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Nanoparticles Based-Plant Protein Containing *Rosmarinus officinalis* Essential Oil; Fabrication, Characterization, and Evaluation

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Abstract: The toxicity risks, instability of essential oil, and complex composition are principal obstacles to using essential plant oil for clinical applications. Solving stability-related problems, providing targeted drug delivery, and decreasing plant essential oil toxicity, encapsulation can be used successfully. Rosemary (*Rosmarinus officinalis*) is a perennial plant of the Lamiaceae family with various healing properties. However, the rosemary essential oil, as volatile oil, is fast evaporated, which limits its applications. This study's goal is to boost the prevent evaporation and bioactivity of rosemary essential oil by developing zein-NPs as a promising NDS (nano-drug-delivery system) and assessing the effect of NPs on the rosemary essential oil efficacy. Scanning electron microscopy (SEM) showed NPs sizes between 70–200 nm. With dynamic light scattering analysis (DLS), the average size of zein nanoparticle-containing rosemary essential oil (NPZLA) was obtained at ca. 154.5 nm. The entrapment efficiency (EE) on rosemary essential oil was ca. 71% inside the zein NPs. The in vitro release suggests that the polymeric barrier can control the rosemary essential oil release. Zein-NPs can be potentially used as NC (nanocarrier) for enhancing the evaporation inhibitor of ether oil of rosemary essential oil to enhance its bioavailability and performance further. It can be concluded that rosemary plant can be used as the core inside the nanoparticle by biological production method due to its medicinal properties and other properties. Based on the stated content, it is clear that in the future, by conducting more extensive research, the necessary platform can be provided for the use of this medicinal plant as much as possible in the pharmaceutical industry.

Keywords: zein; ether oil; *Rosmarinus officinalis*; SEM analysis

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1. Introduction

The presence of natural active ingredients in medicinal plants and the combination of these substances with other substances creates a biological balance and prevents the accumulation of drugs in the human body. The lack of side effects in plants medicines has attracted the attention of people and researchers [1,2]. In other words, all humans have a great desire for drugs and food that have less synthetic substances [3]. More than half of the world's population consumes particular plant essential oil as a remedy for various afflictions [1].

Rosemary (*Rosmarinus officinalis* L.) is a perennial, fragrant, shrub, and evergreen plant member of the mint family Lamiaceae. *R. officinalis* leaves are sword-shaped and needle-shaped [4]. The inflorescence is drooping and has several flowers, and the lower deformed leaves are ovate and noctose with white color appearing as clusters from the end

of the stem. Petals have two tabs, the connection of two petals forms the upper one and the lower female one is formed by the junction of three other petals [3]. Rosemary grows in the Mediterranean region, especially its coastal areas up to Asia Minor [5]. In addition, because it has permanent green leaves and has a special beauty and a pleasant smell, it is cultivated in most areas. The main growers of this plant in the world are North African countries, especially Morocco and Tunisia, and Southern European countries such as Spain, France, Italy, Yugoslavia, and also America [5].

The essential oil (EO) of *R. officinalis* contains substances such as phenolic acids such as neochlorogenic acid, caffeic acid, abietic acid, diterpenes (carnosol and rosmanol), rosemaric acid, chlorogenic acid, and a variety of flavonoids and glycosides such as diosmetin, luteolin, and salicylate. Rosemary has medicinal uses, but if the intention is to prepare essential oil from it, the flowering plant or its dry leaves should be used [6]. The biological activity of this plant is mainly related to its phenolic and volatile compounds such as carnosol and carnosic acid, Carnosol (Rosmarinic acid) found in the extract, and alpha-pinene (α-pinenebortyl acetate) and eucalyptol Camphor, Camphor Bornyl acetate found in the volatile oil of Eucalyptol [7].

Essential oil (volatile oil) is the main component of the leaves and branches of the rosemary plant [7]. The amounts of volatile oils are mentioned as 1% volume in weight, but this amount is reported in different parts of the world as between 5% and 6.5% [7]. The main ingredients include 8-1, cineol, borneol, camphor, borneol acetate, alpha-Pinene, and beta-pinene constitute (Table 1) that depending on the geographical conditions of the place where the plant is grown, the amount and percentage of each of these items is variable [5]. Chemical compounds such as phenolic acids including rosmaric acid, caffeic acid, and salicylate, as well as other natural compounds including flavonoids and phenolic acids, diterpenes, triterpenes, tannins, bitter substances, resin, saponin, protein, fat, carbohydrate, fiber, some salts and vitamins [5,7].

Rosemary has antimicrobial activity in its volatile oil due to the presence of volatile compounds 8-1, cineol, camphor, ignol, and alpha-pinene and the phenolic compound carnosic acid [8] and antioxidant activity of rosemary to phenolic compounds. such as carnosic acid, rosmarinic acid, rosmanol, methyl carnosine, luteolin, betulinic acid, myrcene, and flavonoids such as garlic (Genkwain) and cimartin are attributed to Cimartin [8] Rosmarinic acid has antioxidant medicinal properties, it is anti-inflammatory, anti-allergic and anti-bacterial and viral diseases and its use has been effective in preventing the progression of cancer and treating it [9]. Also, determining the average lethal dose of this drug in mice has shown that the amount of toxicity of this substance has been very low for higher organisms and it is quickly removed from the blood circulation system [10].

Table 1. Chemical composition of rosemary essential oil [11].

No.	Chemical Compound	(%)	No.	Chemical Compound	(%)
1	α-Thujene	0.2	11	P-Cymenene	0.1
2	α-Pinen	18.7	12	Linalool	12.33
3	Camphene	5.19	13	Camphonelal	0.3
4	Verbenone	0.8	14	Camphor	12.9
5	B-Pinen	0.34	15	Cis-Verbenole	0.2
6	Myrcene	1.07	16	Iso-Pinocamphone	0.1
7	3-Carene	0.04	17	Borneol	4.86
8	Limonene	4.6	18	Myrtenole	1.2
9	1,8-Cineole	21.3	19	Verbenone	2.21
10	Trephine	0.1	20	α-Humulene	0.1

In addition, carnosic acid, carnosol, and botulinic acid have been identified as the main anticancer agents in rosemary [12]. Camphor and alpha-pinene in this aromatic plant also have outstanding anti-cancer activities against breast and prostate cancer [12]. Rheumatic diseases, muscle pains, and migraines are used [13]. Also, rosemary increases

blood circulation in the body and can be used to prevent hair loss and treat hair loss [12]. In traditional medicine, the aerial parts of rosemary are mainly used as an anti-inflammatory, headache and Abdominal pain, anti-spasm, arthritis, effects of gout, wound healing, diuretic, analgesic, anti-rheumatism, and anti-depressant are used [14]. Digestive disorders (as an anti-flatulent stimulant for the stomach and intestines), respiratory diseases (as an expectorant and disinfectant), heart pain (as a restorative and stimulant of heart arteries), anti-inflammatory and dizziness and memory enhancement [15].

Essential oil (EO) of plants is chemical compound with biological activities derived usually from the different parts of medicinal plants. Antibiotic, anticancer, antioxidant, antiparasitic, hypoglycemic, antihypertensive, and antifungal properties are the most important and most prominent biological properties of the natural essential oil of medicine plants [16–21]. However, studies show that the use of plant EO faces several challenges [9].

The EOs are also called volatile oils or ether oils because they evaporate faster when air exposure than other oils. In addition, the targeted storage of plant EOs is another challenge in overcoming their potential therapeutic use. In the other hand, most of the plant essential oils are biologically unstable and insoluble in water and bind incompletely to the target sites. In addition, the targeted delivery of plant EOs is another challenge in overcoming their potential therapeutic use [21]. Based on this, it is necessary to discover new techniques to increase antimicrobial activity before using them for treatment.

Currently, the common point of research is the study of the EO's composition, while more attention should be focused on solutions that enable the effective, safe, and direct use of these natural products. One of the most important methods is the use of nanoscience to formulate essential oils to increase the quality of the effect and prolong the biological activities. Encapsulation is a process in which solid, liquid and gas components are included in small capsules and their contents can be released at a controlled rate. Drug-carrying nanoparticles are made from materials such as plant polymers, synthetic polymers, and various materials. Polymeric NPs are one of the newest ways to apply natural essential oils [22]. Biopolymers are one of the types of NMs that are widely used in the pharmaceutical and food industries [22,23]. Lately, NPs based on biopolymers have been widely used in drug delivery systems. In drug delivery systems, the rate of drug dissolution increases with increasing drug levels and associated carriers. Plant protein-based carriers have several advantages over animal lipids, proteins, or synthetic polymers due to their biodegradability, high drug-binding capacity, and availability [24–26]. The methods of producing nanoparticles depend on the material inside the core, which includes organic, aqueous or inorganic phase, and also on the shape of the product. Zein is the primary storage protein of *Zea mays*, which makes up about 45–50% of *Zea mays* protein [27].

Due to their unique properties (e.g., biodegradability, biocompatibility, non-toxicity, and economic justification), this type of protein has a special place in the production of biopolymers, and biodegradable films. Much research is being done to optimize the fabrication, purification and processing of zein and, most importantly, its extraction. Zein plays a functional role in some processes, due to several unique properties including biodegradability and biocompatibility, it can act as strong barrier against oxygen and a heat resistant biopolymer [28].

Therefore, much effort is being made to build it commercially at a low cost [22,29–33]. Zein can be converted into various structures and shapes as microsphere, film, micelles, NPs, fibers, and gels [22,27,33]. Therefore, zein can be an alternative in capsule carriers for food and drug. To previous studies, NPs based on biopolymers (e.g., zein) have more hydrophilic groups, smaller size, and solubility [22,27,34–37]. Encapsulating these compounds (EO) reduces their reaction with environmental factors (water, oxygen, light) and reduces evaporation, increases transmission power, covers taste and causes uniform distribution of the final product [38]. There are many reports that the bioavailability of the compounds encapsulated in nanoparticle (nanomeric dimensions) is more than normal size [39]. Several studies have been carried out on the encapsulation of plant essential oils inside zein NPs [22,28,40,41]. The use of zein for the encapsulation of thymol, citric acid

and nisin has been carried out by Aytac et al. [41]. In another study, thyme essential oil was incorporated into zein nanoparticles [41]. Also, Hosseini et al. have used rosemary essential oil using the zein electrolysis method [28].

Here, the aim was loading the rosemary essential oil into zein nanoparticles using a modified method. Essential oil loaded in zein nanoparticles were evaluated in terms of morphology, physical condition, thermal properties, encapsulation efficiency and essential oil release from zein.

2. Materials and Methods

2.1. Material

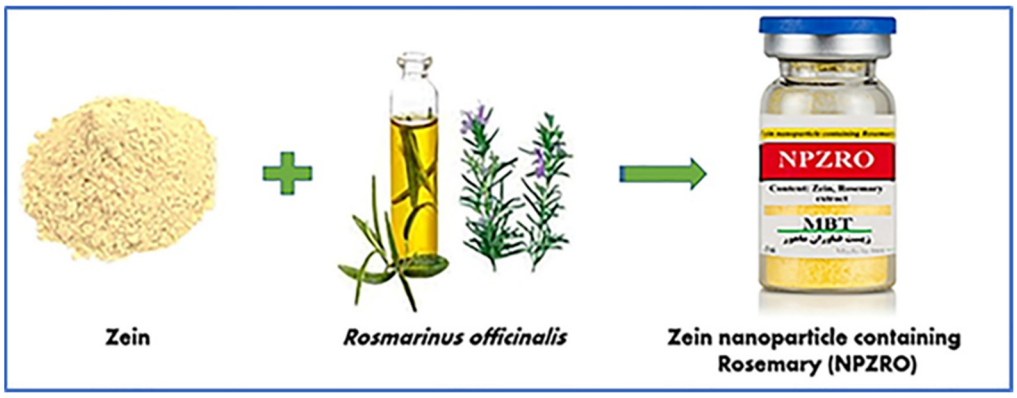
Acetone, ethanol, and Tween 80 were prepared from Merck chemical company. From Acros Organic purified zein was purchased. The dialysis bags were purchased by SERVAPOR.

2.2. Planting and Extraction of Essential Oil of R. officinalis

Uniformly rooted cuttings of rosemary that were obtained from identical mother plants were planted in 2021 at the Zanjan university research field (36°40.976 N, 48°24.263 E, and 1596 m). During the growing season, all agricultural operations were applied to the plants similarly. The plant was harvested were harvested at the full bloom stage and dried in the room shade condition at 25 °C, then pulverized in the form of a homogeneous mixture, and its EO was extracted by the hydro-distillation method via Clevenger apparatus for 3 h. After that, 50 g of powder flower and leaf samples were mixed with 600 mL of distilled water. The prepared sample was poured into a balloon and extracted by Clevenger for 3 h by water distillation. The EO was dehydrated with Na_2SO_4 and then stored in a dark refrigerator at 4 °C.

2.3. Fabrication of Zein-NPs Containing R. officinalis Extract

A homogeneous zein solution was prepared using 0.66 g of zein and 25 mL of acetone and stirred for 24 h at 25 °C. This solution was added 0.033 g of rosemary essential oil (RE) and stirred for several hours (solution I). Also, Tween 80 (0.75 g) dissolved in 25 mL PBS at a concentration of 0.1 M and pH of 7 and stirred for 30 min at 25 °C (solution II). Then the former solution was added dropwise to the later solution under stirring. Finally, by removing acetone under vacuum rotary evaporation, the zein nanoparticles containing RE (NPZRO) was obtained. The freeze-drier was used to gain solid powder (Scheme 1).



Scheme 1. NPZRO formulation in the schematic diagram.

2.4. NPZRO's Physicochemical Characterization

DLS analysis or dynamic light scattering is a method to determine the size of particles and their distribution in liquids. So, the size distribution profile of plant protein containing essential oil (NPZRO) was obtained by DLS (DMP, Beckman Coulter Instruments, Brea, CA, USA) in a solution with a 90° scattering angle at 25 °C.

TGA-DTA thermal analysis or (Thermal Gravimetric Analysis—Differential Thermal Analysis) is one of the widely used methods in material analysis. TGA-DTA thermal analysis measures the changes in the sample due to heat and provides the user with a graph. Takes. By interpreting TGA-DTA thermal analysis, one can find out thermal stability, moisture content, state of crystallization, transformation and many other things in a chemical substance. In this method, determination of melting temperature, vitrification temperature, determination of molecular weight, percentage of crystallinity, thermal degradation and impurities, determination of sublimation and thermal decomposition can be measured. TGA-DTA thermal analysis is currently used to investigate organic and inorganic substances, nanomaterials and drugs. The TGA/SDTA 851 (Switzerland) was used to study the thermal stability of samples, which measured at a heating rate of 10 °C min^{-1} (in a range of ambient temperature to 600 °C) under nitrogen (N2) flow of 20 mL min^{-1}. Scanning electron microscope analysis or SEM analysis is one of the most widely used analysis methods in materials engineering and metallurgy. This microscopic method uses electrons to display surfaces at high magnification. A scanning electron microscope (SEM) with a model Seron Tech (operating at 15 kV) was used to study the surface morphology and particle size. Fourier-transform infrared (FT-IR) analysis with Nicolet 520P spectrometer was applied to characterize functional groups, in which the samples were prepared by the KBr method.

2.5. NPZRO's Entrapment Efficacy (EE%) and Release

The quantity of RE loaded in zein NPs was evaluated by a UV-Vis spectrophotometer (Agilent tech, C60, Santa Clara, CA, USA). For ethanoic RE solutions at various concentrations, a calibration curve was obtained. The obtained NPZRO was then dissolved in 2 mL of ethanol, and after centrifugation, UV-Vis absorption of the supernatant was achieved at 230 nm. Finally, the Equation (1) was used to determine the encapsulation efficiency (EE%):

$$\mathrm{EE}(\%) = \frac{\text{Total content of RO} - \text{free RO}}{\text{Total content of RO}} \times 100 \quad (1)$$

The RE release profile of NPZRO was investigated using the dialysis method. 1 mg of the NPZRO was placed in DB (dialysis bags) then in 30 mL buffer phosphate solution (0.1 M pH = 7) immersed and comprising 3% (v/v) ethanol to ensure sink circumstances. For evaluation, 1 mL of the release medium was withdrawn at specific time intervals and up to 168 h and replaced quickly by a fresh medium (1 mL). The control sample (neat RE) was performed under similar circumstances. By using the obtained standard calibration curve at PBS 0.1 M pH = 7, the release drug concentration was determined. The results were stated as the cumulative release of 3 replications.

3. Results

Dynamic light scattering analysis (DLS) is a technique to determine the particle size distribution of NPs. Figure 1 indicates the average hydrodynamic size of NPZRO, which is about 154.5 nm. This value corresponds to the values reported in the literature [22,28,42–44].

Moreover, scanning electron microscopy (SEM) was used to study the surface topography, and particle size of NPZRO. The SEM pictures of NPZRO are shown in Figure 2. According to these images, the shape of NPZRO particles is spherical and uniform. The results show the formation of capsules with both nano and micro dimensions. In addition, the images show the narrow distribution of nanoparticles, which is in the range of 70 to 200 nm. The results of this study are completely consistent with the results of other researchers [22,28,42–44].

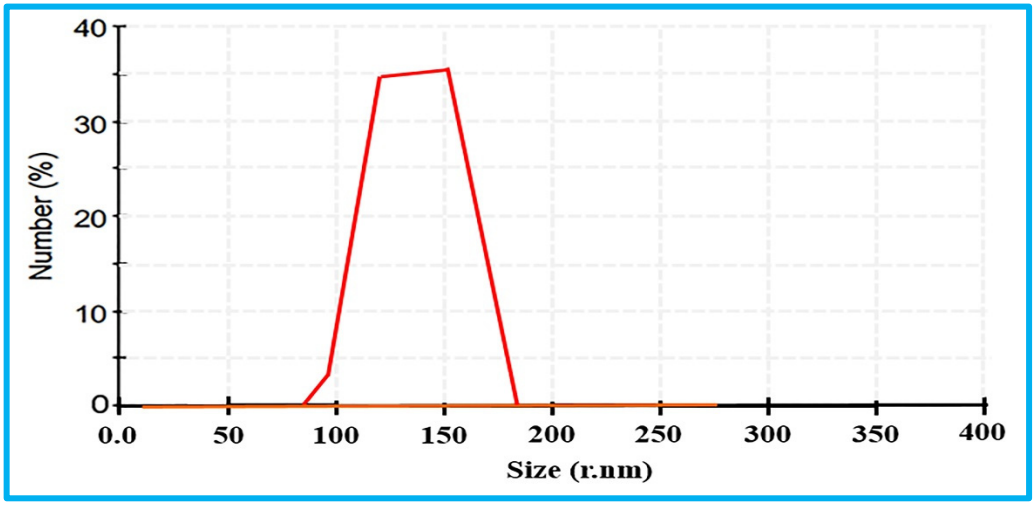


Figure 1. Average hydrodynamic size of NPZRO.

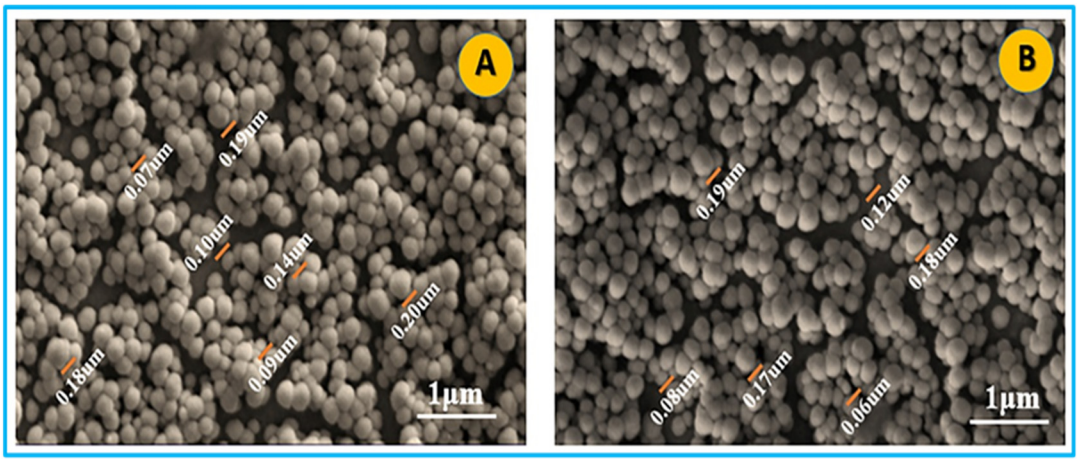


Figure 2. SEM images for NPZLA (**A**,**B**).

The stability of the prepared nanoparticles was investigated by TGA and DSC analyses, which are based on the changes in sample weight by heating at a constant rate. The obtained results of TGA and DSC analysis are shown in Figure 3. As can be seen, thermograms curves of pure zein nanoparticles and NPZRO are very similar and indicate two-weight losses at the temperature range of 30 to 600 °C. The physically adsorbed water and somewhat bound water are removed at 40 to 300 °C, approximately 20% of the sample weight. The severe weight loss of the second stage at 300 to 550 °C, which is about 50% of the total weight of the sample, is related to the destruction of the protein structure of the zein and rosemary. In addition, it is noteworthy that NPZRO shows slightly higher thermal stability than pure zein. It is noteworthy that nanoparticles show slightly higher thermal stability than pure zein. Therefore, the thermal degradation of the second stage starts at about 150 °C.

The interaction between zein nanoparticles and rosemary as well as functional groups was investigated by FT-IR spectroscopy. The observed broad frequencies of all three spectra in the range of 3100–3500 cm^{-1} are attributed to the stretching vibrations of the O-H of alcohols, carboxylic acids, and water (Figure 4). The symmetric, and asymmetric stretching absorption bands of -CH_3 groups of net rosemary can be seen in 2850 and 2925 cm^{-1}, respectively. As can be seen, after interaction between zein nanoparticles and rosemary, these peaks are removed. Due to the presence of C=O groups in all three net rosemary, net zein and NPZRO, the sharp stretching vibrations are observed in 1621–1660 cm^{-1}. However, this frequency in NPZRO has shifted to lower energies. The frequencies observed

in 1457 and 1376 cm^{-1} in rosemary spectrum are attributed to N-H bending vibration and the stretching vibration of C-O (amide), respectively. Moreover, the FT-IR spectra of net zein and NPZRO have similar patterns, indicating no notable changes in the critical bands or functional group's status. The FT-IR spectra can also be used to study particle configuration; with increasing particle size, the peak width decreases, but the intensity increases. As can be seen, the peak intensities of the NPZRO have increased compared with their corresponding absorption bands in net zein.

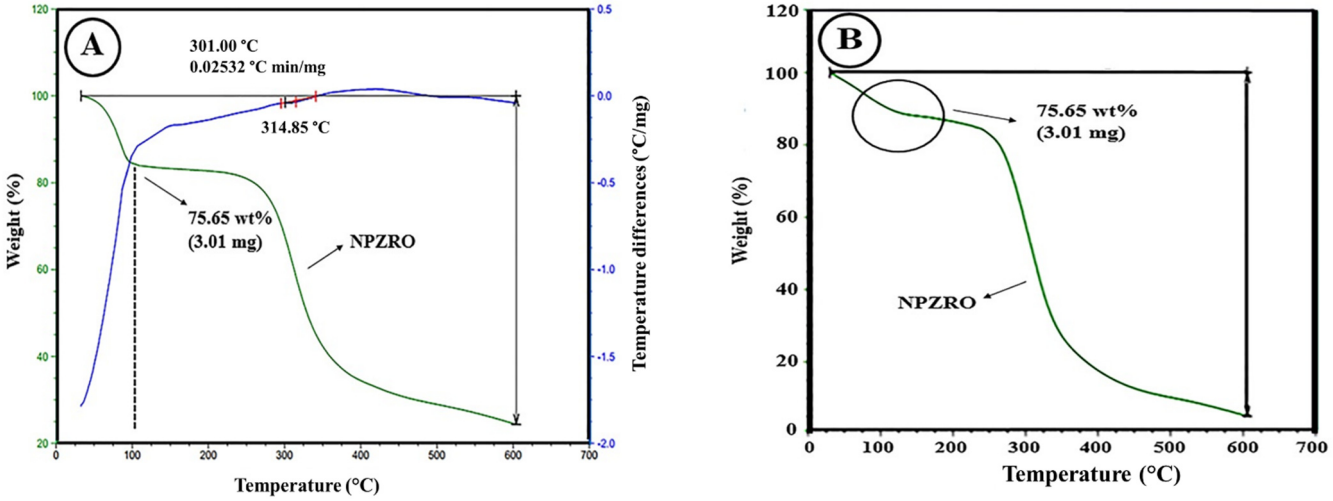


Figure 3. The curves of TGA (**A**) and (**B**) DSC of pure zein and NPZRO at a heating rate of 10 °C min^{-1} under an N2 atmosphere.

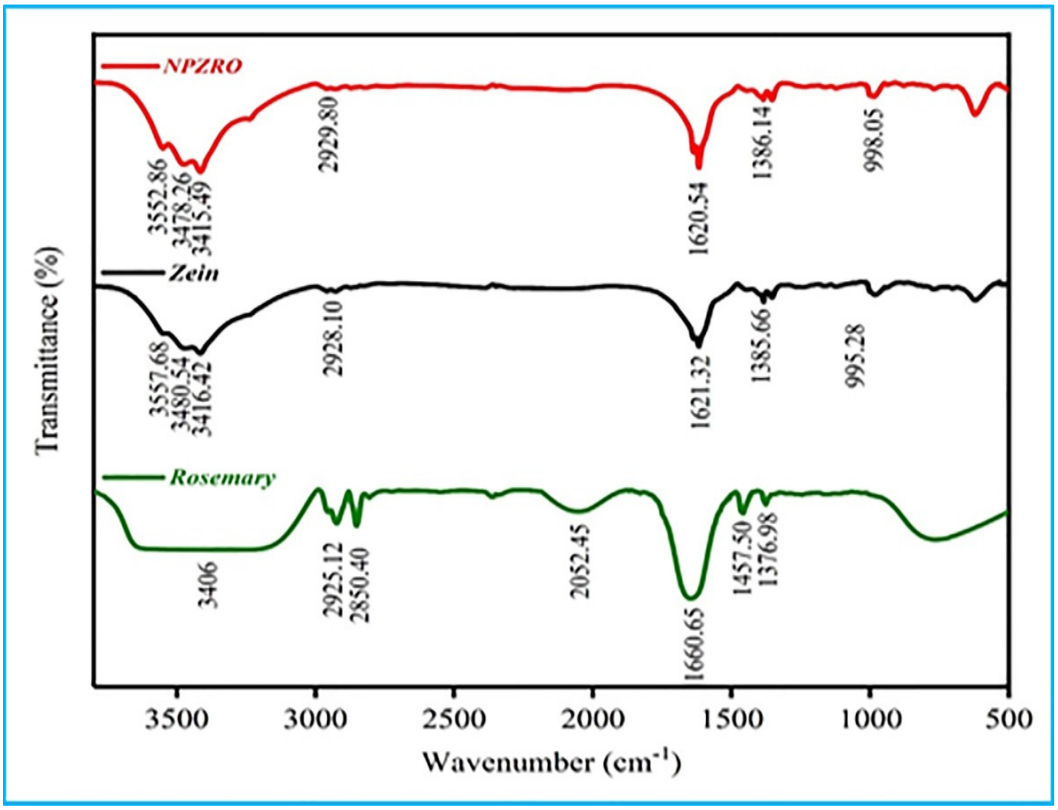


Figure 4. FT-IR spectrum of pure zein, pure RO, and NPZRO.

Ultraviolet–visible spectroscopy (UV-Vis) was applied to determine the amount RE-loaded in zein nanoparticles. To evaluate the amount of essential oil release over time, as a sample, capsules prepared with plant polymer were subjected to release. The release of loaded samples was examined by the dialysis method, in which the samples were placed in dialysis bags and immersed in a PBS solution with a pH of 7 and ambient temperature. To compare the sample release with the control sample, net RE release was also measured under the same experimental conditions. The release results shown in Figure 5 show that the capsules prepared from Zein have the lowest amount of release and the highest retention of essential oil. The obtained results showed that RE was successfully loaded on zein nanoparticles, called NPZRO, which is about 71%. Under in vitro conditions, the RE release of NPZRO is shown in Figure 5. As can be seen, net RE is completely released after 8 h, while in the NPZRO, after 10 h, about 45% of the RE is released. In this case, a two-phase solution is formed, and almost no further release occurs, and it has a fixed pattern.

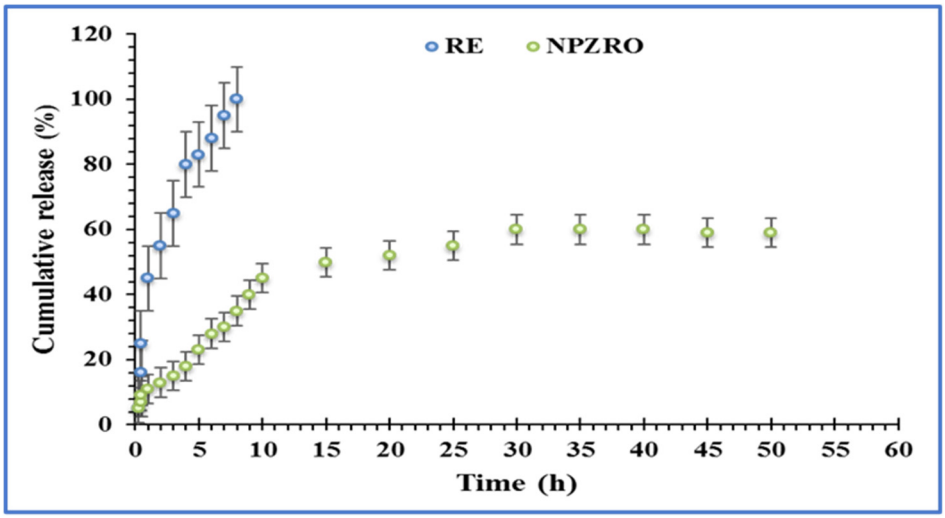


Figure 5. The RE-free and NPZRO's release profiles.

4. Discussion

Oil compounds (essential oils) are sensitive and have useful antimicrobial and antioxidant properties, and in order to increase the stability of these compounds and control their release, the encapsulation process can be used. There are different methods for encapsulating plant essential oils, which differ from each other in terms of process conditions and encapsulation efficiency. In this research, zein, which is a natural polymer with wide properties and applications in the food and pharmaceutical industries, was used.

In summary, the average size of NPZRO was 154.5 nm that measured by DLS analysis. The NPs size were between 70–200 nm based on the SEM results, which was consistent with the results of former studies [21,37,45]. Also, the results of FT-IR appearance of the main distinctive peaks of zein and RE in the spectrum of all the NPZRO indicate a higher ability of zein NPs to encapsulate RE molecules. The RE's drug EE within the nanoparticles was ca. 71%. Many studies reported that the polymeric barrier is able to control the RO release in vitro release suggests [21,46]. Figure 4 shows that the developed NPs enable a more controlled and sustained in vitro release of the loaded EO.

Recently, the high potential of plants EO is well known as a source of various of chemical compounds with important biological activities. However, many of these plant EO have shown poor solubility in aqueous systems, short shelf life, high chemical instability and chemical complexity that affect the biological activity of their various compounds.

These challenges limit their effective application. Controlled release systems such as polymer NPs provide a solution to these problems. Choosing the right encapsulation

method is the key to obtaining a formula (NPs) with suitable properties. Choosing the right encapsulation method is key to obtaining a formula (NPs) with suitable properties different applications. Based on the results of our study and previous studies, the use of biopolymer nanoparticles as carrier of plant extract is most suitable and safe methods [45,47].

The NPs-based biopolymer as carriers of active agents are protection in the application environment, enhancing of cell of tissues internalization, protection against enzymatic degradation, bioavailability improvement, design of sustained-release systems of bioactive compounds, enhancement of solubility, affected area targeting, reduction of toxicity, and masking of unpleasant odor and taste [45,48]. Literature illustrated that mostly dermal and oral routes are employed for the delivery of formulation based of encapsulated plant extracts.

The result suggests that the bioactivity of RE would be influenced by the chemical composition change [45]. The use of biopolymer plant-based can be safe and have minimal change in RE bioactivity. Although studies associated with the useful effects of the lavender oil encapsulated in nanoparticles are still in progress [45], However, due to being inexpensive, safe, and affordable, zein biopolymer can be a promising agent with potential application in essential oil encapsulating systems.

5. Conclusions

By proving the harmful effects of chemical drugs on human health, the attention of researchers as well as people have been drawn towards the use of natural drugs, especially those of herbal origin. Because the general belief among people is that antimicrobial chemicals may threaten their health. For this reason, it is important to use natural materials instead of chemicals. Undoubtedly, the use of plant extracts and essential oils can be a very suitable alternative. In terms of climate and geographical location, Iran is considered one of the best regions in the world for the growth of medicinal plants, and in the past, it has been a source of production and consumption of medicinal plants. In this research, the rosemary medicinal plant, which is one of the native plants available in Iran, was introduced. The rosemary essential oil has been encapsulated by zein and subjected to various physio-chemical characteristics studies. Based on cumulative drug-release, the developed NPs enable a more controlled and sustained in vitro release of the loaded essential oil. Ultimately, encapsulation of RE's essential oil caused increased bioactivity of rosemary extract and slower release.

These results showed that NPs of zein can be potentially used as NCs for enhancing the efficiency of RE, which can further augment its performance and bioavailability. Because of all the favorable physical and biological characteristics presented by the NPs, it is clear that their usage is one of the newest for the delivery of actives with various chemical properties. In the future, nanotech employment in plant essential oil delivery for safe usage in different area among others could be an interesting field for research.

According to the above studies, it can be concluded that rosemary plant can be used as the core inside the nanoparticle by biological production method due to its medicinal properties and other properties. Based on the stated content, it is clear that in the future, by conducting more extensive research, the necessary platform can be provided for the use of this medicinal plant as much as possible in the pharmaceutical industry.

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Abbreviations

NPs	Nanoparticles
EOs	Essential oils
RE	Rosemary essential oil
NCs	Nano-carriers
NPZRO	Nanoparticles of zein containing rosemary essential oil

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