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Physicochemical Characteristics of Nanoliposome Garlic (*Allium sativum* L.) Essential Oil and Its Antibacterial Effect on *Escherichia coli* O157:H7

A. Zabihi [1], A. Akhondzadeh Basti [1*✉], G. Amoabediny [2,3], A. Khanjari [1], J. Tavakkoly Bazzaz [4], F. Mohammadkhan [1], A. Hajjar Bargh [5], E. Vanaki [1]

1. Department of Food Hygiene, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran
2. Department of Chemical Engineering, Faculty of Engineering, University of Tehran, Tehran, Iran
3. Research Centre for New Technologies in Life Science Engineering, University of Tehran, Tehran, Iran
4. Department of Medical Genetics, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
5. Department of Food Sciences and Technology, Faculty of Food Sciences, Islamic Azad University, Science and Research Branch, Tehran, Iran

HIGHLIGHTS

- Entrapment efficiency of prepared nanoliposome garlic Essential Oil (EO) was 64.27±0.78%.
- MIC and MBC of nanoliposome garlic EO against *Escherichia coli* were 0.02% and 0.03%, respectively.
- Nanoliposome encapsulated garlic EO showed enhanced antimicrobial activity against *Escherichia coli*.

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Acronyms and abbreviations
EO=Essential Oil
GC/MS=Gas Chromatography/Mass Spectrometry
PBS=Phosphate Buffered Saline
BHI=Brain Heart Infusion
MIC=Minimum Inhibitory Concentration
MBC=Minimum Bactericidal Concentration

ABSTRACT

Background: *Escherichia coli* O157:H7 is a Gram-negative and facultative anaerobic food-borne bacterial pathogen. The major purpose of this study was to evaluate physicochemical characteristics of nanoliposome garlic Essential Oil (EO) and its antibacterial effect on *E. coli* O157:H7.

Methods: Nanoliposome garlic EO was prepared by ethanol injection method and its physicochemical properties were evaluated. After inoculation of *E. coli* O157:H7 to experimental groups, minimum inhibitory concentration and minimum bactericidal concentration were assessed. Data were analyzed using SPSS software (version 16.0).

Results: The average particle sizes of prepared nanoliposomes were 131.73±14.31 nm with a polydispersity index of 0.212±0.013. The percentage of liposome permeability after 5, 10, 30, and 50 days were 0.46%, 2.47%, 5.63%, as well as 7.29%, respectively, revealing significant difference (p<0.05) among various intervals. Also, the minimum inhibitory concentration as well as minimum bactericidal concentration values of the nanoliposome encapsulated garlic EO against *E. coli* O157:H7 were 0.02% and 0.03%, respectively, and for non-encapsulated EO were 0.03% and 0.04%, respectively.

Conclusion: Nanoliposome encapsulated garlic EO showed enhancing antimicrobial activity against *E. coli* O157:H7 in comparison with the non-encapsulated one.

Introduction

Nowadays, as people tend to consume natural foods due to health concerns, the use of natural preservatives such as Essential Oils (EOs) is increasing (Espina et al., 2012; Khorasany et al., 2016). Several studies indicate that plants EOs have antimicrobial activity against food-borne pathogens (Azhdarzadeh and Hojjati, 2016; Karagozlu et al., 2011; Solomakos et al., 2008; Turgis et al., 2009). However, as some approved safe EOs can

* Corresponding author. ✉ aakhond@ut.ac.ir

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affect the organoleptic quality of foods, their use is limited in the food industry (Oussalah et al., 2007; Solomakos et al., 2008)

Allium is the most important and largest genus of the Alliaceae family and among 450 species of this genus; garlic (*Allium sativum* L.) is a well-known one in most countries as a flavoring agent in foods (Eja et al., 2011; Lu et al., 2014; Razavi Rohani et al., 2011). Also, antimicrobial activity of garlic EO against several food-borne bacteria has been reported in several studies (Daka, 2011; Eja et al., 2011; Karuppiah and Rajaram, 2012; Meriga et al., 2012). As the outer membrane of Gram-negative bacteria contains lipopolysaccharide, this group of bacteria are usually more resistant against EOs in comparison to Gram-positive bacteria (Oussalah et al., 2007). Allicin, a well-known thiosulfinate, is responsible for the pungent smell of garlic EO and its antibacterial activity (Lanzotti, 2006; Lu et al., 2014; Sivam, 2001). Garlic can be useful for treating and preventing of cancer, coronary heart disease, obesity, hypercholesterolemia, diabetes type 2, hypertension, cataract, pulmonary disorders, arthritis, edema, worm infection, and gastrointestinal disorders (Barak et al., 2007; Lanzotti, 2006; Lu et al., 2014).

Applying novel methods like liposome encapsulation of natural compounds such as herbal EOs improves the stability and bioavailability of these compounds. The combination of two Greek words including “lipos” which means fat and “soma” which means body or structure constitute the name of liposome. Liposomes are composed from polar lipids (such as phosphatidylcholine or phosphatidylethanolamine) or the combination of polar fats with cholesterol or ergosterol and are able to encapsulate both hydrophilic and lipophilic compounds (Khatibi et al., 2015). The size of these spherical particles is dependent upon the manufacturing method and can range from tens of nanometers to tens of micrometers. The possibility of encapsulation of both polar and non-polar compounds is one of liposome’s advantages. In addition, the possibility of being produced on an industrial scale by natural constituents such as lecithin from egg yolk, soy beans, and cholesterol make this vesicle non-toxic (Makwana et al., 2014; Mekkerdchoo et al., 2009; Mozafari et al., 2006).

Escherichia coli O157:H7 is a Gram-negative and facultative anaerobic food-borne bacterial pathogen (Djenane et al., 2012; Solomakos et al., 2008). Consumption of contaminated raw or undercooked vegetables, meat, and milk may result in the disease manifested by bloody diarrhea, hemorrhagic colitis, hemolytic uremic syndrome, thrombotic thrombocytopenic purpura, and even death (Hussein and Bollinger, 2005; Solomakos et al., 2008). *E. coli* O157:H7 is estimated to cause 20 deaths, 2138 hospitalizations, and 63153 illnesses per year in the United States (Harris et al., 2012).

The major purpose of this study was to evaluate physicochemical characteristics of garlic (*A. sativum* L.) EO nanoliposome and its antibacterial effect on *E. coli* O157:H7.

Materials and methods

Chemicals

Cholesterol and soybean phosphatidylcholine were purchased from Sigma–Aldrich Company (Germany) and Lipoid GmbH (Germany), respectively. All organic solvents were analytical graded. Deionized water was used throughout the experiment.

Plant material and extraction procedure

Fresh garlic bulbs (*A. sativum* L.) were purchased from local markets of Hamedan province of Iran and identified by the Research Institute of Medicinal Plants, Tehran University of Medical Sciences, Tehran, Iran. After pressing and squeezing the garlic bulbs, they were homogenized in distilled water with a ratio of 1:5 and subjected to steam distillation using a Clevenger-type apparatus for 4 h. The collected EO was dried by adding anhydrous sodium sulfate (Na_2SO_4) and stored at 4 $^{\circ}$C before being used (Khanjari et al., 2013).

Gas Chromatography/Mass Spectrometry (GC/MS) analysis of EO

GC/MS analysis of EO was performed on a gas chromatograph Hewlett-Packard 6890 series II fitted with an HP-5ms column (30 m length×0.25 mm id., 0.25 µm film thickness) and interfaced with an HP 5973 mass spectrometer (Agilent, USA). At first, the column temperature was programmed at 50 $^{\circ}$C for 6 min; the temperature was increased up to 240 $^{\circ}$C at 3 $^{\circ}$C/min. After that, the temperature was increased to 300 $^{\circ}$C at 15 $^{\circ}$C/min and held isothermally for 3 min. For GC/MS detection, an electron ionization mode with ionization voltage of 70 eV was used. Also, Helium was applied as the carrier gas with a flow rate of 1.5 ml/min and the temperature of the ion source was 250 $^{\circ}$C. Identification of the oil components was based on the comparison with their retention indices (C_7–C_{20} *n*-alkanes) as well as mass spectra fragmentation pattern with those of the manufacturer’s database and literature data.

Preparation of nanoliposomes by ethanol injection method

According to the method described by Chiraz et al. (2010), liposomes were prepared by an ethanol injection method as follows. After dissolving EO, phospholipids and cholesterol in ethanol, the resulting solution was

injected by a syringe pump (JMS, model SP-500) in a defined volume of Phosphate Buffered Saline (PBS) under magnetic stirring. Liposomes were formed once the ethanolic solution was in contact with PBS solution. After keeping this suspension under stirring for 15 min at room temperature, rotary evaporation (IKA, rv 10 digital V, Germany) under reduced pressure was used to remove ethanol and part of the water.

Mean particle size and zeta potential measurements

The mean particle size, polydispersity and zeta potential of liposomes were measured by a dynamic light scattering technique (Brookhaven Instruments Ltd., Brookhaven, USA). Samples were diluted with PBS before measurement. All the measures were carried out at 25 °C with a fixed scattering angle of 90 degree and wavelength of 657 nm.

Evaluation of encapsulation efficiency

Dialysis technique was applied against PBS at 4 °C to determine encapsulation efficiency. In order to separate the non-entrapped EOs from nanoliposomes, a cellulose membrane (molecular weight cut off 10 K) was used. After disrupting dialyzed liposomes using methanol, the quantity of encapsulated EOs was determined using a spectrophotometer (Beckman, DU 530, Switzerland) at 275 nm according to Khatibi et al. (2015).

Permeability analysis

As indicated previously by Lu et al. (2014), the prepared nanoliposomes were stored at 4 °C over a period of 50 days for testing their permeability. The changes in entrapment efficiency were investigated over storage periods of 5, 10, 30, and 50 days.

Preparation of E. coli O157:H7 inoculum

E. coli O157:H7 (ATCC 35218) was obtained from Department of Microbiology, Faculty of Veterinary Medicine, University of Tehran and cultured overnight for two consecutive times in tubes containing 10 ml Brain Heart Infusion (BHI) broth medium (Merck, Germany) at 35 °C for 16-18 h. Then, the *E. coli* broth culture was adjusted to optical density of 0.1 at 600 nm in 13×100 mm sterile cuvette using a Spectronic 20 spectrophotometer (Milton Roy Company, Ivyland, USA). This optical density corresponded to 1×10^8 CFU/ml *E. coli*. Double plating of serial dilutions in BHI agar was applied to determine bacterial counts as well as counting colonies after 24 h of incubation at 37 °C.

Minimum Inhibitory Concentration (MIC)

Broth macrodilution technique was used to determine MIC. After preparation of different concentration of free and nanoencapsulated forms of garlic EO (0, 0.01, 0.02, 0.03, 0.04, and 0.06%) in 2 ml tubes containing BHI broth with 5% (v/v) dimethylsulfoxide (as the emulsifier) and 0.05% (w/v) agar-agar (as the stabilizer), 20 µl *E. coli* suspension with 1×10^6 CFU/ml was inoculated to each tube. The tubes were incubated at 35°C for 24 h and finally turbidity in tubes was evaluated to determine MIC.

Minimum Bactericidal Concentration (MBC)

To determine the MBC, 100 µl suspension in each tube with no visible growth (turbidity) was spread over BHI agar medium and incubated at 37 °C for 24 h.

Statistical analysis

The results are expressed as means±standard deviation. Data were analyzed by one-way ANOVA followed by Tukey test using SPSS software for Windows (version 16.0, SPSS Inc., Chicago, IL, USA). All experimental analysis carried out in triplicate.

Results

The main constituents of the garlic EO were diallyl trisulfide (31.23%), diallyl disulfide (25.17%), methyl allyl trisulfide (14.27%), propenyl dithiopropanoate (7.63%), dimethyl trisulfide (3.87%), as well as diallyl tetrasulfide (3.42%).

The average particle sizes of prepared nanoliposomes were 131.73±14.31 nm with a polydispersity index of 0.212±0.013. Also, the zeta potential value was -23.20±0.87 mV. Entrapment efficiency of prepared nanoliposome was 64.27±0.78%. The percentage of liposome permeability after 5, 10, 30, and 50 days were 0.46%, 2.47%, 5.63%, and 7.29%, respectively, revealing significant difference ($p<0.05$) among various intervals.

The MIC and MBC values of the nanoliposome encapsulated garlic EO against *E. coli* O157:H7 were 0.02% and 0.03%, respectively, and for non-encapsulated EO were 0.03% and 0.04%, respectively.

Discussion

More than half of the dominant constituents of garlic EO in this study composed of diallyl trisulfide as well as diallyl disulfide, showing similarity with findings of Razavi Rohani et al. (2011) and Dziri et al. (2014). However, some minor differences in chemical composition of the EO may be due to variation in some factors such as geographical and climate conditions, species, age of the plant, etc. It is stated that the antimicrobial activity of garlic is completely eliminated when the thiosulfinates

(e.g. allicin) are removed from the garlic EO (Hughes and Lawson, 1991). Allicin exhibits its antimicrobial activity primarily through immediate and total inhibition of RNA synthesis; however, DNA and protein syntheses are partially inhibited (Feldberg et al., 1988). Also, the level of lipid content of cell membranes is one of the reasons behind the effect of allicin and other garlic constituents on Gram-negative bacteria (Sivam, 2001).

Our findings demonstrated that the MIC and MBC values of the nanoliposome encapsulated garlic EO against *E. coli* O157:H7 were lower than those of non-encapsulated garlic EO, indicating more susceptibility of the bacteria in nanoliposome encapsulated garlic EO group in comparison with the non-encapsulated one. It is stated that the nanoencapsulation process improves the biological activities of EOs, through development of their bioavailability because of increasing surface to volume ratio by decreasing particle size into nano scale (Ribeiro-Santos et al., 2017). In a similar study, Biddeci et al. (2016) indicated that a bionanocomposite film containing peppermint EO showed enhancing antibacterial effect on *E. coli* and *Staphylococcus aureus*, which is in accordance with the present work. Also, Beyki et al. (2014) found an improved antifungal property of pepper mint EO encapsulated in chitosan-cinnamic acid nanogels. Similar results have been reported by Makwana et al. (2014) and also Mekkerdchoo et al. (2009).

In the current study, the average particle size of prepared nanoliposome was 131.73±14.31 nm with a polydispersity index of 0.212±0.013. Similar findings were reported by Lu et al. (2014), who noted mean size of 145.27±15.19 nm and a polydispersity index of 0.204±0.011 for encapsulated allicin. Also, the zeta potential value of this study was -23.20±0.87 mV, while for Lu et al. (2014)'s study was -40.10±0.96 mV. Normally, a value between -30 mV and +30 mV would be considered as high and acceptable zeta potentials. The entrapment efficiency of prepared nanoliposomes was 64.27±0.78% for this study in comparison with 75.20±0.62% for that of Lu et al. (2014). Gitin et al. (2011) encapsulated *A. sativum* L. with the batch particles from gas saturated solutions system with an encapsulation efficiency of 26.1-48.93% and particle sizes ranging from 71.124 µm to 205.64 µm. Also, Yang et al. (2009) encapsulated *A. sativum* L. using polyethylene glycol with mean size of 233±108 nm. Variations in results of previous researches may be due to the differences in nanoencapsulation method applied.

Conclusion

Nanoliposome encapsulated garlic EO showed enhanced antimicrobial activity against *E. coli* O157:H7 in comparison with the non-encapsulated one. The findings of the present study could be recommended to food industries to use nanoliposome encapsulated garlic EO as a natural and effective food preservative.

Conflicts of interest

There is no conflict of interest in this study.

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