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RESEARCH PAPER

Investigation of Physicochemical Properties of Zein Nanoparticles Loaded with Rabbi Date Seed Extracts Using Antisolvent Precipitation Method

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ABSTRACT

Date seed extracts contain bioactive compounds and many valuable phytochemicals such as phenolic compounds. Using encapsulation, the bioactive compounds of date seed extracts can be preserved against degradation by environmental factors and processing conditions. The aim of this study was to investigate the antioxidant properties of the ethanolic extracts of Rabbi date seed, prepared through ultrasound-assisted extraction (UAE), and to evaluate the physicochemical properties of zein nanoparticles loaded with the extracts. Antisolvent precipitation method was used to encapsulate the extracts at 0.05, 0.1, 0.15, and 0.2 g in zein. The total phenolic content (TPC) and half maximal inhibitory concentration (IC_{50}) of the extracts prepared under the UAE conditions of 45 min, 50°C, and ethanol concentration of 70%, were respectively found to be 275.86 mg Gallic acid equivalent (GAE)/g dry matter and 5.28 µg/ml. By increasing the ratio of the encapsulated extract to zein from 0.1 to 0.4, the encapsulation efficiency (EE) rose from 87.88 to 96.22%, and the size of the zein particles loaded with the ethanolic extracts increased from 140.25 to 195.25 nm, while the zeta potential of the nanoparticles decreased from +17.23 to +10.58 mV. The results of attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) indicated that by raising the ratio of the ethanolic extract encapsulated in the zein carrier, the band of O-H···O stretching vibration was shifted from 3292.80 to 3293.06 cm^{-1}. The images of field emission-scanning electron microscopy (FE-SEM) revealed that both the extract-free and extract-loaded zein nanoparticles had semi-spherical morphology.

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INTRODUCTION

In recent years, novel bioactive phytochemicals derived from medicinal plants have been widely welcomed by societies so as to promote people's health [1]. Functional bioactive compounds are generally categorized into two groups including molecules (vitamins, fatty acids, carotenoids, plant sterols, polyphenols, amino acids, peptides, and proteins) and living cells (probiotics) [2]. Various bioactive substances present in plant extracts and essential oils, such as phenolic compounds, have anti-allergy, anti-inflammatory, antimicrobial, antioxidant, and antidiabetic properties [3]. Low water solubility, volatility, low stability in foods, off-flavor, and negative effects on sensory properties are among the limitations of the direct application of plant extracts and essential oils in the formulation of food products. Moreover, they are prone to environmental stresses such as heat, pH, oxygen, and light, owing to comprising phenolic compounds [4, 5]. Recent developments in nanotechnology have enabled the food industry to surmount the obstacles to the use of plant extracts and essential oils as preservatives with high nutritional value and health-promoting properties. Nanoencapsulation is the encapsulation process of pharmaceuticals and bioactive compounds in a carrier matrix to protect them against biological destruction, extend their shelf-life, prevent their oxidative degradation, modify their diffusion kinetics, biodistribution, and controlled release, as well as improving the sensory and textural properties of food products [6, 7]. Size reduction to nanoscale (less than 1000 nm) can play a key role in the bioavailability, solubility, and targeted delivery of nutrients, due to increasing the surface-to-volume ratio of the particles [8].

In nanotechnology, carrier matrices are divided into three groups, namely lipid, polymer, and lipid-polymer. Potential toxicity, absence of industrial-scale manufacturing processes, limited applications of suitable biopolymers (protein and carbohydrates), and the need for organic solvents have restricted the use of polymer nanocarriers [9].

Zein, a polyamine biopolymer, is the major protein of corn. It is a hydrophobic and thermoplastic polymer. Owing to its hydrophobicity, this protein is transparent and insoluble in water. It is also an inherently free radical scavenger which resistant to bacteria [10]. Among the unique properties of zein as a carrier are its remarkable resistance to digestive enzymes, low digestibility in the digestive system, and wide applications in the delivery of bioactive compounds by simply encapsulating hydrophobic molecules through strong hydrophobic interactions [11].

Nowadays, date seed, one of the major wastes of date fruit, has received considerable attention because of its pharmaceutical properties and comprising a variety of nutritional ingredients including vitamins, carbohydrates, dietary fibers, minerals, amino acids, and lipids [12]. It is rich in protein (5.1%), fat (9%), dietary fibers (73.1%), phenolics (3942 mg/100 g), and antioxidants (80400 µmol/100 g) [13]. Date seed has a low sugar content which approximates to 7.2-7.6% [14]. The phenolic acids identified in date seed include gallic acid, protocatechuic acid, vanillic acid, caffeic acid, p-coumaric acid, ferulic acid, m-coumaric acid, and o-coumaric acid [15]. Date seed extracts are efficient in treating cerebral ischemic damages by reducing the brain oxidative stress [16]. The dietary fibers present in the powder and extracts of date seed play important roles in preventing diabetes, obesity, and hyperlipidemia, as well as protecting consumers against hypertension, coronary artery disease, high cholesterol levels, colon cancer, prostate cancer, and intestinal disorders [17].

Rabbi date is one the native dates of Sistan and Baluchestan province of Iran. It is classified as a semi-dry date with a moisture content of below 14%. A limited number of studies have ever been performed on the antioxidant properties of Rabbi date seed extracts. Radfar et al. investigated the TPC and IC_{50} of the ethanolic (80%) extract of Rabbi date seed, prepared through maceration. They found out that the TPC and IC_{50} were respectively equal to 2423 mg GAE/100 g dry matter and 20.4 µg/ml. They also realized that the phenolic compound profile of Rabbi date seed was composed of gallic acid, vanillic acid, 3,4-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid, cinnamic acid, caffeic acid, and chlorogenic acid [18]. Furthermore, no research has thus far been conducted on the application of zein to encapsulate date seed extracts, as well as examining the properties of the zein particles loaded with the extracts. At the same time, Jivan et al. encapsulated the aqueous extracts of Kabkab date seed using starch nanocrystals in the microemulsion system and evaluated the encapsulation parameters. They declared that the mean size, size distribution, and polydispersity

index (PDI) of the starch nanocrystals loaded with the date seed extracts were found to be 198 nm, 105-376 nm, and 0.162, respectively. The EE of the nanocrystals was equal to 62%, and the morphologies of both the extract-free and extract-loaded nanocrystals were semispherical [19]. Sadeghi et al. encapsulated the aqueous extracts of Kabkab date seed through the microemulsification-cold gel formation of whey proteins and assessed the encapsulation parameters [20]. Considering the role of nanotechnology in preserving the bioactive compounds of date seed extracts, opening up the possibility of enriching nanofoods, raising the bioavailability, targeted delivery of the bioactive compounds, and increasing their effectiveness in restraining and curing diseases, it has been attempted in this research to investigate the potential of zein, as a biopolymer carrier, for the encapsulation of Rabbi date seed ethanolic extract.

MATERIALS AND METHODS

Chemicals and reagents

All the chemicals and reagents used in this study were of analytical grade. Folin-Ciocalteu reagent, gallic acid, anhydrous sodium carbonate (Na_2CO_3), ethanol 96%, methanol 99.9%, and glacial acetic acid were all acquired from Merck Co. (KGaA, Darmstadt, Germany). Double-distilled water was bought from Zolalteb Chemistry Co. (Tehran, Iran). 2,2-diphenyl-1-picrylhydrazyl (DPPH) and zein were supplied by Sigma-Aldrich Co. (Massachusetts, the US). The HPLC-grade trifluoroacetic acid, methanol, and distilled water were purchased from Merck Co., Germany. The HPLC-grade standards of the phenolic compounds (cinnamic acid, caffeic acid, chlorogenic acid, 3,4-dihydroxybenzoic acid, gallic acid, vanillic acid, and 2,5-dihydroxybenzoic acid) were supplied by Sigma-Aldrich Co., the USA.

Date seed powder preparation

The fruits of Rabbi date (*Phoenix dactylifera* L.) were collected from the palm groves of Zabol city (Sistan and Baluchestan province, Iran) in August 2022. After that, the seeds were separated from the pulp and rinsed with tap water to remove the pulp residues. They were then placed on a strainer at ambient temperature for one week to be completely dried. Next, they were ground into a powder under vacuum using an industrial grinder (Flavina Co., Iran) equipped with a cooling system. Eventually, the powder was screened using a sieve with a mesh size of 1 mm.

UAE

25 g of the date seed powder was dispersed in 100 ml of ethanol 70% at a ratio of 1:4 (w/v). The dispersion was thoroughly mixed using a magnetic stirrer (Heidolph, MR Hei-Standard, Germany) at 500 rpm for 30 min. Next, it was poured into a 500 ml capped container to inhibit the vaporization of the solvent during the UAE process which was subsequently carried out using an ultrasonic bath (Bandelin, DT 102H, Germany) at 35 kHz, 480 W, and 50°C for 45 min. Following the process, the resulting extract was centrifuged (ATP, RF14000, Germany) at 7800 rpm for 30 min. Then, it was passed through a Whatman No. 1 filter paper. Afterwards, the solvent was evaporated using a rotary evaporator (Heidolph, Hei-VAP Value Digital, Germany) at 40°C and 200 rpm to prevent the decomposition of the extract phenolics. For evaporating the remaining solvent, the concentrated extract was poured onto a plate and dried in a vacuum oven (SHEL LAB, 1425-2, the US) at 40°C. Finally, the extract was kept at -18°C for further uses [3].

Quantification of antioxidant activity

TPC

The TPC of the date seed extract was determined through the Folin-Ciocalteu method [3]. For this purpose, an ethanolic (70%) solution of the extract was prepared at 1000 ppm. Then, 100 µl of the solution was poured into a test tube using a sampler. Next, 500 µl of the Folin-Ciocalteu reagent and 1000 µl of distilled water were incorporated into the tube. After 1 min of shaking, 1500 µl of sodium carbonate 20% was added, and the mixture was shaken. The tube was stored in the dark at room temperature for 2 h. Finally, the absorbance value of the solution was read using a spectrophotometer (UNICO, 2150, the US) at 760 nm. The standard curve was achieved by drawing the absorbance values against gallic acid concentrations, and the extract TPC (mg GAE/g) was computed using the curve linear equation:

$$Y = 2.9507\ X - 0.039 \qquad R^2 = 0.9951$$

where Y stands for the absorbance value, and X

indicates the TPC (mg GAE/ml).

Free radical scavenging activity

The DPPH test was conducted to measure the antioxidant activity of the extract [3]. To that end, a methanolic solution of DPPH was prepared at 0.004%, 5 ml of which was further combined with 50 µl of different concentrations of the seed ethanolic extracts. The solutions were stored in a dark place at ambient temperature for 30 min. Ultimately, the absorbance values of the samples and the blank were measured spectrophotometrically at 517 nm. The DPPH free radical scavenging activity was quantified by the following equation:

DPPH scavenging activity (%) = Blank absorbance value – Sample absorbance value/ Blank absorbance value × 100

Phenolic compound profile

The phenolic compound profile of the date seed ethanolic extract was obtained through reversed phase-HPLC (RP-HPLC) (Knauer, Smartline 3900, Germany) which was equipped with a UV-diode array detector (UV-DAD) (Knauer, Smartline 2800, Germany) and a C-18 reversed phase column (Waters, Spherisorb S5 ODS2, the USA) with an average particle size of 5 µm and dimensions of 4.6*250 mm^2 [18]. The wavelengths of 254, 275, 305, and 320 nm were regarded for the determination of the phenolic compound profile. 20 µl of the sample was injected into the device. Methanol (99.5% v/v) and 0.05% of trifluoroacetic acid were considered eluent A, and eluent B comprised distilled water and 0.05% of trifluoroacetic acid. The mobile phase had a flowrate of 0.5 ml/min, and the gradient elution conditions were as follows: 5% of eluent A 0-10 min; 5-25% of eluent A 10-20 min; 25-35% of eluent A 20-30 min; 35-45% of eluent A 30-48 min; 45-90% of eluent A 48-50 min; 90-95% of eluent A 50-52 min; 95-5% of eluent A 52-70 min; and 5-100% of eluent A 70-80 min. The entire process took 80 min. The standards of the phenolic compounds included cinnamic acid, caffeic acid, chlorogenic acid, 3,4-dihydroxybenzoic acid, gallic acid, vanillic acid, and 2,5-dihydroxybenzoic acid. Six concentrations of the stock solutions of each standard were prepared and injected into the device. After that, the area under the curve of the chromatograms was plotted against the stock solution concentration to achieve the calibration curves whereby the concentrations of the phenolic compounds were computed. The best linear equation was estimated for each calibration curve, and the concentration of each detected phenolic compound was calculated using the equation.

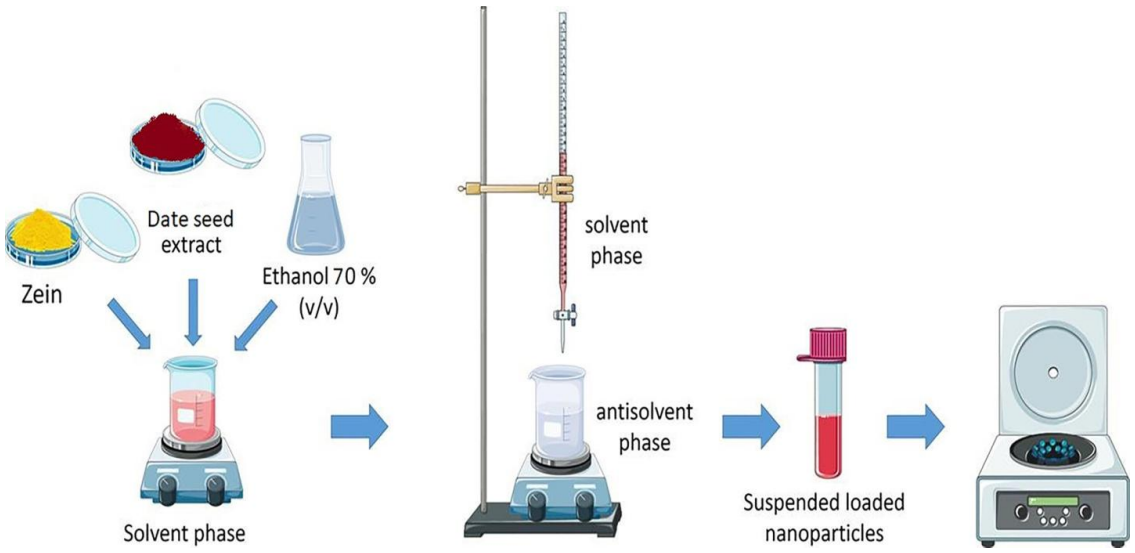

Fig. 1. Schematic diagram of the production process of zein nanoparticles loaded with date seed extract by antisolvent precipitation method.

Encapsulation

The ethanolic extracts of the date seed were encapsulated in zein through the antisolvent precipitation method with some modifications [21]. In order to prepare the solvent phase, 0, 0.05, 0.1, 0.15, and 0.2 g of the ethanolic extract powder were dissolved in ethanol 70% and homogenized using a magnetic shaker (Heidolph, MR Hei-Standard, Germany) at 40°C. The volumes of the solutions were made to 15 ml, so their concentrations would reach 0, 3.3, 6.6, 10, and 13.3 mg/ml. After cooling down the solutions to room temperature, 0.5 g of the zein powder was added (zein concentration became 33.3 mg/ml), and the solution was stirred on the magnetic shaker at 25°C for 1.5 h. It was further homogenized using a digital mechanical homogenizer (IKA, T18 Digital Ultra Turax, Germany) at 12000 rpm for 5 min. The extract-to-zein ratios were equal to 0, 0.1, 0.2, 0.3, and 0.4. To further reduce the particle sizes, the dispersions underwent ultrasonic pretreatment using an ultrasonic homogenizer (Bandelin Sonopuls, HD 2070, Germany) at 20 kHz for 6 cycles (duty cycle = 0.67) in an ice bath. Afterwards, the solvent phase was dropwise added to the antisolvent phase (40 ml of deionized distilled water) at a rate of 1 drop/s under continuous stirring on the magnetic shaker operating at 1500 rpm. With rapid precipitation at ambient temperature, suspensions of zein nanoparticles were obtained, which were immediately centrifuged at 10,000 rpm and 4°C for 20 min. The formed pellets were separated and freeze-dried (Zirbus, VaCo2, Germany) at 0.017 mPa and -57°C for 48 h. They were then ground using an electric grinder and rubbed using a pestle and mortar so as to obtain solid powder nanoparticles. Eventually, the powder samples were kept at 4°C until use.

Encapsulation tests

EE

The amount of the encapsulated extract was determined using the TPC of each powder [22]. To that end, 200 mg of each powder was dispersed in 2 ml of methanol:acetic acid:water mixture at a ratio of 50:8:42 (v/v/v). The dispersion was homogenized on a tube shaker for 1 min and subsequently sonicated in an ultrasonic bath at 480 W, 35 kHz, and 25°C for 20 min. After that, it was centrifuged at 5000 rpm for 10 min. The TPC of the supernatant was quantified through the Folin-Ciocalteu method [3]. EE was calculated using the following equation:

$$EE\ (\%) = \text{Encapsulated TPC} / \text{Total TPC} \times 100$$

Particle size and zeta potential

The size and zeta potential of the nanoparticles were determined through dynamic light scattering (DLS) using a zeta-sizer (Particulate systems, HD NanoPlus, the US). The samples were dissolved in ethanol 70% at 1% (w/v) and subjected to DLS with a scattering angle of 90° at 657 nm and 25°C [23].

ATR-FTIR

The ATR-FTIR spectra of Rabbi date seed ethanolic extract and the extract-free and extract-loaded zein nanoparticles were recorded using an ATR-FTIR device (Bruker, TENSOR II, Germany) in the wavenumber range of 4000-500 cm^{-1} (16 scans for each samples at a resolution of 4 cm^{-1}) [24]. In this method, 5 mg of the nanoparticle sample was completely softened with a pestle and mortar and sprinkled on the ATR diamond crystal surface of the device. The spectra were recorded at 75 psi, and the obtained data were analyzed using the OPUS software.

Nanoparticles microstructure

The microstructure (shape, morphology, and integrity in the capsule) of the particles was evaluated through FE-SEM (KYKY, EM8000F, China) [25]. For this purpose, the lyophilized powder samples were sprinkled on the silicon plate of the microscope and covered with a layer of gold. The images were captured at an accelerating voltage of 10 kV.

Statistical analysis

Mean comparison of the results of the encapsulation tests was carried out using the Tukey's test at 99% confidence level (α=1%) by means of SPSS ver. 28.

RESULTS AND DISCUSSION

TPC

The mean TPC of the ethanolic extracts of Rabbi date seed was found to be 275.86±0.25 mg GAE/g dry matter under the UAE conditions of 45 min, 50°C, and ethanol concentration of 70%. Radfar et al. prepared the ethanolic (80%) extract of Rabbi date seed through maceration and understood that its TPC was equal to 2423 mg GAE/100 g dry matter [18]. Metoui et al. quantified the TPC of the

date seed extracts of twelve Tunisian date varieties in the range of 5.13-9.53 g GAE/100 g dry matter [26]. Dehghanian et al. claimed that the TPC of the methanolic extract of date seed, produced using maceration, was found to be 175.4 mg GAE/g dry matter [27]. Al-Farsi et al. macerated the seed powders of three Omani dates and realized that their TPCs ranged from 3102 to 4430 mg GAE/100 g fresh weight [28]. The TPCs reported in the above-mentioned studies are lower than that of the present research. These differences can highly be due to the effects of the ultrasonic waves on the extraction efficiency of valuable compounds from plant materials in shorter times, as compared to conventional methods. Owing to their high energy content, these waves create shear forces which destroy plant cell walls. As a result, they increase the release of the cell contents into the extraction medium, elevate the mass transfer rate, and reduce the extraction time. With a rise in sonication time, more phenolic compounds diffuse into the extraction medium [3, 29]. Sonication temperature also gives rise to the solubility of polyphenols and accelerates their transfer from plant cells to the extraction medium [30]. Thermal energy improves the extraction efficiency by disrupting the cellular structure of the plant matrix. Additionally, temperature increase can lower the solvent surface tension and, as a result, elevates the wetting of the plant material, thus enhancing the extraction efficiency. High temperatures decrease the viscosity of the extraction medium; therefore, the solvent penetrates into the plant cell more easily, and the extraction efficiency is enhanced [31]. Because of their high affinity with polyphenols, two-phase solvent systems (alcohol-water) give rise to the extraction efficiency of these substances. Furthermore, solvents with high surface tension and polarity and relatively low vapor pressure and viscosity such as the mixtures of ethanol/water and methanol/water facilitate the disintegration of plant cells and the release of the solutes into the extraction medium, thereby increasing the intensity of ultrasonic cavitation and, consequently, the cell permeability [32]. Babiker et al. performed the UAE of phenolics from roasted and unroasted date seeds with methanol 80% as the solvent at 60°C for 30 min. They reported the TPCs of the seeds to lay in the range of 525.35-595.83 mg GAE/100 g dry matter, which are lower than those obtained in the present study. This difference can be caused by the difference in the date varieties as well as the application of thermal treatment at high temperatures (180-220°C) in roasting the date seeds. The intense roasting of date seed coffee can bring about a decline in its antioxidant potential [33]. Afshari et al. macerated Estameran date seed powder in water and various concentrations of water-ethanol. They maintained that the TPCs of the extracts were equal to 742.37 mg GAE/g dry matter for ethanol 75% and 236.07 mg GAE/g dry matter for water [34]. Djaoudene et al. measured the TPC of the seed extract of eight Algerian date varieties as 476 mg GAE/g dry matter [35]. The TPCs reported in these studies were higher than that obtained in the present research. This difference can be attributed to the difference in the date varieties and maturation levels, environment and climate, growing conditions, fertilizers, harvest time, diseases and pests, soil type, processing method, storage conditions, and extraction conditions including solvent type and concentration, time and temperature, and the impact of ultrasound on the extraction efficiency, compared to maceration [36].

IC_{50}

IC_{50} is defined as the concentration of an extract, which can scavenge 50% of DPPH free radicals. Free radical scavenging activity is defined as the capability of reducing agents like phenolic compounds to transfer hydrogen atoms into free radicals. Assessment of this feature is the most common technique for quantifying an extract antioxidant activity, which is according to the discoloration of the DPPH solution by the extract antioxidants. DPPH is a free radical stable at room temperature, and its methanolic solution is purple. If it reacts with an antioxidant, it loses its free-radical character, as its chain is decomposed; hence, its color is converted from purple into bright yellow due to the donation of a hydrogen atom by the antioxidant to the DPPH molecule. Antioxidants reduce DPPH free radicals to a more stable form. Consequently, the higher the TPC and antioxidant compounds of an extract, the lower its IC_{50} [3, 37]. In general, there is a positive correlation between the TPC and antioxidant power of date seed extracts. The mean IC_{50} of the ethanolic extracts of Rabbi date seed, prepared at the UAE time of 45 min, temperature of 50°C, and solvent concentration of 70%, was equal to 5.28±0.07 µg/ml. Radfar et al. prepared ethanolic (80%) extracts of Rabbi date seed using maceration and found

out that their mean IC_{50} was equal to 20.4 µg/ml [18], which is higher than that obtained in the present research, revealing the lower antioxidant power of their extracts. Al-Farsi et al. examined the antioxidant activity of the seed extract of three Omani date cultivars. They reported the IC_{50} of the extracts in the range of 929-580 µmol trolox equivalents/g wet weight [28], which were much higher than that measured in the present study. Afshari et al. macerated Estameran date seed powder in water and different concentrations of aqueous ethanol and stated that the IC_{50} values of the ethanolic (75%) and aqueous extracts were respectively equal to 0.90 and 6.83 mg/ml [34]. The IC_{50} values are higher than that of the ethanolic extract of the present study. Djaoudene et al. prepared the acetonic (75% acetone and 25% water) extracts of eight Algerian date seeds through maceration in the date maturation stage. They cited that the lowest IC_{50} was found to be 37.30 µg/ml [35], which was higher than that of the present research. Ghafoor et al. employed the Soxhlet, supercritical CO_2, and subcritical CO_2 methods to extract the antioxidant components of the seeds of Sukari, Ambara, Majdool, and Sagai dates which are native to Saudi Arabia. They declared that the highest antioxidant activity belonged to Majdool date seed extract produced using subcritical CO_2 (IC_{50} = 109.69 µg/ml), and the lowest antioxidant activity was associated with Sagai date seed extract prepared through Soxhlet (IC_{50} = 353.83 µg/ml). They showed that the IC_{50} values of the extracts produced using supercritical and subcritical CO_2 varied between 109.69 and 228.76 µg/ml [38], which are considerably higher than that achieved in the present study. It can be caused by the difference in the genotypes of the date varieties as well as the climatic and environmental conditions of the dates cultivation.

Phenolic compound profile

The phenolic compound profile of Rabbi date seed ethanolic extract is presented in Table 1. Seven phenolic compounds, including vanillic acid, 3,4-dihydroxybenzoic acid, gallic acid, 2,5-dihydroxybenzoic acid, cinnamic acid, caffeic acid, and chlorogenic acid were identified and quantified in the extract. Cinnamic acid (17.51 mg GAE/g dry matter) was the major phenolic compound of the extract, followed by chlorogenic acid (2.27 mg GAE/g dry matter), caffeic acid (1.93 mg GAE/g dry matter), 3,4-dihydroxybenzoic acid (1.70 mg GAE/g dry matter), vanillic acid (1.47 mg GAE/g dry matter), gallic acid (1.36 mg GAE/g dry matter), and 2,5-dihydroxybenzoic acid which was the minor constituent (1.13 mg GAE/g dry matter). Radfar et al. claimed that the phenolic compound profile of the ethanolic extract of Rabbi date seed included vanillic acid, 3,4-dihydroxybenzoic acid, gallic acid, 2,5-dihydroxybenzoic acid, cinnamic acid, caffeic acid, and chlorogenic acid. Additionally, they declared that cinnamic acid and 2,5-dihydroxybenzoic acid were the major and minor phenolic components of the extract,

Table 1. Encapsulation efficiency of Rabbi date seed ethanolic extracts in zein nanoparticles.

Extract type	Gallic acid	Vanillic acid	-3,4 dihydroxybenzoic acid	-2,5 dihydroxybenzoic acid	Cinnamic acid	Caffeic acid	Chlorogenic acid	Total Polyphenols
Ethanolic extract	1.36±0.09[a]	1.47±0.07[b]	1.70±0.04[c]	1.13±0.08[d]	17.51±0.80[e]	1.93±0.07[f]	2.72±0.09[g]	27.37

The results are expressed as mean ± standard deviation of three replications. There is a significant difference between the values with different letters in each raw ($p<0.01$).

Table 2. Mean size and zeta potential of extract-free and extract-loaded zein particles.

Sample	Extract-to zein ratio	Encapsulation efficiency (%)
Zein loaded with 0.05 g of date seed extract	0.1	87.88±0.11[a]
Zein loaded with 0.1 g of date seed extract	0.2	94.17±0.03[b]
Zein loaded with 0.15 g of date seed extract	0.3	95.12±0.56[b]
Zein loaded with 0.2 g of date seed extract	0.4	96.22±0.02[b]

All the values are the average of three replications. There is a significant difference between the values with different letters in each column ($p<0.01$).

respectively [18]. This conforms to the results of the present research. Majid et al. used aqueous methanol as solvent to determine the phenolic compound profiles of the seeds of three types of Pakistani date. They mentioned that the profiles comprised gallic acid (major phenolic compound), caffeic acid, p-coumaric acid, vanillic acid, sinapic acid, catechin, epicatechin, and chlorogenic acid [39]. Al-Farsi and Lee detected p-hydroxybenzoic acid, m-coumaric acid, and protocatechuic acid as the main phenolics of the seeds of three different varieties of Omani date [40]. Hmidani et al. investigated the phenolic compound profiles of the aqueous methanolic extracts of the seeds of four Moroccan date varieties. They realized that the profiles were composed of p-coumaric acid (major compound), rutin, caffeic acid, and quercetin [41]. El-Mergawi et al. identified p-hydroxybenzoic acid, coumaric acid, protocatechuic acid, and caffeic acid in the seeds of 10 varieties of Saudi Arabian date [42]. Moreover, they detected 5 flavonoids including kaempferol, naringenin, quercetin, luteolin, and isorhamnetin with different contents in the date seeds. Although similar phenolic compounds were detected in the present research, the concentration of each substance differed from that quantified in the afore-mentioned studies, which could be due to several factors including genetic variation among the date varieties, water availability, growth conditions, diseases, ripening stage, harvest time, geographical location, storage conditions, temperature, light, soil, fertilizer, in addition to the methods of extraction and analysis [41].

EE

EE normally refers to the exact number of bioactive compounds truly retained in a system [43]. It can be seen in Table 2 that as the concentration of Rabbi date seed ethanolic extract was increased from 3.3 to 13.3 mg/ml, or in other words, as the extract-to-zein ratio was elevated from 0.1 to 0.4, EE was raised from 87.88 to 96.22% with 93.34±3.74% being the mean EE. In addition, there was a significant difference in EE between the extract-to-zein ratio of 0.1 and the other samples at $P<0.01$. Calliari et al. investigated the encapsulation of *Hibiscus sabdariffa* extract in zein nanoparticles using the antisolvent precipitation method and declared the EE to be 66.1-100%. They also demonstrated that the solvent phase composed of more than 1 g of zein powder in 15 ml of ethanol 80%, was able to encapsulate 0.5-1.5 ml of the extract with an EE of above 91% [21], which conforms to that of the present study considering the use of the same carrier and encapsulation method. Sassi et al. applied freeze-drying to encapsulate date seed polyphenols in a wall of egg yolk protein and Gum Arabic and reported the EE varying from 44.06 to 99.75%. They also obtained the lowest EE when the gum was used alone as the wall material, and as the gum-to-protein ratio rose, the EE declined. The enhancing effect of egg yolk protein and other proteins like zein on the EE of date seed phenolics can be attributed to the strong hydrophobic interactions and non-covalent hydrogen bonds between proteins and polyphenols, relative to those between carbohydrates and polyphenols [44]. Jivan et al. reported an EE of 62% for the aqueous extracts of Kabkab date seed encapsulated using starch nanocrystals in a microemulsion system [19]. This value is lower than the that of the current study, due to employing a carbohydrate carrier, the difference in the TPC of the date varieties, and the type of the microencapsulation method. Liu et al. indicated an increment in the EE of resveratrol in zein-Gum Arabic complex coacervates from 55.85 to 74.20% after sonication. They ascribed this to the steric and non-covalent interactions created between resveratrol, zein, and the gum [45]. The increased EE could be attributed to the ultrasound cavitational, mechanical, and thermal impacts. The lower EE obtained in their research compared to ours, is due to the difference in the sonication time as well as using a carbohydrate carrier. Acoustic cavitation could change the macromolecular structure of zein and other polymers, thereby improving the interactive forces between the macromolecules by enhancing the exposure of the hydrophobic residues of non-covalent active sites. Through their mechanical effects, physical forces can increase the mass transfer rate at interfaces as well as the frequency of contact and collision between zein, other polymers, and the encapsulated compounds, thus giving rise to EE [45]. In conclusion, the increased EE and the desirable encapsulation of Rabbi date seed ethanolic extract in zein nanoparticles with a rise in the extract-to-zein ratio seems to be logical, owing to the creation of strong interactions between the extract polyphenols and the zein nanoparticles, in addition to the boosting effect of ultrasound on improving the EE.

Particle size and zeta potential

The size and charge of particles play key roles in the encapsulation and delivery systems of bioactive compounds. Electrostatic attraction/repulsion between nanomaterials is one of the most essential parameters influencing the colloidal stability of naoformulations and their interactions with the surrounding medium. As a result, zeta potential is a crucial surface parameter in characterizing nanoparticles, which is determined at the relative (diffuse layer) of loosely-bound ions surrounded by the nanoparticle surfaces and free ions in the solution. The ion concentration and pH of the solution impact the composition of the diffuse layer and zeta potential [46]. The mean size and zeta potential of the extract-free zein particles were respectively found to be 109.30±9.75 nm and +21.96±0.05 mV, while those of the extract-

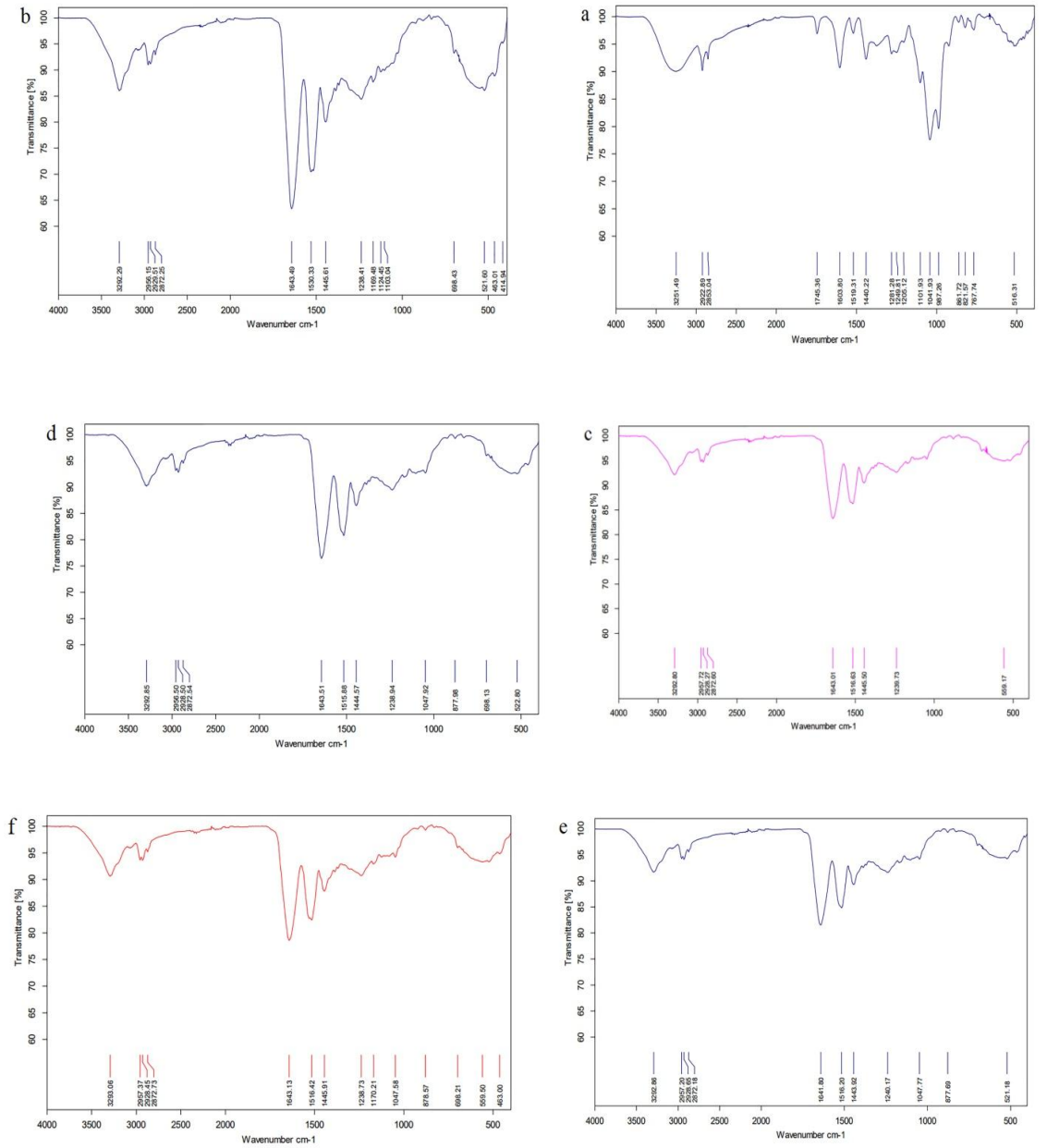

Fig. 2. ATR-FTIR spectrum of: Rabbi date seed pure ethanolic extract (a), extract-free zein nanoparticles (b), and extract-loaded zein nanoparticles at ratios of 0.1 (c), 0.2 (d), 0.3 (e), and 0.4 (f).

loaded ones were equal to 162.67±23.32 nm and +14.13±2.61 mV, respectively. As can be observed in Table 3, with an increase in the extract-to-zein ratio, the nanoparticles enlarged, whereas their zeta potential lowered. The size and zeta potential of the extract-loaded zein nanoparticles lay in the ranges of 140.25-195.25 nm and 10.58-17.23 mV, respectively. Jivan et al. encapsulated the aqueous extract of Kabkab date seed in starch nanocrystals through microemulsification and declared the extract-free and extract-loaded nanocrystal mean sizes to be 164 and 198 nm, respectively [19]. da Rosa et al. reported the zeta potential of zein particles (free of phenolic monoterpenes) in the range of 31.73-19 mV during storage for 1, 30, 60, and 90 days at 6 and 20°C. They also claimed that the zeta potential of the thymol-loaded and carvacrol-loaded zein particles respectively lay in

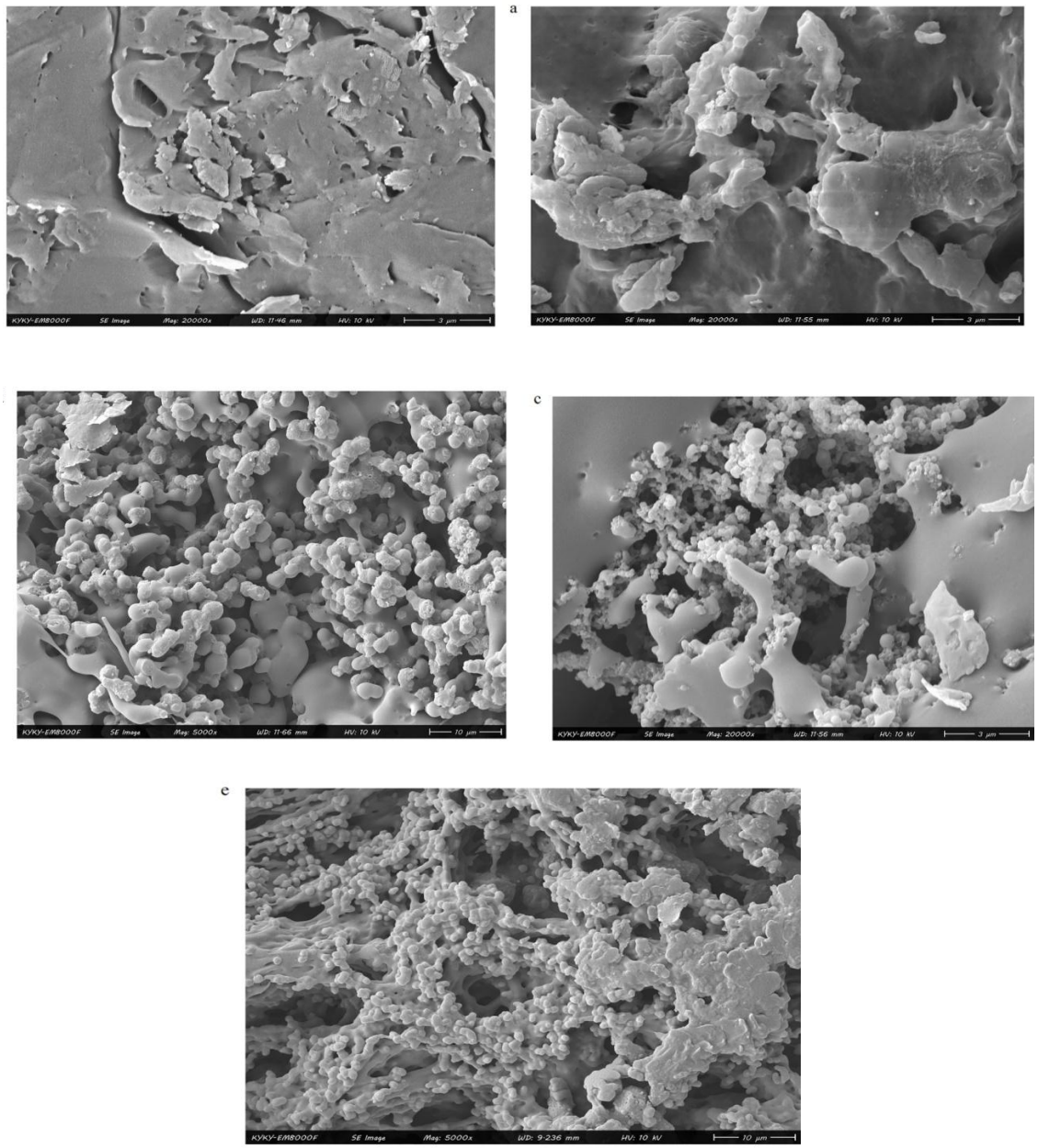

Fig. 3. FE-SEM image of: extract-free zein nanoparticles (a) and extract-loaded zein nanoparticles at ratios of 0.1 (b), 0.2 (c), 0.3 (d), and 0.4 (e).

the ranges of 14.83-9.3 and 16.1-10.7 mV under the same storage conditions [47]. In another research, Sadeghi et al. encapsulated the same extract in whey protein using microemulsification-cold gel formation and reported that the mean diameters of the extract-free and extract-loaded protein particles were respectively equal to 304 and 230 nm [20], which are higher than those obtained in the present study. Calliari et al. claimed that the mean diameter of zein particles loaded with ethanolic extracts of *Hibiscus sabdariffa* using the antisolvent precipitation method varied between 137.9±25 and 257.4±64 nm. They also observed a slight increment in the particle mean diameters with increasing the zein concentration in the solvent phase from 33 to 100 mg/ml [21]. This is in agreement with our findings in the present research, which can greatly be ascribed to the use of the same method (antisolvent precipitation method) for encapsulating the extracts. At the same time, the lower maximum particle size obtained in the present research, can be due to the dramatic effect of ultrasonic pretreatment on reducing the particle sizes, in addition to the lower concentration of the carrier (33.3 mg/ml) in our research than that (33.3-100 mg/ml) in theirs. The slight increase in the particle sizes with raising the extract-to-zein ratio in the zein-containing solvent phase is owing to the nucleation and the growth of the particles as a result of the supersaturation created by the antisolvent effect [48]. As the concentration of solutes is elevated, the nucleation rate is increased, too. An excessive increase in the concentration of the solutes leads to the aggregation of the nuclei and the creation of larger particles [21]. Zou et al. maintained that the mean size and zeta potential of zein particles (free of blueberry procyanidins) were 382.8±9.8 nm and +22.80±1.56 mV, respectively, while those of the zein nanoparticles loaded with the procyanidins were in the ranges of 391.8±9.8-447.2±24.1 nm and 14.24±0.89-20.88±1.38 mV, respectively. They also reported the indirect correlation between the zeta potential and size of the procyanidin-loaded zein nanoparticles [49]. Compared with the present research, the mean size and zeta potential of the procyanidin-free zein particles are higher; however, the variation range of the zeta potential and the indirect relationship between the zeta potential and the particle size are consistent with our research. Luque-Alcaraz et al. reported the mean hydrodynamic diameter and zeta potential of extract-free zein particles to be 159.26±5.96 nm and +22.63±1.52 mV, respectively. They also understood that those of the zein particles loaded with the aqueous extract of orange peel using the antisolvent precipitation method were respectively equal to 199.96±2.87 nm and +11.86±0.63 mV [50]. Compared with our research, the mean size and zeta potential of the extract-free zein particles as well as the mean size of the extract-loaded particles were higher, while the zeta potential of the extract-loaded particles was lower. These differences can be attributed to the differences in the concentrations of zein and the encapsulated extracts. On the other hand, the decrease in the zeta potential with an increase in the extract-to-zein ratio may be owing to the presence of the extract on the particle surfaces. These variations in the surface charge were caused by the hydroxyl groups of the extract polyphenols [50]. The higher the extract TPC, the more its hydroxyl groups (negative charge). This results in a reduction in the positive charge of zein due to the reduced zeta potential. Our results revealed that the zein nanoparticles loaded with Rabbi date seed ethanolic extract were positively charged, which can improve the cellular absorption of the encapsulated extract because of the electrostatic interaction between the particle surface and the cell membrane [51]. The enlargement of the particles with increasing the extract-to-zein ratio was brought about by the spatial arrangement of zein amino acid chains interacting with the

Table 3. Mean size and zeta potential of extract-free and extract-loaded zein particles

Sample	Extract-to-zein ratio	Particle mean size (nm)	Zeta potential (mV)
Extract-free zein	0	109.30±9.75^{a}	+21.96±0.05^{a}
Zein loaded with 0.05 g of date seed extract	0.1	140.25±12.65^{acd}	+17.23±0.04^{b}
Zein loaded with 0.1 g of date seed extract	0.2	149.15±6.43^{acd}	+15.26±0.08^{c}
Zein loaded with 0.15 g of date seed extract	0.3	166.05±7.42^{bcd}	+13.47±0.05^{d}
Zein loaded with 0.2 g of date seed extract	0.4	195.25±5.86^{b}	+10.58±0.08^{e}

All the values are the average of three replications. There is a significant difference between the values with different letters in each column ($p<0.01$).

molecules of the encapsulated extract [52].

ATR-FTIR

FTIR is a potent method for detecting functional groups in the chemical structure of bioactive compounds, which are the key factors in designating their applications. ATR-FTIR has more advantages than FTIR, as it neither requires sample preparation nor degrades the sample [53]. In this research, ATR-FTIR was utilized to determine the presence of phenolic compounds in Rabbi date seed ethanolic extract and their interaction with zein. The spectrum of the pure ethanolic extract (Fig. 2a) shows a broad band in the wavenumber range of 3700-2922 cm^{-1}, characteristic of the stretching vibration of auxochromic hydroxyl (-OH) groups of phenolic compounds [54]. The band at 3251 cm^{-1} may be associated with the hydroxyl group of the carboxylic group (COOH) on the benzene ring of phenolic acids [55]. The signal ranging from 2922 to 2853 cm^{-1} is related to the stretching vibration of the CH_2 group of the pure ethanolic extract. The band between 1101 and 987 cm^{-1} pertains to the aromatic groups (C=O) present in the phenolic compounds of the extract. These results are in line with those previously presented by González Cruz et al. for the phenolic compounds of Biloxi blueberries extracted with acidic methanol [54]. The ATR-FTIR peaks observed in the spectrum of the pure ethanolic extract at 1603, 1519, and 1440 cm^{-1} represent the C=C stretch in the aromatic rings of the functional groups of phenolic compounds. The peaks at 1281 and 1041 cm^{-1} are respectively associated with the flavonoids C-O and asymmetrical C-O of the extract polyphenols. The bands at 767 and 516 cm^{-1} are characteristic of the out-of-plane C-H bend present in the benzene rings of polyphenols. These results conform to those of the aqueous extract of Kabkab date seed reported by Bagheri et al. [55]. The ATR-FTIR spectrum of the extract-free zein nanoparticles (Fig. 2b) have signals at 1643 and 1530 cm^{-1} respectively related to the amides type I and II in the amino acid structure of zein protein. The band of amide type I pertains to the axial stretching vibration of C-O, which is located along with the amide type II band with an asymmetrical angulation of N-H bond. The peaks in the range of 2956-2872 cm^{-1} indicate the C-H bonds of the CH_2 and CH_3 radicals in the zein structure. The band ranging from 3600 to 3014 cm^{-1} belongs to the axial stretch of –OH. These findings almost conform to those previously obtained by Zheng et al. for zein nanoparticles produced through the pH-driven method [56]. The high similarity between the ATR-FTIR spectra of the extract-loaded zein nanoparticles (Fig. 2c-f) for the extract-to-zein ratios of 0.1, 0.2, 0.3, and 0.4, respectively and that of the extract-free one showed that the extract was mainly physically entrapped in the cross-linked nanoparticles, and the probable chemical interactions between the extract and the zein matrix included hydrogen, non-covalent, and van der Waals bonds in addition to hydrophobic interactions [19]. It was also realized that as the extract-to-zein ratio was elevated from 0.1 to 0.4, the band of O–H···O stretch was shifted from 3292.80 to 3293.06 cm^{-1}, while the spectrum of the extract-free zein particles had an O-H stretch band at 3292.29 cm^{-1}. The displacement of the -OH band may be due to the formation of strong hydrogen bonds between the amide bonds of zein and the hydroxyl groups of the extract. In other words, the displacement may be caused by the increased activity of hydrogen bonds between the date seed extracts and the zein nanoparticles. Zhang and Han reported that with an increase in the loading amount of rutin into zein-sodium caseinate nanoparticles (from 0.05 to 0.2 g), the peak of the stretching vibration of O–H···O was shifted from 3423.89 to 3443.78 cm^{-1}. They ascribed this to the more potent activity of hydrogen bonding between the zein-sodium caseinate nanoparticles loaded with rutin [57]. More compatibility of the zein nanoparticles loaded with Rabbi date seed extracts was achieved owing to hydrogen, van der Waals and hydrophobic interactions. The emergence of the bands at 1238 cm^{-1} for the extract-free zein nanoparticles and at 1240-1238 cm^{-1} for the extract-loaded ones can be attributed to the β-sheet structure of the zein protein [55].

Microstructure

Scanning electron microscopy (SEM) is utilized to analyze the microstructure and morphology of biological materials on micro- and nanoscales. It provides accurate and complete details of biological materials [53]. The FE-SEM images of the extract-free and extract-loaded zein nanoparticles are depicted in Fig. 3. It was observed that both the extract-free and extract-loaded nanoparticles had semi-spherical shapes attached to each other and/or overlapped in the dry state [19, 55]. The

crosslinks between or the aggregation of the extract-free and extract-loaded zein particles were obvious in the FE-SEM images, which were improved as the extract-to-zein ratio was increased from 0.1 to 0.4. This confirmed the enhanced EE and the increased possibility of the entrapment of the extract in the polymer network of zein with the rise in the extract-to-zein ratio. The elevated number of the extract-loaded zein particles can be attributed to the non-covalent, van der Waals, hydrogen, and hydrophobic interactions between the extract and the protein matrix of the capsules [20].

CONCLUSION

Application of non-thermal processes like ultrasound not only raises the EE of phenolic and antioxidant compounds of date seed, but also reduces the extraction time and retains heat-sensitive compounds, compared to conventional extraction methods. The phenolic compound profile of Rabbi date seed ethanolic extract consisted of cinnamic acid (17.51 mg GAE/g dry matter), chlorogenic acid (2.27 mg GAE/g dry matter), caffeic acid (1.93 mg GAE/g dry matter), 3,4-dihydroxybenzoic acid (1.70 mg GAE/g dry matter), vanillic acid (1.47 mg GAE/g dry matter), gallic acid (1.36 mg GAE/g dry matter), and 2,5-dihydroxybenzoic acid (1.13 mg GAE/g dry matter). The hydrophobic nature of zein biopolymer caused it to bind with the phenolics of Rabbi date seed extract through non-covalent, van der Waals, hydrogen, and hydrophobic interactions. Therefore, it can be a strong carrier for the encapsulation of date seed extracts. As the extract-to-zein ratio was increased, the EE of Rabbi date seed extracts and the size of the extract-loaded zein nanoparticles were raised, whereas particles' zeta potential lowered. The ATR-FTIR results and the FE-SEM images confirmed the excellent potential of zein for encapsulating date seed extracts, creating powerful interactions with hydrophobic core materials, formulating food products, producing nano foods, and nanomedicines.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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