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Nano-encapsulation of thyme essential oil in chitosan-Arabic gum system: Evaluation of its antioxidant and antimicrobial properties

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ABSTRACT

In this study, nano-capsules based on chitosan (CS) and Arabic gum (AG) involving thyme essential oil (TEO), as an active ingredient, were prepared using the emulsion method. The nano-capsules were characterized by their encapsulation efficiency (EE), morphologies, particle size distributions, zeta potential and release (RE). The obtained results showed that nano-capsules produced using a relative ratio of CS:AG (1.5%:8.5%) clearly showed the highest encapsulation efficiency (77.67%) and zeta potential value (+ 43.17 mV). *In vitro* release study demonstrated a slow release for the samples with larger CS ratio. In addition, scanning electron microscopy (SEM) images showed that nano-capsules sizes were over the range 385.2-756.1 nm with a rough surface shape for all samples. Moreover, quantitative values of antioxidant activity of free TEO and nano-encapsulated TEO were studied using the free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. The essential oil (EO) was investigated for its antibacterial activity against common Gram-positive and Gram-negative pathogenic microorganisms including *Staphylococcus aureus* and *Escherichia coli* by disk diffusion and dilution (MIC) methods. Our results accounted for higher antioxidant and antibacterial activities of encapsulated TEO compared to those of free TEO. Finally, the CS:AG ratio of 1.5%:8.5% was found to be suitable wall material for TEO encapsulation.

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1. Introduction

Natural products such as plant essential oils (EOs) have antioxidant and antimicrobial potential as well as beneficial effects on human health (Akhlaghi, 2017; Camilo et al., 2017; Mahdavi, 2017; Nazemizadeh Ardakani and Masoudi, 2017). It has been well-documented that plant EOs as safe additives can be used in foods, pharmacy, medicine, nutritional supplements and cosmetic products (Burt, 2004; FDA, 2014; Mohammadhosseini et al., 2017). The antioxidant and antimicrobial activity of natural compounds have been extensively reported in the literature (Lucera et al., 2012; Jayasena and Jo, 2013; Mohammadhosseini, 2017).

TEO is a good source of bioactive components such as phenolic and terpenoid compounds with antimicrobial and antioxidant properties as an alternative for synthetic antioxidants to lower lipid oxidation (Gutierrez et al., 2008; Barbosa et al., 2009; Gutierrez et al., 2009; Jayasena and Jo, 2013; Bensid et al., 2014; Rezaei and Mohammadhosseini, 2014; Hashemi-Moghaddam et al., 2015). The TEO is more sensitive towards environmental parameters like high temperature, light, the presence of oxygen, etc. To overcome these challenges, an EO needs to be encapsulated in a suitable wall material to decrease oxidative stability, controll release and improve the shelf life of these ingredients (Jafari et al., 2008). In this technique, liquid materials such as EOs are encapsulated in a shell material in order to obtain a dry powder. Encapsulation could be an adequate technique to protect aroma from degradation and evaporation. Therefore, it is of prime importance to achieve the best formulation of wall combination for the prevention of

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flavor loss as well as to elevate the controlled release of the core during the storage or processing (Chiu et al., 2007).

Numerous investigations have been recently evaluating the application of chitosan (CS) as an encapsulating agent (Peng et al., 2010a, 2010b; Estevinho et al., 2013; Nuisin et al., 2013). CS is a cationic polymer composed of linear beta-(1-4)-linked D-glucosamine and *N*-acetyl-D-glucosamine with biodegradable and biocompatible characteristics (Liu, 2014). In this research, CS was selected as a biopolymer due to its ability to control the release of active ingredient, easily available free amine groups for cross-linking, and suitable electro potential (Forssell, 2004; Liu, 2014). Arabic gum (AG) is a heteropolysaccharide with excellent emulsifying properties, film-forming, good ability to produce nanosize particle and low viscosity at high concentration. However, it is not appropriate for encapsulation of monoterpenoids when it is used alone (Bertolini et al., 2001; Kaushik and Roos, 2007). Accordingly, CS and AG were chosen as proper polymeric wall materials considering their ability to form electrostatic complexes at routine pH ranges of food products (Bansal et al., 2012). Furthermore, many studies have reviewed the various methods of EO encapsulation (Bertolini et al., 2001; Bae and Lee, 2008; Estevinho et al., 2013; Jayasena and Jo, 2013; Asprea et al., 2017; Shamaei et al., 2017; Shrestha et al., 2017), but there are no data, to the best of our knowledge, on structural properties, antioxidant and antimicrobial activities of nano-encapsulated TEO.

The aim of this study was to produce nano-encapsulated EOs, evaluate the structural properties, and determine the antioxidant and antimicrobial activities related to TEO nano-capsules.

2. Experimental

2.1. Materials

Thyme EO was purchased from the Barij Essence Pharmaceutical Company. The coating materials used (CS), with low molecular weight having 75-85% degree of deacetylation, along with sodium tripolyphosphate (TPP) and Tween 80 were all prepared from Sigma-Aldrich (St. Louis, MO, USA). In addition, Arabic gum was provided from Saadatchemieazma Co., Tehran, Iran. In all of our experiments, distilled and deionized water were used for the preparation of the solutions.

2.2. Preparation of emulsion

The emulsions were prepared according to the previously published papers by Hosseini et al. (2009) and Saloko et al. (2013) with slight modification. Briefly, CS (0.5%, 1% and 1.5% w/v) and AG (9.5%, 9 and 8.5 w/v) were dispersed in a glacial acetic acid solution (1% v/v). After the solution was completely dissolved, Tween 80 at 0.1% concentration was added, as a surfactant, and the resulting solution stirred for 2 hours until a completely uniform solution was obtained. In this study, all of the combinations were prepared using 1 g of Tween 80, 100 g of EO and 1 mg/mL of tripolyphosphate (TPP) in the mixture. The total concentration of the dissolved solid involving wall material and oil was adjusted at 20% (w/w). The EO was added in a ratio of 1:4 (w/w) to all of the prepared solutions. Then, each solution was incubated at room temperature with a magnetic stirrer and centrifuged for 30 minutes in 50 mL test tubes at 3000 rpm. The solution was heated at 50 ºC for 15 minutes and homogenized with a 5200 rpm homogenizer for 2.5 minutes (Saloko et al., 2013). In order to achieve the smaller particle mean size of the capsules and increase the encapsulation efficiency (EE) of the EO, the sonication approach was used. In this regard, the emulsion was subjected to a sonication process in an ice bath for 7 min, 1 second on and 1 s off, using a probe (200 UPS, Dr. Heischler, Germany) until the emulsion became completely clear (Najaf Najafi et al., 2010).

2.3. Drying of emulsion by lyophilization (freeze drying procedure)

The prepared emulsions were frozen at -70 ºC overnight, and dried in a freeze dryer for 72 hr. When the freeze-drying process was performed, the obtained powder was kept in moisture-impermeable plastic bags and stored at -20 ºC for further characterization of its properties.

2.4. Physical properties of EO nano-capsules

2.4.1. Determination of encapsulation efficiency (EE)

Encapsulation efficiency (EE) was performed according to the method given by Bae and Lee (2008) with slight changes. Accordingly, 1.5 g of encapsulated powder was shaken with 15 mL of ethanol (98%) in a glass jar for 10 minutes. In the next step, the mixture was filtered by Whatman paper No.1 with medium porosity. The remained powder was washed with ethanol three times and the solvent was evaporated from the reaction medium until a constant weight was obtained at room temperature. The nanocapsules were weighed and dissolved in distilled water. The encapsulated oil content was measured by Clevenger distillation. The non-encapsulated oil was determined regarding the difference between the total essential oil and the content of encapsulated oil. The encapsulation efficiency (EE %) was calculated as (Eqn. 1):

$$EE\ (\%) = \frac{T_0 - S_0}{T_0} \times 100 \qquad \text{(Eqn. 1)}$$

Where T_0 is the total EO content and S_0 is the non-encapsulated EO content.

2.4.2. *In vitro* release

In vitro release (RE) was carried out according to the Wang and Weller (2006) method. Following this method, 100 mg of each sample was first dissolved in 10 mL of distilled water and placed in a dialysis bag on stirrer under a constant temperature (25 ºC). Then, at regular intervals, an aliquot of 1 mL was removed, diluted and tested using UV-Vis at 275 nm. The yield of RE was expressed in percentage (%).

2.4.3. Particle size and zeta potential

The hydrodynamic size and zeta potential of nano-capsules were determined by a commercially available Malvern Zetasizer 3000HS (Malvern Instruments Ltd., UK) instrument. Zeta potential basically provides information about the potential difference between the dispersion medium and the stationary layer of the fluid attached to the dispersed particle, which leads to a deeper insight into the stability of the nano formulations (de Oliveira et al., 2018; Marín et al., 2018; Tan et al., 2018; Xue et al., 2018). Measurements were made using aqueous diluted samples (2:1 ratio). Basing on the principle of photon correlation spectrometry, this instrument also provides the possibility of the measurement of particle-size distributions in the range.

2.4.4. Scanning electron microscopy (SEM)

Morphology of the freeze-dried nano-capsules was examined to study the surface structures of powders by scanning electron microscopy (SEM; Leo EVO-40 VPX, Carl Zeiss SMT, Cambridge, UK). The samples were glued onto an adhesive tape mounted on the specimen stub and the corresponding particles were covered with gold-palladium prior to the analysis. Finally, the representative SEM images (×100) were recorded and characterized.

2.5. Chemical properties of TEO nano-capsules

2.5.1. Determination of total phenolic content (TPC)

The TPC of encapsulated and non-encapsulated EOs were distinguished by using the Folin- Ciocalteu method according to the given method by Ebrahimzadeh et al. (2008). Briefly, diluted samples (0.05:1 g/mL) were mixed with Folin-Ciocalteu reagent (2.5 mL, 1:10 diluted with distilled water) and left at room temperature (25 ºC) for 3-4 minutes. Then, 3 mL of Na_2CO_3 (1 M) was added to the mixture. Tubes were allowed to stand in a dark room for 1 hour. In the final step, the levels of total polyphenols were determined using a UV-VIS spectrophotometer at 765 nm. The total phenolic content was determined as mg of Gallic acid equivalent using an equation obtained from the standard Gallic acid calibration graph (Slinkard and Singleton, 1977).

2.5.2. Determination of the antioxidant activity by DPPH method

The DPPH radical scavenging activity was determined through a standard method (Ojeda-Sana et al., 2013) with some modifications. Briefly, 20 µL of each treatment in six different concentrations and 180 µL of DPPH solution in ethanol were added. An alcoholic (EtOH) solution was used as the blank sample. The plate was incubated for 24 h and the corresponding absorbance was measured at 515 nm at 10 min intervals. The antioxidant activity of the samples, described as percentage inhibition of DPPH, was calculated according to the following equation (Eqn. 2).

$$\%\text{Inhibition} = ([A_b - A_s] A_b) \times 100 \quad \text{(Eqn. 2)}$$

Where A_b and A_s respectively account for the absorbance of blank sample and sample at the end of the reaction (Delgado Adámez et al., 2012; Ramos et al., 2012; Ojeda-Sana et al., 2013).

2.6. Antimicrobial activities of EO nano-capsules

2.6.1. Minimum inhibitory concentration (MIC)

The lowest inhibitory concentration (MIC) was calculated according to the described procedure by Duarte et al. (2015). Broth dilution method was used to determine the minimum inhibitory concentration (MIC) of samples. Experiments were performed on two different microbial strains, namely *Escherichia coli* and *Staphylococcus aureus* which were grown in sterile Mueller Hinton Broth (Merck, Germany) at 37 ºC for 18-24 to get a bacterial suspension of 10^5 cfu/mL. The serial dilutions of samples were prepared using a 96-well microtiter plate. In order to prepare different concentrations of essential oils, 128 µL of samples was added to the first six rows. Then, 100 µL of solution was transferred from the well 1 to the well 2. This procedure was continued up to the well 8. Then, 100 µL inoculum was added to each well. The final concentration of bacteria in each microwell was 10^5 cfu/mL which was estimated using the surface plate counting method. As a positive control, 100 µL bacterial stock was added to 100 µL of nutrient broth lacking EOs (0%). A well containing only 200 µL of broth was prepared as a negative control. Final solutions were incubated at the temperature as mentioned earlier. The MIC value was determined as the lowest concentration of the antimicrobial agent that inhibited the visible growth of the test microorganism. The experiments were carried out in three replicates.

2.7. Statistical analysis

One-way analysis of variance (ANOVA) was performed using SPSS (ver.15) software. Differences

among mean values were examined by Duncan's test ($p \leq 0.05$) significance level.

3. Results and Discussion

3.1. Physical properties of EOs nano-capsules

3.1.1. Encapsulation efficiency

The EE results of the treatments evaluated are shown in Table 1. The encapsulation efficiency values varied from 62.67% to 77.67% depending on the CS:AG ratio. There were significant differences ($p<0.05$) between samples using CS:AG (1.5:8.5%) which showed the highest value (77.67 ± 4.16%) compared with the other samples. The lowest EE was obtained in nano-capsules with wall material CS:AG (0.5:9.5%). CS and AG complex developed emulsifying characteristics and formed a suitable membrane at the oil-water interface, since it can cover the EO with a better quality of nano-encapsulated material. Many researchers have reported EE values from 0 to 95% depending on the type and composition of wall material, the ratio of core material to wall material, the stability and physicochemical properties of the emulsions (Hardas et al., 2000; Hogan et al., 2001; Baik et al., 2004; Klinkesorn et al., 2006). In addition, the investigation results showed that the ratio of wall material had a very significant effect ($p<0.05$) on the surface oil content of essential oil encapsulated powders. This trend could be explained by the droplet size, similar to the results of Jafari et al. (2008). In previous studies, considerable lower levels of encapsulation efficiency values have been reported for zein particles containing EO (Padua and Wang, 2009) and flax oil (Omar et al., 2009).

Table 1

Wall materials composition, encapsulation efficiency (EE%) and zeta potential (mV) values of thyme essential oil nano-encapsules.

Sample	CS: AG	Matrix:oil	EE%	Zeta potential
F1	0.5%:9.5% (w/v)	4:1	62.67 ± 2.08^{b}	9.5 ± 1.32^{c}
F2	1%:9% (w/v)	4:1	66.33 ± 3.22^{b}	21 ± 2^{b}
F3	1.5%:8.5% (w/v)	4:1	77.67 ± 4.16^{a}	43.17 ± 1.76^{a}

Reported means (± standard deviations) derived from 3 replications with 3 samples per replication. Means with in column followed by different superscripts are significantly different at $p \leq 0.05$.

3.1.2. Particle size and zeta potential

The size distribution of nano-capsules containing TEO was shown in Fig. 1. As can be seen, the mean diameter of nano-capsules ranged from 385.2 to 756.1 nm that is similar to other findings which reported size range of EO nano-capsules (Bae and Lee, 2008; Jafari et al., 2008; Marcuzzo et al., 2010). Polydispersity index is usually used for the determination of particles diameter distribution in suspension. The smaller polydispersity index expresses the more homogenous particle size

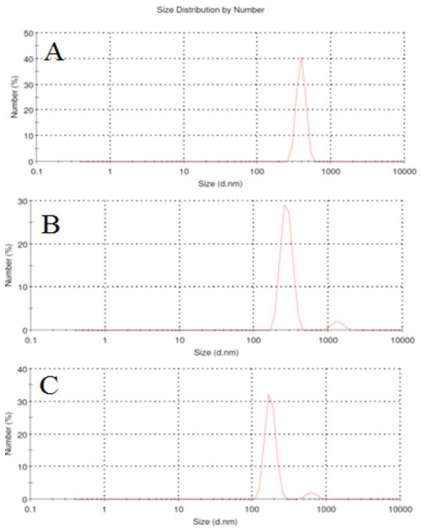

Fig. 1. Size distribution of thyme essential oil (TEO) nano-capsules produced from different ratio of wall material combinations: (a) F1 sample: CS:AG (0.5:9.5 w/v%); (b) F2 sample: CS:AG (1:9 w/v%); (c) F3 sample: CS:AG (1.5:8.5 w/v%).

distribution and therefore represents a desirable uniformity in diameter (Hasani et al., 2015). Our study revealed that the polydispersity index of nano-capsules were ranging from 0.44 to 0.66 indicating nano-capsules were monodisperse, stable and had a uniform distribution. According to the results, the use of CS:AG wall materials ratio (1.5%:8.5%) resulted in a smaller size compared to other formulations prepared with different wall materials ratios. The use of the different ratios of wall materials had a significant effect on particle size (Fig. 1). The selection of wall material combinations and their concentrations affect both the emulsion properties and the particles characteristics after drying. It is well-described that powder properties such as encapsulation efficiency, particle size, morphology and oxidative stability are greatly influenced by the type of encapsulating agent used (Jafari et al., 2008). The particle size of the encapsulated fish oil by the same method was also in nano scales (Bejrapha et al., 2010). In addition, zeta potential measurements is a good clue to predict the stability of nano-capsules. The values of TEO nano-capsules were more than +43 mV in CS:AG (1%:9%) samples. This result is in agreement with previous studies (Raja et al., 2013; Kumar et al., 2015).

3.1.3. Morphology

SEM images of the TEO nano-capsules are shown in Fig. 2. They provide useful information about both the particle size and morphology. Morphological studies on dried particles provide valuable perspective in relation to the drying particles, chemical and physical factors affecting the particle structure. As anticipated, freeze dried microcapsule particles appeared to be composed of an irregular shaped state. The edges of the nano-capsules were considerably sharper.

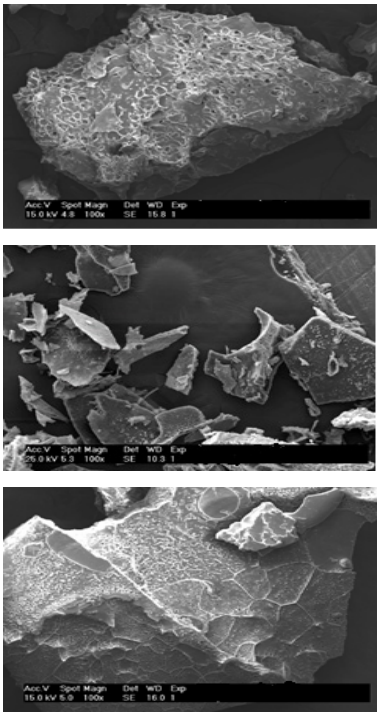

Fig. 2. SEM images showing the morphology of dried thyme essential oil (TEO) nano-capsules (powder form) produced from different ratio of wall material combinations: (a) F1 sample: CS:AG (0.5:9.5 w/v%); (b) F2 sample: CS:AG (1:9 w/v%); (c) F3 sample: CS:AG (1.5:8.5 w/v%).

Anandharamakrishnan and Karthik (2013) reported a spray-dried microencapsulated powder which displayed spherical shape with a smooth surface as compared with nonspherical freeze dried microencapsulated particles. However, in our study, the freeze dried powder exhibited the cakelike structure with uneven surfaces (Fig. 2). As seen, nano-capsules appeared to be largely free of cracks and essential oil in surface because of higher encapsulation efficiency (EE %, Fig. 2: c), but the presence of some cracks and greater pores were also noted in Fig. 2 (a and b).

3.1.4. Release study

The release profile of the nano-encapsulated TEO in CS:AG is shown in Fig. 3. The release profile would, therefore, be a valuable way to describe the antioxidant activities of TEO nano-capsules. Compared to the release profile of essential oils encapsulated in zein particles in polar medium reported by Parris et al. (2005), the maximum amount of the TEO (>50%) was released rapidly after about 5 hours. In the release results, encapsulates using CS:AG (0.5:9.5%) and samples using CS:AG (1.5:8.5%) had the highest and lowest rate, respectively. Rapid release in the first 5 hours of the test is most probably due to the absorption of the oil from the surface of the nanocapsules. The slow release of core compounds has been ascribed to the release of the core material through the wall compounds, the

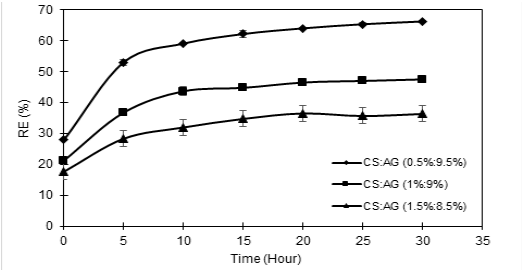

Fig. 3. Kinetics of *in vitro* release from different thyme essential oil (TEO) formulations prepared with various ratios of wall material.

hydrocarbon portions of layers, and the pores contained within the surface.

3.2. Chemical properties of EOs nano-capsules

3.2.1. Determination of total phenolic content (TPC)

The quantities of total phenolics in different nano-encapsulated and non-encapsulated TEOs varied from the lowest value of 202.65 mg gallic acid equivalent indicated for free EOs to the highest level of 219.29 mg GA equivalent attributed to samples using CS:AG (1.5:8.5%) (Table 2). The applications of chitosan to be used as an antioxidant compound for foodstuffs have been widely reported in the literature (Chien et al., 2007; Badawy and Rabea, 2009). Therefore, an increase in TPC may be related to the presence of chitosan and Arabic gum in formulation system which can result in the interaction of their hydroxyl groups with Folin-Ciocalteu reagent leading to the formation of a colored complex with a series of non-phenolic compounds of these adjuvants.

3.2.2. Determination of antiradical activity by DPPH assay

The Table 2 shows the antioxidant activity values of encapsulated and non-encapsulated TEO compared with that of the synthetic antioxidant (BHT). As shown, that DPPH radical scavenging was higher in encapsulated TEO than free TEO. This result may be related to the presence of chitosan and Arabic gum in nano-capusles as wall materials. They contain many hydroxyl groups (OH) in their chemical structures which contribute strongly to the antioxidant activity by donation of a free electron (hydrogen). Radical scavengers can be reacted and inhibited with peroxide radicals to limit the peroxidation chain reaction. It was confirmed that chitosan acts as an antioxidant by scavenging oxygen-containing radicals such as hydroxyl, superoxide, alkyl as well as highly stable DPPH radicals tested *in vitro*. The nano-encapsulated sample prepared with a chitosan (1.5%) has a higher antioxidant activity than those of other samples. Furthermore, a similar antioxidant activity was observed with BHT and formulation using 1% of chitosan.

Table 2

Total phenolic compounds (TPC) and antioxidant activity of different thyme essential oil formulations compared to those of free thyme essential oil and standard BHT.

Sample	CS:AG	Matrix:oil	TPC (mg gallic acid eq. g^{-1})	DPPH radical scavenging (%)
Free TEO	–	-	202.65 ± 2.06^{c}	74.6 ± 1.35^{d}
F1	0.5%: 9.5% (w/v)	4:1	206.56 ± 2.42^{bc}	82.91 ± 3.09^{c}
F2	1%: 9% (w/v)	4:1	209.42 ± 1.74^{b}	88.53 ± 0.93^{b}
F3	1.5%: 8.5% (w/v)	4:1	219.29 ± 2.11^{a}	93.82 ± 3.62^{a}
BHT	–	–	–	92.54 ± 1.19^{ab}

Reported means (± standard deviations) derived from 3 replications with 3 samples per replication. Means with in column followed by different superscripts are significantly different at $p \leq 0.05$.

Table 3

Antibacterial activity of the different thyme essential oil formulations compared to those of free Thyme essential oil and standard (streptomycin).

Microorganism	Thyme Essential Oil				Antibiotic
	free	Formulated (CS:AG) (w/v)			Streptomycin
		0.5%:9.5%	1%:9%	1.5%:8.5%	
	MIC	MIC	MIC	MIC	MIC
Staphylococcus aureus	1.5	1.8	1.3	0.95	2
E. coli	2.5	2	1.9	1.2	1.5

MIC: Minimum inhibition concentration (values in mg/mL)

3.3. Antimicrobial activity

Both free TEO and it's encapsulated from have a high antimicrobial activity which may be related to the presence of high concentration of thymol in unencapsulated EOs and chitosan as an antibacterial agent within encapsulated EOs. In our study, the antimicrobial activity of TEO in free and encapsulated forms was determined against two bacterial strains. The dilution method was done in order to determine MIC of the EOs samples. The results of antimicrobial activities of TEO in free and encapsulated forms were shown in Table 3. In all strains tested, the antimicrobial activity of TEO in nano-encapsulated form was greater than that of uncapsulated form. In contrast, the numerical values of MIC of free essential oils were found to be higher than those of encapsulated form. This is because of the fact that the release of TEO from particles takes much more time to show antimicrobial activity. This study also revealed that nano encapsulated EO samples using CS:AG (1.5: 8.5%) had significant differences with free EOs due to the presence of chitosan in higher rate in the formulation. TEO, as well as CS:AG capsules containing this oil, showed significant antibacterial activity against the tested representative strains. It was also noted that antibacterial impact of nano-capsules was developed by increase in chitosan rate in formulation giving rise to an enhancement of the inhibition zones.

4. Concluding remarks

This study demonstrated that the wall material ratio used in the preparation of TEO formulation has an important impact on the physico-chemical properties of obtained nano-capsules from stability, size, and release points of view. In addition, freeze drying of formulation is an important criterion to conserve nano-capsules in a powder form and longer against physical damages involving light, pH, temperature, etc. However, this technique may cause structural damages in capsule morphology as shown by SEM images. Moreover, the release of TEO from nano-capsules was still well-controlled and its antim icrobial activity was more remarkable than that of free TEO against Gram-positive and Gram-negative pathogenic microorganisms like *Staphylococcus aureus* and *Escherichia coli*. In fact, the release property is sought in the agri-food sectors in order to preserve a broad spectrum of food products as much as possible against bacteria pathogens. This study can suggest that the production of TEO nano-capsules with chitosan/Arabic gum as adjuvants followed by a freeze drying process may be a successful method to develop the chemical stability, antioxidant and antimicrobial activity of this TEO for a variety of purposes.

Conflict of interest

The authors declare that there is no conflict of interest.

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