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Encapsulation of Thyme essential oils in chitosan-benzoic acid nanogel with enhanced antimicrobial activity against *Aspergillus flavus*

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ABSTRACT

This study was set to investigate the encapsulation of the Thyme essential oils using chitosan and benzoic acid-made nanogel in order to enhance its antifungal properties and half-life. To achieve this, the self-assembled polymer of chitosan and benzoic acid nanogel (CS-BA) was synthesized, its size and shape were confirmed by spectrometric (FTIR) and microscopic methods (TEM and SEM) and was then used in encapsulating the essence. Under sealed condition, the minimum inhibitory concentration of the CS-BA encapsulated essential oils was recorded at 300 mg/l while the free Thyme extract could only completely prevent the growth of *Aspergillus flavus* at an elevated concentration of 400 mg/l. Under non-sealed condition, higher concentration of encapsulated Thyme essential oils (500 mg/l) was required to cause complete fungi inhibition and free oils failed to lead to full inhibition even at concentrations as high as 1000 mg/l. *In vivo* analysis also revealed significant anti-fugal properties of the encapsulated oils at concentrations above 700 mg/l. Overall, due to the volatility and instability of free essential oils, CS-BA nanogel encapsulation was found to have significantly increased half-life and the anti-fungal properties of Thyme essential oils.

1. Introduction

The fact that fungal attacks cause a huge damage in agricultural outturn and jeopardize billions of dollars in this industry every year is undeniable. Mycotoxins can decrease the quality and value of the agricultural products and more importantly consumption of mycotoxin-contaminated products could lead to injury or death in animals and human (Yu Cleveland, Nierman, & Bennett, 2005; Zain, 2011). Numerous studies have been conducted in order to restrain the production of these compounds and especially aflatoxins (Groopman Kensler, & Wild, 2008; Jouany, 2007; Lowe & Arendt, 2004). The results of such investigations have shown that some herbal extracts and essential oils are capable of preventing the growth of the aflatoxin-producing fungi and therefore, could be safely used in food and pharmaceutical industries (Baratta et al., 1998; Beyki et al., 2014; Passone, Girardi, Ferrand, & Etcheverry, 2012; Ribeiro et al., 2013). Among these beneficial extracts, Thyme essential oils due to its anti-fungal, anti-viral and anti-bacterial properties has attracted a great deal of attention during the last decades (Kumar, Shukla, Singh, Prasad, & Dubey, 2008; Lopez-Leon, Carvalho, Seijo, Ortega-Vinuesa, & Bastos-González, 2005). Its properties have been previously investigated against various pathogenic agents such as *Botrytis cinerea*, *Salmonella enteritidis*, *Salmonella typhimurium*, *Yersinia enterocolitica*, *Shigella flexneri*, *Listeria monocytogenes*, *Shigella sonnei*, *Salmonella choleraesuis* and *Aspergillus niger* (Rota, Carraminana, Burillo, & Herrera, 2004; Soylu, Soylu, & Kurt, 2006).

On the other hand, essential oils are essentially volatile compounds and easily degradable at ambient temperature. As a result, increasing their activity and stability using different strategies such as encapsulation has always been considered crucial (Herrero, Carmona, Jiménez-Colmenero, & Ruiz-Capillas, 2014; Marques, 2010; Parris, Cooke, & Hicks, 2005). Nanogels, networks of synthetic/bio polymers, are divided into two different types; nano-hydrogels and nano-organogels (micelle nanogels). The former

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are composed of hydrogels at nanoscale which could absorb a significant amount of water easily (high degree of inflation in water) while the latter are hydrophobic nanogels with a tendency for oily substances. In fact, nano-organogels are micelle-like nanoparticles which form aggregates in contact with water while sequestrating their hydrophobic regions at the center. The hydrophobic region includes the oily substances attached to polymeric backbones containing functional groups such as carboxyl, amine, aldehyde and so on.

Nanogels in general have high capacity and consistency. Encapsulation of antimicrobial essential oils within an nano-organogel has other benefits such as controlled and sustained release of a certain amount of oils from the carrier e.g. nanogel as well. This would allow the nutrients to be exposed to specific and adequate amounts of essential oils for a longer time (Chacko, Ventura, Zhuang, & Thayumanavan, 2012; Herrero et al., 2014; Raemdonck, Demeester, & De Smedt, 2009).

In the past decade, a variety of polysaccharide nanoparticles have been investigated for encapsulation of compounds with biological activity. Among those polysaccharide structures, chitosan (CS) which is produced by partial deacetylation of chitin, and is a major compound of marines shells such as shrimp and crab has been frequently used mainly due its biocompatibility (Borges, Cordeiro-da-Silva, Romeijn, Amidi, & Borchard, 2006; Yu et al, 2005; Zain, 2011). Several studies have shown that CS-based nanogels could be used in tissue engineering, drug delivery and as carriers of various macromolecules (Dang & Leong, 2006; Raemdonck et al., 2009). In a study conducted by Zivanovic and Chi (2005), CS films enriched with different essential oils were found to possess more antimicrobial activity in comparison with CS films and free essential oils during a 5-day period. In fact, CS owes its unique features as a suitable carrier for pharmaceutical and other biological molecules to the existing amine groups on its polymeric structure and the resulting positive charges (Agnihotri, Mallikarjuna, & Aminabhavi, 2004; Pedro, Cabral-Albuquerque, Ferreira, & Sarmento, 2009; Uzun, 2006).

The present study was set to produce nanogel through the formation of amid linkages between the existing amino groups of CS (α(1-4)-2-amino-2-deoxy β-D-glucan), and the carboxyl group of BA (C_6H_5COOH). Moreover, the synthesized CS-benzoic acid (BA) nanogel was used to encapsulate Thyme essential oils in order to evaluate their potential synergistic effects in elimination of *Aspergillus flavus*.

2. Materials and methods

2.1. Materials

CS and BA were purchased from Sigma (Germany). Ethylene dichloride (EDC) was obtained from Fluka. Acetic acid, tween 80 and ethanol were purchased from Merck (Germany). The essential oils of *Thymus vulgaris* (Thyme) was provided by Barij Essence Co. (Iran) (Table 1). A. flavus (ATCC5004) was provided by Pasture Institute (Iran). Potato Dextrose Agar (PDA) and Potato Dextrose Broth (PDB) were purchased from Himedia (India).

2.2. Preparation and storage of A. flavus

The standard lineage of *A. flavus* (ATCC 5004) was obtained from the Pasture Institute of Iran and was cultured in falcon tubes containing PDA medium and stored in an incubator for 3–5 days to develop spores. For long-term preservation of spores, glycerol was added to the tubes.

Table 1
Composition of the thyme essential oils.

Component	Composition (ml/l)
R-pinene	0.4
R-terpinene	1.2
p-cymene	20
ç-terpinene	5.4
â-caryophyllene	1.5
Borneol	1.7
Terpinen-4-ol	0.7
Carvacrol	2.7
Thymol	65.7

2.3. Preparation and numeration of spores

The PDA medium was incubated with spores for 3–5 days in order to obtain fungal masses. Some spores were then transferred to a medium containing physiologic serum and Tween 80 and vortexed. The spores were counted using neobar lamella and a standard stock was prepared (150 spores/ml) and was stored at 4 °C.

2.4. Nanogel formulation and analysis

BA was coupled to CS by the formation of amide linkages through an EDC-mediated reaction following the method proposed by Chen, Lee, and Park (2003) and Yuta, Ikeda, Yamaguchi, Aoyama, and Akiyoshi (2003). Nanogels were prepared by adding 3.125 mol of CS to 100 ml of acetic acid (10 ml/l). Then, a mixture of 1.562 mol of parahydroxy-benzoic acid and 4.686 mol EDC in 10 ml ethanol was slowly added to the CS solution drop-wised and stored in dark for 24 h before use. The pH was adjusted to 8.5–9 by adding 0.1 N NaOH. The white nanogel sediments were washed by ethanol and centrifuged three times (5 min, 9000 × *g*). Nanogel particles were dispersed in acetic acid (10 ml/l), followed by a filtration through a 0.2 μm-pore size filter. To confirm the formation of nanogels, Fourier Transformation Infrared (FTIR) spectrum at 20 °C and at the range of 500–4000 $cm^{-1}$was performed using a FTIR-430 (Jascow, Japan).

CS-BA nanogel morphology was analyzed using scanning electron microscopy (SEM) on a Philips: XL30 (Netherlands, http://www.philips.com). One drop of freshly prepared nanogel suspension was deposited on carbon stickers, dried with air and coated with gold. Morphological characteristics of the CS-BA nanogels were also examined by high resolution Transmission Electron Microscopy (TEM) on an H-600; Philips (Netherlands, http://www.philips.com). Specimens were prepared as described by Beiki et al. (2014). The size distribution of the nanoparticles was checked by particle size analyzer.

2.5. Encapsulating of Thyme essential oils in CS-BA nanogel

The Thyme essential oils was dissolved in ethanol (1:1, v/v) and mixtures of nanogels (500 mg/l) and essential oils (5000 mg/l), were prepared by sonication (70 kHz) for 5 min. The encapsulation efficiency was measured by a Shimadzu spectrophotometer (Japan, http://www.shimadzu.com), based on the optical density spectra of the essential oils (400–650 nm). The spectroscopic readings were performed after precipitating the nanogel by centrifugation (10,000 × *g*, 15 min).

2.6. Determination of minimum inhibitory concentrations (MIC) of CS-BA nanogel, free and CS-BA nanogel encapsulated Thyme essential oils

Sterile Erlenmeyer flasks containing 25 ml of PDB medium, 150 spores and different concentrations of CS-BA nanogel, as well as free and CS-BA nanogel-encapsulated Thyme essential oils were