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Preparation, characterization, and enhanced antimicrobial activity: quercetin-loaded PLGA nanoparticles against foodborne pathogens

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Abstract: The use of quercetin as a bioflavonoid is becoming increasingly common in food industries even though poor water solubility, instability, absorption, and permeability have limited its application. The oil-in-water single-emulsion solvent evaporation method to synthesize highly stable and soluble quercetin-encapsulated nanoparticles (NPs), in which the reaction yield, particle size, and polydispersity of the NPs are varied greatly within the process parameters of the synthesis method, has been optimized. NPs with different initial quercetin amounts were used to determine how the quercetin amount affected nanoparticle properties and antimicrobial efficiency. *Listeria monocytogenes*, *Salmonella typhimurium*, *Escherichia coli*, and *Staphylococcus aureus* were chosen as model bacteria due to their being foodborne pathogens. The results of antimicrobial activity evaluated by three different methods showed that the antimicrobial activity of both quercetin NPs and free quercetin was effective on gram-positive strains (*L. monocytogenes and S. aureus*). Additionally, it was detected that Q31 NPs have more effective antimicrobial activity than other synthesized quercetin nanoparticles depending on the amount of substance and release. Furthermore, on the basis of assessing the antibacterial effects by scanning electron microscopy, it was detected that bacteria cells lost their integrity and became pale with the release of cytoplasm and decomposed after treatment with Q31 NPs.

Key words: Antimicrobial activity, nanoparticle, pathogens, PLGA, quercetin

1. Introduction

Quercetin (3,3,4,7-pentahydroxyflavone), a well-known bioflavonoid, has generated considerable interest within the food and drug industries due to its beneficial properties including antioxidant, antiinflammatory, antimutagenic, anticarcinogenic, antibacterial, and antiviral effects (Budhian et al., 2007; Bhutada et al., 2010; Kumari et al., 2010a; Chitkara et al., 2012). It is found abundantly in fruits and vegetables that are essential parts of the human diet (Chen et al., 2010; Kumari et al., 2010a; Nathiya et al., 2014). Notwithstanding this wide spectrum of phytochemical properties, poor water solubility, absorption, permeability, instability, and light-induced decomposition of quercetin limit its use in food formulations (Kumari et al., 2010a). Moreover, direct application in food matrices generally results in reduced activity because of its interactions with other components such as proteins and lipids. Therefore, quercetin presents a challenge for antimicrobial activity because interaction of quercetin with microorganisms is difficult in aqueous media (Vashisth et al., 2013; Silva et al., 2014).

Throughout the world, many researchers are exploring efficient drug delivery vehicles that offer safe and efficient transmission of therapeutic drugs (Stevanovic and Uskokovic, 2009; Boimvaser et al., 2016). Poly(lactic-co-glycolic acid) (PLGA) is one of the most effectively developed biocompatible and biodegradable polymers that can be utilized for medicine and pharmaceuticals (Hans and Lowman, 2002; Kumari et al., 2010b). Recently, several reviews have summarized the developments of nanoencapsulation techniques and discussed the important factors for bactericidal activity, such as the influence of very small particle sizes and high surface-to-volume ratios (Derman et al., 2015). For biodegradable polymeric nanoparticles, PLGA is an appropriate system for encapsulation of antimicrobial compounds, especially given the hydrophobic structure in consequence of its high hydrophobicity (Kumari et al., 2010b), strong

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mechanical strength, slow drug release (Vashisth et al., 2013), biodegradability, biocompatibility, and low toxicity. Several natural compounds with antimicrobial activities have been encapsulated in nanoparticles. For instance, Gomes et al. (2011) stated that growth inhibition of *Salmonella* spp. (gram-negative bacteria) and *Listeria* spp. (gram-positive bacteria) was observed when eugenol and trans-cinnamaldehyde-loaded PLGA nanoparticles (NPs) were used at different concentrations between 10 and 20 mg/mL. Darvishi et al. (2015) reported that the minimum inhibitory concentration (MIC) of 18-β-glycyrrhetinic acid (GLA)-loaded NPs was approximately four times lower for *Pseudomonas aeruginosa*, three times lower for *Staphylococcus epidermidis*, and two times lower for *Staphylococcus aureus* when compared to free GLA solution regardless of the drug-to-polymer ratio. Pool et al. (2012) showed that encapsulation of different flavonoids in PLGA nanoparticle systems increased the antiradical and chelating properties of quercetin and catechin (Pool et al., 2012).

In the literature, the antimicrobial effect of quercetin was assessed by using both gram-positive and gram-negative bacteria, and, in particular, selective antimicrobial activity was revealed against *S. epidermidis*, *S. aureus*, and methicillin-resistant *S. aureus* (MRSA) (Ramadan et al., 2009; Hirai et al., 2010). Although quercetin has been tested against gram-positive and gram-negative bacteria and several studies have been published about the antimicrobial properties of quercetin (Ramadan et al., 2009; Hirai et al., 2010), its antimicrobial activity in PLGA NP systems has not been clearly explained in terms of the mechanism of microorganism inhibition, in which it plays an essential role against pathogens (Nathiya et al., 2014).

Therefore, the aims of this work were to synthesize and characterize quercetin-loaded

PLGA nanoparticles with different drug/polymer ratios in detail and to assess the antimicrobial efficiency of PLGA-loaded quercetin nanoparticles against foodborne pathogens (*Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium*, and *Listeria monocytogenes*). For this purpose we synthesized highly stable and soluble quercetin-encapsulated NPs using the oil-in-water (o/w) single-emulsion solvent evaporation method. Quercetin-loaded PLGA NPs differed from each other in terms of encapsulation efficiency, reaction yield, particle size, and polydispersity. Subsequently, to assess the antimicrobial activity, agar well diffusion, broth dilution, and time-kill assay methods were performed and the obtained results were supported by scanning electron microscopy (SEM) investigation of bacteria before and after exposure to NPs.

2. Materials and methods

Quercetin, polyvinyl alcohol (PVA), PLGA (lactide:glycolide = 50:50; Mw ~ 38–54 kDa), and dichloromethane (DCM) were purchased from Sigma-Aldrich (St. Louis, MO, USA). For microbiological tests, Mueller-Hinton broth (MHB) and Mueller-Hinton agar (MHA) media (both from Difco, BD Diagnostic Systems, Hunt Valley, MD, USA) were used. *Listeria monocytogenes* (ATCC 35152), *Salmonella typhimurium* (ATCC 14028), *Staphylococcus aureus* (ATCC 25923), and *Escherichia coli* (ATCC 25922) were provided by the Atatürk University Biology Department (Erzurum, Turkey).

2.1. Preparation of quercetin-loaded PLGA nanoparticles

In this study, nanoparticle preparation was carried out in three different parts. In the first part, quercetin-loaded nanoparticles were synthesized using three different methods, which including nanoprecipitation, salting-out, and single emulsion-solvent evaporation.

In the nanoprecipitation method (Budhian et al., 2007), PLGA and quercetin were dissolved in 5 mL of acetone and added to 50 mL of PVA (1% w/w) solution drop by drop. In contrast to the second and third experiments, in the first experiment (Q1 NP) external power was not applied. On the other hand, in the second (Q2 NP) and third (Q3 NP) experiments, 120 s of sonication (80% amplitude) and 5 min of homogenization at 24,000 rpm were applied, respectively.

In the salting-out method (Sah and Sah, 2015), PLGA and quercetin were dissolved in 5 mL of acetone and added to 20 mL of PVA (2% w/w), which included 60% $MgCl_2$. For Q4 NP and Q5 NP, 120 s of sonication (80% amplitude) and 5 min of homogenization at 24,000 rpm were applied, respectively.

In the single emulsion-solvent evaporation method (Derman, 2015), quercetin and PLGA were dissolved in DCM (1.5 mL) and ethanol (0.750 mL), respectively (Q6 NP). The organic mixture was then emulsified with an aqueous PVA solution (4 mL, 3% w/w). For emulsification, 120 s of sonication (80% amplitude) was applied, and then this solution was added to PVA solution (35 mL, 0.1% w/w).

In all experiments, 100 mg of PLGA and 10 mg of quercetin were used and the organic solvent was removed by mixing the solution at room temperature and 700 rpm for 4 h. The obtained NPs were characterized in detail and the best results were achieved with the single-emulsion solvent evaporation method. Therefore, the optimization study was carried out by o/w single-emulsion solvent evaporation method.

In the second part, which was the optimization study of single-emulsion solvent evaporation method, different homogenization or sonication power (Table 1) was applied for obtaining the water and aqueous phase emulsion. For all syntheses, obtained NP suspensions were collected by centrifugation at 12,000 rpm for 30 min at 4 °C. Thereafter, the NPs were washed 3 times with ultrapure water to

Table 1. The effect of different synthesis methods and synthesis parameters on encapsulation efficiency, reaction yield, particle size, PDI, and zeta potential .

Sample	Method	Time (s)	Homogenization speed (rpm)	Sonication power (W)	RY (%)	EE (%)	Particle size (nm)	PDI	Zeta potential (mV)
Q1 NPs	Nanoprecipitation	-	-	-	50.1	75.7	297.75 ± 6	0.211 ± 0.016	–25.5 ± 1.2
Q2 NPs	Nanoprecipitation	120	-	80	76.2	78.2	421.85 ± 28	0.472 ± 0.008	–18.1 ± 0.8
Q3 NPs	Nanoprecipitation	300	24,000	-	56.5	72.7	1457 ± 18	0.256 ± 0.001	–6.5 ± 0.1
Q4 NPs	Salting-out	120	-	80	74.5	63.6	1906 ± 77	0.251 ± 0.004	–2.7 ± 0.1
Q5 NPs	Salting-out	300	24,000	-	51.7	44.7	1107 ± 46	0.324 ± 0.010	–3.4 ± 0.2
Q6 NPs	Oil-in-water single emulsion	120	-	80	86.8	89.1	529.3 ± 14	0.218 ± 0.020	–13.7 ± 0.8
Q7 NPs	Oil-in-water single emulsion	180	24,000	-	55.2	88.2	334.4 ± 8	0.228 ± 0.009	–12.3 ± 0.2
Q8 NPs	Oil-in-water single emulsion	180	18,000	-	64.9	90.1	329.2 ± 8	0.148 ± 0.003	–15.1 ± 0.1
Q9 NPs	Oil-in-water single emulsion	180	12,000	-	66.9	91.9	387.6 ± 11	0.188 ± 0.200	–15.9 ± 0.1
Q10 NPs	Oil-in-water single emulsion	180	6000	-	67.0	95.1	1101 ± 56	0.763 ± 0.300	–19.9 ± 0.2
Q11 NPs	Oil-in-water single emulsion	180	3000	-	67.1	95.1	1138.5 ± 166	0.403 ± 0.020	–27.2 ± 0.8
Q12 NPs	Oil-in-water single emulsion	120	-	20	75.4	93.9	763.05 ± 1	0.456 ± 0.010	–13.7 ± 4.2
Q13 NPs	Oil-in-water single emulsion	120	-	40	87.6	94.3	541.8 ± 2	0.151 ± 0.120	–11.1 ± 1.0
Q14 NPs	Oil-in-water single emulsion	120	-	60	89.8	95.3	403.2 ± 15	0.238 ± 0.015	–12.4 ± 1.2
Q15 NPs	Oil-in-water single emulsion	120	-	80	74.5	92.1	291.6 ± 7	0.245 ± 0.140	–15.0 ± 0.2
Q16 NPs	Oil-in-water single emulsion	120	-	100	76.7	91.7	479.55 ± 5	0.148 ± 0.080	–13.1 ± 0.6

remove any unbound PVA and quercetin. Finally, the pellets were freeze-dried and stored at –80 °C until used.

In the third part, three NP formulations with different drug/polymer ratios (10/100, 30/100, and 50/100) were synthesized for antimicrobial activity studies using optimized process parameters. In the first formulation (Q27 NP), 10 mg of quercetin was dissolved in ethanol and 100 mg of PLGA was dissolved in DCM. The mixed organic phase was emulsified in PVA solution by microtip probe sonicator for 120 s (80% amplitude) and the following preparation steps continued as described above. The second and third formulations (Q29 NP and Q31 NP, respectively) were prepared following the same procedure as that for Q27 NP, except that 30 or 50 mg of quercetin was used in these formulations instead of 10 mg. Free nanoparticles were synthesized with the same protocol without quercetin.

2.2. Characterization of polymeric nanoparticles

2.2.1. Reaction yield and encapsulation efficiency

To detect the encapsulation of quercetin, the amount of quercetin within the solution obtained after centrifugation was calculated using UV-Vis spectroscopy at 374 nm in triplicate.

Quercetin concentration in the supernatant was determined by comparing the concentration to a previously constructed standard calibration curve. The reaction yield (RY) and encapsulation efficiency (EE) of quercetin-loaded nanoparticles were calculated using the formulas given below.

EE % = (amount of quercetin encapsulated in NPs/initial quercetin added) × 100 (1)

RY % = (amount of weighed NPs/amount of initial PLGA+quercetin) × 100 (2)

2.2.2. Particle size, polydispersity index, and zeta potential

Particle size (Z-Ave), polydispersity index (PDI), and zeta potential (ζ) of the NPs were determined with photon correlation spectroscopy using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) equipped with 4.0 mV He-Ne laser (633 nm) (Derman, 2015). A 1/30 dilution of the fresh NP solution in distilled water at 25 ± 0.1 °C was used for measurements and each measurement was performed in triplicate using 0.8872 cP viscosity and 1.330 refractive index for the solutions at a dielectric constant of 79; f(ka), 1.50 (Smoluchowski value).

For determination of the zeta potential of bacterial cell suspensions, overnight cultures of bacteria cell suspensions were washed three times in 0.01 M sodium phosphate buffer (pH 7.4), resuspended in PBS, and adjusted to an OD600 of 0.1 in buffer. The zeta potentials of these solutions were determined under similar conditions as mentioned above.

2.2.3. Scanning electron microscopy

SEM was used for visualization of the shape and size of NPs. Freeze-dried NPs were fixed on metallic studs and then coated with gold under a vacuum (Arasoglu et al., 2015). A JSM-7001FA (JEOL, Tokyo, Japan) scanning electron microscope was used with an acceleration voltage of 7.00 kV.

Furthermore, the interactions of the NPs with bacterial cells were examined using SEM. For this purpose, the bacteria were incubated with the Q31 nanoparticles on MHB for 24 h, then collected by centrifugation, washed three times, and resuspended in 0.01 M sodium phosphate buffer (pH 7.4). The cells were diluted (approximately 10^4 CFU/mL) and fixed on metallic studs. The SEM analyses were carried out under similar conditions as mentioned above.

2.2.4. In vitro quercetin release

The in vitro release profiles of Q27 NPs, Q29 NPs, and Q31 NPs were conducted to determine the release of quercetin from the PLGA NPs according to a previously published dissolution method (Derman et al., 2015). Lyophilized NPs were suspended in the release medium, phosphate-buffered saline with 0.01% sodium azide, and incubated at 37 °C in a shaking incubator (60 rpm), from which at predetermined time intervals up to 120 h (1, 2, 3, 4, 12, 24, 48, 72, 96, and 120 h) medium was fully removed and analyzed for quercetin contents. The quercetin concentration in the supernatant was measured with UV-Vis spectroscopy at 374 nm by comparing the concentration to a previously constructed standard calibration curve.

2.2.5. Evaluation of antibacterial activity

The antibacterial activity of the free quercetin and three different quercetin NPs consisting of different drug/polymer ratios (Q27, Q29, and Q31) was evaluated by three different methods including agar well diffusion, broth dilution, and time-kill assay for four different foodborne microorganisms (*Listeria monocytogenes*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Escherichia coli*).

2.2.5.1. Preparation of bacterial suspensions

All strains were grown on MHB at 37 °C and 200 rpm in a shaking incubator for an overnight period. The bacterial cultures were diluted with sterile buffer solution until the optical density was adjusted to 0.1 at 600 nm (corresponding to 10^8 CFU/mL). This concentration was used as the bacterial working dilution in the methods applied in this study.

2.2.5.2. Evaluation of antibacterial activity by agar well diffusion

The antimicrobial susceptibility tests of the free quercetin and quercetin-loaded

NPs (Q27 NPs, Q29 NPs, and Q31 NPs) were performed as described previously by Irshad et al. (2012). Briefly, petri plates were inoculated with bacterial cultures and then wells were punched in the plates. After adding 100 µL of test sample to each well, petri plates were aerobically incubated at 37 °C for 24 h. The concentrations of samples were determined as 2 mg/mL for NPs and 0.225 mg/mL, 0.589 mg/mL, and 0.733 mg/mL for free quercetin (based on the encapsulation efficiencies of the NPs). The antimicrobial activity was evaluated by measuring the inhibition zones surrounding wells (mm). Empty PLGA nanoparticles and antibiotic disks (gentamicin, 10 µg/disk; ofloxacin, 5 µg/disk; vancomycin, 5 µg/disk; ampicillin, 10 µg/disk) were used as negative controls and positive controls, respectively.

2.2.5.3. Assessment of antibacterial activity according to broth dilution method

This method was carried out in accordance with the relevant 2012 CLSI standard (Cockerill et al., 2012). MIC values of test samples were determined by broth dilution method using micro- and macrodilution techniques.

In the microdilution method, 100 µL of test samples (concentrations as mentioned in Section 2.2.5.2.) were added to the first wells of 96-well plates after adding 95 µL of MHB to each well. Subsequently, 100 µL from their serial dilutions was transferred to five consecutive wells (ranging from 100 µg/mL to 3.125 µg/mL). The last three wells were used as positive (ampicillin antibiotic solution), negative (only bacteria), and blank (only broth) controls, respectively. Finally, 5 µL from each bacterial suspension was inoculated into wells (except blank control). The final volume of each well was adjusted to 200 µL and then the plate was incubated at 37 °C for 24 h. MIC values were obtained spectrophotometrically at OD570 nm.

In the macrodilution method, eight tubes were prepared to contain 8.0 mL of MHB, 1.0 mL of test samples (ranging from 200 µg/mL to 3.125 µg/mL), and 1.0 mL of bacterial working dilution for each sample and each bacterial strain. After 24 h of incubation at 37 °C, a series of diluted solutions were prepared from the test samples into tubes. Each dilution was spread on MHA and incubated at 37 °C for 24 h. The viable bacteria cells were counted. For each bacterial strain, MIC values of free quercetin and quercetin NPs were determined by correlated spectrophotometric readings at OD570 nm and standard plate counts.

2.2.5.4. Evaluation of bacterial growth inhibition by time-kill assay

This method was carried out based on ASTM standard E2149-13a (ASTM International, 2013). In this method, which is designed under dynamic contact conditions and depends on direct contact of bacteria with the drug, antimicrobial activity of a test material is determined by shaking in a bacterial working dilution depending on the time.

In brief, the test samples (concentrations as mentioned in Section 2.2.5.2.) were put into tubes containing 50 mL of MHB and then 0.5 mL of bacterial working dilution was added and the mixture was incubated at 37 °C in a shaking incubator. At selected time points (0 h, 3 h, 6 h, 24 h, 48 h, and 72 h), 100 µL of samples was spread on MHA and then incubated 37 °C for 24 h. Viable colonies were quantified. The results were evaluated in two ways, growth-inhibiting efficiency and bactericidal activity, according to the formulas below:

$$\text{Growth-inhibiting efficiency, \%, CFU/mL} = ((B - A)/B) \times 100 \quad (3)$$

$$\text{Bactericidal activity, \%, CFU/mL} = ((C - A)/C) \times 100 \quad (4)$$

Here, A is CFU/mL for the flask containing the quercetin-loaded nanoparticles after the predetermined time, B is CFU/mL for the flask containing the free quercetin after the predetermined time, and C is the initial number of colonies in the culture broth containing only bacteria.

2.2.6. Statistical analysis

All results were expressed as mean ± standard deviation. SPSS 15.0 was used for statistical analyses (SPSS Inc., Chicago, IL, USA). ANOVA was applied for analyses of variance and the difference between mean values was compared by Tukey's test. A level of probability of $P < 0.05$ was taken as indicating statistical significance (Özbek et al., 2008).

3. Results

3.1. Characterization of nanoparticles

In this study, first, three different synthesis methods including nanoprecipitation, salting-out, and o/w single-emulsion solvent evaporation were compared in terms of encapsulation efficiency, reaction yield, particle size, polydispersity index, and zeta potential.

After detailed characterization of nanoparticles, the o/w single-emulsion solvent evaporation method was found to be most effective based on EE. Thus, the o/w single-emulsion solvent evaporation method was used for the preparation of quercetin-encapsulated nanoparticles. The effect of homogenization (time and speed) and sonication (time and power) processes on the NP characteristics were studied in detail. All experimental conditions and the obtained physicochemical properties of the quercetin-encapsulated PLGA NPs are presented in Table 1.

3.2. Effect of synthesis method, homogenization speed, and sonication power

In the present study, when we examined the results of the three methods used for synthesis of quercetin-encapsulated nanoparticles, the lowest particle size

(297.75 ± 6 nm) was obtained by the nanoprecipitation method with 50.1% RY and 75.7% EE. Otherwise, the best RY (86.8%) and EE (89.1%) values were achieved with the o/w single-emulsion method. In the salting-out method, nanoparticles size were measured in micrometers. On the other hand, in the nanoprecipitation method, application of sonication was more effective than homogenization for low particle size, high EE, and RY.

After achieving the best results with the single-emulsion solvent evaporation method, this method was optimized at different homogenization speeds (3000, 6000, 12,000, 18,000, and 24,000 rpm) and different sonication powers (20, 40, 60, 80, and 100 W) on the basis of minimum particle size, maximum encapsulation efficiency, and high reaction yield (Table 1).

From the results, it was observed that homogenization speed also plays a significant role on particle size and encapsulation efficiency. A linear decrease in particle size was observed with increasing homogenization speed. As mentioned in the literature, high-speed homogenization leads to increased shear force intensity, which overcomes the intraforces acting in the particles (Quintanar-Guerrero et al., 1996; Kushwaha et al., 2013). The encapsulation efficiency and the reaction yield also decreased linearly with decreasing homogenization speed. At 24,000 rpm homogenization speed the RY and EE were calculated as 55.2% and 88.2%, respectively. These results, obtained in parallel with the literature, were probably caused by the decrease in particle size (Song et al., 2008a; Kushwaha et al., 2013; Derman, 2015).

When examining the effect of sonication power, it can be seen in Table 1 that the power of the sonication has a remarkable effect on the particle size but has no significant effect on encapsulation efficiency and reaction yield. The particle size was significantly decreased by increasing the sonication power (from 20 W to 80 W), and then increased sharply for 100 W power of sonication. This result indicates that sonication energy at 80 W for 120 s was enough to resist the interfacial tension. A further increase in sonication power (100 W) led to an increase in particle size. This can be explained by the possible increases of external energy leading to agglomeration of the NPs (Halayqa and Domańska, 2014) and our results are generally in agreement with the literature (Halayqa and Domańska, 2014; Naeem et al., 2015). On the other hand, both EE and RY first increased up to 60 W and then decreased slowly at 80–100 W. When the particle size, EE, and RY results were evaluated, it was concluded that best results were achieved at 80 W.

Based on the best results for the lowest particle size with high entrapment efficiency, the single-emulsion solvent evaporation method with 80 W sonication power for 120 s was chosen for synthesis of Q27, Q29, and Q31 NPs.

3.3. Detailed characterization of Q27 NPs, Q 29 NPs, and Q31 NPs

In this study we determined the RY, EE, size distribution, zeta potential, and PDI for the detailed characterization of Q27, Q29, and Q31 NPs. The results show that higher reaction yield was achieved by increasing the initial quercetin amount (Table 1). Additionally, in contrast to the literature (Song et al., 2008b), the encapsulation efficiency was increased by increasing the initial quercetin amount and was calculated

as 93.4%, 96.2%, and 97.8% for Q27 NP, Q29 NP, and Q31 NP, respectively.

Additionally, dynamic light scattering (DLS) results showed that the sizes of the NPs are very close to each other and vary from 244.3 ± 4 to 262.8 ± 7 nm. Furthermore, PDI results showed that all NPs have narrow particle size distribution with a small PDI value (Figure 1; Table 1).

Moreover, SEM images of Q27 NPs, Q29 NPs, and Q31 NPs (Figure 1) were in agreement with DLS results and all NPs showed smooth and spherical shapes with uniform particle distribution.

3.4. In vitro release study

In the present study, the in vitro release of optimized NPs was carried out at pH 7.4 in PBS buffer for up to 120 h and the release profiles are shown in Figure 2. The Q27, Q29, and Q31 NPs with different quercetin contents (1.15 mg, 3.01 mg, and 3.70 mg, respectively) presented a three-phasic release profile characterized by burst release, lag phase, and sustained release. It was observed in the release study that the certain initial burst release increases as the quercetin content increases.

In the first step of release, which was caused by diffusion of the drug and continued for 4 h of incubation, burst releases amounted to 0.39 mg, 1.08 mg, and 1.65 mg for Q27 NPs, Q29 NPs, and Q31 NPs, respectively. In the second phase, caused by drug diffusion and cleavage of polymer chains, 0.98 mg, 2.33 mg, and 2.79 mg quercetin release was observed for Q27 NPs, Q29 NPs, and Q31 NPs, respectively. In the last step of quercetin release, a near-linear and continuous release was observed over 72–120 h that reached 0.95 mg (82.6%), 2.42 mg (80.4%), and 2.88 mg (77.8%), respectively, at the end of 120 h.

From the release results, it can be concluded that the increase in encapsulated quercetin

in the particles affects the release profile such as initial burst release or the cumulative amount of released drug at any time. This situation has been described by other researchers; the increase in encapsulated drug increases the amount of drug close to the surface and in the core of NPs (Budhian et al., 2008; Derman, 2015).

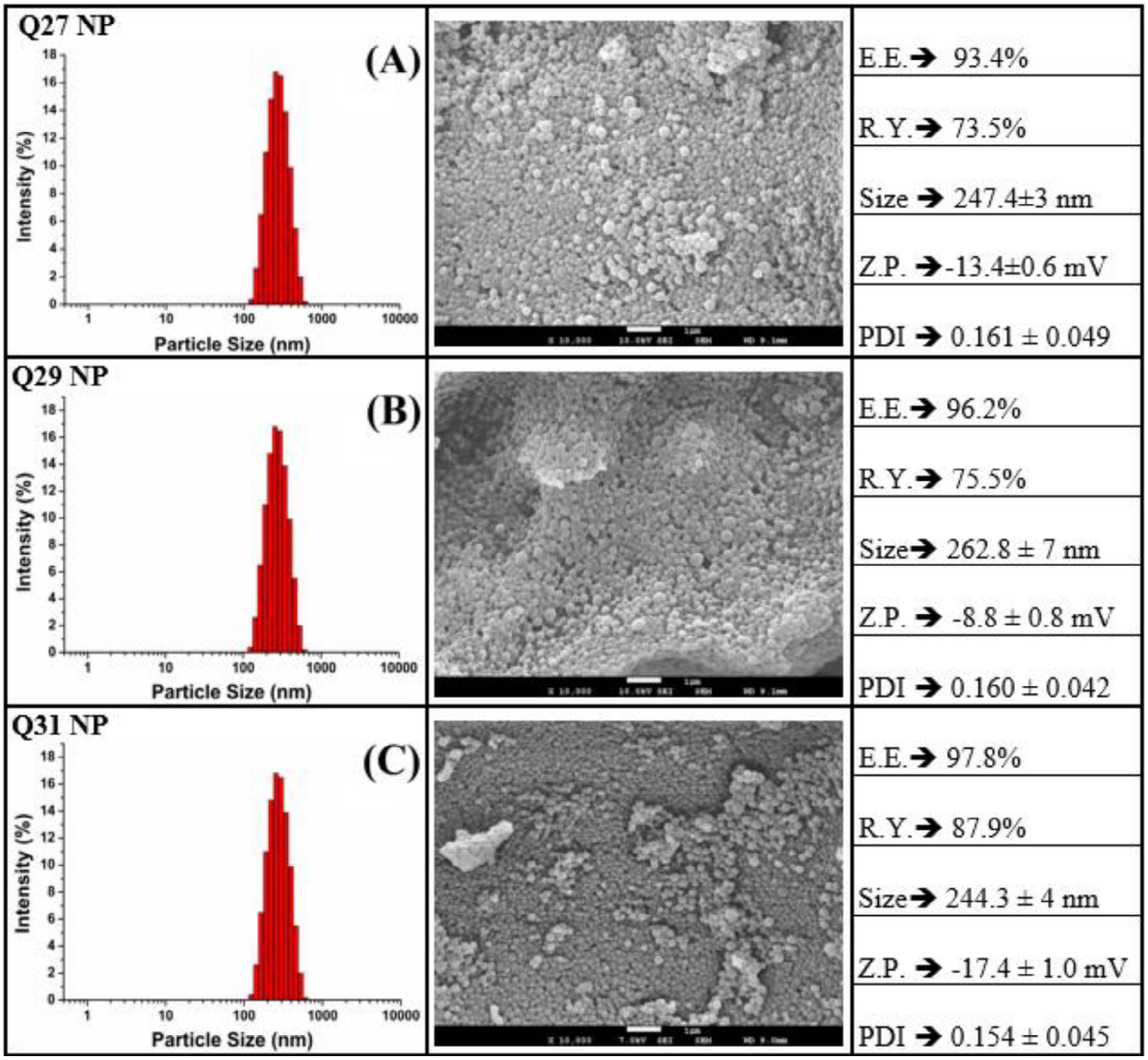


Figure 1. Particle size distribution, SEM images, and physicochemical properties of Q27 NPs (A), Q29 NPs (B), and Q31 NPs (C).

3.5. Antibacterial activity of nanoparticles

3.5.1. Evaluation of antimicrobial activity by agar well diffusion method

In this method, the antimicrobial potentials of free quercetin and three different NPs were evaluated with regard to their zone of inhibition against foodborne pathogens. In the agar well diffusion method, none of the test samples showed antimicrobial activity at the tested concentrations (Figure 3). However, it was observed that the low water solubility of quercetin was increased by encapsulation in PLGA and the precipitate formation was reduced in NP formulations (Figure 3).

3.5.2. Evaluation of antimicrobial activity by broth dilution method

In this study, the MIC values of free quercetin and quercetin-loaded nanoparticles were evaluated by micro- and macrodilution techniques. In the broth microdilution method, MIC values could not be determined spectrophotometrically due to turbidity formation, which gave absorbance at 570 nm caused by insoluble quercetin compounds (data not shown).

The high optical density resulting from the increase in turbidity is considered as bacterial growth in this method; thus, it was detected that the free quercetin gave false positive results by causing visible turbidity due to the insolubility of quercetin. However, this situation was not observed for quercetin-encapsulated PLGA NPs. There is no doubt that NP systems are good solvent systems as alternatives to other organic solvents such as ethanol, methanol, and DMSO.

To obtain reliable MIC values in the broth macrodilution method, standard plate counts were performed together

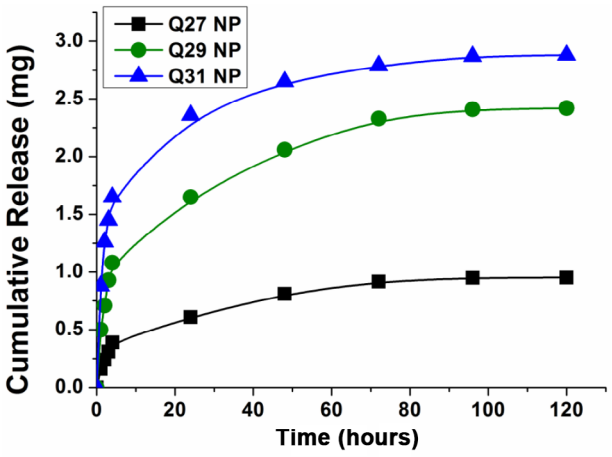


Figure 2. Time-dependent release profile of different amount quercetin encapsulated nanoparticles (Q27 NPs, Q29 NPs, and Q31 NPs).

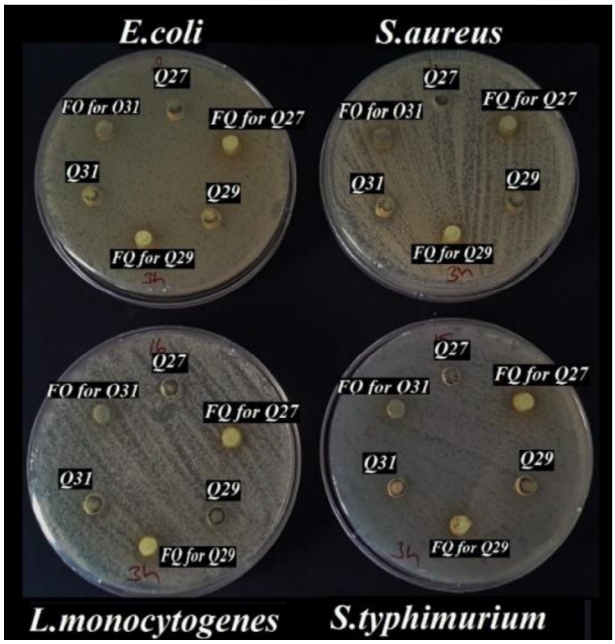


Figure 3. The images obtained by agar well diffusion method of the free quercetin and its nanoparticle formulations (Q27, Q29, and Q31 NPs) against *E. coli*, *S. aureus*, *L. monocytogenes*, and *S. typhimurium* pathogens.

with spectrophotometric measurements at an OD of 570 nm (ELISA reader) for each strain. Table 2 shows the MICs of quercetin and quercetin-loaded nanoparticles against various foodborne pathogens. According to these results, based on the increase in quercetin amounts, the MIC value decreases and the antimicrobial activity increases. However, it was observed that PLGA NP systems did not cause a remarkable decrease in MIC values. Hence, it was determined that there was no significant difference between Q27, Q29, and Q31 NPs and free quercetin in terms of MIC values, except for the decrease provided by Q31 NPs against *L. monocytogenes* when compared to free quercetin (respectively 100 µg/mL and 200 µg/mL). This can be explained by the controlled and sustained release of quercetin from NPs. In parallel with the literature (Hill et al., 2013), a more sustained release of quercetin after the initial burst gives rise to more inhibitory activity over a longer period, and thus recovery time for damaged pathogens during the initial burst is extended. As a result, it was thought that the activity depends on the dose. Additionally, MIC values of both quercetin-loaded NPs and free quercetin were not determined at the tested concentrations for *E. coli* and *S. typhimurium*.

3.5.3. Determination of antimicrobial activity by time-kill assay

The time-kill studies were performed over a period of 72 h with bacteria being exposed to quercetin and its NP formulations. In the time-kill assay, the decrease in the number of bacterial colonies was interpreted as growth-inhibiting or bactericidal activity. According to our results, neither quercetin NP formulations nor free quercetin showed bactericidal activity for any of the strains used (data not shown). In addition, the test samples did not show a significant inhibition effect on the growth of *E. coli* and *S. typhimurium* bacteria when compared with the control group containing bacteria only, nor on *L. monocytogenes* and *S. aureus*.

Figure 4A shows that quercetin NPs (Q27 NPs, Q29 NPs, and Q31 NPs) have an inhibition effect on the growth of *L. monocytogenes*. As shown in the figure, the strongest inhibition effect was found with the Q31 NPs (96.48%). Q27 NPs showed a 17.98% decrease in colony counts compared to free quercetin after 6 h and this value reached 63.48% at the end of the first day. However, regrowth of 53.10% was observed after 48 h. For Q29 NPs, the decrease in bacterial counts due to the inhibition effect of the NPs reached 85.78% on the first day and then showed a gradual increase on subsequent days. However, bacterial regrowth in the broth containing Q29 NPs was observed to occur more slowly than in the broth with Q27 NPs. In the study performed with Q31 NPs, it was observed that at end of the sixth hour antimicrobial activity began (reaching 45.63%), then rose to over 96.48% on the first day and lasted for 3 days.

Figure 4B demonstrates the time-kill curves of quercetin NPs for *S. aureus*. As shown in the figure, Q29 NP and Q31 NP formulations of quercetin-loaded PLGA exhibited a noticeable inhibition effect on the growth of *S. aureus*, but the growth inhibition effect of Q31 NP was slightly higher (a highest reduction rate of 79.12%) than that of Q29 NP

Table 2. Minimum inhibitory concentration (MIC) of quercetin loaded nanoparticles (Q27 NP, Q29 NP, and Q31 NP) and free Quercetin (at different amounts) agaınst foodborne pathogens through broth macrodilution method.

Test samples	Drug/ polymer ratio	MIC values (μg/mL)			
		Microorganisms			
		Listeria monocytogenes	*Salmonella typhimurium*	*Escherichia coli*	*Staphylococcus aureus*
Q27 NPs	10/100	>200	>200	>200	>200
Free Q for Q27 NPs		>200	>200	>200	>200
Q29 NPs	30/100	200	>200	>200	200
Free Q for Q29 NPs		200	>200	>200	200
Q31 NPs	50/100	100	>200	>200	100
Free Q for Q31 NPs		200	>200	>200	100

(a highest reduction rate of 53.70%). Q29 NPs showed a 30.77% drop in bacterial counts compared to free quercetin at 6 h, and on the first day the reduction percentage reached over 53.70 %, then decreased. For Q31 NPs, 38.40% growth inhibition was achieved within 6 h. On the first day, this increased to 79.12%. After that, it returned to the bacterial growth stage while the inhibition rate decreased.

According to the results obtained with this method, the antimicrobial activity related to growth inhibition by quercetin NPs was slightly higher and longer for *L. monocytogenes* than *S. aureus*.

3.5.4. Effect of bacterial surface charge on antimicrobial activity of nanoparticles

In this study, bacterial surface charges of *S. aureus*, *S. typhimurium*, *L. monocytogenes*, and *E. coli* were measured at –13.1 ± 0.57 mV, –17.9 ± 0.62 mV, –7.04 ± 0.82 mV, and –2.05 ± 0.12 mV, respectively. Surface charge results showed that *E. coli* and *S. typhimurium* were significantly more negatively charged than *L. monocytogenes* and *S. aureus*. Additionally, *L. monocytogenes* was generally less negatively charged than all other bacteria. For this reason, *L. monocytogenes* presented a lower resistance and highest susceptibility to the anionic NPs because the zeta potential of Q31 NPs is –17.4 ± 1.0 mV.

3.5.5. Evaluation of bacteria cell integrity damaged by quercetin nanoparticles

In the present study, the best antimicrobial activity was observed for Q31 NPs against *L. monocytogenes* and *S. aureus*. We therefore investigated the morphological changes of these cells before and after exposure to Q31 NPs by SEM. The obtained SEM micrographs of *L. monocytogenes* and *S. aureus* cells are shown in Figure 5.

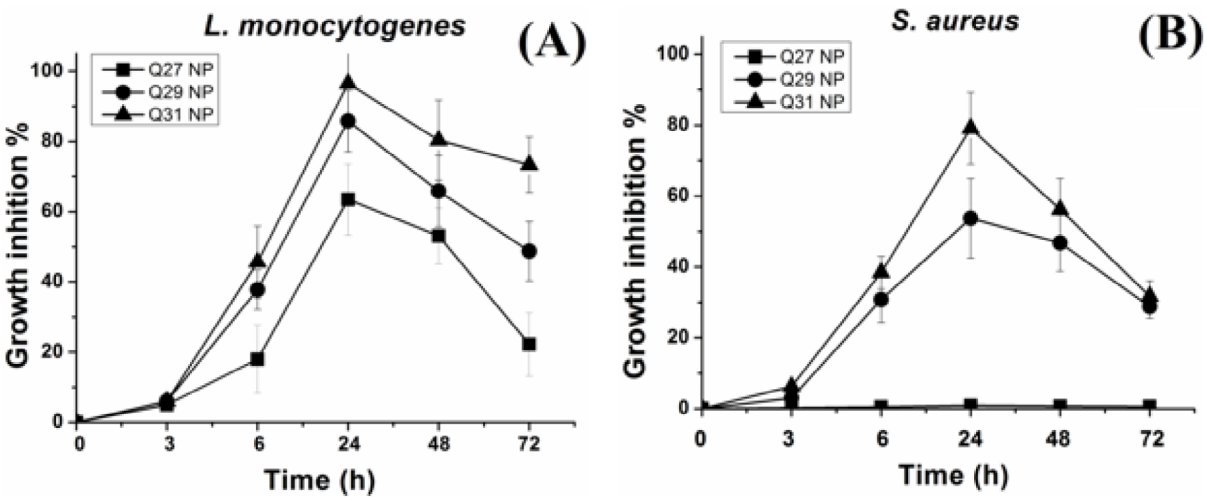


Figure 4. The time-dependent growth inhibition effect of quercetin nanoparticles on cell viability of *L. monocytogenes* (A) and *S. aureus* (B) during 72 h of exposure. The growth inhibition % determined by Eq. (3) is a comparison of free quercetin and its nanoparticle formulations (Q27, Q29, and Q31).

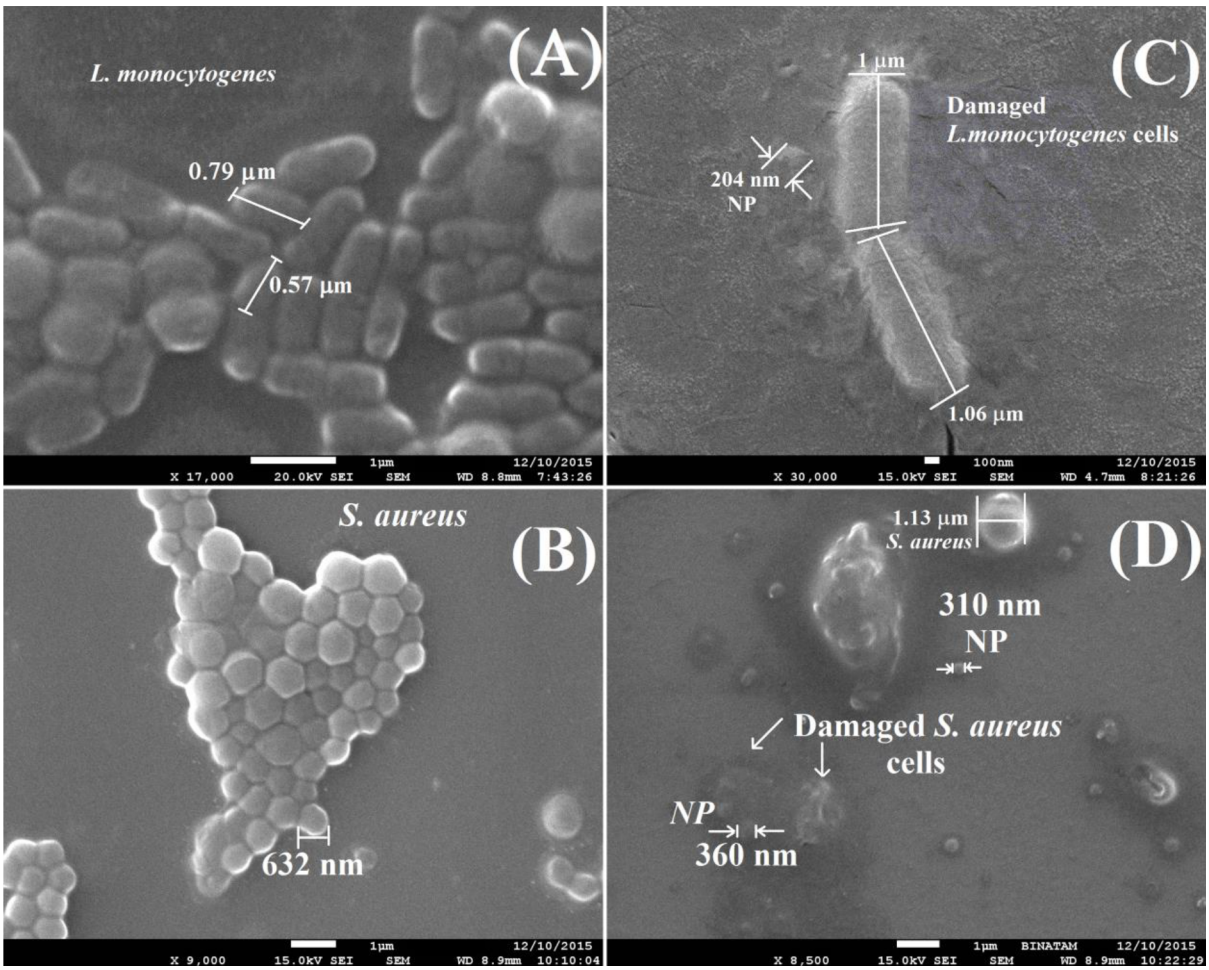


Figure 5. Typical scanning electron microscope images of untreated *L. monocytogenes* (A), untreated *S. aureus* (B), quercetin nanoparticle (Q31 NP)-treated *L. monocytogenes* (C), and quercetin nanoparticle (Q31 NP)-treated *S. aureus* (D).

Untreated *L. monocytogenes* cells were typically rod-shaped (Figure 5A) and untreated *S. aureus* cells were cocci bacteria, namely staphylococcal (Figure 5B), both with smooth and intact cell walls. After 24 h of exposure to Q31 NPs, the quantity of both strains greatly decreased in parallel with the results of the time-kill assay. As observed in the SEM images, the cell walls became wrinkled and damaged, as in previous reports (Figures 5C and 5D) (Tang et al., 2013; Li et al., 2015). However, it should be noted that *S. aureus* cells that dispersed in a cluster were destroyed as groups rather than individually due to the antibacterial effect of quercetin NPs. Additionally, it can be seen in Figures 5C and 5D that bacteria cells were surrounded by Q31 NPs.

4. Discussion

In this research, the antimicrobial activity of quercetin-encapsulated PLGA nanoparticles against four different bacterial strains (*Listeria monocytogenes*, *Salmonella typhimurium*, *Escherichia coli*, and *Staphylococcus aureus*) was evaluated by three different methods and using three NP systems that differed in drug/polymer ratios (Q27 NPs, Q29 NPs, and Q31 NPs).

According to the previous research in the literature, the antibacterial mechanism of quercetin could be attributed to the inhibition of nucleic acid synthesis, cytoplasmic membrane function, bacterial cell wall synthesis, or energy metabolism (Cushnie and Lamb, 2005; Hirai et al., 2010). In this research, conducted with the purpose of increasing antibacterial activity by encapsulating quercetin into a NP system, the effects of the bacterial strains, methods, and drug/polymer ratios on antimicrobial activity were analyzed using the PLGA NP systems that are commonly preferred in drug delivery.

First of all, it was realized that method selection is extremely important because when the qualitative agar well diffusion method was used, false negative results were obtained. This could result from the insolubility of

quercetin in water and its insufficient diffusion due to its hydrophobic structure. However, in the literature it has been shown by the agar well diffusion method that quercetin molecules have antimicrobial activity. In these studies, the antimicrobial activity study was carried out after the solubility of free quercetin was enhanced using different solvents such as methanol, DMSO, or ethanol (Puupponen-Pimiä et al., 2001; Ramadan et al., 2009). This method works on the principle that antimicrobial agents diffuse through the agar medium and therefore it may give false results due to the lack of diffusion for hydrophobic compounds (Darvishi et al., 2015). In the research conducted by Park and Ikegaki (1998), the effect of solvents on antimicrobial activity was evaluated and it was determined that the solvent used directly affects the antimicrobial activity results. Therefore, while investigating the antimicrobial properties of compounds with hydrophobic characteristics without using any solvent, it is suggested that diffusion methods such as agar well diffusion are not suitable (Arasoglu et al., 2015; Wiegand et al., 2015). In broth dilution, one of the qualitative methods, and especially in the microdilution technique, it is thought that the formation of turbidity due to low solubility of quercetin caused false readings by spectrophotometer and a low total reaction volume resulted in false negative results. The high optical density resulting from the increase in turbidity is considered as bacterial growth in this method; thus, it was detected that the free quercetin gave false positive results by causing visible turbidity due to the insolubility of quercetin. The same result was observed by Sivaranjani et al. (2014). Thus, high working volumes in the macrodilution technique that is used for determining MIC values and in the time-kill method that is used for evaluation of bacterial growth by living cell counts have considerable influence over antimicrobial activity results. The reason for this could be the increase in bacteria–active substance surface interaction when the volume is high. This is because, especially in the time-kill method, quercetin-loaded nanoparticles inhibited microbial growth more effectively when compared to free quercetin. This could be because of the high surface/volume ratio due to the relatively small sizes of NP systems.

When choosing methods for antimicrobial activity studies of PLGA NP systems, the physical and chemical properties of active substances to be loaded into PLGA should be in accordance with the working principles of the method. Moreover, two or more methods should be evaluated together. Nevertheless, when the literature was reviewed it was seen that different antimicrobial activity results were obtained from NP systems even though chemical compounds and the methods used were the same. The reasons for this could include parameters such as methods that are not standardized, use of active substances with different chemical compositions and uncertain amounts of active substances encapsulated in NPs, bacterial concentration, and total volume.

In this study, the antimicrobial activity results between bacteria strains showed significant differences. The first reason for this is the variation between the cell wall structures of bacteria. In our study, it was shown that quercetin and quercetin-loaded NPs have antimicrobial activity only against gram-positive bacteria (Hirai et al., 2010). The complex cell wall structure of gram-negative bacteria acts as a barrier against quercetin and quercetin-loaded nanoparticles (Nikaido, 1996; Duffy and Power, 2001; Ramadan et al., 2009; Espitia et al., 2012; Arasoglu et al., 2015; Li et al., 2015; Suleiman et al., 2015). The second reason is the differences in surface charges due to cell wall structures of microorganisms (Croll et al., 2004; Dillen et al., 2008; Rao, 2008; Rawlinson et al., 2011; Espitia et al., 2012; Arasoglu et al., 2015). According to the zeta potential results of our study, *L. monocytogenes* and *S. aureus* (–2.05 ± 0.12 mV and –7.04 ± 0.82 mV) have more positive surface charges than *E. coli* and *S. typhimurium* (–17.9 ± 0.62 mV and –13.1 ± 0.57 mV). Synthesized quercetin NPs have negative surface charges (zeta potential: –17.4 ± 1.0 mV), so it was thought that their interaction with microorganisms having a positive surface charge is stronger. SEM images also supported this idea. The gathering of NPs around clusters of bacteria is observed in these images. Pore sizes of the cell membranes are also important because it has been stated in the literature that due to the smaller pore structure of *E. coli* than *S. aureus* (Sun et al., 2016), penetration of active substances released from NPs into cells is limited. The disruptive effect of intracellular material released subsequent to the degradation of the cell membrane is the third reason (Li et al., 2015). In SEM images, the release of cytoplasmic constituents of damaged bacteria cells into the environment and thus the formation of translucent cytoplasm zones was observed. Accordingly, we thought that quercetin NPs cause degradation of the cell membrane by forming pores due to interaction with the cell membrane and so intracellular matrix release from cells led to cell death or damage.

Lastly, it was shown in our study that the drug/polymer ratio affects the antimicrobial activity. While the MIC value of Q27 NPs loaded with lower amounts of the drug could not be determined at the tested concentrations, it was observed that antimicrobial activity increased in parallel with the drug ratio increase in Q29 and Q31 NPs. Dose-related antimicrobial activity of quercetin, increase in zeta potentials of NPs (Q31 NPs have the highest antimicrobial activity and lowest negative zeta potential), and time-dependent increase in released material amount are the reasons for this. In particular, after the initial

burst, a more consistent release of quercetin causes more inhibitory activity over a longer period of time and thus recovery time for pathogens damaged during the initial burst is extended (Hill et al., 2013).

There are similarities and also some differences between the antimicrobial activity results of our study and previous research both for quercetin and quercetin NPs. Similar results relating to antimicrobial activity of quercetin on *L. monocytogenes* were found in a study conducted by Vaquero et al. In that study, it was determined that the anti-*Listeria* activity of the flavonoid group including quercetin and rutin was more effective than that of other flavonoid groups and nonflavonoid groups (Vaquero et al., 2007; Rodríguez Vaquero et al., 2011). Hirai et al. demonstrated that quercetin possesses antibacterial activity against *S. aureus* and *S. epidermidis* and significantly enhanced the antibacterial activity of various antibiotics in the treatment of MRSA. Similar results for *E. coli* were found by Hirai et al., in which gram-positive cocci and gram-negative rods, such as *Streptococcus* spp., *P. aeruginosa*, *E. faecalis*, and *E. coli*, did not show susceptibility to any concentration of quercetin (Hirai et al., 2010). Additionally, in the literature free quercetin exhibits antimicrobial activity against several bacterial species including *Staphylococcus* spp., *Bacillus* spp., and *Pseudomonas* spp. (Ramadan et al., 2009; Hirai et al., 2010). However, its effective concentrations for these microorganisms were variable because of the fact that the method selected for determination of antimicrobial effects can influence the results obtained. Therefore, some of the results related to quercetin in this study are consistent with those obtained before. As indicated previously, it is thought that the precipitation arising from the solvent used in dissolving flavonoids is likely to cause diminished contact between bacterial cells and flavonoid molecules and may lead to false negative results of antibacterial activity (Cushnie and Lamb, 2005). Our results differ notably from the antimicrobial study of quercetin-loaded PLGA nanoparticles performed by Sun et al. (2016). This could be caused by differences in the methods used for antimicrobial activity tests and nanoparticle synthesis, solvents used for quercetin aside from water, amounts of encapsulated quercetin in NPs, zeta potentials, and release profiles. Hence, we thought that the consequential differences of this and similar studies in the literature can be reviewed much more effectively when standardized methods are used or adequate method information is given.

In conclusion, during the last decade, it has been emphasized that encapsulation of active agents in PLGA NP systems protects sensitive bioactive compounds, increases their solubility, and improves their bioavailability in medicinal, pharmacological, and food industries. Additionally, the factors that limit the use of quercetin bioflavonoid, which is found in abundance in fruits and vegetables consumed in daily human life, such as poor water solubility, instability, absorption, and permeability, were aimed to be overcome by encapsulating PLGA NP systems in this study. Hence, highly monodispersed quercetin-encapsulated PLGA NPs were successfully developed by o/w single-emulsion solvent evaporation and their antimicrobial activity was evaluated, compared to free quercetin, on the foodborne pathogens *L. monocytogenes*, *S. typhimurium*, *E. coli*, and *S. aureus*. The antimicrobial activity of quercetin NPs and the effects of the bacterial strains, methods, and drug/polymer ratios on antimicrobial activity were investigated. According to the results, quercetin-loaded PLGA NPs increased the duration and effectiveness of antimicrobial activity only against gram-positive bacteria.

Although the antimicrobial mechanism of quercetin NPs has not been clearly

determined yet, their antimicrobial activity has been attributed to three main factors: cell wall structure of microorganisms, surface charges of microorganisms, and the domino effect of the released intracellular matrix from damaged or dead cells. Additionally, the increase in zeta potentials of NPs and in time-dependently released quercetin from NPs improved the antimicrobial activity of quercetin NPs.

In addition, it was determined once again that the method of selection in the antimicrobial studies of PLGA NP systems is extremely important. However, the factors that affect antimicrobial activity, such as NP preparation methods, solvents of NPs or active agents, zeta potential and sizes of particles, release properties of NPs, antimicrobial activity methods, concentration of NPs used, active ingredient amounts in NPs, and the bacteria concentration used, should be stated. Thus, it is considered that more realistic comparisons between the same studies for antimicrobial activity of PLGA NP systems can be achieved.

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