

The inhibitory effect of garlic (*Allium sativum* L.) essential oil nanoliposomes on Shiga-toxin 2 expression in *Escherichia coli* O157:H7

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ABSTRACT

Foodborne diseases are considered as one of the main problems of public health. *Escherichia coli* O157H7 are responsible for major outbreaks of bloody diarrhea and hemolytic uremic syndrome (HUS) throughout the world. The mortality is originated from the production of a Shiga toxin (Stx) by these bacteria. Garlic essential oil (GEO) has antibacterial effects on many food-borne pathogens. Therefore, this study was aimed to evaluate the antibacterial activity of the *Allium sativum* L. EO and its nanoliposomal form on the virulence of *E. coli* O157:H7. Reverse passive latex agglutination test was used to detect Shiga toxin2 (Stx2) production after exposure to sub-inhibitory concentrations of free and nanoencapsulated EO. Also, the effect of sub-inhibitory concentrations of free and nanoliposomal form of GEO was evaluated on Stx2 gene expression and the relative transcriptional level of Stx2 gene was determined by real-time PCR. It was found that the sub-inhibitory concentrations of liposomal form of EO (50 and 75%) had a significantly higher inhibitory effect on Stx2 titer than its free form ($p < 0.05$). Also, increasing the concentration of EO and nanoencapsulated EO significantly reduced Shiga toxin 2 gene expression according to control. Using 75% sub-inhibitory value of free and nanoliposome GEO, the relative transcriptional level of Stx2A gene was reduced from 0.938 to 0.667 and 0.931 to 0.659, respectively. Based on our findings, different methods of nanoencapsulation should future study to improve nanoliposome efficacy to suppress toxin production on expression level.

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1. Introduction

Shiga toxin-producing *E. coli* (STEC) serogroup O157:H7 is an important foodborne pathogen that is associated with severe bloody diarrhea, hemolytic uremic syndrome (HUS) and death (1). It has caused a great number of outbreaks in the world, which had been due to the consumption of ground beef, ready-to-eat salad, cheese, apple juice, and other foods (2-5). Shiga toxin (Stx) is a major virulence factor of STEC O157:H7. Production of Stx in the gastrointestinal tract contributes to the development of hemorrhagic colitis. The sufficient absorbance of Stx into the blood circulation system can lead to vascular endothelial damage and dysfunction and finally, chronic kidney damage and neurological disorders such as HUS. The Stx family is divided into two major

branches, Stx1 and Stx2. STEC is capable of producing both Stx1 and Stx2 but Stx2 producing strains are related to the more severe disease (HUS) in humans (6, 7). Stx2 consists of an A subunit in associated with five identical B subunits. The A-subunit, an active component of the Stx, injures the eukaryotic ribosome and prevents protein synthesis in target cells. The role of B subunit is an attachment to the specific endothelial cell receptor, globotriaosylceramide, Gb3. Both types of Stx are encoded by *stx*-genes located on temperate prophages integrated into the *E. coli* O157:H7 chromosome (8, 9). The activation of Stx prophage, results in phage replication, cell lysis and finally Stx release. Importantly, released Stx phages are able to be integrated into the commensal *E. coli* genome and may alter it to new Stx-producing *E. coli* (STEC), facilitating the spread of STEC strains (10). The development

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of potent antimicrobial agents to control these bacterial pathogens and neutralize virulence toxins should be prioritized in food and drug industries. Although antibiotic therapy is generally forbidden for treatment of STEC infections in humans, due to the indirect selection of multi drug resistant strains pave the way for increasing of antibiotic resistant pathogens and facilitating the spread of these mobile resistance agents to other bacteria. However, an alternative antimicrobial intervention is urgently required that remarkably inhibit not only *E. coli* O157:H7 growth, but also Stx production. Recently, natural products such as essential oils (EOs) have been widely utilized for bactericidal, virucidal, fungicidal, antiparasitical, insecticidal, and other medicinal properties (11, 12). EOs are mixture of a variety of volatile molecules such as terpenoids, phenol-derived aromatic components, and aliphatic components have gained strong attention in pharmaceutical and food industries (13). Nowadays, Garlic, one of the plants belonging to the Liliaceae, is used as one of the most popular and most commonly used seasonings and spices in many parts of the world. Garlic essential oil has antimicrobial activity on many food-borne pathogens, including *Escherichia coli*. Antimicrobial activity of garlic is due to thiosulphates, especially Thiosulphinates and mainly Allicin, which is the main cause of antimicrobial activity as well as taste and aroma of garlic (14, 15). Like other EOs, the EO of *Allium sativum* L, due to its lipophilic nature is insoluble in water, highly volatile, easily decomposes and distributes defectively to the desired sites. Encapsulation of these compounds has been proposed as an effective way to control their release, improve their stability, water solubility and maintain their bioactivity (16). Recently, nanoencapsulation is one part of nanotechnology that has been growing application in food and drug industries. Nanoencapsulation of EOs particularly nanoliposomal one has been recognized as a promising strategy for overcoming EOs limitations, lowering their dose and increasing long term safety of these constituents (17). Our previous study showed that nanoliposomal encapsulated GEO enhanced antibacterial activity against *E. coli* O157:H7 in comparison with the non-encapsulated one (18). In addition, related studies stated that the nanoencapsulation process amends the biological activities of EOs, through enhancement of their bioavailability because of increasing the surface to volume rate (19, 20). Since there is no information available regarding the effect of nanoliposomal GEO on the expression of key virulence factors in *E. coli* O157:H7. Accordingly, the current study was aimed to evaluate sub-inhibitory concentrations of nanoliposomal encapsulated *Allium sativum* L EO on *E. coli* O157:H7 Stx2 production and related gene expression using real-time PCR.

2. Materials and methods

2.1. Nanoliposomes preparation and characterization

Nanoliposome GEO was prepared by ethanol injection method described by Chiraz et al. (21) and its physicochemical properties; Mean particle size, zeta potential measurements,

Encapsulation efficiency, and Permeability, were evaluated according to the protocol described in detail in a previous paper (18).

2.2. Evaluating the effect of sub-inhibitory concentrations of GEO on the production of Shiga toxin 2

The reverse passive agglutination method was carried out to evaluate the effects of sub-inhibitory concentrations of the essential oil on the production of Shiga toxin 2. As evaluated before (18), microbial suspensions were exposed to the inhibitory and sub-inhibitory concentrations of EO and nanoencapsulated EO and then were centrifuged at 4000 rpm at 4°C for 20 minutes. The presence or absence of Shiga toxin 2 in the supernatant was determined using a VTEC-RPLA kit (Oxoid, UK).

2.3. RNA extraction and purification

E. coli (ATCC 35218) was cultured in BHI agar with subinhibitory levels of EO and nanoencapsulated EO at 35 °C for 72 h. RNA preparation was done with the use of TriPure isolation reagent (Roche Applied Science, Germany) according to the manufacturer's instructions for 18, 24, 48 and 72 h. Briefly, cells were obtained through centrifugation at 12,000 ×g for 15 min at 4 °C in polypropylene centrifuge tubes. The supernatant was removed and discarded. In order to ensure complete dissociation of nucleoprotein complexes, 1 ml of TriPure was added to the cell pellet and homogenized samples underwent incubation for 5 min at 15 to 25 °C. Afterwards, 0.2 ml chloroform (Merck, Germany) was added to the samples; tubes were shaken vigorously for 15 seconds and incubated at 15 to 25 °C for 15 min. Tubes were then centrifuged at 12,000 ×g for 15 min at 2 to 8 °C in order for the mixture to be separated into three phases.

RNA was precipitated from the colorless aqueous phases follows: 0.5 ml isopropanol (Merck, Germany) was mixed with the aqueous phase. After that, samples were shaken 7-8 times and incubated for 5–10 min at 15 to 25 °C to allow the formation of RNA precipitate. Samples were then centrifuged at 12,000 ×g for 10 min at 2 to 8 °C. The supernatant was removed and discarded. The RNA pellet was washed with 1 ml of 75% ethanol twice. RNase-free DNase I (Qiagen, Hilden, Germany) treatment was carried out to eliminate contaminating DNA. RNA quantification was done by measuring the absorbance at 260 nm. The purity of nucleic acid was tested by measuring the A260nm/A280 nm ratio with a NanoDrop Spectrophotometer 2000 (ThermoScientific, USA). Ethidium bromide staining was used to visualize RNA quality and integrity following RNA electrophoresis on 1% agarose gel. DNA-free RNA was dissolved in DEPC-water (diethyl pyrocarbonate treated double-distilled water) and stored at –70 °C.

2.3.1. cDNA synthesis

Using the Omniscript Reverse Transcription kit (Qiagen,

Hilden, Germany) according to the manufacturer's instructions, RNA was reverse transcribed into cDNA and the obtained cDNA was stored at -20°C until required.

2.3.2. Real-Time PCR

For the premix solution, 2.5 μl of magnesium chloride, 1 μl dNTPs, 1 μl forward primer, 1 μl reverse primer, 1 μl single polymerase enzyme, 2.5 μl 10x buffer and 1 μl BSA were mixed and the final volume reached 24 μl by adding ddH₂O. All phases were carried out on the ice. Each primer was applied twice and all reactions had a negative control. The final volume was transferred to PCR vials and the surface of the solution was covered with 25 μl of mineral oil to prevent vaporization during the thermal cycles. Afterwards, 1 μl DNA template (cDNA of the control sample) was added to the solution below the oil layer and vortexed. The primer pairs used for 2A Shiga toxin and GAPDH (as the internal control or reference gene) are presented in Table 1. Applied thermal cycling conditions were as follows: one cycle at 95°C for 4 min, 35 cycles at 94°C for 30 s, various temperatures for 30s, 72°C for 1 min, 72°C for 5 min and cooling to 4°C . For SYBR Green-based amplicon detection it is important to run a dissociation curve following real time PCR. This is because of the fact that SYBR Green will recognize any double-stranded DNA including primer dimers, contaminating DNA, and PCR products from misannealed primers. Therefore, the derivative plot of the melting curve of each gene in the reaction was investigated as such. $\Delta\Delta\text{Ct}$ method described in Applied Biosystems User Bulletin No.2 was employed to determine the relative expression levels. All experiments were performed in triplicate and each experiment had three replicates. The results are expressed as mean \pm standard error. Data analysis was

conducted by using the SPSS software, version 16. Statistical differences were evaluated using one-way analysis of variant (ANOVA). The p-value less than 0.05 were considered to be statistically significant.

Table 1. Primers used for Real-time PCR.

| Primer | Sequence (5' → 3') | Amplicon length (bp) |
|----------|----------------------------|----------------------|
| Stx2A-F* | TTG-CTG-TGG-ATA-TAC-GAG-GG | 84 |
| Stx2A-R | TCC-GTT-GTC-ATG-GAA-ACC | |
| GAPDH-F | TCC-GTG-CTG-CTC-AGA-AAC | 78 |
| GAPDH-R | CAC-TTT-CTT-CGC-ACC-AGC | |

F*: Forward; R: Reverse

3. Results

3.1. Effect of sub-inhibitory concentrations of free and nanoliposomal GEO on Shiga toxin 2 production

Data regarding the production of Shiga toxin 2 by the microorganism on BHI medium containing various dilutions of *Allium sativum* L. EO are demonstrated in Table 2. The exposure of *E. coli* to the EO and nanoliposomal EO reduced the production of Shiga toxin 2. *Allium sativum* L. EO at 25% inhibitory concentration had no suppressive effect on Shiga toxin 2 production. The increment of the EO concentration to 50 and 75% inhibitory concentrations could reduce the Stx2 production; however, there was no significant difference between these two levels on toxin production.

EO incorporated into nanoliposomes increasingly inhibited Shiga toxin 2 production at all tested sub inhibitory levels (25, 50, and 75%). The highest suppressive effect on Shiga toxin2 production ($p>0.05$) was observed by 75% inhibitory level of nanoliposomal EO.

Table 2. Effect of various levels of the EO and nanoencapsulated EO on Shiga toxin 2 production.

| EO concentration | Toxin dilutions | | | | | | |
|-----------------------------------|-----------------|-----|-----|------|------|------|-------|
| | 1/2 | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 | 1/128 |
| Positive control of Shiga toxin 2 | + | + | + | + | + | + | + |
| Control (no EO) | + | + | + | + | + | - | - |
| 25% MIC EO | + | + | + | + | + | - | - |
| 50% MIC EO | + | + | + | + | - | - | - |
| 75% MIC EO | + | + | + | - | - | - | - |
| MIC EO | - | - | - | - | - | - | - |
| Control (no nanoencapsulated EO) | + | + | + | + | + | - | - |
| 25% MIC nanoencapsulated EO | + | + | + | + | - | - | - |
| 50% MIC nanoencapsulated EO | + | + | + | + | - | - | - |
| 75% MIC nanoencapsulated EO | + | + | + | - | - | - | - |
| MIC nanoencapsulated EO | - | - | - | - | - | - | - |

3.2. Effect of sub-inhibitory concentrations of free and nanoliposomal GEO on Stx2 gene expression.

Fig.1 depicts Stx2 gene expression relative levels in *E. coli* O157:H7 subjected to 25, 50 and 75% inhibitory values of the EO and its nanoliposomal form. Stx2 gene relative expression in samples affected by 25, 50 and 75% subinhibitory levels of free EO were 0.938, 0.824 and 0.667 and these values about

25, 50 and 75% subinhibitory levels of nanoliposomal EO obtained 0.931, 0.832 and 0.659, respectively. According to the results, exposure to subinhibitory levels of both free EO and nanoliposomal EO remarkably decreased Stx2 gene expression compared with control (untreated sample) ($p<0.05$). Comparison of relative expression of Stx2A gene between free and nanoliposome enveloped EO revealed no significant difference ($p>0.05$).

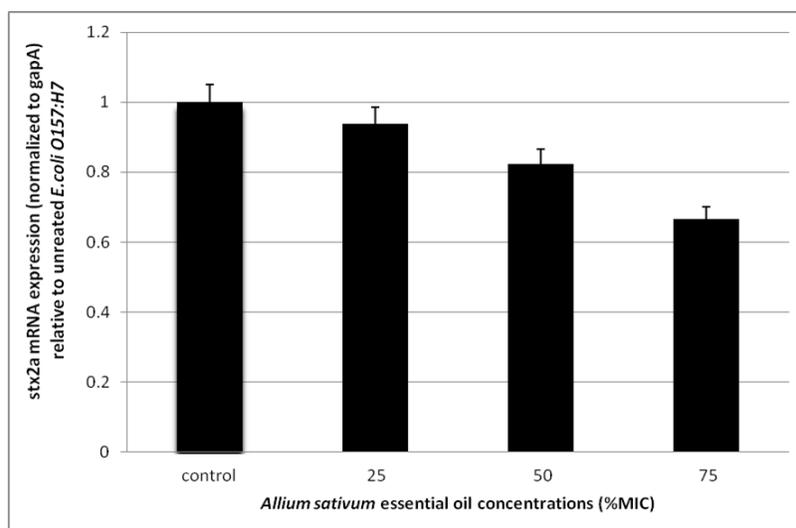


Fig 1. The effect of sub MIC levels (25, 50 and 75% MIC) of EO on relative expression of Stx2A gene in *Escherichia coli* O157:H7. The level of Stx2A expression between the treated and untreated samples was compared using the housekeeping gene GAPDH. Values are expressed as the mean \pm standard error.

4. Discussion

In a recent paper, we conducted the GC/MS analysis of garlic essential oil and the main constituents were reported. In addition, the formation of nanoliposome containing GEO and its physical properties such as particle size, polydispersity index, zeta potential, encapsulation efficiency, the percentage of permeability were studied. Furthermore, as mentioned before, the MIC levels of GEO (0.03%) and its nanoliposomal form (0.02%) were measured (18). In the current study, the effect of inhibitory and subinhibitory (25, 50 and 75%) concentrations of GEO and its nanoliposomal form were evaluated on *stx2* mRNA expression and Stx2 production, which is an important factor involved in virulence of *E. coli* O157:H7. Reverse passive latex agglutination test for detection of Stx2 showed that 50 and 75% subinhibitory levels of free EO could significantly reduce the Stx2 production in *E. coli* O157:H7 ($p < 0.05$) except for 25% level. Our result was in agreement with Azizkhani et al. (22) and Khatibi et al. (17) findings who reported that *Zataria multiflora* Boiss (ZMEO) below 25% MIC showed no inhibitory effect on *Staphylococcus aureus* enterotoxin and *E. coli* O157:H7 Stx2 production, respectively, but increased concentrations of the EO (50 and 75% MIC) notably ($p < 0.05$) inhibited the toxin production (17, 22). Also, Sheng et al. (23) stated that the sub-MIC of cinnamon oil and its major component cinnamonaldehyde inhibited *E. coli* O157:H7 Stx2 protein production. Another similar study by Smith-Palmer et al. (24) showed the ability of subinhibitory concentrations of plant essential oils (bay, clove, cinnamon, nutmeg and thyme) to influence the production of enterotoxins A and B and α -toxin by *Staphylococcus aureus*. Furthermore, the suppressive effect of other plant products such as spices on the Verotoxin production of EHEC O157 has been reported (25). In addition, the current study revealed that inhibition of Stx2 production occurred in all dilutions of EO loaded nanoliposomes and 75%

inhibitory level of that was obviously more effective than other levels. More importantly, nanoliposomal EO had a remarkably higher inhibitory effect on Stx2 titer than its free form. These findings correlate with Khatibi et al. (17) study. Several studies represented that nanoliposome increases the antibacterial activity of essential oils due to stabilizing volatile molecules, shielding them from evaporation and also by increasing cellular interactions between them and the microbes as a result of their very small size (26-28). Comparison of the expression of Stx2 gene between test (in presence of subinhibitory levels of the EO and nanoencapsulated EO) and control cultures revealed that all the under study dilutions of the EO and nanoencapsulated EO resulted in a reduction in Shiga toxin 2 gene expression compared to the control group (no EO) ($p < 0.05$) (Fig.1). Statistically, significant differences were noted in the efficacy of different sub-inhibitory concentrations of EO and the nanoencapsulated EO in this respect ($p < 0.05$). It seems that EOs could have inhibited at transcription, translation, and export from the cell or direct inactivation of the toxin (24). Comparison of the effect of free EO with nanoencapsulated EO on Shiga toxin 2 gene expression indicated that the effect of various sub-inhibitory concentrations of the EO on reducing Stx2 gene expression was almost equal in EO and nanoencapsulated EO and no statistically significant difference was noted in this respect ($p > 0.05$). In contrast, in a similar study was undertaken using *Zataria multiflora* Boiss EO incorporated into nanoliposome prepared by the thin film hydration method, represented that the Sub-MIC values of nanoliposomal EO had a better activity in the reduction of Stx2A gene expression than free EO (17). This inconsistency is due to the difference in liposome preparation methods. Another study conducted by these authors (29) and some other studies (30, 31) showed that the preparation method of liposome can affect the encapsulation efficacy such as the maintenance and release of essential oils embedded. There have been no reports on the inhibitory effect

of GEO and its nanoencapsulated form on toxin gene expression. Some other studies examined only the effect of free EO and plant secondary metabolites as a suppressive agent on *stx2* gene expression or other *E. coli* toxin genes. Sheng et al. (23) checked the effect of cinnamon oil or its main component cinnamaldehyde on *E. coli* Shiga toxin 2 gene expression, revealed that the MIC and sub MIC_s (0.25 and 0.75 MIC) of cinnamon oil inhibited *stx2* mRNA expression in *E. coli* O157:H7. Lee et al. (32) showed esculetin, a plant secondary metabolite, effectively repressed the Shiga-like toxin gene, *stx2* in *E. coli* O157:H7.

5. Conclusion

Our study showed that both GEO and its liposomal form suppressed the *stx2* gene expression and STX2 production via the use of nanotechnology, especially lipid based nanoencapsulation as one of the safest methods of encapsulation. Due to the higher inhibitory activity of the nanoencapsulated GEOs, lower antimicrobial concentrations are required for a bactericidal action and we can more effectively benefit from the antibacterial effects of garlic with less adverse scent and taste.

References

- Jacob ME, Foster DM, Rogers AT, Balcomb CC, Shi X, Nagaraja TG. Evidence of non-O157 Shiga toxin-producing *Escherichia coli* in the feces of meat goats at a U.S. slaughter plant. *Journal of Food Protection*. 2013;76:1626-1629.
- King LA, Loukiadis E, Mariani-Kurkdjian P, Haeghebaert S, Weill FX, Baliere C, et al. Foodborne transmission of sorbitol-fermenting *Escherichia coli* O157:H7 via ground beef: an outbreak in northern France, 2011. *Clinical Microbiology Infection*. 2014;20:1136-44.
- McCullum JT, Williams NJ, Beam SW, Cosgrove S, Ettestad PJ, Ghosh TS, et al. Multistate outbreak of *Escherichia coli* O157:H7 infections associated with in-store sampling of an aged raw-milk Gouda cheese. *Journal of Food Protection*. 2010;75:1759-65.
- Vojdani JD, Beuchat LR, Tauxe RV. 2008. Juice-associated outbreaks of human illness in the United States, 1995 through. *Journal of Food Protection*. 2005;71:356-64.
- Marder EP, Garman KN, Ingram LA, Dunn JR. Multistate outbreak of *Escherichia coli* O157:H7 associated with bagged salad. *Foodborne Pathogenesis and Disease*. 2014;11:593-95.
- Mainil JG, Daube G. Verotoxigenic *Escherichia coli* from animals, humans and foods: who's who? *Journal of Applied Microbiology*. 2005;98:1332-44.
- Smith JL, Fratamico PM, Gunther NW, IV. Shiga toxin-producing *Escherichia coli*. *Advanced Applied Microbiology*. 2014;86:145-97.
- Fraser ME, Fujinaga M, Cherney MM. Structure of Shiga toxin type 2 (Stx2) from *Escherichia coli* O157:H7. *Journal of Biological Chemistry*. 2004;279:27511-7.
- Robins-Browne RM. The relentless evolution of pathogenic *Escherichia coli*. *Clinical Infectious Disease*. 2005; 41:793-4.
- Gamage SD, Patton AK, Hanson JF, Weiss AA. Diversity and host range of Shiga toxin-encoding phage. *Infection and Immunity*. 2004;72:7131-9.
- Kuorwel K K, Cran M J, Sonneveld K, Miltz J, and S. W. Bigger, "Essential oils and their principal constituents as antimicrobial agents for synthetic packaging films. *Journal of Food Science*. 2011;76(9):R164-R177.
- Adorjan B, Buchbauer G. Biological properties of essential oils: an updated review. *Flavour and Fragrance Journal*. 2010;25(6):407-26.
- ShankarRaut J, Karuppayil S. A status review on the medicinal properties of essential oils. *Industrial Crops and Products*. 2014;62:250-64.
- Kumar, M., Berwal, J.S. Sensitivity of food pathogens to garlic (*Allium sativum*). *Journal of Applied Microbiology*. 1998;84:213-5.
- Medellin-Pena M, Wang H, Johnson R, Anand, S, Griffiths M. Probiotics affect virulence-related gene expression in *Escherichia coli* O157:H7. *Applied and Environmental Microbiology*. 2007;73:4259-67.
- Ortan A, Campeanu G, Dinu-Pirv C, Popesc L. Studies concerning the entrapment of *Anethum graveolens* essential oil in liposomes. *Roman Biotechnology Letters*. 2009;14:4411-7.
- Khatibi S A, Misaghi A, Moosavy MH, Akhondzadeh Basti A, Mohamadian S, Khanjari A. Effect of nanoliposomes containing *Zataria multiflora* Boiss. essential oil on gene expression of Shiga-toxin 2 in *Escherichia coli* O157:H7. *Journal of Food Quality and Hazards Control*. 2017;4(1):24-8.
- Zabihi A, Akhondzadeh Basti A, Amoabediny G, Khanjari A, Tavakkoly Bazzaz J, Mohammadkhan F, Hajjar Bargh A, Vanaki E. Physicochemical characteristics of nanoliposome garlic (*Allium sativum* L.) essential oil and its antibacterial effect on *Escherichia coli* O157:H7. *Journal of Applied Microbiology*. 2017;124(2):389-97.
- Biddeci G, Cavallaro G, Di Blasi F, Lazzara G, Massaro M, Milioto S, et al. Halloysite. nanotubes loaded with peppermint essential oil as filler for functional biopolymer film. *Carbohydrate Polymers*. 2016;152:548-57.
- Makwana S, Choudhary R, Dogra N, Kohli P, Haddock J. Nanoencapsulation and immobilization of cinnamaldehyde for developing antimicrobial food packaging material. *LWT-Food Science and Technology*. 2014;57:470-76.
- Chiraz J.M., Diab R., Andrieu V, Elaissari A, Fessi H. Ethanol injection method for hydrophilic and lipophilic drug-loaded liposome preparation. *Journal of Liposome Research*. 2010;20:228-43.
- Azizkhani M, Basti A.A, Misaghi A, Tooryan F. Effects of *Zataria multiflora* Boiss., *Rosmarinus officinalis* L. and *Mentha longifolia* L. essential oils on growth and gene expression of enterotoxins C and E in *Staphylococcus aureus* ATCC 29213. *Journal of Food Safety*. 2012;32:508-16.
- Sheng L, Rasco B, Zhu M. Cinnamon oil inhibits Shiga toxin type 2 phage induction and Shiga toxin type 2 production in *Escherichia coli* O157:H7. *Applied and Environmental Microbiology*. 2016; 82(22):6531-40.
- Smith-Palmer A, Stewart J, Fyfe L. Influence of subinhibitory concentrations of plant essential oils on the production of enterotoxins A and B and α -toxin by *Staphylococcus aureus*. *Journal of Medical Microbiology*. 2004;53:1023-7.
- Takemasa N, Ohnishi S, Tsuji M, Shikata T, Yokoigawa K. Screening and analysis of spices with ability to suppress verocytotoxin production by *Escherichia coli* O157. *Journal of Food Science*. 2009;74(8):M461-6.
- Jain KK. Nanodiagnostics: application of nanotechnology in medical diagnostics. *Information Health Care*. 2003;3(2):153-61.
- Maryam I, Huzaifa U, Hindatu H, Zubaida S. Nanoencapsulation of essential oils with enhanced antimicrobial activity: A new way of combating antimicrobial resistance. *Journal of Pharmacognosy and Phytochemistry*. 2015;4(3):165-70.
- Wattanasatcha A, Rengpipat S, Wanichwecharungruang S. Thymol nanospheres as an effective anti-bacterial agent. *International Journal of Pharmaceutics*. 2012;434:360-5.
- Khatibi S.A, Misaghi A, Moosavy M.H, Amoabediny G, Akhondzadeh Basti A. Effect of preparation methods on the properties of *Zataria multiflora* Boiss. essential oil loaded nanoliposomes: characterization of size, encapsulation efficiency and stability. *Pharmaceutical Sciences*. 2015;20:141-8.
- Arabi M.H, Mirzapour A, Chabok H, Shafiee Ardestani M, Saffari M. Preparation methods of nanoliposomes containing *Zataria multiflora* essential oil: A comparative study. *Bioscience and Biotechnology Research. Community*. 2017;10(1):151-60.
- Maestrelli F, Capasso G, González-Rodríguez ML, Rabasco AM,

Ghelardini C, Mura P. Effect of preparation technique on the properties and in vivo efficacy of benzocaine-loaded ethosomes. *Journal of Liposome Research*. 2009;19(4):253-60.

32. Lee J.H, Kim YG, Cho H.S, Ryu S.Y, Cho M. H, Lee J. Coumarins reduce biofilm formation and the virulence of *Escherichia coli* O157:H7. *Phytomedicine*. 2014;21(8):1037-42.