

Evaluation of *Zataria multiflora* Boiss. and *Carum copticum* L. Essential Oil Based Nanoemulsions in Inhibition of *Byssoschlamys fulva* Growth in Apple Juice

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ABSTRACT

Byssoschlamys fulva is a heat-resistant fungus whose growth causes significant economic losses since it is mostly implicated in the spoilage of processed fruits (e.g., apple juice). Essential oils have received an increasing attention for use in food products to prevent mold growths. In this study, the ultrasonic emulsification method was employed to prepare *Zataria multiflora* Boiss. Essential Oil (ZEO) and *Carum copticum* L. Essential Oil (CEO) based NanoEmulsions (NEs) separately using a mixture of components including *Z. multiflora* and *C. copticum* oils, each as an organic phase, as well as the surfactant Tween 80 at a ratio of 1:4 v/v. The *Z. multiflora* NanoEmulsion (ZEO-NE) formulated with a droplet diameter of 19.42 ± 1.66 nm and a PolyDispersity Index (PDI) of 0.377 and the *Carum copticum* NanoEmulsion (CEO-NE) with a droplet diameter of 15.13 ± 0.56 nm and a PDI of 0.253 was found to remain stable for more than 9 months at 25 °C. The *in vitro* evaluation revealed that the the ZEO-NE at a concentration of $5 \mu\text{L mL}^{-1}$ and CEO-NE at $25 \mu\text{L mL}^{-1}$ gave rise to inhibition effects of $84.23 \pm 0.006\%$ ($P < 0.05$) and $86\% \pm 0.012$ ($P < 0.05$) against *B. fulva*, respectively. The *in situ* assessment of the nanoemulsions in apple juice revealed a significant ($P < 0.05$) reduction in the inoculated fungal population. Results indicate that the ZEO-NE and CEO-NE can be used as antifungal compounds in beverages.

Keywords: Antifungal compounds, Polydispersity index, Spoilage molds, Ultrasonic emulsification.

INTRODUCTION

Apple is a fruit with a great potential for industrial processing and of much commercial value. Its juice is one of the most favorable products among consumers as a source of many beneficial components such as antioxidant substances (Longhi *et al.*, 2014). One challenge facing the apple juice industry, however, is the presence of toxigenic and spoilage molds that can survive pasteurization (Tremarin *et al.*, 2015; Longhi *et al.*, 2014).

Byssoschlamys sp. is almost uniquely associated with food spoilage, particularly with the spoilage and depreciation of heat-processed acid foods such as fruit juices and fruit-based products since they can grow in acidic environments and under partial oxygen pressures (Kotzekidou, 2014). Furthermore, some species have been reported as mycotoxin producers. *Byssoschlamys* is mostly implicated in the spoilage of apple juice since clarified apple juice can be easily spoiled by *B. fulva* even at very low initial concentrations (Sant'Ana *et al.*, 2010). Ascospores of *B. fulva* are

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extremely heat resistant and are able to withstand processing treatments normally applied to certain fruits and potentially toxic products (Kotzekidou, 1997; Kotzekidou, 2014). The antifungal properties of Essential Oils (EOs) have been known for a long time and studies have been conducted to investigate their effects on postharvest phytopathogens (Kouassia *et al.*, 2012; Basak and Guha, 2017b). Plant essential oils are reportedly rich sources of bioactive compounds like benzaldehyde, carvacrol, carvone, cineole, cinnamaldehyde, citral, cymene, estragole, eugenol, geraniol, limonene, menthol, pinene, terpenene, terpineol, thymol, and vanillin all of which exhibit antibacterial, antifungal, anti-giardia, antioxidant, anti-ulcer, anti-yeast, anti-atherosclerotic, insecticidal, and insect-repellent properties (Ghosh *et al.*, 2014; Basak and Guha, 2015). *Zataria multiflora*, which is a thyme like plant belonging to the Lamiaceae family that is extensively used in a wide variety of fields in its native region. Modern pharmacological studies have shown that *Zataria* has a wide range of biological properties including antimicrobial and anti-oxidative. In this context, the *Zataria multiflora* Essential Oil (ZEO) has played an important role in pharmaceutical as well as in food industries (Bordbar *et al.*, 2017). *Carum copticum* (Ajowan) is an annual, grassy plant belonging to the Apiaceae family, which grows in India, Iran, and Egypt (Goudarzi *et al.*, 2010). It has been reported that *C. copticum* Essential Oil (CEO) has diuretic, carminative, analgesic, anti-dyspnoea, antibacterial, antifungal, anthelmintic, bronchodilator, hypocholesterolaemic and anti-inflammatory compounds (Gilani *et al.*, 2005; Goudarzi *et al.*, 2010; Mohagheghzadeh *et al.*, 2005).

The major problem with the antimicrobial components of plant essential oils is their sparing solubility in aqueous phases. Hence, these lipophilic antimicrobials are seen at low concentrations in the solvent phase where pathogens exist (Ghosh *et al.*, 2014). NanoEmulsions (NEs) serve as delivery

agents for hydrophobic bioactive compounds such as antimicrobial compounds. (Ghosh *et al.*, 2013; Basak and Guha, 2017a).

Nanoemulsions are liquid-liquid dispersions of two immiscible liquids (e.g., oil and water) with one phase dispersed in the second in the form of droplets that contain nanodroplets with mean droplet radii of around $10 \leq r \leq 100$ nm (Weiss *et al.*, 2009; Sugumar *et al.*, 2014; McClements and Rao., 2011). Nanoemulsions can be prepared via such energy intensive methods as ultrasonication, microfluidization, shearing, and homogenization. Ultrasound cavitation uses high frequency (greater than 20 kHz) sound waves to reduce the emulsion droplet size (Ghosh *et al.*, 2014). The main objectives of the present study were to formulate individual nanoemulsions of *Z. multiflora* and *C. copticum* oils by ultrasonic emulsification and investigate the antifungal activity of the nanoemulsions thus prepared against *B. fulva*. Also, we aimed to evaluate the nanoemulsions for apple juice preservation against fungal spoilage.

MATERIALS AND METHODS

ZEO and CEO were purchased from Zardband Pharmaceuticals-Medicinal Plants Production Company (Tehran, Iran). Sesame oil was obtained from a local supermarket (Isfahan, Iran). Tween 80 was purchased from Merck Chemical Company (Germany).

Microorganisms and Preparation of Spore Suspensions

Lyophilized pure cultures of *Byssoschlamys fulva* PTCC 5062 were obtained from Persian Type Culture Collection of Iranian Research Organization for Science and Technology (IROST), Tehran, Iran. To activate the lyophilized cultures, the ampoule containing *B. fulva* was broken under sterile conditions, cultured in the Potato Dextrose Agar medium (PDA,

Merck, Germany) at pH 5.6, and incubated for 5-7 days at 30°C.

The preparation of *B. fulva* spores was started by pre-sporulation in Petri dishes containing the PDA at pH 5.6 for 7 days at 30°C. The collected spores were then added to plates containing the Malt Extract Agar medium (MEA, Merck, Germany) and incubated at 30°C for 30 days. After this period, 1.0 mL of sterile distilled water was added to each plate which was then scraped using a rubber spatula. The entire plate content was filtered through four layers of sterile gauze and centrifuged at 2,000×g for 15 minutes. Spore counts and the absence of mold hyphae were determined under the microscope. The final suspensions were prepared with the precipitate in a minimum volume of water sufficient to obtain highly concentrated suspensions (around 10⁵ spores mL⁻¹). The *B. fulva* suspensions were transferred to a flask and kept at 4°C until use (Tremarin *et al.*, 2015).

Preparation of Nanoemulsions

Oil-in-water nanoemulsions of ZEO and CEO were prepared in two stages according to the method described in Ghosh *et al.* (2014); that of ZEO-NE was formulated using 6% ZEO, 24% Tween 80, and 70% Water (based on a v/v proportion) and that of CEO-NE was formulated using 4.5% CEO, 1.5% sesame oil, 24% Tween 80, and 70% Water (based on a v/v proportion). Coarse emulsion of each essential oil was prepared by mixing the oil and the surfactant followed by water addition and agitation using a magnetic stirrer at 250 rpm for 10 minutes. Then, the coarse emulsions were subjected to ultrasonic emulsification using a 20 kHz Sonicator (Ultrasonics arico, Iran) with an utmost power output of 300W. Heat is normally generated during the process of high energy emulsification as observed during the ultrasound irradiation. The heat thus generated was reduced by keeping the emulsion sample in a comparatively bigger beaker containing ice.

Nanoemulsion Particle Size Measurements

The droplet size distributions and PolyDispersity Indices (PDI) of the ZEO-NE and CEO-NE were determined using a Zetasizer nano (Model ZEN3600, malvern, England). Droplet size was determined using the Dynamic Light Scattering technique (DLS).

Nanoemulsion Turbidity Measurements

Turbidity analysis of the formulated nanoemulsions was carried out by measuring the absorbance of undiluted samples at a wavelength of 600 nm using a UV-Visible Spectrophotometer (UV-Visible Spectrophotometer 2100, unico, USA).

Stability of the Nanoemulsions

The nanoemulsions thus prepared were subjected to centrifugation at 10,000 rpm (HERMLE Z36HK, German) for 30 minutes to study their resistance to phase separation. Intrinsic stability was investigated by storing the nanoemulsions at room temperature before visual observation for phase separation, creaming, or flocculation.

In vitro Antifungal Activity of the Nanoemulsions on *Byssoschlamys fulva*

The nanoemulsions were evaluated for their antifungal activity using the contact phase method (Soylu *et al.*, 2010). For this purpose, different concentrations of each nanoemulsion (5, 25, 50, 75, and 100 µL mL⁻¹) were prepared in 20 mL of PDA melted at a temperature of 40°C. After PDA solidification, *B. fulva* was immediately inoculated by placing a piece of the fungus colony (4 mm diameter) cut with a sterile cork borer from the edge of actively growing cultures of *B. fulva* in the center of each plate and used as a source of



inoculum. Plates without nanoemulsions were used as control. All the plates were then incubated for 5–7 days at 30°C (Al-Reza *et al.*, 2010). When fungal growths were observed on the whole surface of the control plate, inhibition concentrations of nanoemulsions were determined by measuring the colony diameter grown in the treatment plates. The anti-fungal activity of each nanoemulsion was determined using the following formula due to Lee *et al.* (2007):

$$IP = (d_c - d_t) \times 100 / d_c \quad (1)$$

Where, *IP* is inhibition percentage, d_c is the colony diameter in the untreated plates (control), and d_t is the diameter size in the plates treated with individual nanoemulsions at each concentration tested. Finally, the values of percent inhibition were reported for the nanoemulsions.

The fungicidal/fungistatic nature of the nanoemulsions was tested by observing the revival of the growth of an inhibited mycelium disc following its transfer to the non-treated PDA. A fungicidal effect was defined as no growth, whereas a fungistatic effect was defined as the temporary inhibition of microbial growth. The agar disc of *B. fulva*, which had failed to grow, was transferred onto the agar medium lacking the nanoemulsions and then incubated at 30 °C. The activity of each concentration of the nanoemulsions was considered fungicidal if the pathogen did not grow, or fungistatic if the pathogen growth occurred (Zamani-Zadeh *et al.*, 2014).

Turbidity Measurement

Apple juice samples were heat treated as described in previous section. Briefly, the juice tubes were divided into three equal groups (n= 21). To the first group, 5 $\mu\text{L mL}^{-1}$ of the ZEO-NE, and to the second, 25 $\mu\text{L mL}^{-1}$ of the CEO-NE was added, and the last group was left blank to be used as the control. All the groups were kept at 25°C for 21 days. Using a turbidimeter (martini Mi 415), the turbidity of

each sample was measured in NTU units on days 0, 1, 2, 3, 7, 14, and 21.

Effects of ZEO-NE and CEO-NE on *B. fulva* Growth Inhibition in Apple Juice

The juice obtained from a commercial clarified apple juice concentrate (70 °Brix) was reconstituted in commercial non-carbonated mineral water using a 1:7 dilution (v/v) (Muñoz *et al.*, 2012). The juice sample was poured into well-capped glass tubes 20 mL in volume and placed in a water bath (Memert, Germany) for heat treatment to raise the juice temperature to 90°C (within approximately 1-2 minutes) at which it was maintained for 2 minutes (Pala and Toklucu., 2013). After pasteurization, the samples were immediately cooled to room temperature by plunging into an ice water bath (Pala and Toklucu, 2013).

The juice tubes were divided into three equal groups (n= 21). All the groups were inoculated with 10^5 spores mL^{-1} of *B. fulva*. To the first group, 5 $\mu\text{L mL}^{-1}$ of the ZEO-NE, and to the second, 25 $\mu\text{L mL}^{-1}$ of the CEO-NE was added, and the last group was left blank to be used as the control. All the groups were then immediately incubated at 25°C for 21 days. Samples were then taken from each group on days 0, 1, 2, 3, 7, 14, and 21 to determine *B. fulva* populations in each tube using the standard plate count method.

Turbidity Measurement

Apple juice samples were heat treated as described in previous section. Briefly, the juice tubes were divided into three equal groups (n= 21). To the first group, 5 $\mu\text{L mL}^{-1}$ of the ZEO-NE, and to the second, 25 $\mu\text{L mL}^{-1}$ of the CEO-NE was added, and the last group was left blank to be used as the control. All the groups were kept at 25°C for 21 days. Using a turbidimeter (martini Mi 415), the turbidity of each sample was measured in NTU units on days 0, 1, 2, 3, 7, 14, and 21.

Statistical Analysis

Statistical Analysis Of the data Variance (ANOVA) was performed using the SAS statistical software and the means were separated using the Least Significant Difference (LSD) test at $P \leq 0.05$. All the experiments were performed in three replicates.

RESULTS AND DISCUSSION

Particle Size Distribution and Polydispersity Index

Droplet size and PolyDispersity Index (PDI) of the ZEO-NE and CEO-NE after 30

minutes of ultrasonic emulsification are shown in Figures 1(a-b), respectively. Clearly, the ZEO-NE recorded a droplet size of 19.42 ± 1.66 nm with a *PDI* of 0.377 while the droplet size of the CEO-NE was 15.13 ± 0.56 nm with a *PDI* of 0.253. The appearance of the emulsion changed from milky white to transparent with increasing sonication time from 0 to 30 minutes (Figure 2) Moreover, a direct correlation was observed between the steady decrease in droplet size and emulsification time. Previous study has shown that emulsion appearance is highly dependent on particle size and, further, emulsions become transparent when droplet diameter falls below a critical value (i.e., $d < 90\text{--}100$ nm) (Weiss *et al.*, 2009 ; Basak and Guha, 2017a).

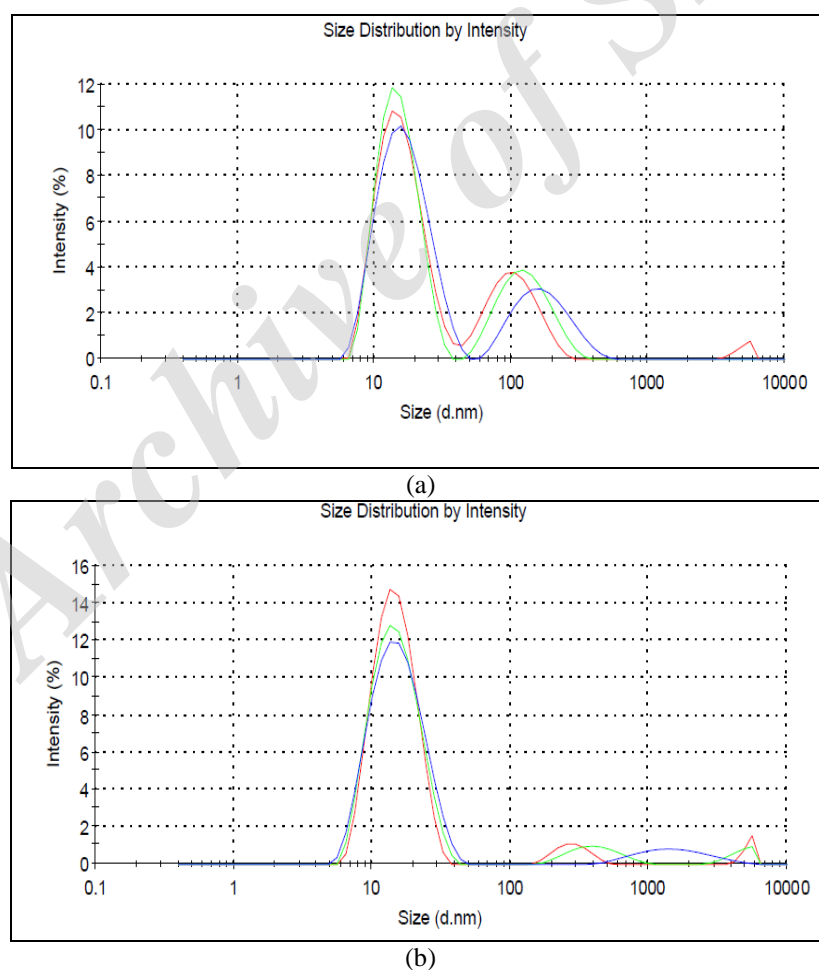


Figure 1. Light scattering image showing (a) droplet size distribution of the ZEO-NE, (b) droplet size distribution of the CEO-NE.

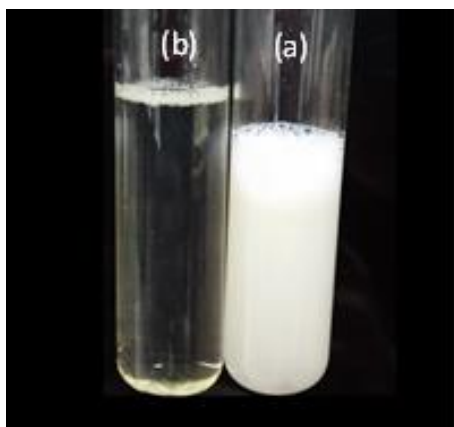


Figure 2. Visual appearance of nanoemulsion: (a) Before, and (b) After (30 minutes) sonication.

Polydispersity is a measure of droplet homogeneity and stability in the emulsion. The relatively lower values of PDI for nanoemulsions can be correlated with a higher stability upon storage (Sari *et al.*, 2014). The small droplets obtained by the emulsion technique offer a larger surface area that allows for the rapid penetration of active components (Sugumar *et al.*, 2014). In this study, Tween 80 was used as the surfactant for its high hydrophilic-lipophilic balance (HLB-15), which makes it favorable

for oil-in-water emulsions. Moreover, being a small molecule surfactant, Tween 80 is comparatively more effective in minimizing droplet diameter than most polymers which are rapidly adsorbed onto the droplet surface (Ghosh *et al.*, 2013). It has been reported found that the factors determining the final droplet size of the nanoemulsion are emulsifier type, concentration, and emulsification time such that droplet size decreases with increasing emulsification time and surfactant concentration (Ghosh *et al.*, 2013; Ghosh *et al.*, 2014).

Optical Property

Emulsion turbidity is expressed as the absorbance of the sample at 600 nm sonicated for 0 to 30 minutes. Quantitative measurements of the optical transparency of nanoemulsions in both visible and ultraviolet wavelengths are shown through transmission measurements. The effect of sonication time on the formulated nanoemulsions for different time intervals is shown in Figure 3. A sharp decline was observed in absorbance with increasing emulsification time. Turbidity of the formulated nanoemulsion was size dependent (Ghosh *et al.*, 2014).

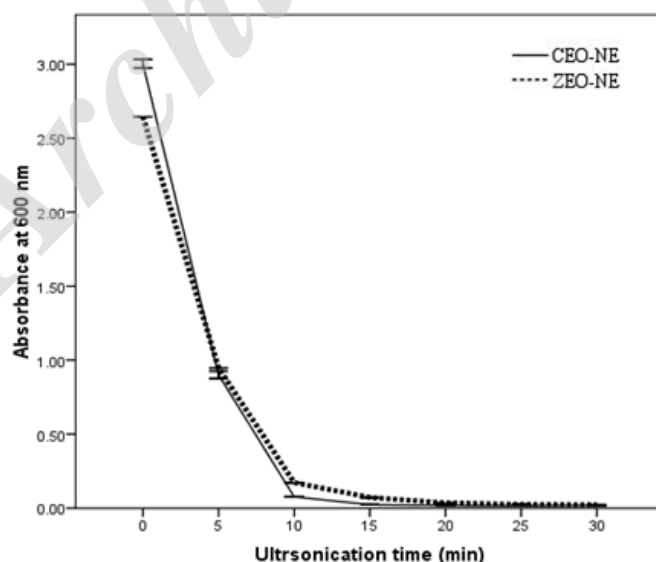


Figure 3. Relationship between light absorbance and sonication time. (* Each number is based on three replicates).

After sonication, the appearance of the nanoemulsion changed to transparent with light scattering, which might have been due to the Rayleigh scattering effect caused by the nano-sized droplets (Sugumar *et al.*, 2014).

Emulsion Stability

All the nanoemulsions were stable after centrifugation at 10,000×g for 30 minutes, and no phase separation or creaming was observed even after more than 9 months of storage at room temperature. Eid *et al.* (2013) reported that the optimal storage condition for olive oil nanoemulsion is 4°C for 6 months of storage.

In vitro Antifungal Activity of the Nanoemulsions

Antifungal activities of the ZEO-NE and CEO-NE were tested against *B. fulva*. Both nanoemulsions were capable of preventing the radial growth of *Byssochlamys fulva* on PDA plates at all experimental concentrations (5, 25, 50, 75, and 100 µL

mL⁻¹). The highest (84.23%±0.006 and 86%±0.012) (P< 0.05) inhibition rates were obtained at a concentration of 5 µL/mL for the ZEO-NE and 25 µL/mL for the CEO-NE. The CEO-NE showed a greater inhibitory effect than the ZEO-NE at all the concentrations tested (Figures 4 and 5).

Results indicated that, by increasing CEO-NE concentration, there were no significant differences in prevention percentage. This can be related to the fact that the differences between the concentrations were not sensible. Shahabi *et al.* (2017) reported that Minimum Inhibitory Concentration (MIC) of ZEO-NE against *Listeria monocytogenes* and *Salmonella Typhimurium* was 2,500 and 5,000 µg L mL⁻¹, respectively.

The difference between the inhibition percentages of the ZEO-NE and CEO-NE might be attributed to differences in the fatty acid profiles of the essential oils of the two nonemulsions. The ZEO contained monoterpenes, *p*-cymene, thymol, linalool, carvacrol, and 1,8 cineole (Zamani-Zadeh *et al.*, 2014), whereas the major components of the EO of the *C. copticum* fruits were thymol (54.50%), *c*-terpinene (22.96%), *p*-cymene (19.38%), and carvacrol (0.46%)

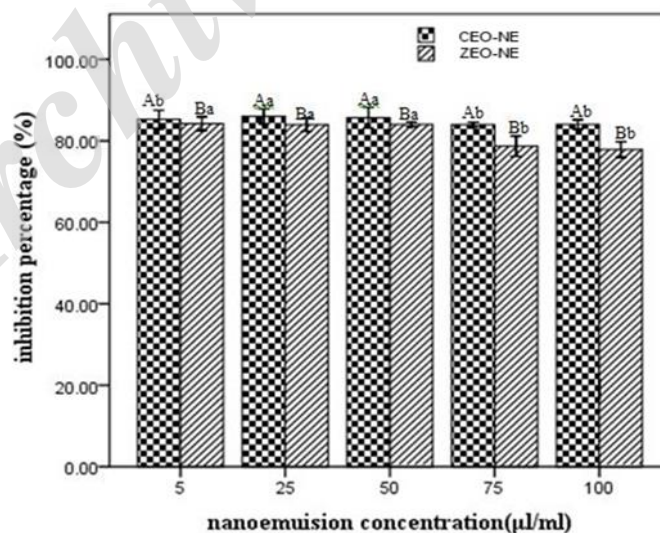


Figure 4. Inhibitory effects of the ZEO-NE and CEO-NE nanoemulsions on *Byssochlamys fulva*. (* Each number is based on three replicate plates; ** Mean values followed by different capital letters within the column and different lowercase letters between columns are significantly different according to different letters within the Least Significant Difference Test (P < 0.05).

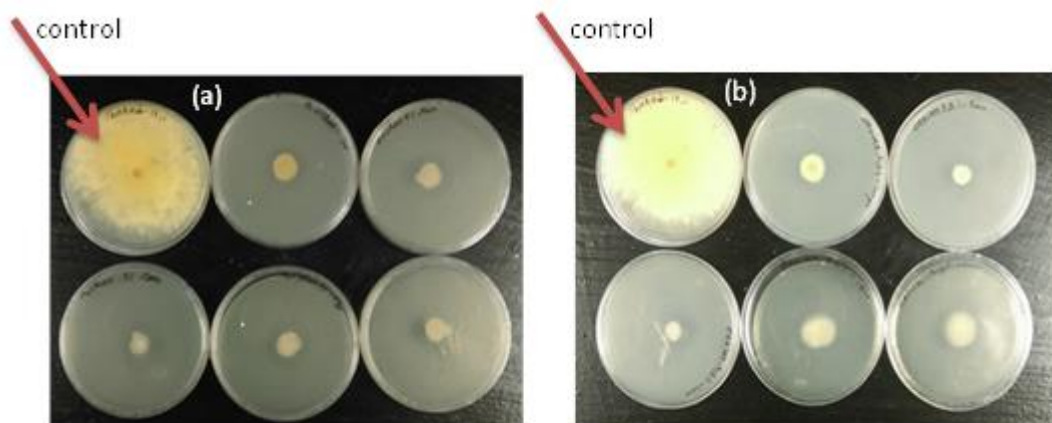


Figure 5. Inhibition percentages for different concentrations of the nanoemulsions against the fungus *Byssoschlamys fulva*: (a) ZEO-NE, and (b) CEO-NE, the diameters of the colonies of both the control and treated samples were measured once fungal growths had covered the whole surface of the control plate. The values obtained were then used to evaluate the inhibition percentage of each nanoemulsion according to the formula(1) (these plates were photographed after 10 days).

(Mohagheghzadeh *et al.*, 2007).

In the current study, the fungicidal/fungistatic effects of the nanoemulsions were determined after mold plugs were transferred from the nanoemulsions treated with the PDA media to the nontreated media; and when mycelial growths were observed, this indicated the fungistatic effects of the nanoemulsions on *B. fulva*. In many cases, the antifungal activity of the essential oils results from the interaction between different kinds of compounds such as phenols, aldehydes, ketones, alcohols, esters, ethers, and hydrocarbons present in these oils. Several studies have found that a number of these compounds exhibited significant antifungal properties when tested separately (Bassolé and Juliani, 2012).

Effects of the Nanoemulsions on Apple Juice Turbidity

In order to evaluate the effects of ZEO-NE and CEO-NE on the appearance of apple juice, the samples were examined for changes in their turbidity over a three-week period. No significant differences were

observed between the treatment and the control samples at time zero (i.e., immediately after the addition of the nanoemulsions), nor on days one, two, three, or seven. On the fourteenth day, however, significant differences appeared between the samples treated with the CEO-NE and the control. An even significantly greater difference ($P < 0.05$) was observed between the samples treated with the ZEO-NE and the control. Finally, both nanoemulsions were noted to reduce apple juice turbidity after twenty-one days (Figure 6).

Effects of the ZEO-NE and CEO-NE Nanoemulsions on *B. fulva* Growth Inhibition in Apple Juice

According to the results, the highest inhibitory effects were achieved at a concentration of $5 \mu\text{L mL}^{-1}$ for the ZEO-NE and $25 \mu\text{L mL}^{-1}$ for the CEO-NE. These concentrations were used to evaluate the antifungal effects of the two nanoemulsions on *B. fulva* inoculated in the apple juice medium. At time zero i.e., immediately after inoculation of *B. fulva*, no differences were observed between the control and the

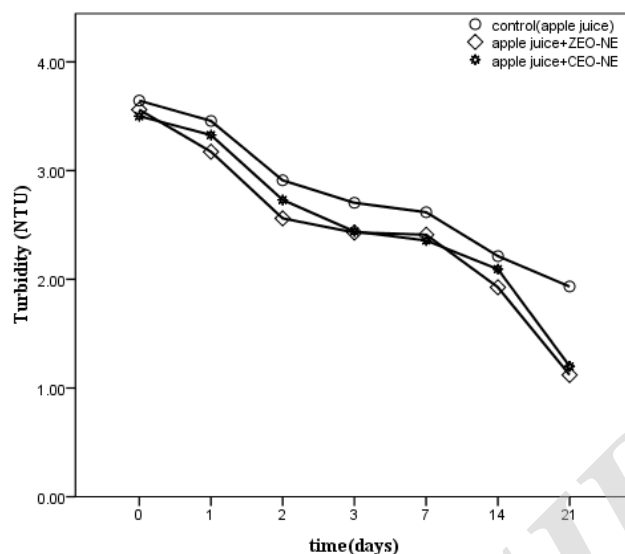


Figure 6. Effects of the ZEO-NE and CEO-NE on apple juice turbidity. (* Each number is based on three replicates).

treatment samples; however, significant differences ($P < 0.05$) emerged between the treatment and control samples throughout the storage period (Figure 7).

CONCLUSIONS

This study investigated the preparation of ZEO-NE and CEO-NE and the possibility of

their use to enhance the shelf-life of apple juice. The results obtained revealed that the ZEO-NE and CEO-NE were capable of inhibiting the growth of the heat-resistant mold *B. Fulva*, which is the main cause of spoilage in fruit juices, especially in apple juice. Further study is, however, required to verify their efficiency.

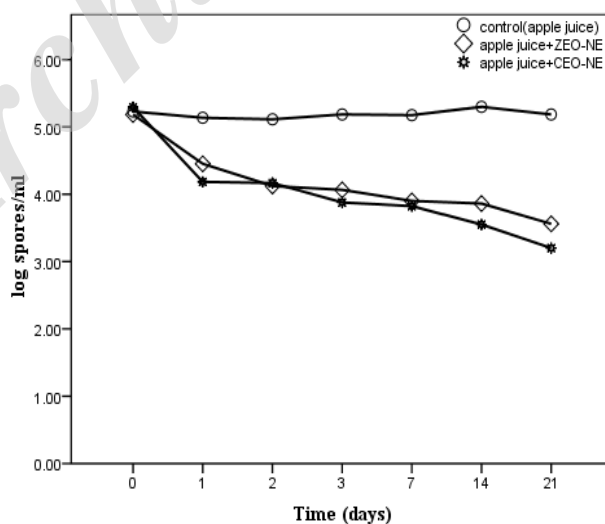


Figure 7. Antifungal effects of ZEO-NE and CEO-NE against *Byssoschlamys fulva* in apple juice. (* Each number is based on three replicate plates).



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ارزیابی نانوامولسیون اسانس آویشن و زنیان در جلوگیری از رشد قارچ بایسوکلامیس فولوا در آب سیب

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چکیده

قارچ بایسوکلامیس فولوا با تولید آسکوسپورهای مقاوم به حرارت، در طی پاستوریزاسیون آب سیب زنده میماند و یکی از عوامل اصلی فساد قارچی آب سیب است. امروزه تمایل زیادی به استفاده از اسانس های گیاهی به عنوان محافظت کننده های طبیعی در مواد غذایی وجود دارد. وارد کردن مستقیم



اسانس گیاهان در مواد غذایی به دلیل طبیعت آب‌گریز، فعال و فرار مولکول‌های زیست فعال آن محدودیت‌های تکنولوژیکی دارد. هدف از انجام این پژوهش، تولید نانوامولسیون روغن در آب اسانس آویشن و اسانس زنیان با استفاده از امواج فراصوت است. نانوامولسیون هریک از اسانس‌های زنیان و آویشن با فرمولاسیون اسانس زنیان و اسانس آویشن به عنوان فاز روغنی، توپین ۸۰ به عنوان امولسیفایر با نسبت 1:4 (v/v) روغن به سورفاکتانت و آب، تهیه شدند. اندازه ذرات نانوامولسیون زنیان $19/42 \pm 1/66$ نانومتر با PDI 253/0 و برای ذرات نانوامولسیون آویشن $19/42 \pm 1/66$ نانومتر با PDI 377/0 به دست آمد. بالاترین میزان بازدارندگی $0/05 < P < 0/05$ (P) و $0/12 < P < 0/05$ به ترتیب در غلظت 5 $\mu\text{l/ml}$ نانوامولسیون آویشن و غلظت 25 $\mu\text{l/ml}$ نانوامولسیون اسانس زنیان علیه قارچ بایسوکلامیس فولوا به دست آمد. با توجه به نتایج به دست آمده از نانوامولسیون اسانس زنیان و نانوامولسیون اسانس آویشن می‌توان به عنوان یک عامل ضد قارچ در صنعت نوشیدنی‌ها استفاده کرد.