%$), and iron trichloride ($FeCl_3$) were procured from CTR SCIENTIFIC (Monterrey, NL, MEX); Tryptic Soy Broth and Oxford Agar were purchased from MCD LAB (Tlaneplanta de Baz, EM, MEX); MacConkey Sorbitol Agar and Mueller–Hinton Agar were from Difco (Sparks, MD, USA); Ultrapure water was obtained using a purification system (Merck, Darmstadt, HE, GER).

2.3. Preparation of the Extract of Bitter Orange Peel

The initial solutions were prepared with 1.5 g of orange peel and 10 mL of absolute ethanol. These solutions were then homogenized, subjected to sonication for 20 min, and subsequently centrifuged at 1500 rpm for 15 min (Compact II Centrifuge, BD, East Rutherford, NJ, USA). The supernatants were collected, and the residues underwent a second extraction [14]. Thus, the supernatants were combined and concentrated with the rotary evaporator R-100 V (Flawil, SG, CH) at 200 rpm, 40 °C, and 90 kPa. The concentrated samples were suspended in ethanol until a known concentration was obtained. This process was conducted in two batches.

2.4. Preliminary Test for Nanoencapsulation

Uniaxial electrospray was used to test the production of gelatin nanoparticles [15,16]. For the feed solution, a concentration of 10% *w/v* gelatin was considered. This solution was prepared with 20% aqueous acetic acid, then sealed with parafilm, and stirred under ambient conditions until complete dissolution was achieved, approximately 1 h. In the electrospray process, a flow rate in the pump (KD Scientific, Holliston, MA, USA) of 0.1 mL/h was maintained for the polymer solution. The voltage applied using a CZE 1000R high-voltage power supply (Spellman, Hauppauge, NY, USA) was 15 kV, and the distance to the collector varied from 5, 10, and 15 cm. The distance to produce the gelatin nanoparticles was selected based on the lowest mean diameter (MD) and polydispersity index (PI). MD was measured using ImageJ 1.53 k (Wayne Rasband, Bethesda, MD, USA), while PI was calculated according to Estrella-Osuna et al. [17] and a frequency analysis.

$$PI = \sigma / \bar{X}, \quad (1)$$

where σ corresponds to standard deviation $\bar{X}$ refers to the mean diameter of nanoparticles.

2.5. Preparation of Gelatin Nanoparticles Containing an Extract of Bitter Orange Peel

The coaxial electrospray process was conducted considering the optimal distance for gelatin nanoparticle production, a voltage of 15 kV, a flow rate of 0.1 mL/h for the polymer solution (outer layer material), and a flow rate of 0.08 mL/h for the extract of bitter orange peel, which was the inner material.

2.6. Morphological and Structural Characterization of Nanoparticles

The morphological characterization of the nanoparticles was conducted by scanning electron microscopy (SEM) using a JEOL 5410 LV equipment (Arkishima, Tokyo, Japan). An acceleration voltage of up to 15 kV and samples were positioned onto double-sided conductive carbon tape affixed to the aluminum pin stub of the SEM [18].

The infrared spectrum was obtained using an FT-IR spectrophotometer (Frontier, Perkin Elmer, Waltham, MA, USA). The spectra were recorded using the attenuated total reflectance (ATR) technique in transmittance mode. A spectrum scan from 4000 to 400 cm^{-1} was considered [19]. The determinations were performed three times.

2.7. Preparation of Nanoparticle Suspension

The empty nanoparticles and those that included the ethanolic extract of bitter orange peel were suspended in absolute ethanol for approximately 12 h. Subsequently, these solutions were centrifuged at 1500 rpm for 15 min (COMPACT II CENTRIFUGE, BD, NJ, USA). The supernatants at a concentration of 2000 $\mu\text{g/mL}$ were used to determine the antioxidant and antibacterial activity.

2.8. Determination of Total Phenolic and Flavonoids Contents

The total phenolic content was determined in accordance with Garzón-García et al. [20]. A total of 10 μL of samples (extract of bitter orange peel, solutions of empty gelatin nanoparticles, and nanoparticles containing the extract) and 25 μL of 1 N Folin solution were added to the microplate and refrigerated for 5 min. After refrigeration, 25 μL of 20% Na_2CO_3 and 140 μL of distilled water were added to the wells. The microplate was put to rest for 30 min, and the absorbance was measured at 760 nm (Multiskan GO, Thermo Fisher Scientific, Waltham, MA, USA). A calibration curve was plotted with the gallic acid standard, considering concentrations from 0.06 to 1 mg/mL ($R^2 = 0.998$). The results were reported as mg of gallic acid equivalents per gram of sample (mg GAE/g DS).

The determination of flavonoid content was performed according to Del-Toro-Sánchez et al. [21]. A total of 80 μL of each sample and 80 μL of an ethanolic solution of AlCl_3 (20 g/L) were added to wells of the microplate, shaken for 30 s, and left in the dark at room temperature for 1 h. Subsequently, it was shaken again for 30 s, and the absorbance was measured at 415 nm. A calibration curve was plotted with the quercetin standard at concentrations from 0 to 0.3 mg/mL ($R^2 = 0.998$). The results were reported as mg of quercetin equivalents per gram of sample (mg EQ/g DS).

2.9. Antioxidant Capacity Essays

For the measurement of antioxidant capacity by scavenging 2,2-diphenyl-1-picrylhydrazyl radical ($\text{DPPH}^\bullet$), 1.5 mg of $\text{DPPH}^\bullet$ radical was dissolved in 50 mL of methanol. The absorbance of the solution was adjusted to 0.7 ± 0.01 at a wavelength of 515 nm. A total of 200 μL of the solution and 20 μL of extract and sample solutions were added to the microplate wells, left to rest in the dark for 30 min, and the absorbance was measured at a wavelength of 515 nm [22]. A calibration curve was plotted with the Trolox standard, considering concentrations between 0 and 0.08 mg/mL ($R^2 = 0.993$). The results were expressed as μM Trolox equivalents per gram of sample ($\mu\text{M TE/g DS}$).

For the determination of antioxidant capacity by 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) ($\text{ABTS}^{\bullet+}$) assay, 19.3 mg of ABTS were dissolved in 5 mL of distilled water.

Separately, 0.0378 g of potassium persulfate and 1 mL of water were mixed. Subsequently, 88 µL of the potassium persulfate solution was added to the ABTS solution and left to rest in the dark for 12 h under refrigeration. A total of 1 mL of this last solution was added to 88 mL of ethanol, and the absorbance was adjusted to 0.7 ± 0.01 at a wavelength of 734 nm. For measurements, 270 µL of the adjusted solution and 20 µL of each sample were added to the microplate wells. The absorbance was measured at 734 nm in a microplate reader after 30 min of rest [23]. A calibration curve was plotted with the Trolox standard at concentrations from 0 to 0.1 mg/mL ($R^2 = 0.992$). The results were expressed in µTrolox equivalents per gram of sample (µM TE/g DS).

To measure the ferric-reducing antioxidant power, three stock solutions were prepared: a sodium acetate buffer solution (300 mM/L at a pH of 3.6), a 20 mM $\text{FeCl}_3 \times 6\text{H}_2\text{O}$ solution, and a 10 mM TPTZ in 40 mM HCl solution. The working solution was obtained by adding the three stock solutions in a 10:1:1 ratio (buffer/ $\text{FeCl}_3 \times 6\text{H}_2\text{O}$ /TPTZ-HCl). For measurements, 20 µL of samples (extract of bitter orange peel, solutions of empty gelatin nanoparticles, and nanoparticles containing the extract) and 280 µL of the working solution were added to the microplate wells and the absorbance was measured at 638 nm after 30 min of rest. A calibration curve was plotted with the Trolox standard, considering concentrations between 0 and 1 mg/mL ($R^2 = 0.995$). The results were expressed as µM Trolox equivalents per gram of sample [22].

2.10. Antimicrobial Activity

Escherichia coli O157:H7 (ATCC 43890) and *Listeria monocytogenes* (ATCC 7664) strains were used in this study. An aliquot of each strain was added to 3 mL of tryptic soy broth and left to incubate at 37 °C for 18 h. To obtain pure cultures, a loop of *E. coli* O157:H7 was streaked on Sorbitol MacConkey Agar and *L. monocytogenes* on Oxford Agar. The plates were left incubated at 37 °C for 24 h. For each bacterium, a colony from the plates was inoculated in 9 mL of trypticase soy broth [24]. The population of the suspension was adjusted to 1.5×10^8 cells/mL using the McFarland standard. Serial decimal dilutions were performed to obtain inoculums with a population of 10^3 CFU/mL. For the agar well diffusion method, 100 µL of each inoculum were streaked on plates with Mueller–Hinton Agar. Subsequently, three 6 mm wells were made per plate, into which 50 µL of each sample was added [25]. The plates were incubated for 18 h at 37 °C. The wells with 50 µL of ethanol were considered as negative control. After incubation, the inhibition zones were measured with a vernier caliper.

2.11. Statistical Analysis

The results of the antioxidant and antibacterial activity determinations were performed in triplicate and were presented as mean $\pm$ standard deviation. A one-way analysis of variance (ANOVA) and a Tukey test were carried out to determine whether there was a significant difference in the antioxidant and antibacterial activity of the extract of bitter orange peel, the empty gelatin nanoparticles, and the nanoparticles containing the extract ($p < 0.05$) [26]. The statistical analyses were performed using Minitab 17 (State College, PA, USA).

3. Results and Discussion

3.1. Preparation of Bitter Orange Peel Extract

After the ultrasound-assisted extraction process and the concentration of the extract by evaporation under reduced pressure, an average yield of 1.076% of ethanolic extract of bitter orange peel was estimated considering the two batches. A concentration of 70 mg/mL was chosen for electrospray nanoencapsulation and subsequent analyses.

3.2. Obtaining of Nanoparticles

The determination of the best conditions of nanoencapsulation was based on the values of mean diameter (MD) and polydispersity index (PI) for the empty particles produced