RESEARCH PAPER

Nanoencapsulation of Thyme Extract with Antifungal and Antioxidant Effects

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ABSTRACT

Antifungal bioactive compounds in Thyme extract are prone to degradation by oxidation, heating or light. For preventing of this problem, encapsulation process is a valuable method to protect these natural products. The aim of this study was to encapsulate of Thyme extract via chitosan as a biodegradable polymer to keep antifungal effect and other chemical bioactive compounds. In this study, extraction of Thyme was carried out by probe ultrasonic- assisted extraction (UAE) system with total solid content (TSC) 64% and then synthesis of chitosan nanoparticles containing Thyme extract was performed by ionotropic gelation technique. Morphology of chitosan nanoparticles was identified through field emission scanning electron microscopy (FE-SEM). The related images showed a spherical shape of the nanoparticles. Particles size of the encapsulated polymers was 75.07 nm with single modal particle size distribution which was measured by dynamic light scattering (DLS) method. Infrared spectroscopy (IR) of both encapsulated with and without extract indicated that the synthesized nanoparticles could involve the bioactive compounds to their vacancies, successfully. This ecofriendly procedure could be represented as a suitable alternative for biological control of the plant pathogens instead of chemical fungicides.

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INTRODUCTION

The last decade, indiscriminate use of synthetic herbicides, pesticide and fungicides have caused environmental pollutions and development of resistance in many species of weeds, plant pests and plant pathogens [1]. Also by affecting non-target organisms, they cause disruption of biological control systems. Herbal medicines consist of some bioactive compounds which are able to prevent and treat many diseases. Their applications scope has ranged from traditional and popular drugs in each country [2]. The use of herbs as medicine is the oldest method for human health care. Easy-to-consume and availability for human were caused that herbal plants have been used up to now. They have lower price in comparison to chemical drugs. Since medicinal herbs are taken from nature, they exclude the harms of using chemical drugs [3]. These plants contain some poly phenol compounds that can be affected as antifungal effectiveness [4]. Thus, herbal extracts or essential oils can be used as alternatives for control of plant pathogens. Thyme is one of the medicinal plants which is found in many parts of the world, especially Iran. Because of the highest concentration of the bioactive compounds like phenols and flavonoids, Thyme has positive effect on health of the consumers. It

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is used as herbal tea or is added in the food. There are some encapsulation processes. The choice of encapsulation method depends on the type and physical properties of the core and shell material. The chosen encapsulation technique should give high encapsulation efficiency and loading capacity. Capsules should not exhibit aggregation or adherence together. They should have a narrow particle size distribution and the process should be suitable for industrial scale production. Depending on the initial physical state of the core phase, two different fabrication routes can be distinguished: (i) coating solid particles by shell-forming material in a fluidized bed and (ii) dispersing a capsule-forming material in the form of droplets in another immiscible liquid (emulsion) and then followed by droplet solidification [5]. For example, many components of essential oils (EOs) are volatile or some of the bioactive compounds in the extracts are sensitive so they cannot be active for several days without protection. Thus, they should be kept for usage more times [6]. To resolve these problems encapsulation procedure in different surfactant or polymer structures is proposed to increase their durability [7]. Researches showed that emulsions of EOs commonly fail to show a proper duration of action while polymeric carriers like encapsulation are more stable [8]. The genus Thymus consists of various species of herbaceous perennials. Thymus daenensis Celak (Avishane-Denaei in Persian) is one of the known thymus species in Iran which grows wildly in the central and northwest of Iran [9]. The essential oil in thymus showed inhibitory properties on Alternaria solani, Fusarium solani, Rhizoctonia solani and also has a high antifungal activities. Also, the antifungal activity of the essential oil and its major compounds was reported against standard and clinically isolated strains of yeasts and filamentous fungi [10]. Chitosan was recognized as a safe material and approved as a food additive by the food and drug administration [11]. Several advantages including nontoxicity, mucoadhesivity, biodegradability, antibacterial activity and antioxidant effects of chitosan were caused the researchers use this material as a ecofriendly polymer in their researches [12]. These advantages made chitosan-based carrier not only suitable for use in medicine molecules but also in food preparation [13]. Encapsulating of Tarragon essential oil in chitosan nanocapsules with larvicides effect was reported [8]. Chitosan nanoparticles loaded with cinnamon, Thyme, and Schinus molle essential oils were effective against some foodborne pathogens [14]. Enhancement of the thermal stability and antioxidant activity for chitosan nano capsules containing Thyme essential oil was reported [15]. Due to the mild processing condition and the aqueous environment, the ionotropic gelation technique is the most widely used among the methods to prepare chitosan nanoparticles. In this technique, a poly anion such as sodium tripolyphosphate is used to interact by electrostatic interaction with the positively charged primary amino groups on chitosan to provide spherical chitosan nanoparticles. In this study encapsulation of Thyme extract by chitosan as a biodegradable polymer was performed. At first Thyme extract was prepared. Ultrasound-assisted extraction is a green and economically viable alternative to conventional techniques for food and natural products [16]. So, this approach was used as a suitable method to prepare Thyme extracts in this research. Then capability of the prepared chitosan nanoparticles by the ionotropic gelation technique for encapsulation of Thymus extracts was investigated. All of the processes in this research are eco-friendly and compatible to environment.

MATERIALS AND METHODS

Thyme leaves (Thymus vulgaris L.) were collected from Firoozkooh Mountains and were identified based on the flora of Iranica and authenticated by taxonomist. Fungal plant pathogen (Macrophomina phaseolina) was obtained from Mazandaran Agricultural Research Center. Chitosan with low molecular weight (80% degree of deacetylation), acetic acid, sodium hydroxide and sodium tripolyphosphate (STPP) were prepared from Sigma-Aldrich products. Distillated water was used for the preparation of the solutions during all of the procedures. Whatman papers No.40 was used in Buchner funnel for filtering of the extract. UV/Visible spectrometer Jenway model 6305 was used to determine amount of the total phenolic content in 760 nm wavelength. Fourier transform infrared spectra (FTIR spectra) of the nanoparticles were recorded on a Perkin Elmer 400 FTIR Spectrometer (Perkin Elmer, Beaconsfield,UK). All spectra were recorded in absorption mode from 600–4000 cm^{-1} with a resolution 4 cm^{-1}. Surface morphological information of the encapsulated particles was obtained by scanning electron microscope

(SEM) model VEGA\\TESCAN-XMU (Canada). Ultrasonic- assisted extraction (UAE) system 400 w model VGT-1730QTD (South Korea) with probe diameter 7 mm was used for Thyme extraction. Thyme extract was dried by vacuum freeze-dryer model Alpha11-2 LD Plus (South Korea). Pre-concentration of the extract was carried out by rotary evaporator model IKA RV10 (Germany). Size of the nanoencapsulated particles was measured by dynamic light scattering (DLS) model VASCO (Cordouan Tech- France).

Extraction Process

200 mL of hydro alcoholic mixture (70% EtOH/H_2O, v/v) was used for extraction of 10 g dried leaves powder of Thyme with ratio 1:20 g/mL (solid to solvent). Extraction process was carried out during 20 minutes by probe ultrasonic- assisted extraction system [17]. The highest concentration was collected at room temperature with 5.51% of Thyme concentration yield. Thyme extract was pre-concentrated by rotary evaporator at 40°C and then separated via vacuum freeze-dryer device at -50 °C.

Antifungal effect of the Thyme extract

Investigation of antifungal effect of Thyme extract against Macrophomina phaseolina (causal agent of soybean charcoal rot) was conducted [18].

Encapsulation Procedure

For encapsulation of Thyme extract, 50 mg of chitosan powder was put in a round flat bottom flask and was dissolved in a 50 mL mixture of acetic acid solution (1% AcOH/H_2O, v/v). The mixture was stirred 2 hrs. on speed of 600 rpm. 50 mL of the extract solution (1mg/mL concentration) was added once to the mixture. After 20 minutes stirring, 33 mL of STPP solution which have been already stirred separately during 20 minutes was added by rate of 1mL/min. After 40 minutes stirring, the mixture was put 24 hrs. in refrigerator. After that the mixture was centrifuged and encapsulated Thyme extract was separated by vacuum freeze-drying system at -50 °C [19].

Total solid content (TSC) efficiency

1 mL of Thyme extracts was poured into a watch glass and placed in an oven at 40 °C. After 48 hours, solid contents of the glass were measured [20]. Total solid content percentage was calculated according to equation (1):

$$\% TSC = (m_{extract} \times V_t / m_{raw\ material}) \times 100 \qquad (1)$$

Where $m_{extract}$ introduce contents of the glass, $m_{raw\ material}$ is initial mass powder of Thyme (10g) and V_t denotes total volume of the hydro alcoholic solvent (200 mL).

Total phenolic content

Main reason of many health issues like cancer and heart diseases are caused by free radicals. Researches showed that plant extracts containing phenolic compounds exhibited high antioxidants properties because of their phenolic compounds. These plants are very rich in phenolic compounds, such as flavonoids, phenolic acids and phenolic diterpenes. These chemical compounds protect the body against the produced harmful compounds during metabolism of fats and can protect us against major diseases such as coronary heart disease and cancer in human. These compounds

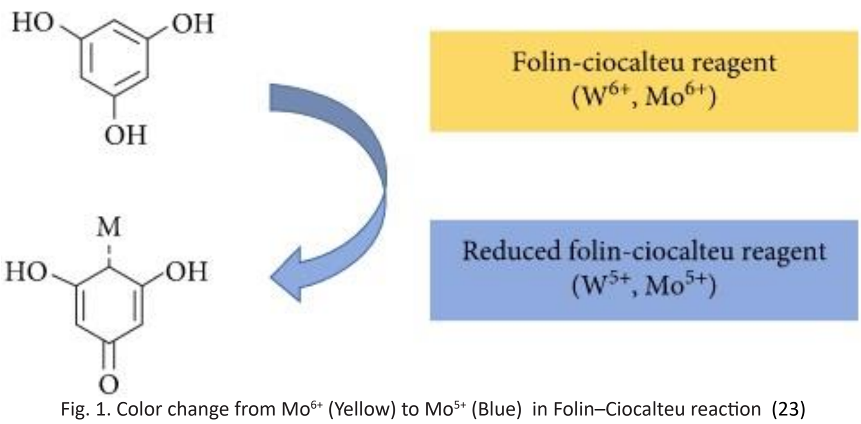

Fig. 1. Color change from Mo^{6+} (Yellow) to Mo^{5+} (Blue) in Folin–Ciocalteu reaction (23)

are produced as secondary metabolites which cause high antioxidant activity for this herbal plant. So, measuring of total phenolic contents is important [21]. To check percentage of phenolic contents, colorimetric method was used [22]. Total phenolic content was determined using Folin–Ciocalteu method according to oxidation-reduction procedure. In this process, tungsten (W) and molybdenum (Mo) react with polyphenol groups in an oxidation-reduction reaction in presence of sodium carbonate (Fig. 1).

In this method, 0.5 mL of Thyme extract and 2.5 mL of Folin–Ciocalteu reagent were poured in a test tube and then 1 mL of sodium carbonate 75 g/L was added to the solution after 5 minutes. After mixing at room temperature and incubation for 1.5 hour, the absorption of mixture was measured at the wave length of 765 nm. Gallic acid was used as a standard for calibration curve at concentrations of 0 to 0.25 g/L. So, at first calibration curve according to color change mechanism was drawn (Fig. 2).

Result of total phenolic content was expressed in mg of Gallic acid per g of dried extract (mg GA/g extract) [24].

Characterization of the encapsulated polymers

Morphological Study

The morphology of chitosan nanoparticles was evaluated through field emission scanning electron microscopy (FE-SEM). The average size value of the nanoparticles was measured by DLS measurements.

Infrared Spectroscopy

Encapsulated polymers in absence of the Thyme extract were synthesized to compare of its IR-Spectrum with encapsulated polymer containing of Thyme extract. IR spectrum of both encapsulated with extract and without extract obviously indicated that the synthesized nanoparticles were contained some other functional groups which had absorbance in different wavenumbers.

RESULTS AND DISCUSSION

Total solid content efficiency

According to three times measuring, the mean extraction efficiency of solids equal to 64 % was reported (Table 1).

Antifungal effect of Thyme extract

Inhibition of mycelial growth of M. phaseolina was observed by Thyme extract in in-vitro

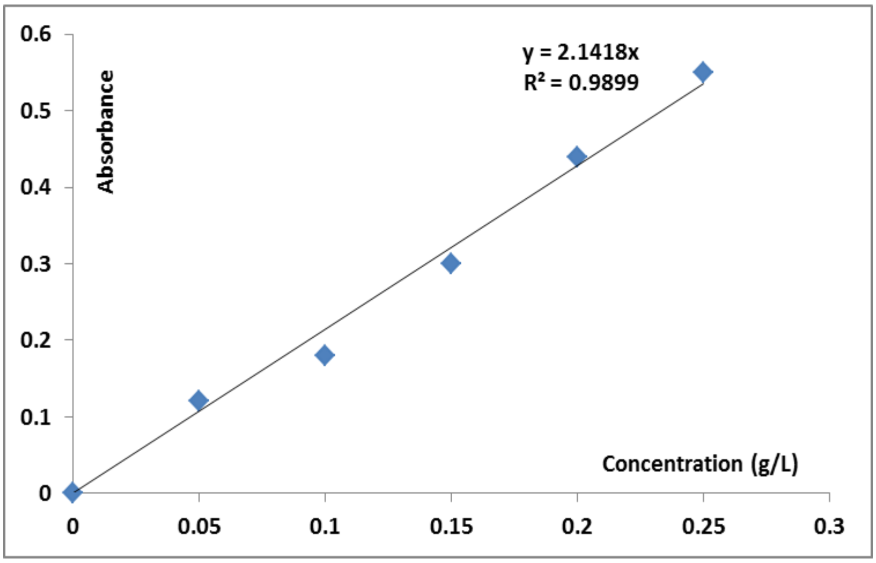

Fig. 2. Standard Curve for GA (Absorbance Vs. Concentration)

Table 1. Measuring of total solid content efficiency in three experiments

NO.	Solid in 1 mL of the extract	TSC (%)	Mean (%)
1	0.028 g	56	
2	0.032 g	64	64
3	0.036 g	72	

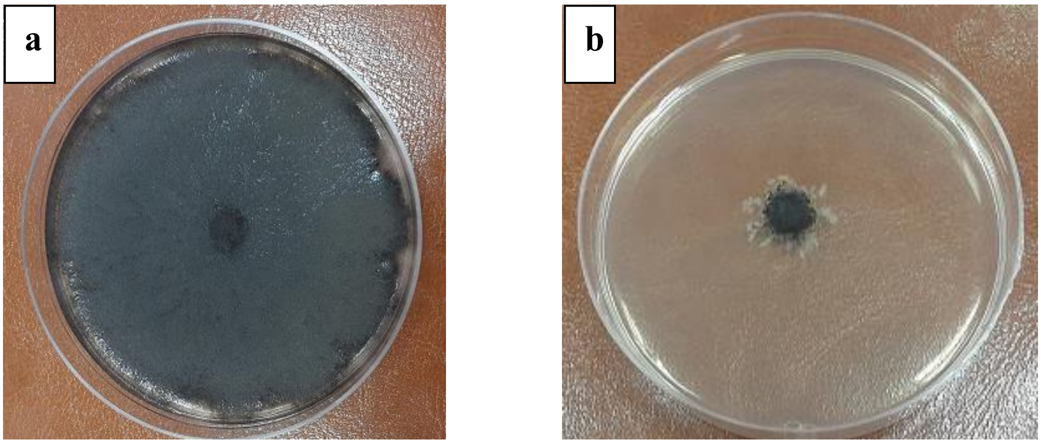

Fig. 3. Antifungal effect of Thyme extract against M. phaseolina (b) in comparison with the control (a)

conditions (Fig. 3).

Total phenolic content

The total phenolic content was determined as mg of Gallic acid equivalent using an equation (2) which was obtained from the standard Gallic acid calibration graph.

$$Y = 2.1418\,X \qquad R^2 = 0.9899 \qquad (2)$$

High phenol content in Thyme extract equal to 18.6 mg gallic acid equivalent (GAE) /g dry weight of Thyme we observed. Total phenolic content equal to 8.1 mg GAE /g dry weight of Thyme was reported in a research that Thyme extraction was carried out in methanol solution [25].

Morphology of the encapsulated particles

The surface morphology of the nanoparticles containing Thyme extract was determined using FE-SEM (Fig. 4). As it was shown in this figure, the obtained nanoparticles were almost spherical

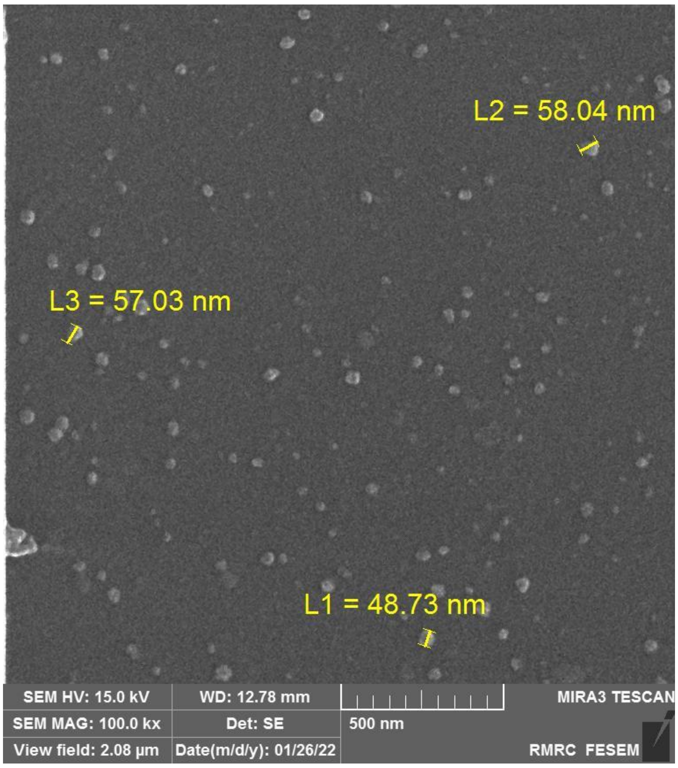

Fig. 4. FESEM image on surface of the nanoparticles containing thyme extract.

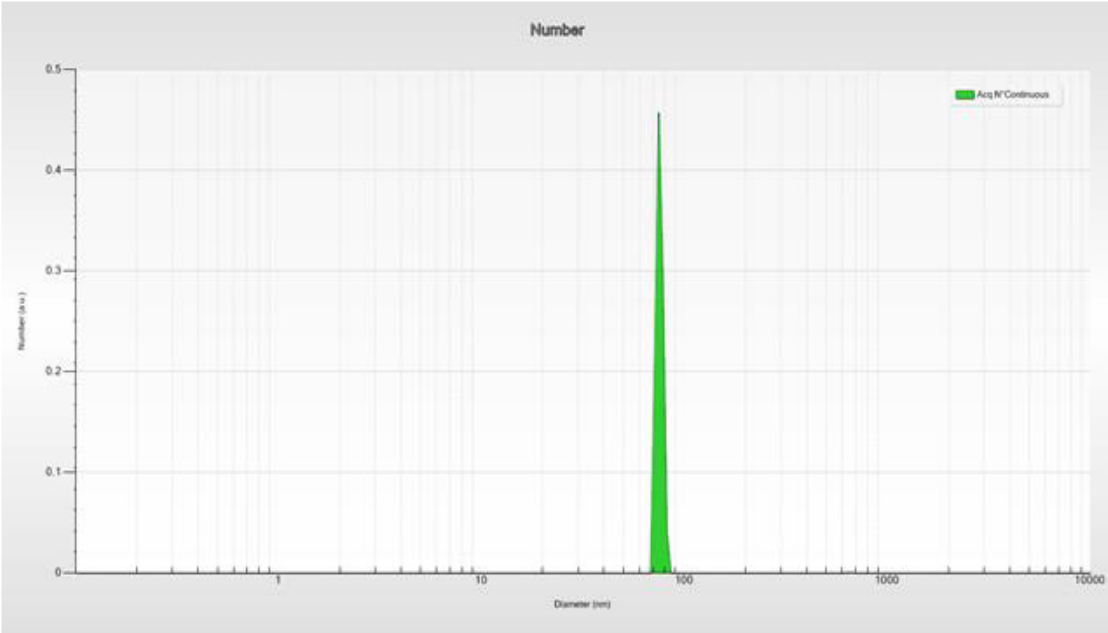

Fig. 5. Particles size distribution of the encapsulated polymers by DLS measuring technique

Table 2. Distribution statistics and Mean diameters of the polymers via DLS measuring technique

Mean particles size	Mean diameter of the particles (Intensity 10 %)	Mean diameter of the particles (Intensity 50 %)	Mean diameter of the particles (Intensity 90 %)
75.07 nm	71.02 nm	74.39 nm	77.91 nm

shape and had a smooth surface with a diameter closely equal to 58 nm. The FE-SEM images indicated spherical shape of the nanoparticles. The average size values of the nanoparticles in FE-SEM imaging agree quite well with those obtained by DLS measurements.

DLS measuring technique showed a single modal size distribution of the nanoparticles. Particle size distribution with DLS analysis showed that average particle size of the polymers was 75.07 nm (Fig. 5).

The polydispersity index (PdI) was closely 0.2 for these particles. It means that some of the particles were agglomerated and dispersion was not carried

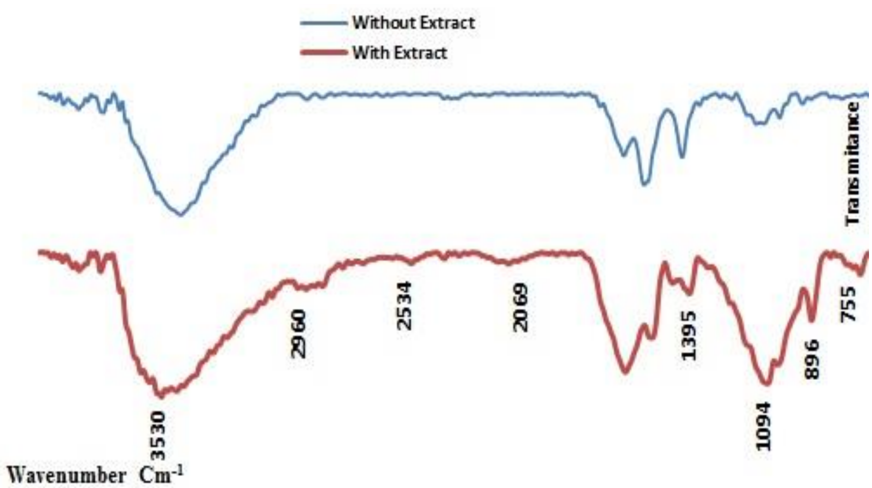

Fig. 6. IR spectrum comparison of the encapsulated polymers contain extract and without extract

out completely. Table 2 illustrates distribution statistics of the nanoparticles size.

Infrared Spectroscopy

Fig. 6 shows IR spectrum of the encapsulated both containing Thyme extract and without extract of the nanoparticles. This figure obviously indicate that the synthesized nanoparticles contain some other functional groups which had absorbance in different wavenumbers such as 755 cm^{-1}, 896 cm^{-1}, 1094 cm^{-1}, 1395 cm^{-1}, 2069 cm^{-1}, 2534 cm^{-1}, 2960 cm^{-1} and etc. It means that the encapsulated polymers could involve the bioactive compounds to their vacancies successfully. The specific broad bonds 3400-3500 cm^{-1} are related to O-H stretching groups which may be attributed to phenols, alcohols or N-H functional group of amides or amines. The absorption peak at about 2930 cm^{-1} belongs to C-H stretching. The bands at 1625 cm^{-1} was assigned to the C–O group of the carboxylic acid and the bands at 1390 cm^{-1} was assigned to the C–C groups of the alkenes. The band at 1060 cm^{-1} is related to the C-O in ethers, alcohols and esters. The characteristic absorption bands of chitosan nanoparticles including 1650 cm^{-1} is attributed to the carbonyl group of the amid in chitosan compound. The band at 1094 cm^{-1} belongs to symmetric and antisymmetric stretching vibrations in PO3 group of tripolyphosphate [26].

CONCLUSION

There are a lot of bioactive compounds in plant extracts with pathogenic and antioxidant properties. Because of their instability in environmental conditions, the researchers are attending to increase the efficacy of these natural products especially antifungal and antioxidant compounds. Using encapsulation of plant compounds in various nanoparticles could be the answer for such complications. Since using the chemical fungicides have many adverse effects on the human health, this idea is valuable work. Recently researches have been focused on finding ecofriendly and safe fungicide for controlling of the pathogens in agriculture industry. In this study, Thyme extract as a case study with antifungal effect were encapsulated in chitosan nanoparticles. The obtained particles sizes were in the range of 75 nm along with a PDI value of 0.2 validate the physical stability of nano system and prevent agglomeration of the particles. This study demonstrated that ecofriendly nanoencapsulation of herbal extracts containing antifungal bioactive compounds can be introduced as a suitable alternative for synthetic antifungal toxins and chemical fungicides in terms of long activity, efficiency and stability in various environmental conditions.

CONFLICT OF INTEREST

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

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