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In vitro Evaluation of the Antibacterial Potential of Niosome-Encapsulated Olive Leaf Extract

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Abstract
Olive leaf extract (OLE) loaded niosomes as an antibacterial agent delivery system against *Escherichia coli* (ATCC 25922), *Salmonella* Typhimurium (ATCC 14028), *Listeria innocua* (ATCC 33090) and Methicillin-resistant *Staphylococcus aureus* (MRSA) were investigated by comparing with the antibacterial activity of free OLE. Niosomes were prepared using a lipid film hydration method followed by ultrasound-assisted size reduction technique and characterized in terms of morphology, entrapment efficiency (41.92±0.81%), average particle size (2.66±1.25 μm) and zeta-potential (-34.16±6.48 mV). The minimum inhibitory concentrations (MIC) of free OLE and OLE loaded niosomes against strains were determined. The most susceptible strain against free OLE was the MRSA with MIC value of 2.34 mg/mL OLE. However, the MIC value of OLE loaded niosomes against MRSA was 100 mg/mL. Unfortunately, OLE loaded niosomes (150 mg) with max capacity did not show any antibacterial effect against other three strains due to entrapment efficiency. In conclusion, OLE loaded niosomes has promising value as a novel antimicrobial delivery system to control MRSA.

Keywords: Olive leaf extract, microencapsulation, noisome, oleuropein, antibacterial activity.

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1.Introduction

Recent advances in nanotechnology ensured that using lipid-based encapsulation technologies including liposomes and niosomes, for encapsulation of bioactive compounds has overgrown (Shishir *et al.* 2018). Biocompatible and degradable niosome developed as an alternative to the liposome is self-assembled vesicles in the presence of non-ionic surfactants and cholesterol (Khan and Irchhaiya 2016). Niosomes and liposomes are nearly the same from the point of their physical properties and formulations. Niosomes have some advantages such as higher chemical stability, adaptability of industrial production at low cost, ease of storage and sterilization to compare with liposomes (Aditya *et al.* 2017). It has been reported that using the niosome for encapsulating active compounds can improve the antimicrobial (Patel *et al.* 2015) and antioxidant (Liang *et al.* 2016) activities and the stability (Ruksiriwanich *et al.* 2014) of the compounds. Liposome-like technology can also mask the unpleasant taste of the therapeutic agent (Sajal *et al.* 2015). On the other hand, the use of suitable microencapsulation techniques for many purposes, such as increasing the stability of natural antimicrobials, masking bitter tastes, improving the diffusion properties of antimicrobials to the foods or pathogenic bacteria cells, are also among favorite research topics.

The olive tree (*Olea europaea* L.) is recognized for its economic importance in the Mediterranean

countries. Rich phenolic content in their fruits and leaves with broad distribution exists (Silva *et al.* 2006). Because of interest to the biofunctionality of the phenolic compounds, the olive fruit and other parts of the olive tree have been investigated. Oleuropein is one of the major phenolic compounds that have antimicrobial activity in olive leaves (Esti *et al.* 1998). Plant-based antimicrobial compounds rise to prominence in the food industry with their therapeutic effect, and they can be utilized for food preservation and various pathogen-induced diseases (Tiwari *et al.* 2009). On the other hand, foodborne pathogens such as *Escherichia coli,* Salmonella spp., Campylobacter spp., *Listeria monocytogenes,* and *Staphylococcus aureus* are reported as a significant public health concern (Boskovic *et al.* 2015). The overuse of antibiotics against pathogen causing infections has made common the form of antibiotic-resistant strains, like methicillin-resistant *Staphylococcus aureus* (MRSA). MRSA threat human health in hospital and community, and cause of severe healthcare-associated infections, furthermore community-associated MRSA, livestock-associated MRSA, and healthcare-associated MRSA can be found in foods. Farms, livestock and people working in animal husbandry can be a significant source for the contamination of MRSA to foods, especially animal-based foods (Sergelidis and Angelidis 2017).
The major purpose of this study is to microencapsulate OLE using niosomal encapsulation technique and to determine the antibacterial activities of OLE and OLE loaded niosomes against food-borne pathogens including *Listeria innocua* (ATCC 33090), *Escherichia coli* (ATCC 25922), *Salmonella* Typhimurium (ATCC 14028) and Methicillin-resistant *Staphylococcus aureus* (MRSA).

2. Materials and Methods

2.1. Plant Material and Preparation of the Samples: Homogeneous powder from fresh leaves of Peranzana olive cultivar was prepared following the method suggested by Can *et al.* (2018). Powdered leaves were mixed with 80% aqueous ethanol (1:30 w:v) and exposed to an extraction process using a water bath (Memmert WNB 10, Germany) at 80°C for 10 minutes. The ethanolic phase of the mixture was evaporated using a rotary evaporator (Büchi Rotavapor R-300, Switzerland) and lyophilized (Christ, Germany). The lyophilized sample was mixed with distilled water (1:7) (w/v) and centrifuged at 14000 rpm for 5 minutes (Centurion Scientific, UK). The supernatant was filtered through a 0.20 µm PTFE filter and was the lyophilized second time. Samples were kept in at -22 °C until the subsequent analyses were acquired.

2.2.Oleuropein Analysis: Oleuropein content of leaves was determined by using the methodology previously described by Al-Rimawi (2014). Oleuropein analysis was performed using an analytical HPLC system (Shimadzu LC 20A, Japan), equipped with a DAD (diode array detector) and an Inertsil C18 ODS 3 (250 x 4.6 mm, 5 µm particle size) reverse phase column. Oleuropein content was expressed as "mg oleuropein/g DW (dry weight) leaves."

2.3.Total Phenolic Content (TPC): Total phenolics in the samples were analyzed following the Folin–Ciocalteu method (Singleton and Rossi 1965) and expressed as mg GAE (gallic acid equivalents)/g DW leaves.

2.4. Preparation of Vesicles: Preparation of niosomes were done using the lipid film hydration method followed by ultrasound-assisted size reduction technique with minor modifications reported by Ruckmani and Sankar (2010). 100 mg/mL OLE in PBS (pH 7.4) was prepared under sterile condition. OLE was filtered through a 0.20 µm PTFE filter and stored at 4°C. 500 mg Span 60 and 500 mg cholesterol were dissolved in 8 mL chloroform, and the organic solvent was removed under vacuum at 150 rpm, 60°C for 2 hours using the rotary evaporator to form the thin film. The dried film hydrated with 5 mL OLE (100 mg/mL) prepared before, followed by shaking at 150 rpm, 60°C for 2 hours using shaking incubator (Biochemical Incubator SPX-70S, China) and ultrasonic bath (Isolab, Germany) at 60°C for 20 minutes. For the preparation of empty niosomes, the dried film was hydrated with sterile PBS (pH 7.4) without OLE with same hydration procedure. The niosomal suspension was maturated night long at 4°C. The maturated niosomes were sonicated at 30°C for 30 min by ultrasonic bath sonicator to achieve a niosomal dispersion containing both free and entrapped OLEs of varying size and stored at 4°C for further analysis.

2.5. Characterization of Niosomes

2.5.1. Entrapment Efficiency: The unentrapped OLE was separated from the niosomal dispersion by centrifugation method with modifications reported by Pawar *et al.* (2016). OLE loaded niosomes were washed with PBS and dispersion was centrifuged at 4°C, 15000 rpm for 30 min using a refrigerated centrifuge (Hettich Universal 320R, Germany). The concentration of the free OLE in the supernatant was

analyzed by the Folin–Ciocalteu method. The percentage of OLE entrapment in niosomes was calculated as:

$$\text{Entrapment efficiency (\%EE)} = [(Ct - Cf)/Ct]x100 \quad \text{(Eq. 1)}$$

Where *Ct* is the total and *Cf* is the free OLE concentration

2.5.2. Vesicle Diameter: The niosomal dispersion was prepared at 0.5 mg/mL concentration with 0.01 M potassium chloride for use in the dynamic light scattering (DLS) method. A Particle Size Analyzer (90 Plus, Brookhaven Instrument Corp., USA) was used to determine the average particle sizes of niosomes.

2.5.3. Zeta Potential: The niosomal dispersion was prepared at 0.25 mg/mL concentration with 0.01 M potassium chloride. Zeta potential measurement of niosomal dispersion was performed using the ZetaPals Zeta Potential Analyzer BIC (Brookhaven Inst. Corp., USA).

2.5.4. Optical Microscopy: The niosomal dispersion was viewed under an optical microscope (Zeiss, PrimoVert, Germany) to observe the shape and lamellar nature of the vesicle before and after sizing.

2.5.5. Transmission Electron Microscopy (TEM): A drop of niosomal dispersion was placed onto a carbon-coated grid and allowed to air dry to a thin film. The effective formation of the vesicles was investigated at 120 kV using TEM (JEOL JEM 1400 Plus TEM, Japan).

2.5.6. Scanning Electron Microscopy (SEM): A drop of niosomal dispersion was placed on clear glass stub and air dried. The sample was coated with Palladium-Gold. Surface morphology of samples was viewed at 10 kV using SEM (FE-SEM JFM 7100F EDS, JEOL, Japan).

2.6. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC): The MICs of free and OLE loaded niosomes were determined against four bacteria using standardized broth microdilution technique (Kuete and Metuno 2007). 750 mg/mL OLE in PBS (pH 7.4) was prepared under sterile condition. 750 mg/mL stock OLE solution was diluted with PBS to prepare concentrations (750-37.5 mg/mL) against *L. innocua, E. coli* and *S.* Typhimurium, and concentrations (75-0.29 mg/mL) against MRSA. 300 mg/mL stock niosomal dispersion was diluted with PBS to prepare seven concentrations (300-6.25 mg/mL) against *L. innocua, E. coli* and *S.* Typhimurium, six concentrations (200-6.25 mg/mL) against MRSA. 100 μL of each sample solution (OLE or niosomes) was added to the sterile 96 wells that contained 95 μL Mueller-Hinton Broth and 5 μL bacterial suspensions (10^7 CFU/mL). The final inoculum concentration in the well is 10^5 CFU/mL. In order to control the growth of the microorganism, 195 µL MHB and 5 µL of inoculum (10^7 CFU/mL) were pipetted into well. For sterility control, 200 µL MHB was incubated. Bacteria were incubated in a spectrophotometer at 37°C for 18 hours and absorbance was measured at 620 nm per hour. Experiments were performed in duplicate. The MBC value was higher than the MIC value and indicated that there was no colony growth from samples incubated at 37°C for 18 h.

2.7. Statistical analyses
Statistical Analysis Systems Version 9 (SAS Institute Inc, Cary, NC, USA) with MİXED procedure was used to analyze the data.

3. Result and Discussion
3.1. Characterization of OLE Loaded Niosomes
The ratio of surfactant, cholesterol and other additives are important parameters as well as the ratio of the active substances to the total lipids added to the formulation (Mokhtar *et al.* 2008). Span 60: Cholesterol (1:1 w/w) ratio was selected since it gives the optimal encapsulation efficiency according to many studies (Balakrishnan *et al.* 2009, Shilakari *et al.* 2016). It has been reported that Span 60 (HLB 4.7) enables higher encapsulation efficiency compared to Span 20, due to its high phase transition temperature. On the other hand, the permeability of biomolecular the membranes and structure of the hydrocarbon chain in surfactant are principal for encapsulation of hydrophilic molecules in lipid-based capsules. Span 60 has saturated acyl chains in the semi-solid gel state under normal ambient conditions, so leakage of the hydrophilic active substance from the vesicles prepared with span 60 is less (Abdelkader *et al.* 2010). Cholesterol has a significant influence on membrane properties. Nematollahi *et al.* (2017) reported that while membrane stability increase with cholesterol usage in the niosomal formulation, membrane fluidity decrease.

Characterization of the prepared niosomes was achieved by determining encapsulation efficiency, vesicle diameter, and zeta potential. Light microscopy, SEM, and TEM were also used to examine the morphology of niosomes. Also, the encapsulation efficiency of the niosomes is the most essential parameter in terms of pharmacy with its potential use in drug delivery systems (Balakrishnan *et al.* 2009). The encapsulation efficiency of the OLE loaded niosomes was 41.92±0.81% after free OLE was removed from the capsules. Particle size which varies with the HLB value and the chain length of the surfactant is an essential factor for encapsulation efficiency and release of the active substance (Uchegbu and Vyas 1998). The ultrasonic method (30°C, 30 min) was applied in order to reduce the size of niosomes, and the average vesicle diameter of the prepared OLE loaded, and empty niosomes were 2.66±1.25 µm and 2.37±1.41 µm, respectively.

Similarly, the diameter of niosomes prepared by Aswathy and John (2014) was reported between 2.58 µm and 3.13 µm. The zeta potentials of OLE loaded, and empty niosomes were measured as -34.16 ± 6.48 mV and -70.68 ± 4.75 mV, respectively. The charge on the surface of the vesicles produces a homogeneous dispersion because of producing a repulsive force between the vesicles. Values of zeta potential above 30 mV (positive or negative) are adequately high for electrostatic stability and a stable dispersion (Mahajan *et al.* 2012). Patel *et al.* (2015) reported that the zeta potentials of propolis-loaded and free niosomes were -33.2 mV and -38.8 mV, respectively.

As shown in Figure 1, niosomes are seen in different shapes and sizes after maturation. Vesicle sizes prominently decreased, and the distribution of vesicles became more homogenous after sizing with the ultrasound application. SEM micrographs reveal the empty and OLE loaded niosomes are spherical and TEM micrographs confirm the presence of homogeneously dispersed vesicles. However, while the shapes of the empty niosomes are generally spherical, different forms were also observed in OLE loaded niosomes.

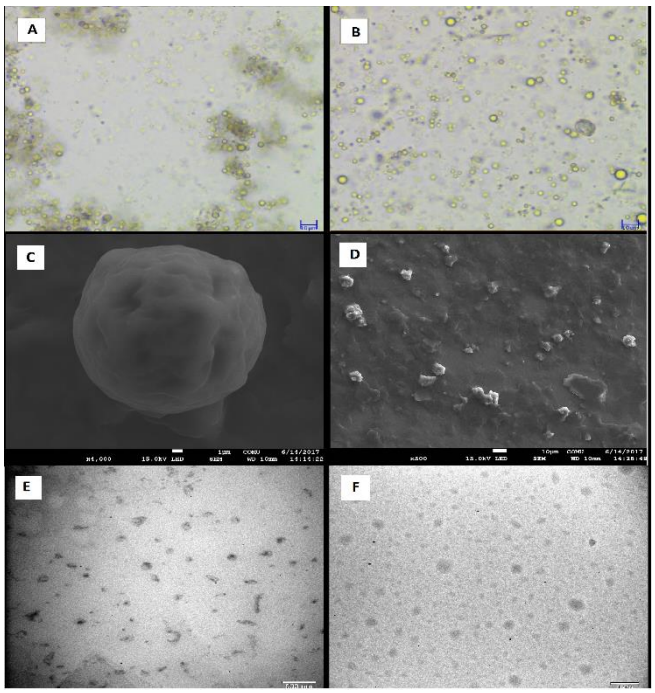

Figure 1. (A) OLE loaded niosome after maturation (x10), (B) OLE loaded niosome after sizing (x10), (C) SEM micrograph of OLE loaded niosomes (4000x), (D) SEM micrograph of OLE loaded niosomes (x4000) (E) TEM micrograph of OLE loaded niosomes (x500) (F) TEM micrograph of empty niosomes.

3.2. *In Vitro* Antibacterial Activity of free OLE and OLE Loaded Niosomes

The MIC and MBC values of free OLE and OLE loaded niosomes for each test strain are given in Table 1. MICs of free OLE were 2.34 mg/mL (including 70.60±3.68 µg oleuropein) against MRSA and 150 mg/mL (including 3380.00±169.71 µg oleuropein) against *E. coli, S.* Typhimurium and *L.innocua*. The growths of bacteria have slowed but not stopped at OLE concentrations lower than 2.34 mg/mL for MRSA and lower than 150 mg/mL for *E. coli* and *S.* Typhimurium, compared to inoculum growth (Figure 2). The antimicrobial activity of free OLE depends on some parameters such as leaf origin, harvesting time, climate, extract preparation methods (Ranalli *et al.* 2006) and also oleuropein content of extract as

we observe in this study. The antibacterial activity of oleuropein results from the presence of the ortho-diphenolic system due to the number and location of hydroxyl groups of the phenolics (Cowan 1999). Pereira *et al.*(2007) reported that OLE damages the cell membrane permeability due to the protein denaturation. Moreover, isolated phenolic compounds can be less active than plant extracts because of the interaction of phenolics each other.

Table 1. Viable counts in samples of OLE and OLE loaded niosomes inoculated with listed strains incubated at 37°C for 18 hours

Inoculum	Sample	Conc. (mg/mL)	Oleuropein content (µg/mL)	$t_{initial}$ (log_{10} CFU/mL)	$t_{18\ h\ (inoculumcontrol)}$ (log_{10} CFU/mL)	$t_{18\ h}$ (log_{10} CFU/mL)	Difference** (log_{10} CFU/mL)
MRSA	OLE	2,34	70,60±3,68	6,02±0,03*	9,17±0,09	≤1	-9,17
	Niosome	50	76,53±2,79	5,89±0,05	9,17±0,09	8,75±0,10	-0,42
		100	153,10±7,41	5,87±0,05	9,17±0,09	5,57±0,09	-3,60
		150	229,60±3,61	5,99±0,01	9,17±0,09	≤1	-9,17
	Empty Niosome	50	-	6,09±0,08	9,17±0,09	8,91±0,07	-0,26
		100	-	5,93±0,03	9,17±0,09	8,98±0,07	-0,19
		150	-	5,93±0,03	9,17±0,09	9,15±0,11	-0,02
E. coli	OLE	150	3380±169,71	5,37±0,07	9,01±0,15	≤1	-9,01
	Niosome	150	229,60±3,61	5,65±0,05	9,01±0,15	9,02±0,09	0,01
	Empty Niosome	150	-	5,61±0,02	9,01±0,15	9,03±0,11	0,02
S. Typhimurium	OLE	150	3380±169,71	5,81±0,08	9,24±0,05	≤1	-9,24
	Niosome	150	229,60±3,61	6,24±0,01	9,24±0,05	9,19±0,06	-0,05
	Empty Niosome	150	-	6,49±0,07	9,24±0,05	9,28±0,06	0,04
L. innocua	OLE	150	3380±169,71	6,50±0,06	7,65±0,07	≤1	-7,65
	Niosome	150	229,60±3,61	6,49±0,14	7,65±0,07	8,23±0,08	0,58
	EmptyNiosome	150	-	6,64±0,13	7,65±0,07	7,21±0,24	-0,44

*Values are expressed as means ± S.D. (n=2), Detection limit ≤ 1 log_{10}CFU/mL
**Difference =$t_{18\ h\ (inoculum\ control)} - t_{18\ h}$

The MIC values of OLE loaded capsules against *E. coli, S.* Typhimurium and *L. innocua* were lower than150 mg/mL, as seen in Figure 3. However, according to the results of the MBC for the verification of the microdilution method, no inhibition was observed for these pathogens at 150 mg/mL of OLE loaded niosomes. Therefore, deviations in the graphs obtained by broth microdilution method may be the milky color of highly concentrated niosomes analyzed spectrophotometrically.

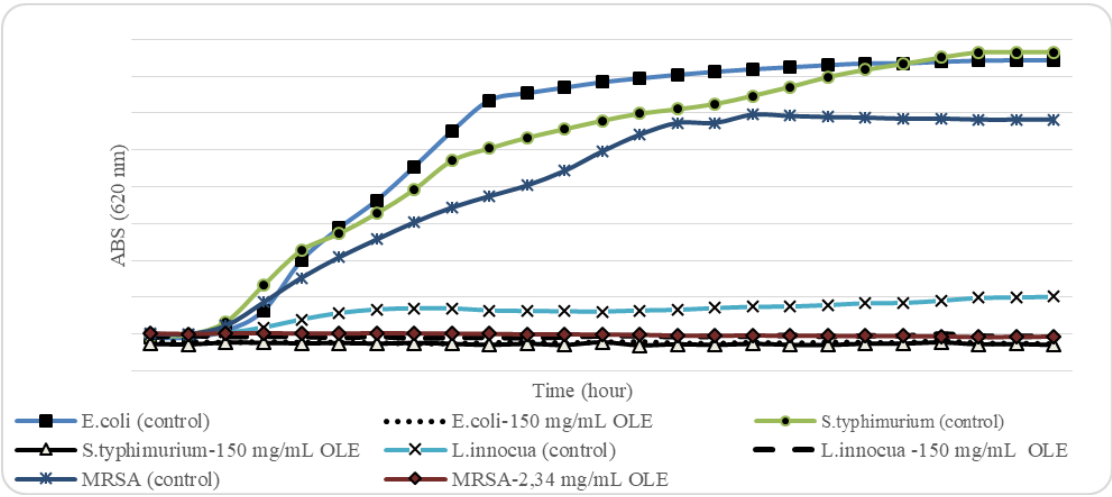

Figure 2. OLE concentrations against *E. coli, S.* Typhimurium, *L. innocua* and MRSA.

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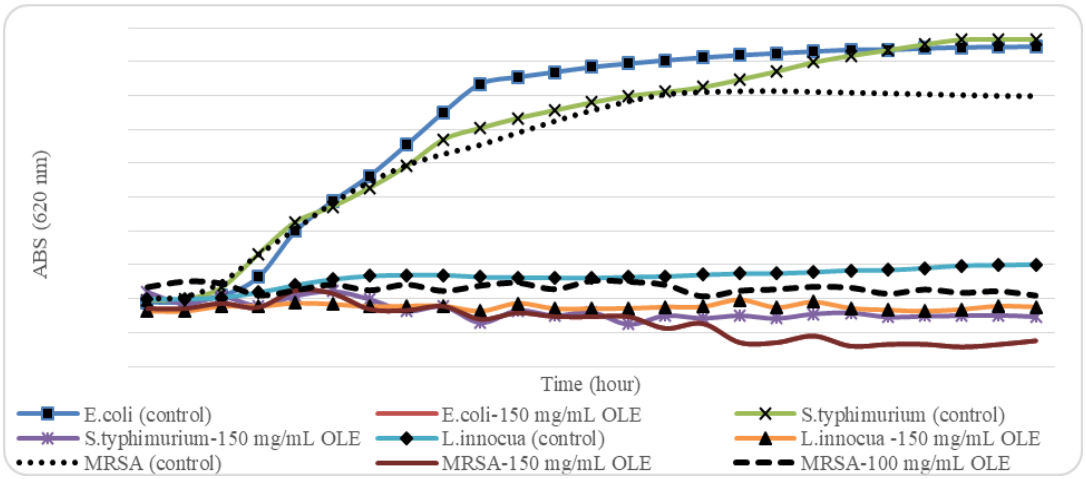

Figure 3. OLE loaded niosome concentrations against *E. coli, S.* Typhimurium*, L. innocua* and MRSA.

For post-incubation samples (t_{18}, Table 1), neither the difference between the count of *E. coli* treated with OLE loaded niosomes and count of control inoculum is not significant (P> 0.05) nor the difference between S.Typhimurium treated with OLE loaded niosomes and control. *Moreover,* OLE loaded niosomes (150 mg/mL) increased the growth of *L. innocua* by 0.58 log (P<0.005). The encapsulated OLE is not sufficient for the inhibition of *E. coli,* S.Typhimurium *and L. innocua*. Also, empty capsules do not show any antibacterial effect against strains (Table 1). On the other hand, the growth of MRSA was not observed at 150 mg (with 229.60±3.61 µg oleuropein)/mL of OLE loaded capsule treatment (P<0.0001). The MIC and MBC of OLE loaded niosomes against MRSA were 100 mg (containing 153.10±7.41µg oleuropein)/ml and 150 mg (containing 229.60±3.61 µg oleuropein)/mL respectively. OLE-loaded niosomes show potential as an antimicrobial agent only against MRSA among pathogens involved in this research.

OLE content of niosomes decreased because of low encapsulation efficiency (41.92±0.81%). For this reason, the OLE loaded niosomes (150 mg/mL) used in the antibacterial activity assays against *E. coli, S.* Typhimurium and *L. innocua,* did not contain enough oleuropein for inhibition. Besides, when OLE and OLE loaded niosomes are compared quantitatively against MRSA, about 3.25 times more oleuropein-containing niosomes seem to be active on inhibition of MRSA when compared to oleuropein content. In literature, results of the agar diffusion method have shown that gatifloxacin loaded niosomes have similar antibacterial activity with aqueous gatifloxacin against *Pseudomonas aeruginosa* and *S.aureus* (Mahajan *et al.* 2012). In another study, MIC values of the free and niosomal moxifloxacin were found the same at 0.87 µg/mL against *S.aureus*, although MIC values of the free moxifloxacin and loaded niosome were found at 6.25 µg/mL and 3.125 µg/mL respectively against *P. aeruginosa* (Sohrabiet *et al.* 2016). Patel *et al.* (2015) prepared propolis-loaded niosomes and has reported that MIC values of propolis loaded noisome and ethanolic propolis solution against *S. aureus* was 0.3 mg/mL and 0.5 g/mL, respectively. The MIC values of free vancomycin and vancomycin-loaded niosome against MRSA were reported as 8 µg/mL and 1 µg/mL, respectively (Barakat *et al.* 2014).

Conclusion

In recent years, using phenolic compounds has attracted considerable attention from the food and pharmaceutical industries because of their functional properties. In this study, *in vitro* use of OLE as an antibacterial agent has shown a significant potential when assessed against *L. innocua* ATCC 33090, *E. coli* ATCC 25922, *S.* Typhimurium ATCC 14028 and methicillin-resistant *S. aureus* (MRSA). MRSA has susceptible deficient concentrations of OLE that can reduce potential risks particularly in the field of health. On the other hand, niosomal form of OLE was developed to offer useful and promising antimicrobial agent carrier system in order to enhance the food shelf-life and safety applications. *In vitro* antibacterial potential of OLE loaded niosome can be improved by using new formulations and techniques. These results warrant further research on oleuropein purification from OLE to increase the loading efficiency of niosome formulations to improve antimicrobial efficacy.

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Conflict of interest
All of the authors declare that there is no conflict of interest.

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