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Encapsulation of *Satureja khuzestanica* Essential Oil in Chitosan Nanoparticles with Enhanced Antifungal Activity

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***Abstract*—**During the recent years the six-fold growth of cancer in Iran has led the production of healthy products to become a challenge in the food industry. Due to the young population in the country, the consumption of fast foods is growing. The chemical cancer-causing preservatives are used to produce these products more than the standard; so using an appropriate alternative seems to be important. On the one hand, the plant essential oils show the high antimicrobial potential against pathogenic and spoilage microorganisms and on the other hand they are highly volatile and decomposed under the processing conditions. The study aims to produce the loaded chitosan nanoparticles with different concentrations of savory essential oil to improve the anti-microbial property and increase the resistance of essential oil to oxygen and heat. The encapsulation efficiency was obtained in the range of 32.07% to 39.93% and the particle size distribution of the samples was observed in the range of 159 to 210 nm. The range of Zeta potential was obtained between -11.9 to -23.1 mV. The essential oil loaded in chitosan showed stronger antifungal activity against *Rhizopus stolonifer*. The results showed that the antioxidant property is directly related to the concentration of loaded essential oil so that the antioxidant property increases by increasing the concentration of essential oil. In general, it seems that the savory essential oil loaded in chitosan particles can be used as a food processor.

***Keywords*—** Chitosan, encapsulation, essential oil, nanogel.

I. Introduction

SUPPLY of healthy foods was associated with the public health and is considered as one of the most important human needs. One method for decreasing the diseases caused by consuming the food and controlling the pathogenic microorganisms in the food is using the chemical preservatives that due to the carcinogenic properties of some of these materials, the use of natural preservative is one of the important approaches to promote the community health. One of the natural compounds that can be used as a preservative in the food is essential oil. The extracts and essential oils obtained from aromatic plants have the anti-bacterial, anti-fungal, anti-oxidant and anti-cancer properties and are able to control the pathogen growth and toxin production by microorganisms [1], [2]. Essential oils include the phenolic compounds, terpenes, aliphatic alcohols, aldehydes, ketones, acids and isoflavonoids. These compounds, especially the phenolic materials, are the main cause of antimicrobial and anti-oxidation properties of essential oils [3], [4]. The U.S. Food and Drug Administration has recognized the use of essential oils as a food additive that is generally safe [5]. The mechanism of antibacterial effect of essential oils is related to their hydrophobic nature that causes the penetration of these materials into phospholipids of bacterial cell membrane, causing disruption in the structure and the increase in permeability. This causes the outflow and leakage of ions and other cell contents that ultimately will lead the cell death. In some cases, the essential oils affect the enzymes responsible for the energy production or the synthesis of structural compounds of cell [3], [6]. One of the plants whose essential oil can be used in the food industry is savory. Gamma-terpinene is one of the dominant chemical compounds in Savory essential oil that is an antimicrobial agent due to the benzene ring and hydroxyl group. Essential oils can be the alternatives to chemical preservatives used in the food industry. But given that essential oils mainly are the volatile compounds and sensitive to light, heat, evaporation and oxidation, for using them in the food industry a complementary process is considered necessary to protect the essential oils. In the meantime, the encapsulation of essential oil increases the antimicrobial power and its controlled release while protecting the essential oil [7]-[10]. In this case, the bioactive compounds are loaded in polymer nanocarriers. Chitosan is one of nanocarriers that are used in food and medical industries due to antimicrobial properties [10], [11]. The study aims to produce the *Satureja Khuzestanica* essential oil loaded in chitosan nanoparticles and study its antimicrobial properties.

II. Material and Methods

A. Materials

Essential oil and Tween 60 were gift from Flavor and Color Freer Co., Isfahan, Iran. Chitosan (MW≈648000 Da) was purchased from Sigma, Germany. Pentasodium tripolyphosphate (TPP), Ethanol, Acetic aicd and Hydrochloric aicd were provided from Merck, Germany. *R. stolonifer* was obtained from Iranian Research Institute of Plant Protection (IRIPP).

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B. Preparation of Chitosan Nanoparticles Loaded with Satureja Khuzestanica Essential Oil

Savory-loaded chitosan particles produced according to the method described by [12] with little modified. Shortly, 50 ml of chitosan solution (0.3% w/v) was prepared by dissolving the chitosan flakes in aqueous acetic acid solution (1% v/v) at an ambient temperature during the night. Then, Tween 60 was added to the solution and stirred at 60 ˚C for 2 h to achieve a homogeneous mixture. Savory was gradually dropped into the stirring mixture, and it was added for 20 min. The different contents of savory, i.e., 0, 0.0375, 0.075, 0.1125, 0.15, and 0.1875 gr (0, 750, 1500, 2250, 3000, 3750 ppm respectively) were used to obtain the different weight ratios of chitosan to savory of 1:0, 1:0.25, 1:0.50, 1:0.75 1:1.00 and 1:1.25, respectively. Then, TPP solution (0.5% w/v, 40 ml) was slowly dropped into an O/W emulsion while stirring. The particles were collected by centrifugation at 10000 rpm for 10 min at 25 ˚C and washed with aqueous Tween 60 solution (1% v/v) and distilled water several times to remove free essential oil. The wet particles obtained were dispersed in 25 ml of distilled water.

C. Determination of EE and LC

The amount of essential oil in chitosan nanoparticles were determined by UV-Vis spectroscopy. Each sample (100 µl) boiled with an aqueous hydrochloric acid solution (2 M, 5 ml) at 95 °C for 30 min. After cooling down, 1 ml of ethanol was added to the mixture. The mixture was centrifuged at 25 °C for 2 min at 9000 rpm. In the wavelengths ranging 220 to 400 nm, the amount of essential oil in supernatant was measured by UV-Vis spectroscopy.

D. Particle Size Distribution

The particle size distribution of essential oil loaded in chitosan was determined by using DLS technique and using a nanoparticle analyzer (Malvern Instrument, ZEN3600 ,UK). The zeta potential was measured to determine the stability of the colloidal dispersion.

E. Textural Analysis

Tomato texture was assessed by a universal testing machine (Instron 1140, Instron, UK). Flat probe of 2 mm diameter was used for penetration test and compressed twice at a constant speed of 1 mm/s. The start of penetration test was the contact of the probe and tomato surface, and 8 mm of penetration distance were used. Eight replicates were taken per sample each test.

F. Differential Scanning Calorimetry (DSC)

The thermal analysis of sample was performed by DSC device. 4 to 8 mg of each sample was accurately flooded in a DSC aluminum pan and heated from 30 to 400 °C with a constant rate of 10 °C/ min under the flow rate of 20 mL/min Nitrogen.

G. Determination of Antioxidant Activity

Antioxidant activity (AOA) of chitosan nanoparticles loaded with savory was determined according to the method described by [12] against DPPH radical. For this purpose, 10 mg of the sample was combined with 100 ml of 0.1 M DPPH solution (in ethanol) and the mixture was kept in dark at ambient temperature for 2 h. Then, the absorbance was measured at 517 nm by using UV-Vis Spectrophotometer. Chitosan nanoparticles prepared without adding essential oil was used as control. Percent of AOA was calculated by using the following formula:

$$\%\text{AOA} = \frac{Acontrol - Asample}{Acontrol} \times 100$$

where $A_{control}$ and A_{sample} are absorbance of the control and nanoparticles sample containing essential oil.

H. Effect on Growth of Fungus

Antifungal test was carried out by the pour-plate method as described by [13]. In this method, the solutions of serial concentrations of each treatment were mixed with sterilized potato dextrose agar (PDA) in petri dish to obtain the final concentrations as follows: 750, 1500, 2250, 3000 and 3750 ppm for essential oil, chitosan nanoparticles loaded with savory. After inoculating the mycelia of fungus onto the center of agar, the dishes were sealed with parafilm and incubated at 25 ˚C for 5- 7 days, until the growth in the control plates (without the treatments) reaches the edge of the plates. Then, the antifungal index of treatment was calculated as follows:

$$\text{Antifungal index (\%)} = [(C-T)/C] \times 100$$

where C and T are the radial growth (mm) of fungus in the control and treated plates, respectively. Then, the results obtained in vitro study were carried out on tomatoes. The uniform and physical damage-free fruits were purchased from the local market and sterilized by sodium hypochlorite solution. The fruits were immersed in the respective treatment for 20 minutes after washing with distilled water. They were sprayed by *R. stolonifier* suspension and were stored for 9 days at 25 ° C. 25 tomatoes inoculated without coating were used as control. During the storage, the percentage of decayed tomatoes was recorded in each treatment.

I. Statiscal Analysis

Data was reported as mean±standard deviation (SD). SAS statistical software release 9.1 (SAS Institute Cray, NC) was used for the statistical analysis. The Least significant difference (LSD) test was conducted to determine the differences between the samples at significant level of $p<0.05$.

III. Results and Discussion

A. Particle Size Distribution and Zeta Potential

Keawchaoon and Yoksan had reported the average particle size between 532 nm to 716 nm that it could be due to higher concentrations of chitosan in making nanogel [12]. As observed in Table I, increasing the essential oil concentration decreases the particle size but the final concentration of essential oil leads to increase the average particle size that is consistent with the results of [14]. The results of zeta potential are shown in Table I. In all treatments, the results of negative zeta potential were

observed that it represents the negative charge on the surface. As observed, zeta potential is significantly decreased by increasing the essential oil concentration.

TABLE I
PHYSICOCHEMICAL PROPERTIES OF ESSENTIAL OIL LOADED CHITOSAN NANOPARTICLES

Sample	CS:EO mass ratio (w/w)	EE (%)	LC (%)	AOA (%)	Z-average diameter (nm)	Zeta potential (mV)
T1	1:0.00	0	0	0	210.27	-11.9
T2	1:0.25	32.07 ± 0.12^{e}	11.42 ± 0.3^{d}	48.7 ± 0.33^{e}	189.64	-10.32
T3	1:0.50	32.48 ± 0.08^{d}	18.79 ± 0.2^{c}	58.01 ± 0.08^{d}	195.38	-14.48
T4	1:0.75	$37.56\pm0.05_{b}$	23.04 ± 0.1^{b}	60.43 ± 0.15^{c}	171.87	-19.87
T5	1:1.00	39.93 ± 0.02^{a}	25.38 ± 0.06^{a}	67.67 ± 0.07^{b}	149.76	-21.22
T6	1:1.25	36.1 ± 0.1^{c}	25.41 ± 0.11^{a}	70.22 ± 0.09^{a}	159.61	-23.1

Letters within a column indicate significantly different values ($p < 0.05$).

B. Encapsulation Efficiency

The amount of essential oil loaded was determined by absorption in the wavelength of 287nm. The encapsulation efficiency is according to the results in Table I between 32.07% to 39.93% that indicates when the essential oil fed was 100 gr, the amount of essential oil loaded on nanoparticles was 32.07 to 39.93 gr. To weight ratio of chitosan to essential oil of 1:1, increasing the concentration of essential oil leads to increase the encapsulation efficiency but at the final concentration of essential oil, the encapsulation efficiency is decreased that it can be due to the limitation of encapsulation. In addition, LC tends to increase by increasing the concentration of essential oil. 100 gr of nanoparticles containing essential oils include 11.42 to 25.41 gr.

C. Textural Analysis

As can be observed in Fig. 1, the firmness is significantly decreased during the storage of control samples but it increased by increasing the concentration of essential oil loaded in chitosan nanoparticles so that there is no significant difference in the firmness in the ratio of essential oil to chitosan of 1:1 compared to the control samples on the first day. It seems that the inhibitory effect of nanoparticles containing essential oils on *R. stolonifer* prevents from the fungal growth and leads to maintain the firmness in the texture of tomato. According to the results obtained, we can conclude that the use of nanoparticles containing essential oil can improve the physical properties of product during the storage.

D. Thermal Analysis

DSC can show the encapsulation efficiency by polymeric materials. As observed in Fig. 2, DSC thermogram of chitosan nanoparticles shows an endothermic peak in 123.4 °c representing the evaporation of water. Also, an exothermic peak can be observed in 273.8 °c representing the decomposition of nanoparticles. In the samples of chitosan nanoparticles containing the essential oil, the endothermic and exothermic peaks are observed at higher temperature. The endothermic peak at higher temperature represents high water holding capacity and stronger water-polymer interaction and the formation of exothermic peak at higher temperature indicates a better thermal resistance of nanoparticles loaded by essential oil.

E. Antioxidant Properties

In control sample, the antioxidant properties were not observed but they increased by increasing the concentrations of essential oil in the chitosan nanoparticles that the highest AOA% was observed at the highest concentration of essential oil showing the antioxidant properties in essential oil. Many authors have reported the antioxidant activity of essential oils that the mentioned effect is related to the present composition.

F. The Effect of Essential Oil Coated on the Antifungal Characteristic

The results suggest that the weight ratio of chitosan to essential oil of 1:1 causes the complete inhibition of mold growth and in the ratio of 1:0.75 has a suitable inhibition capacity that can be observed in Fig. 3. In the lowest concentration, the inhibition capacity of essential oil is lower than chitosan nanoparticles containing essential oil. It seems that during encapsulation and protection of antimicrobial compounds, the inhibitory efficiency of essential oil increases. To study the effect of encapsulated essential oil on tomato, the weight ratio of chitosan to essential oil of 1:1 was used and the results were recorded during 9 days. As seen in Figs. 4 and 5, the amount of rotting the tomatoes is decreased by using the chitosan nanoparticles containing the essential oil. The results show that the use of chitosan nanoparticles containing essential oil leads to decrease the rottenness and control the growth of *R. stolonifer* in the tomato. In the chitosan nanoparticles containing essential oil sample, the rottenness began on 8 day but in the control sample the rottenness began on 4 day.

IV. CONCLUSION

In this study, the nanoparticles of chitosan containing savory essential oil were used to increase the resistance of essential oil to heat, light, oxygen and increase the potential of using it to control the growth of *R. stolonifer*. The success of loading the essential oil in chitosan particles was confirm by observing the band absorption in the UV-Spectrophotometry and the temperature of decomposition in DSC. Chitosan nanoparticles containing essential oil show the strong antioxidant and antimicrobial properties that it depends on the concentration of essential oil. In our study, the optimum weight ratio of chitosan to essential oil of 1:1 was observed that has an appropriate

inhibitory effect on controlling the growth of *R. stolonifer* in vivo. It seems that the essential oil coated by chitosan can be an appropriate alternative to synthetic fungicide to prevent the growth of microorganisms in the food.

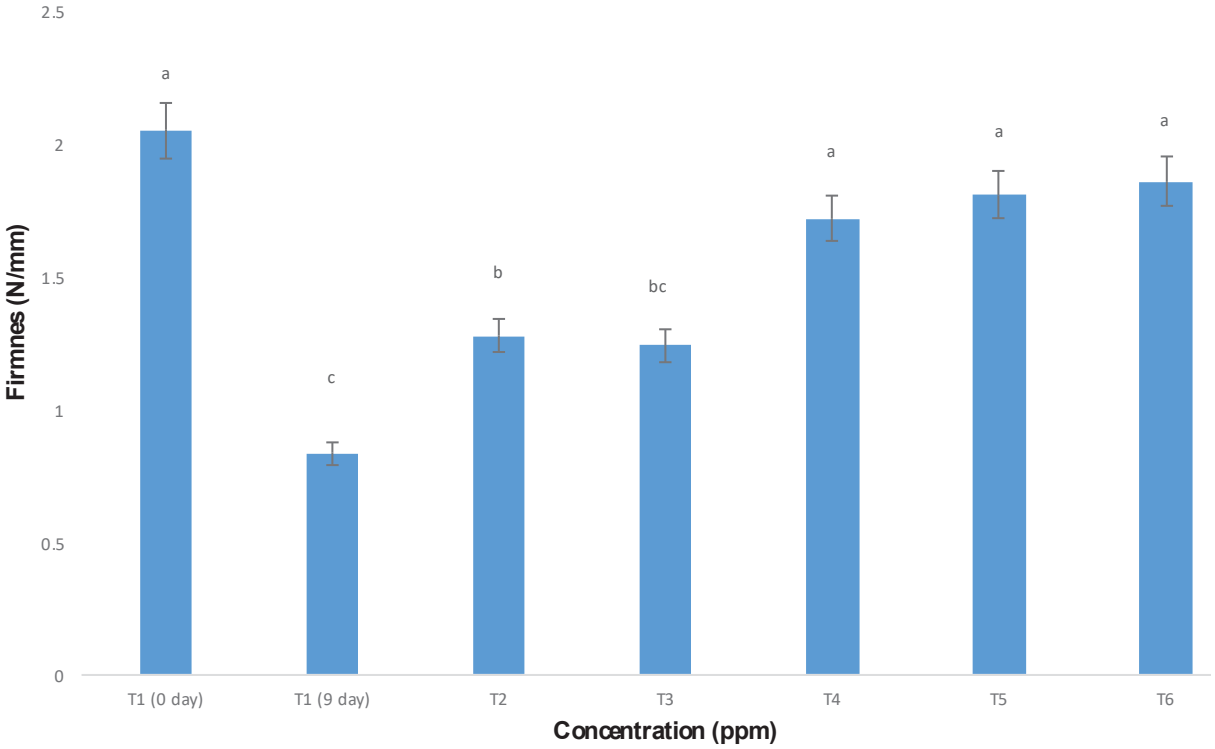

Fig. 1 Comparison changes of firmness with different ratio of CS:EO

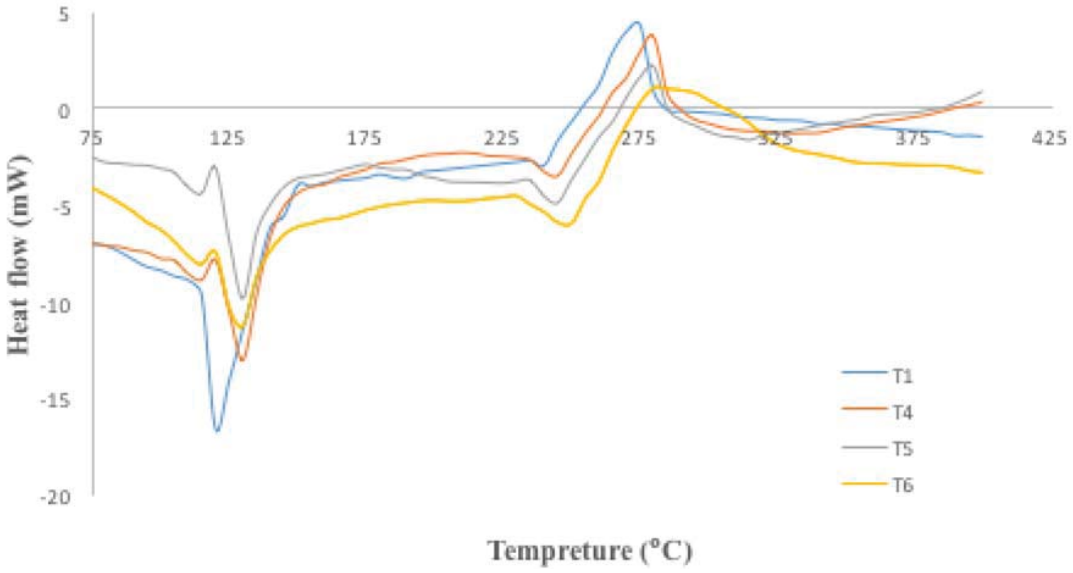

Fig. 2 DSC Thermograms of different ratio of CS:EO. (T2 and T3 not determined)

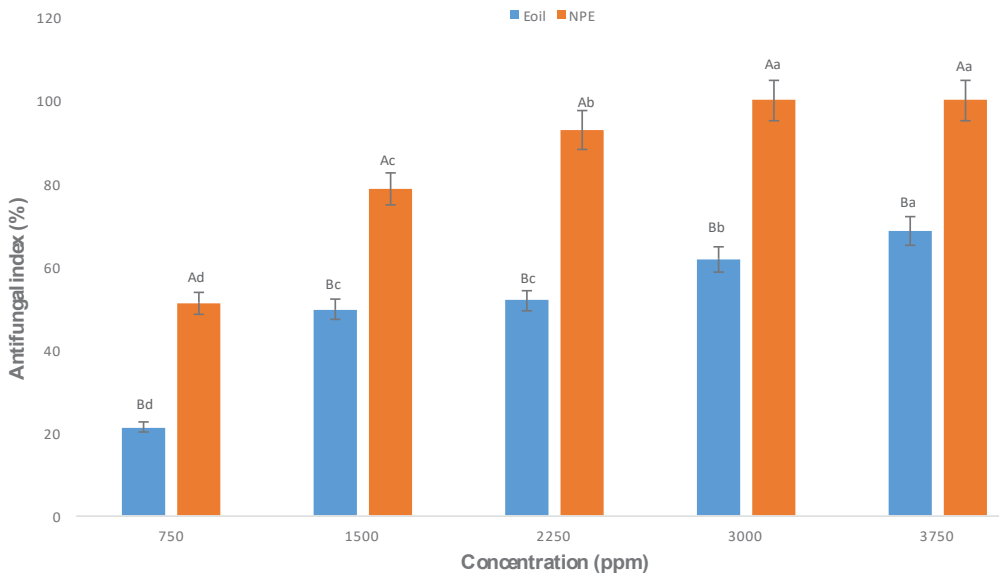

Fig. 3 Effect of different ratio of essential oil encapsulated and free essential oil on antifungal index (%) under in *vitro* condition

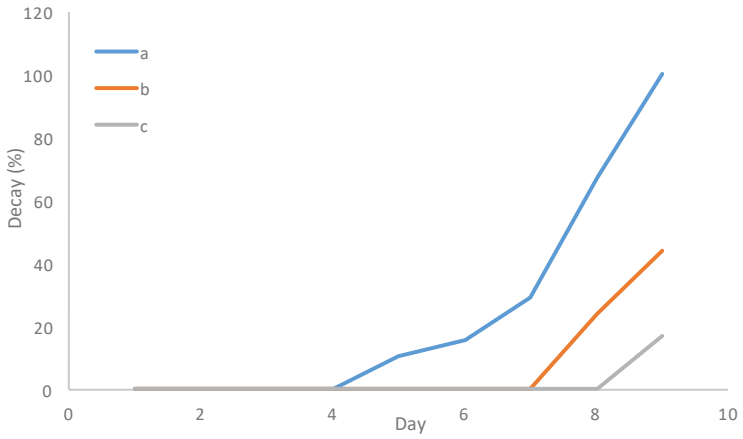

Fig. 4 Effect of different ratio of essential oil encapsulated and free essential oil on disease incidence (a) Control (b) Essential oil (c) Essential oil encapsulated

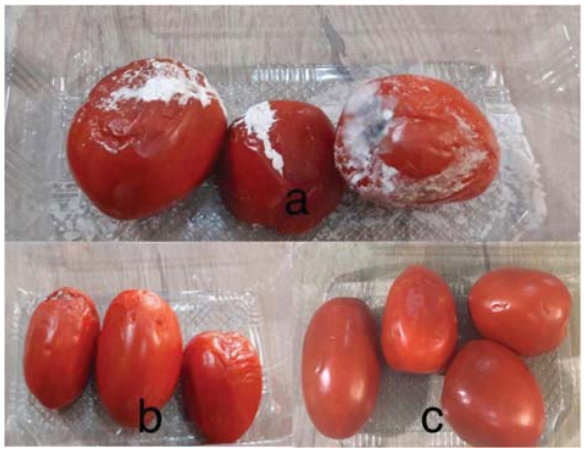

Fig. 5 Appearance of tomatoes treated with essential oil encapsulated and free essential oil during storage (a) Control (b) Essential oil (c) Essential oil encapsulated

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