JFBE 2(2): 147-154, 2019

Journal of Food and Bioprocess Engineering

Journal homepage: https://jfabe.ut.ac.ir

Original research

Development of nanoemulsion-based antimicrobial activity of cinnamon prepared with soy protein isolate-lecithin

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ABSTRACT

The objective of the present research was to develop cinnamon oil nanoemulsions using soy protein isolate and lecithin as food grade natural emulsifiers. For this purpose, stable cinnamon oil nanoemulsion (droplet diameter of 141.2 nm) prepared by ultrasonic emulsification. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of free and nanoemulsified cinnamon oil against *Escherichia coli, Enterococcus faecium, Bacillus cereus, Salmonella typhi, Pseudomonas aeruginosa* and *Staphylococcus aureus* were determined. According to the results, *E. coli* was more resistant to cinnamon. Also, the nanoemulsion exhibited high inhibitory effects than the pure essential oil. The antibacterial activity of free and nanoemulsified cinnamon oil against *E. coli, E. faecium* was evaluated by measuring nucleic acid, protein and potassium leakage from the cells. Investigation of the kinetics of microbial deactivation showed that after 24 h incubation, recovery was observed for all treatment and recovery rate of nanoemulsion treatment was slower than that of free cinnamon oil treatment.

Keywords: Cinnamon, Nanoemulsion, Killing kinetics, Cell membrane, Potassium leakage

Received 8 May 2019; Received 15 October 2019; Accepted 28 October 2019

1. Introduction

Essential oil elements or spice essential oils (EOs) are the secondary metabolite of herbal plants naturally occurring antimicrobials and antioxidant properties, as well as food flavoring agents and, have been reported to be used as an antimicrobial agent in food products (Azhdarzadeh et al., 2017). Cinnamon (*Cinnamomum zeylanicu*) is an agent of evergreen tree and shrubs and belongs to *Lauraceae* family containing various essential oils usually grown in South and South-East Asia, which is used for their medicinal properties. Cinnamaldehyde is the main compound accounting for more than 60% of all the components in the cinnamon oil that was found to have anti-inflammatory and antimicrobial activity.(Ranjbar et al., 2007; Shahverdi et al., 2007).

The greatest challenge in using essential oil as a bioactive compound is its low solubility in water and short-term stability. In recent years, various studies have been conducted to solve this problem and various solutions have been proposed. In this regard, nanoemulsion is one of the emergents and encouraging technologies that created nanosize oil droplets in the surfactant-containing aqueous phase, being capable of solving these problems. High energy methods such as sonication are commonly used to produce nanoemulsions.(Almadiy et al., 2016; Ghosh et al., 2013; Shams & Sahari, 2016; Topuz et al., 2016; Xue et al., 2015).

Forming nanoemulsions, emulsifiers and surfactants play an important role. These compounds decrease the interfacial tension between the aqueous and oil phase and maintain the nanoemulsions. Recently, the use of polysaccharide or protein (food grade ingredients) instead of synthetic surfactant (the potential risk of toxicity) in the preparation of the nanoemulsions has increased. The nanoemulsions having small particle size (dimensions as low as 200 nm) may develop interactions between the active ingredients with biological membranes, improving antimicrobial activities. Therefore, we can use them as food preservatives in the food industry. In addition, nanoemulsions due to low turbidity and high kinetic stability, being suitable for a wide range of commercial use, are used as preservatives in foods, beverages and pharmaceuticals (Hu et al., 2016; Lu et al., 2017; Ma et al., 2016; Topuz et al., 2016).

Some studies have reported that the antimicrobial activity of EOs has decreased after nanoemulsions (Jumaa et al., 2002; Sznitowska et al., 2002). For example, the use of pea protein and soy lecithin for the production of carvacrol nanoemulsion, only slightly enhanced antimicrobial activity of carvacrol against *E. coli* (Donsì et al., 2012).

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To our knowledge the effect of cinnamon oil nanoemulsion on bacterial pathogens have not been studied. Therefore, the purpose of this study was to evaluate the antimicrobial features of stable cinnamon oil nanoemulsion prepared with soy protein isolate and lecithin against *E. coli* and *E. faecium* by measuring the release of nucleic acids and potassium (cell components), and the kinetics of inactivation of bacterial. This research reports new and important information about the mode of action of cinnamon nanoemulsions on an important bacterial species by evaluating the release of nucleic acids and potassium (cell components), and the kinetics of inactivation of bacterial growth**.**

2. Material and Methods

2.1. Materials

Soy protein isolate (SPI) and soy lecithin of food grade were purchased from Safir gosht Co (Shiraz, Iran) and Dairy Co (Shiraz, Iran), respectively. Cinnamon oil was purchased from Zarband Co (Tehran, Iran). Sunflower oil was purchased from local supermarket and used without further purification. Analytical grade hydrochloric acid (ACS reagent, 37%) and sodium hydroxide (ACS reagent, >98.0) were purchased from Sigma chemical company. To prepare of all solutions, deionized and distilled water was used.

2.2. Microorganisms and culture media

The Persian type culture collection of the Iranian Research Organization for Science and Technology (IROST, Tehran, Iran) were provided the bacteria used in this research Gram-negative (*Escherichia coli* ATTC9763, *Pseudomonas aeruginosa* and *Salmonella typhi* PTCC1609) and Gram-positive (*Enterococcus faecium*, *Bacillus cereus* and *Staphylococcus aureus* ATCC25923). Culture media of nutrient agar (NA) and Mueller Hinton agar (MHA) and peptone water were purchased from Merck Chemical Co. (Darmstadt, Germany).

2.3. GC-MS analysis

The GC-MS analysis (Agilen 6890A, China) equipped with mass selective detector was used to identified the component of cinnamon oil. Carrier gas used was helium at constant flow rate of 1 ml/min and an injection volume of 0.5µl was employed, injector temperature 250 °C, Ion-source temperature 280 °C. The oven temperature was programmed from 50 °C (isothermal for 5 min), with an increase of 3 °C/min, to 280 °C and held for 10 min at isothermal (280°C). Total GC running time was 90 min (Khadri et al., 2008).

2.4. Solution preparation

By dispersing SPI powder in deionized water and stirred for 5 min, the stock solution of SPI (3 wt%) was prepared. Then the solution exposed to ultrasonic bath 500 W (Elmasonic S 60 H, Germany) for 5 min at 50 °C and then centrifuged at 5000 g for 10 min. This solution was stored at room temperature for 24 h to ensure complete dissolution of SPI (Noshad et al., 2016)

2.5. Emulsions preparation

The method of Noshad et al. (2016) was used to prepare the emulsion. Briefly, first homogenizing 2 wt% cinnamon oils with 98% aqueous emulsifier solution (1% w/v SPI and 0.05% wt lecithin) in high- speed blender (UltraTurrax T-25, IKA Instruments, Germany) followed by sonication (probe-type ultrasonic) for 2 min at a frequency of 25 kHz, amplitude of 60% (UP 200H, Germany). Also, the solution container was placed in cooling bath during sonication to maintain the temperature constant. A series of preliminary experiments were carried out to select this formulation (Noshad et al., 2016).

2.6. Droplet size measurement

Droplet size and particle size distribution of nanoemulsions were measured by the Dynamic Light Scattering (DLS) instrument (ZEN3600, England). To have a specified particle count range, the samples were diluted with deionized water (1:100).

2.7. Antimicrobial activity

2.7.1. Determination of MIC and MBC

The microbroth dilution method was used to evaluate the minimum inhibitory concentration (MIC) of pure and nanoemulsified essential oils (Swathy et al., 2018). Tryptic soy broth (TSB) as diluent was used to dilute the nanoemulsion and control samples at various concentrations. The culture media was diluted to 10^6 colony- forming unit (CFU)/mL bacteria using TSB. To well of sterile 96-well microtiter plates were first added 120 µL of bacterial culture followed by 120 µL of the antimicrobial sample. For positive and negative control, TSB with and without bacterial culture was used. Absorbance of wells at 625 nm was measured using a (Pishtaz Teb, Iran) Microplate Reader before and after 24 h incubation at 37 °C. The cinnamon oil concentration that allowed less than or equal to a 0.05 raise in absorbance after 24 h incubation defined MIC. To evaluate the minimum bactericidal concentration (MBC), 20 µl of the broth from wells containing no growth were plated onto Mueller Hinton Agar (MHA) and were incubated at 37 °C for 24 h. The lowest cinnamon oil concentration corresponding to MBC.

2.7.2. Killing kinetics assay

The decrease in the numbers of CFU /mL over 72 h was used to measure the antibacterial activity of the nanoemulsified and pure cinnamon oils against *E. coli* and *E. faecium*. For this purpose, 4.5 ml of bacteria media (the absorption of it at the OD_{600} was 0.5- 0.6 cm^{-1}, that indicates to the exponential phase of bacterial growth) was added to 0.5 ml of sterile normal saline solution (0.085%) consist of the MIC and MBC values of the nanoemulsified cinnamon oil and equal amount of pure cinnamon oil. All samples were kept at 37 °C under agitation condition. Then for analysis,100 µl portion were removed and diluted several times for colony counting after, 0, 4, 8, 24, 48, 72 h from the time of incubation (Moghimi et al., 2016).

2.7.3. Integrity of the cell membrane

The method described by Moghimi et al. (2015), was used to measuring the release of cell constituents into supernatant which indicated the integrity of *E. coli* and *E. faecium*. First, make 150 ml of bacterial culture then incubated at 37 °C under agitation until the

OD_{600} of the bacterial suspension achieved 0.5 to 0.6 cm^{-1} (corresponds to the exponential phase of bacterial growth). The medium was centrifuged for 10 min at 4000 g, washed three times with sterile normal saline (0.085% NaCl). Then, 5 ml of microbial cell suspension (containing approximately 10^{12} to 10^{13} CFU/ml) comprising pure or nanoemulsified cinnamon oil were then incubated at 37°C under agitation for 24h. To compare the pure and nanoemulsified cinnamon oil, the equal concentration of cinnamon oil that was available in MIC and MBC concentrations of the nanoemulsion were used. The mixture was centrifuged for 10 min at 4000 g and 10 µl supernatant was removed to measure the UV absorption at 260 nm using a UV-visible spectrophotometer (WPA, England)(Moghimi et al., 2016).

2.7.4. *Potassium release assay*

The method explained by Bouhdid et al. (2010), with some modification was used to evaluate the concentration of free potassium ions in the bacterial suspensions. First, 60 ml cultures were grown in NB medium and as long as the OD_{600} of bacteria reached about 0.5 cm^{-1} was incubated at 37°C under agitation. The medium was centrifuged for 10 min at 4000g, washed three times with sterile normal saline (0.085% NaCl). By mixing 1 ml bacterial suspension with 9 ml normal saline solution containing either pure or nanoemulsified cinnamon oil, the cell suspension was prepared. 10 ml of cell suspension was then incubated at 37°C under agitation for 60 min, with cinnamon oil concentration equal to the control and the MIC and the MBC. Using an atomic absorption spectrophotometer to evaluate the potassium concentration in the supernatant (Bouhdid et al., 2010).

2.8. *Statistical analysis*

The one-way analysis of variance (ANOVA) test was carried up using the SPSS 16.0 statistical analysis software (SPPS Inc., Chicago, USA). To evaluate the difference of mean values, the Duncan test was used at the significance level of 0.05.

3. Results and Discussion

3.1. *Composition of cinnamon oil*

The concentration of the volatile components in cinnamon oil was analyzed with GC MS. Peak area normalization method was used to calculate the relative contents of the components. The concentration of the volatile components in cinnamon oil were as follows (shown as percentage peak areas of GC-MS), cinnamaldehyde (66.81%), trans- cinamyl acetate (8.52%), eugenol (4.73%), 1,8- cineole (3.04%), linalool (2.6%), p-cymene (2.53%), terpinepl (1.51%), benzene propanal (1.16%), limonene (1.12%), n-Docosane (1.04%), n-docosane (1.04%), α-pinene (1.03%), benzyl benzoate (1.01%), phellandrene (0.61%), 4-terpineol (0.56%), benzaldehyde (0.48%), α-terpinolene (0.48%), acetoeugenol (0.42%), trans- caryophyllene (0.25%), β- pinene (0.24%), camphene (0.23%), α- propanal,2-methyl-3-phenyl (0.11%), β-myrcene (0.06%), α-terpinene (0.05%). As can be observed from the results, cinnamaldehyde is a major component of the cinnamon oils as its main cause of antimicrobial activity.

3.2. *Nanoemulsion characteristics*

The sonication process was used to prepare nanoemulsion and SPI was used as an emulsifier to facilitate nanoemulsion constitution and stability. To avoid droplet growth, lecithin was used as a ripening inhibitor. Ostwald ripening is observed in nanoemulsions describing the change of an inhomogeneous structure over time. This is because the oil phase becomes slightly soluble in the surrounding aqueous phase, as a result, small droplets of oil become larger. The main problem in oil in water nanoemulsion containing essential oils is Ostwald ripening. Thus, to retarded droplet growth, a ripening inhibitor was used. In the study, we used lecithin as a natural water-insoluble surfactant for retarding Ostwald ripening. After three months, measuring the particle size nanoemulsion showed a negligible increase from 141.2 ± 1.1 nm to 145.1± 1.3 nm that was not significant ($p<0.01$) and no phase separation was observed in the three months, indicating the stability of nanoemulsion toward creaming (Fig. 1 & 2).

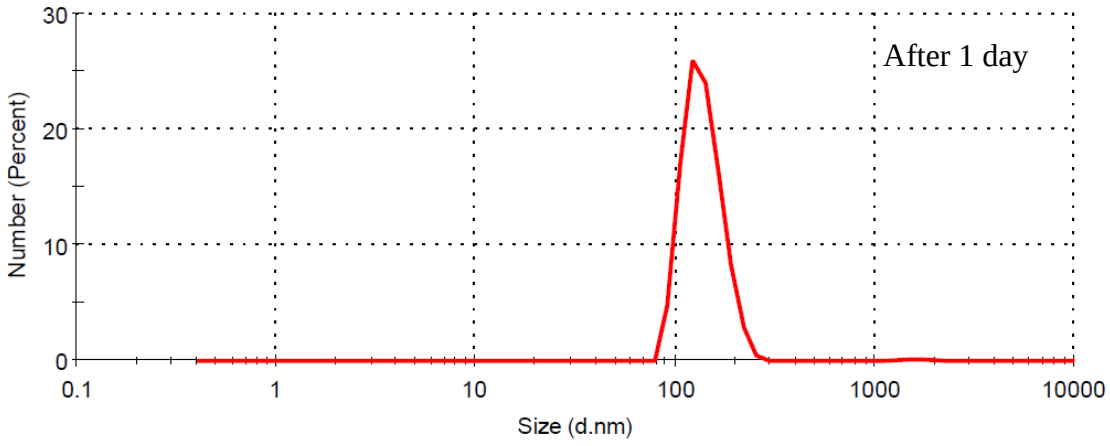

Fig. 1. Particle size distribution for cinnamon oil nanoemulsion after 1day

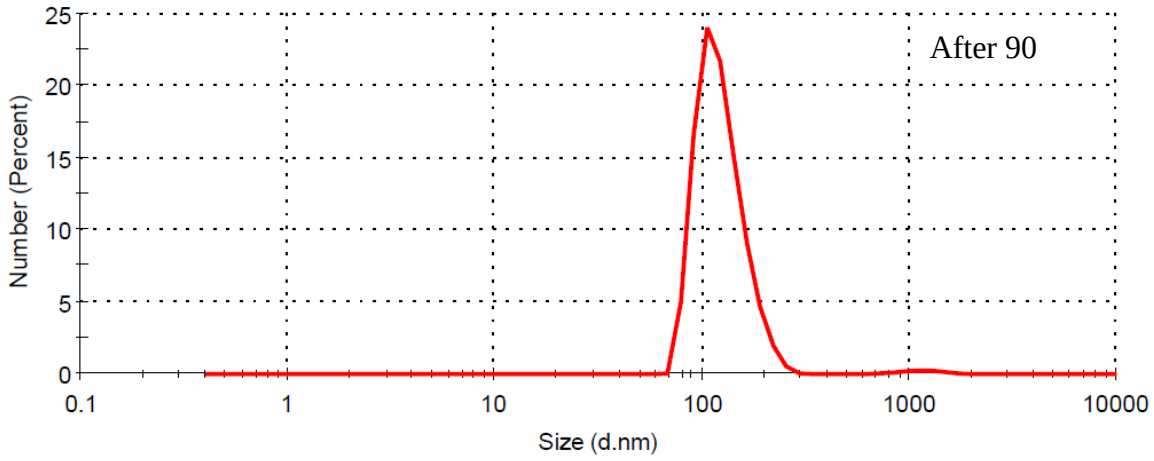

Fig. 2. Particle size distribution for cinnamon oil nanoemulsion after 90 day

3.3. MIC and MBC

Table 1 shows the MIC and MBC of free and nanoemulsified cinnamon oil against *E. coli, E.faecium, B.cereus, S.typhi, P.aeroginosa* and S. *aureus*. According to the results presented in Table 1, *E. coli* was more resistant to cinnamon oils and the highest amount of MIC and MBC. The higher resistance among Gram-positive bacteria than Gram-negative bacteria could be probably related to the differences in the cell membrane of these bacterial groups and clearly more thickness of their peptidoglycan wall. Furthermore, the nanoemulsion showed high inhibitory effects with MICs and MBCs than the pure essential oil. These results indicate that emulsification of cinnamon oil considerably affects the antimicrobial activity of cinnamon oil in the pathogen and greatly improves its bactericidal activity. Recent results indicated that change of essential oils into nanoemulsions increased their antimicrobial activity, being in agreement with our results (Moghimi et al., 2016).

Table 1. Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) of free, and nanoemulsified cinnamon oil

Bacteria	MIC (ppm)		MBC (ppm)	
	Free oil	Emulsion*	Free oil	Emulsion
S. faecium	1400	600	5000	2500
B. cereus	1400	600	5500	3500
E. coli	1600	700	6000	3500
S. typhi	1500	600	4500	3500
P. aeroginosa	1500	600	4500	3500
S. aureus	1500	600	5000	3500

* 2% Cinnamon oil emulsified by 1.5% isolated soy protein and 0.01% lecithin.

3.4. Growth kinetics

The effect of the MIC and MBC of nanaoemulsion and pure cinnamon oils on the growth kinetics of *E. coli* and *E. feause* was determined. Figure 3 shows *E. coli* and *E. faseu* growth for a period of 72 hours for both bacteria, except for untreated control, a continuous decrease of viable cells viewed for nanoemulsion and pure oil treatments with MIC. After 24 hours of incubation, recovery was observed for all treatments. A recovery rate of nanoemulsion treatment was slower than that of free cinnamon oil treatment. This result is consistent with previous reports indicating that nanoemulsions are able to increase antibacterial activity and they were significantly effective in deactivating the bacteria (Ghosh et al., 2013; Sugumar et al., 2013). Moreover, when cinnamon oil concentration raised to the MBC, the complete deactivation of *E. coli* was observed in 72 hours for nanoemulsion and free cinnamon oil treatments. Hence, it can be concluded that the emulsification technique employed in this study increases the antibacterial activity of cinnamon oils.

3.5. Release nucleic acids

One of the most important antimicrobial mechanisms of essential oils is damage to the cell membrane of bacteria and rise in membrane permeability. Increased permeability decreases the integrity of the membrane and thus increasing the leakage of intercellular contents. Therefore, measuring the release of proteins and nucleic acids is one strategy to evaluate the efficacy of antimicrobial essential oils. The loss of intracellular materials is commonly quantified by absorbing at a wavelength of 260 nm (Moghimi et al., 2016). Materials absorbing at 260 nm show nucleic acid. Thus, the absorption mostly indicates that major membrane damage has occurred and thus large molecules have been lost from the cell interior. Figure 4 showed nucleic acids release for a period 72 (h), When *E. coli* and *E. feacium* cells were treated with essential oil nanoemulsion at the MIC concentration, a significant increase in the cell components release was observed compared to the control and pure essential oils. There was an approximately 2- fold rise in the optical density of the bacterial cell culture filtrates of nanoemulsion sample compared to the pure and the control samples. With increased cinnamon oil concentration, the absorption of free cinnamon oil was higher than the control. The increased absorption with increased cinnamon oil concentration (up to MBC) indicating damage is greater membrane integrity, indicating the death of the bacteria.

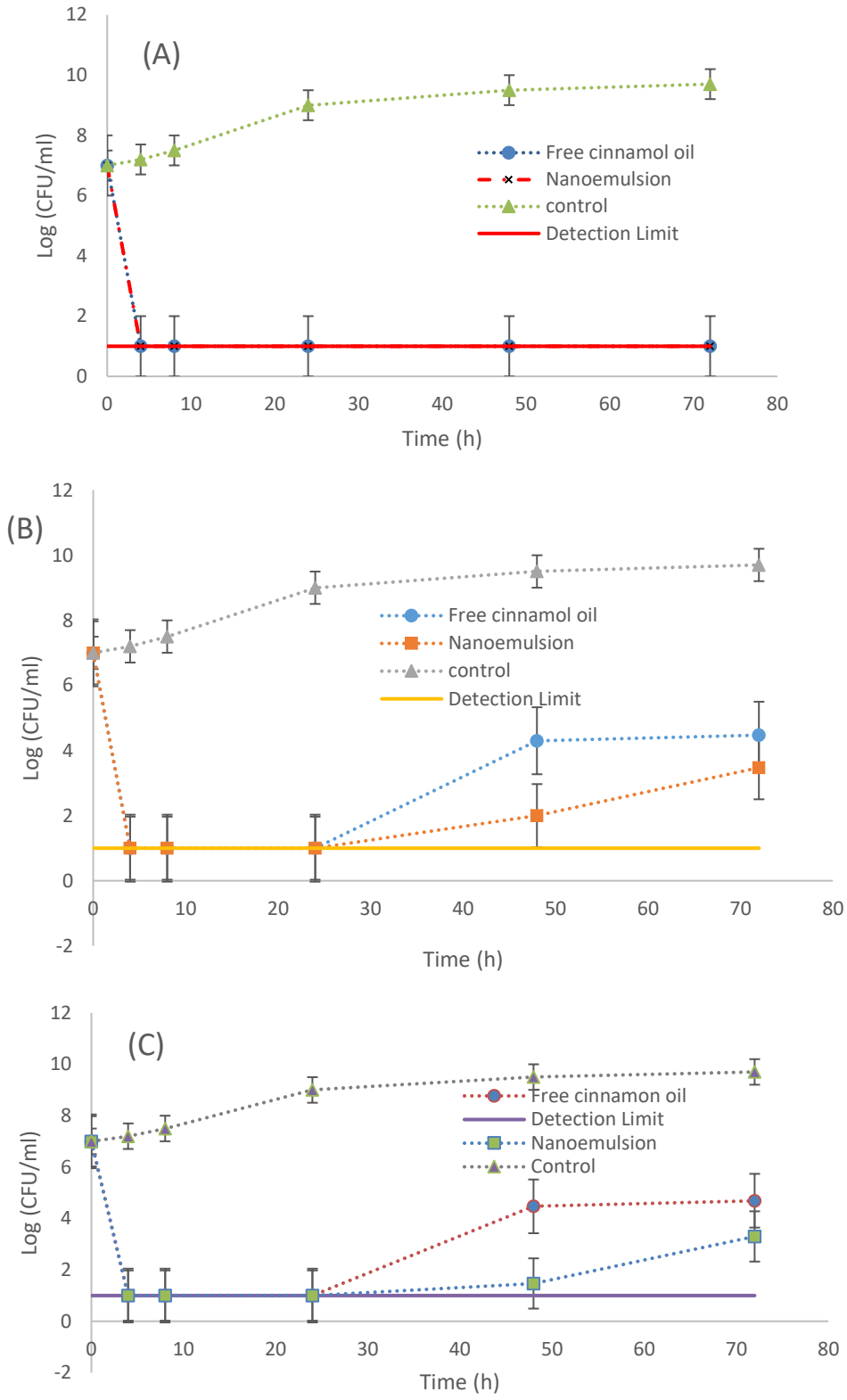

Figure 2. Kinetics of bacterial killing after treatment with control, pure cinnamon oil and cinnamon oil nanoemulsion over 72 h period at MBC of *E. coli* & *E. feacium* (A), MIC of *E. coli* (B) and MIC of *E.faecium* (C)

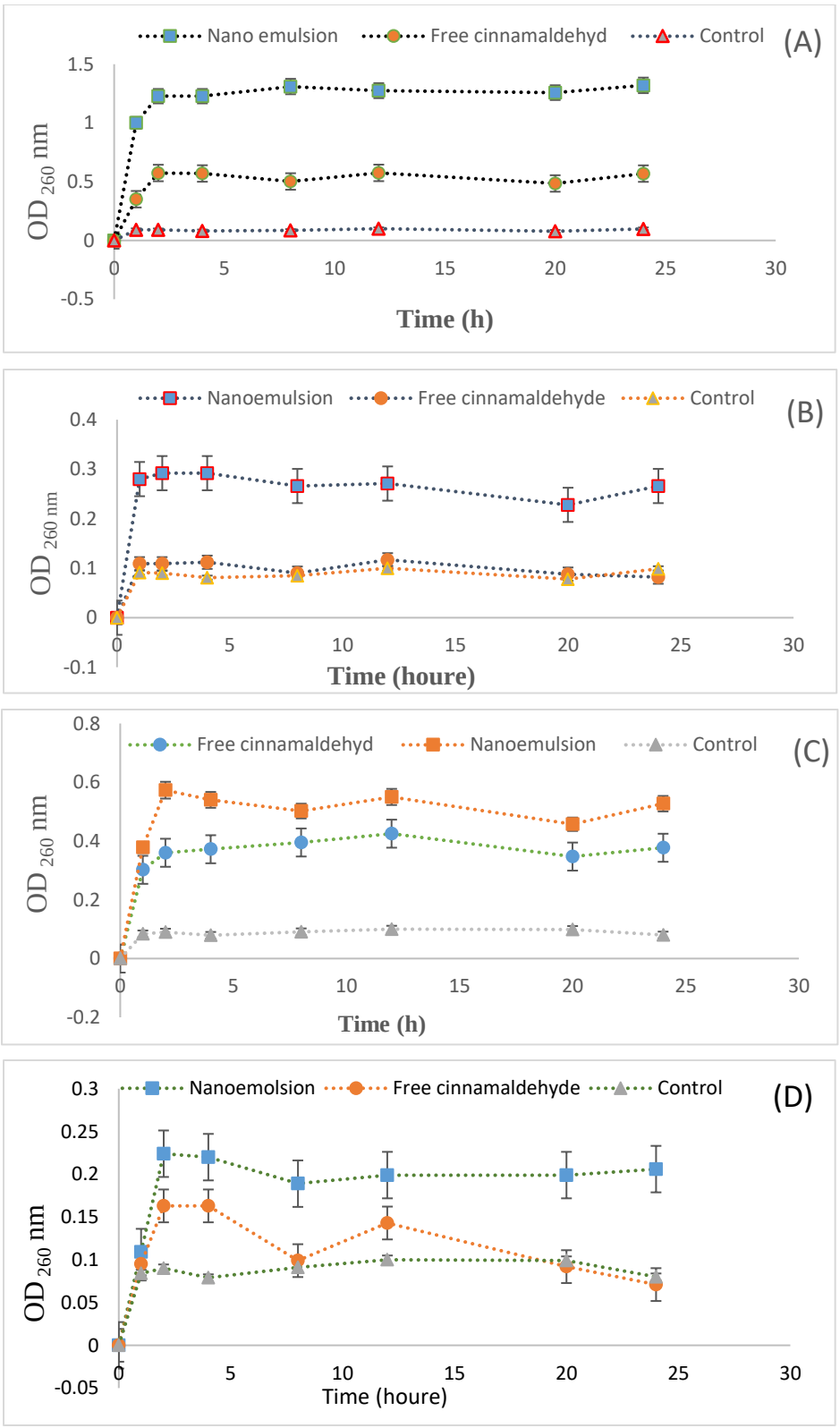

Fig. 4. Nucleic acids release after treatment with control, pure cinnamon oil and cinnamon oil nanoemulsion over 24 h period at MBC (A) and MIC (B) of *E. coli* and MBC (C) and MIC (D) of *E. feacium*.

Table 2. Determination of potassium leakage (ppm) for the control and essential oil nanoemulsion samples

Time(min)	*E. coli*					*S. faseium*				
	Control	Pure Oil		Nanoemulsion		Control	Pure Oil		Nanoemulsion	
		MIC	MBC	MIC	MBC		MIC	MBC	MIC	MBC
0	14.1±0.08	15.9±1.1	21.6±0.4	16.1±0.26	23.4±0.1	12.7±0.8	14.4±1.0	18±0.2	14.7±0.1	18.3±0.1
5	14.3±0.74	17.1±0.04	22.9±0.51	17.1±0.83	25.8±0.34	13.2±1.1	14.4±0.7	18.7±0.6	14.8±0.3	18.7±0.2
15	15 ±1.21	17.5±0.48	25 ±0.71	17.4±0.83	27.3±0.11	15±0.1	15.7±0.4	21.5±0.3	15.9±0.1	18.2±0.3
30	15.1±0.47	17.1±0.61	25.2±1.05	17.4±1.3	26.5±0.21	15.2±0.6	15.8±0.1	21.2±0.1	15.6±0.3	17.3±0.1
45	14.9±1.14	16.7±0.09	24.6±0.25	16.7±0.07	25.7±0.1	15.4±0.2	15.8±0.1	20±0.8	15±0.4	17±0.2
60	15.2±0.04	16.2±1.09	24.1±0.61	17.3±0.05	26±0.74	15.1±0.3	15.1±0.1	20.2±0.2	15±0.8	16.9±0.3

Note. Difference between data's in any columns were not significant ($p<0.05$)

3.6. Potassium leakage

Measuring potassium leakage was used as a method to collect more information about the disruption of cell membranes. Thus, the atomic absorption spectrophotometry was used to measure potassium leakage. According to the results presented in Table 2, Potassium concentration was 15.2± 0.04 for the control sample, as for the essential oil nanoemulsion, this value increased to 17.3± 0.05 and 16.9±0.3 ppm for *E. coli* and *E.faecium*, respectively. The death of some the bacteria during their normal life cycle leads to cell lysis duet and observed potassium leakage in the control group. Additionally, by increasing the concentration of MIC to MBC, potassium leakage also increased, representing the greatest damage to the wall and increased cell permeability to potassium leakage. The reason is that the nano-droplets of cinnamon oils were able to enter the cell membrane surface, while pure cinnamon oils due to low solubility in water could not easily bring to the cell membrane surface (Moghimi et al., 2016). Entered nano-droplets of cinnamon oil into the cell membrane, by changing the phospholipid bilayer integrity or by interfering with active transport protein fixed in the phospholipid bilayer to ruptured the cell membrane. As a result of increased cell membrane permeability to potassium and nucleic acid. The results confirmed that change of essential oils into nanoemulsions increased their antimicrobial activity. Measuring the amount of potassium leakage can be also used as a method to evaluate the antimicrobial of essential oils.

4. Conclusion

Natural food- grade emulsifier (lecithin and SPI) were used to successfully prepared cinnamon nanoemulsions. After 90 days, nanoemulsions had very good stability and no phase separation or particle growth was observed in nanoemulsions. According to results, the nanoemulsion indicated high inhibitory effects with MICs and MBCs than the pure essential oil. So that, *E. coli* and *E.faecium* cells were treated with essential oil nanoemulsion at the MIC concentration a significant raise in the cell compounds release compared to the control and pure essential oils were observed. There was an approximately 2- fold increase in the optical density of the bacterial cell culture filtrates of nanoemulsion sample compared to the pure and the control samples. Potassium concentration was 15.2± 0.04 for the control sample, as for the essential oil nanoemulsion, this value was increased to 17.3± 0.05 and 16.9±0.3 ppm for *E. coli* and *E.faecium*, respectively. The results confirming that conversion of cinnamon oils into nanoemulsions increased their antimicrobial activity of them. Inclusion, the nanoemulsion studied can potentially be used as to antimicrobial preservatives ton enhance food safety due to their ability to increase antimicrobial efficacy.

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