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ORIGINAL ARTICLE

Synthesis, characterization and antibacterial activity of juglone encapsulated PLGA nanoparticles

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Abstract

Aims: The aim of the study was to examine the effect of different process parameters which including; initial juglone amount, initial poly(D,L-lactide co-glycolide) amount, polyvinyl alcohol volume and polyvinyl alcohol concentration on encapsulation of juglone to poly(D,L-lactide co-glycolide) nanoparticles.
Methods and Results: The synthesized nanoparticle formulations were analyzed for reaction yield, encapsulation efficiency, particle size, polydispersity, zeta potential and juglone release. In conjunction with the highest encapsulation rate, the highest amount of juglone release was obtained for F4 formulation, which has 281·8 nm particle size, 0·217 polydispersity index, and −19·55 mV zeta potential. After the detailed physicochemical characterization of this formulation, the four different kinetic models were used and it was found that juglone release mechanism controlled by Fickian diffusion method. According to antimicrobial activity results, minimal inhibitory concentration (MIC) values of both F4 and free juglone is higher for Gram negative bacteria than Gram positive bacteria. Inhibition zone diameters in the quantitative methods are found 15 and 16 mm for *Staphylococcus aureus*, 9 and 7 mm for *Bacillus cereus*, respectively, for F4 and free juglone. Moreover, the MIC values for qualitative methods were found 31·5 μg ml^{-1} for two bacteria strains.
Conclusions: It was found that the antibacterial activity of the juglone nanoparticles was higher and longer than the free juglone. Additionally, a similar antimicrobial effect with a lower juglone amount (obtained from controlled release study) indicates that nanoparticle formulation is more effective.
Significance and Impact of the Study: The use of nanoparticle formulations of juglone in biological systems and applications could be more beneficial than its free form due to its toxicity.

Introduction

Polymers that convert into nontoxic degradation products are usually preferred for producing sustained release systems with long term effect (Arasoglu *et al.* 2015). Biodegradable polymers, that are most commonly used in nanoparticle synthesis to provide sustained release of encapsulated active substances, are aliphatic polyesters such as polylactic acid, polyglycolic acid and poly (D,L-lactic-co-glycolic acid), which is copolymer of these (Kumari *et al.* 2010; Akagi *et al.* 2011). PLGA breaks into lactic acid and glycolic acid monomers and they degrade into carbon dioxide and water by citric acid cycle (Chakrapani 2006). Because of its inert properties in physiological environment, which are biodegradability, biocompatibility and nontoxic degradation components, PLGA is one of the FDA approved biopolymers used frequently for drug delivery systems, and appropriate carrier to prepare nanoparticle systems with substances soluble in water or oil (Gunatillake and Adhikari 2003; Bala *et al.* 2004).

Water solubility of PLGA depends on its molecular weight and lactic-glycolic ratio. Biodegradability increases

with high glycolic acid content and low molecular weight, by this way, it is possible to extend and control the drug release time (Parveen and Sahoo 2006). It has been reported in literature that loading of antimicrobial agents into PLGA nanoparticular systems increased pharmacokinetics properties, therapeutic index and biocompatibilities of active substances in comparison with their free form (Lemoine *et al.* 1996; Arasoglu *et al.* 2015).

Juglone (5-hydroxy-1,4-naphthoquinone), the active substance used in this research, is a naphthoquinone structured allelochemical that can be isolated from bark, peel, fruits and leaves of walnut tree (*Juglans* sp.). The most important feature of this compound shows allelopathic effects by inhibiting essential enzymes for metabolic functions of other plants (Kocacaliskan 2006; Altikat *et al.* 2013). In previous researches, it was shown that juglone has herbicidal (Dama *et al.* 1998), antibacterial (Chuan Tan *et al.* 2012; Xu *et al.* 2013; Zakavi *et al.* 2013), antiviral (Montenegro *et al.* 2010), antifungal (Bonjar 2004; Bonjar *et al.* 2004), and antioxidant (Zakavi *et al.* 2013) effects. It was also stated that the antimicrobial activity of juglone stems from its toxic effect. Juglone's toxicity was explained with mechanisms such as cell death, cell cycle disruption, DNA modifications (predominantly rapid dividing cells), inhibition of mRNA synthesis, alkylation of thiol or amine groups of essential proteins, and production of ROS radicals (Strugstad and Despotovski 2013; Xu *et al.* 2013). Besides, it is known that walnut had been used as a medicinal plant in many countries. Nowadays, juglone is used as a colouring agent in food, cosmetics and textile industry (Strugstad and Despotovski 2013).

In our research, the aims are (i) to find suitable synthesis parameters and to characterize the nanoparticles detail and (ii) to assess antibacterial activity of juglone-PLGA nanoparticles in comparison with free juglone. In this present study, the antibacterial activity of juglone-PLGA nanoparticles was performed for the first time. Activity studies were performed detail by using four different methods and four micro-organisms (*Bacillus cereus, Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli*).

Material and methods

Synthesis of nanoparticles

Juglone loaded PLGA nanoparticles were synthesized using the modified single emulsion solvent evaporation method which was described in our previous studies (Arasoglu *et al.* 2015; Derman 2015). A certain amount of juglone and PLGA (Table 1) were dissolved in 1·0 and 2·5 ml of dichloromethane, respectively. These oil phases were mixed to ensure that all of the material was dissolved. The organic mixture was emulsified with PVA solution using the microtip probe sonicator (output power 70 W, power of 80% and 2 min; Bandelin Sonopuls, Berlin, Germany). The o/w single emulsion was then added dropwise to 35 ml of PVA solution (0·1% w/v) and left on gentle stirring overnight at room temperature to allow for evaporation of organic solvent. The obtained nanoparticles were then collected by centrifugation at 10 285 ***g*** for 40 min (Hettich-Universal 32 R, Andreas Hettich GmbH & Co. KG, Gartenstraße, Tuttlingen, Germany) at 4°C. The nanoparticles were washed three times with ultrapure water and then lyophilized. To obtain the free nanoparticles (F16), the similar method was conducted without Juglone and lyophilized nanoparticles were stored at −80°C until used. In the present study, the effect of various process parameters including the initial juglone amount, initial PLGA amount, PVA concentration in aqueous phase PVA volume (aqueous-to-organic phase volume ratio) on nanoparticle properties were investigated. In these experiments, only one parameter was changed and all the other processing parameters were kept constant.

Characterization of nanoparticles

Reaction yield (RY) and encapsulation efficiency (EE)
The reaction yield (Eqn 1) and encapsulation efficiency (Eqn 2) of nanoparticles were determined in triplicate. The reaction yield was calculated gravimetrically using the formula given below (Arasoglu *et al.* 2015).

$$\mathrm{RY\%} = \frac{\text{Amount of nanoparticle produced}}{\text{Amount of initial polymer+Juglone}} \times 100 \quad (1)$$

Encapsulation efficiency of nanoparticles was detected via indirect quantification methods by analyzing the supernatant of nanoparticle formulation using UV-Vis Spectroscopy at 424 nm in triplicated. Previously, the constructed standard calibration curve was used for determine the concentration of juglone. The encapsulation efficiency of juglone was calculated using the formula given below:

$$\mathrm{EE\%} = \frac{\text{Amount of Juglone encapsulated in NPs}}{\text{Initial Juglone added}} \times 100 \quad (2)$$

Size distribution and zeta potential of nanoparticles
Particle size distribution and zeta potential (ζ) of nanoparticles were determined by a Zetasizer (Zetasizer Nano ZS, Malvern, UK) instrument using dynamic light scattering and electrophoretic light scattering techniques,

Table 1 Various formulation parameters for Juglone loaded nanoparticles

Nanoparticle number	Initial Juglone and PLGA amount		Polyvinyl alcohol (PVA)		Solvent type Dichloromethane (ml)
	Juglone (mg)	PLGA (mg)	%	ml	
F1	10	100	3	4	3·5
F2	20	100	3	4	3·5
F3	30	100	3	4	3·5
F4	40	100	3	4	3·5
F5	20	100	0·1	4	3·5
F6	20	100	0·5	4	3·5
F7	20	100	1	4	3·5
F8	20	100	2	4	3·5
F9	20	100	3	6	3·5
F10	20	100	3	8	3·5
F11	20	100	3	10	3·5
F12	20	100	3	20	3·5
F13	20	200	3	4	3·5
F14	20	300	3	4	3·5
F15	20	400	3	4	3·5
F16	–	100	3	4	3·5

respectively (Derman and Akdeste 2015). Measurements were carried out in triplicate, at 25 ± 0·1°C with using 0·8872 cP of viscosity, 1·330 of refractive index, 79 of dielectric constant and *f* (ka) 1·50 (Smoluchowski) (Derman *et al.* 2015). The number of runs and run durations were chosen automatically in both size and zeta potential analysis.

Morphology of nanoparticles

The morphology and size distribution of nanoparticles were investigated by scanning electron microscopy (SEM). High resolution SEM images of nanoparticles were taken by Field Emission JEOL SEM 7001F under 7–10 kV acceleration voltages (Arasoglu *et al.* 2016). Samples were homogeneously coated with 7 nm Au by DC Sputter to prevent the charging effect.

Differential scanning calorimetry studies of nanoparticles

Thermal transition properties of juglone loaded nanoparticles, juglone, PLGA and their physical mixture (Juglone-PLGA) were investigated using a Perkin Elmer Pyris 6 type differential scanning calorimeter (DSC). The test was conducted according to ISO/IEC 17025 standard. The thermograms were obtained at a scanning rate of 10°C min^{-1} in 30–150°C temperature range and performed under the N_2 atmosphere.

In vitro release and release kinetics of nanoparticles

The *in vitro* release of juglone from different nanoparticle formulations was studied using a modified dissolution method in phosphate buffer solutions (PBS) at pH 7·4 (Arasoglu *et al.* 2015). 5 mg of selected nanoparticles were weighed and suspended in 2 ml of 150 mmol l^{-1} PBS and incubated at 37°C in a shaking incubator (150 rev min^{-1}). At specified time intervals (up to 30 days), the samples were centrifuged at 10 285 ***g*** for 20 min, then the supernatant was collected and the precipitates were suspended with 2 ml of fresh PBS. The amount of released juglone was determined by UV-VIS spectroscopy as described in the previous section.

Various kinetic models were used to analyze the juglone release mechanism from nanoparticular system. The juglone release data was fitted to zero order kinetic (Eqn 3, explains the drug release from the systems, where the release rate is not dependent on juglone concentration), first order kinetic (Eqn 4, describes the systems where the rate of drug release is concentration dependent), Higuchi kinetic (Eqn 5, defines the drug release from insoluble matrix based on Fickian diffusion), and Korsmeyer–Peppas kinetic (Eqn 6, explains the drug release from polymeric system).

$$Q_t = k_0 \cdot t \tag{3}$$

$$\ln Q_t = \ln Q_0 - k_{1\cdot t} \tag{4}$$

$$Q_t = k_h \cdot t^{1/2} \tag{5}$$

$$M_t/M_\infty = k_P \cdot t^n \tag{6}$$

where Q_t is the release percentage of drug at time t, t is the time, Q_0 is the initial concentration of the drug, k_0, k_1, k_h and k_p are the rate constants and n is the release exponent.

To find out the drug release mechanism, the data which corresponded to $M_t/M_\infty = 0{\cdot}6$ were fitted in Korsmeyer–Peppas model (Korsmeyer *et al.* 1983; Dash *et al.* 2010). In Korsmeyer–Peppas model the n value characterizes a drug release mechanism for a cylindrical-shaped matrix (Chourasiya *et al.* 2015).

Antibacterial activity analyses of the produced juglone nanoparticles

The antibacterial activity of the F4 nanoparticles was evaluated comparatively with the free juglone by both qualitative and quantitative analyses. Gram positive (*B. cereus* ATCC 10876, *S. aureus* ATCC 25923) and Gram negative (*P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922) bacterial strains were used as model organisms.

Micro-organisms were cultured aerobically at 37°C for 24 h in Mueller Hinton Broth (MHB) and the turbidity was adjusted to 10^8 CFU per ml (0·5 McFarland standard). Also, 2 mg ml^{-1} F4 nanoparticle and 0·555 mg ml^{-1} free juglone solutions (equivalent to the F4 concentration in

terms of active ingredient) were prepared as working stock solutions.

Qualitative analyses

Disc diffusion and agar well diffusion analyses were performed according to the standards of EUCAST/April 2013 and the literature published by Irshad *et al.* (2012), respectively. In the disc diffusion assay, sterile 6-mm discs were impregnated with 10 μl of F4 and free juglone and placed on the Mueller Hinton Agar (MHA) plates inoculated with bacterial suspensions. In agar well diffusion assay, wells were opened on the pre-inoculated MHA plates and loaded with 100 μl of the stock solutions. All plates were incubated at 37°C for 18–24 h. For both methods, the antibacterial activity was determined by measuring the inhibition zone diameter in mm. The data used were the mean of three replicates. Gentamicin (10 μg per disc), Oflaxocin (5 μg per disc) and Vancomycin (0·5 μg per disc) were used as positive controls. Zone diameters greater than or equal to 6 mm were considered as antibacterial activity and were classified as follows: 6–10 mm (weak effect), 11–16 mm (moderate effect), 17–25 mm (strong effect).

Quantitative analyses

The broth microdilution assay, firstly preferred method for quantitative analysis, was carried out in accordance with the CLSI standard, January 2012 (Riffel *et al.* 2002), to determine the lowest concentration (minimal inhibitory concentration (MIC) of F4 nanoparticles and free juglone that inhibits the growth of bacteria. In this method, each well of a 96-well plate is prepared to contain 100 μl working stock solution, 95 μl MHB and 5 μl bacterial suspension (*c.* 5×10^5 CFU per ml). Starting from the first well, the next five wells were prepared by diluting the test substance. The last three wells were used as positive (ampicillin solution), negative (only bacteria) and blank (only broth) control, respectively. The plate was incubated at 37°C for 24 h. Microbial growth was determined by reading the respective absorbance at 600 nm using the Shimadzu/UV-1800 universal microplate reader (Shimadzu Corporation, Japan). Additionally, to avoid the possibility of misinterpretations due to the turbidity of insoluble compounds, the minimum bactericidal concentration (MBC = the lowest concentration required to demonstrate the bactericidal effect of a test substance) was determined by subculturing the MIC dilutions onto the sterile MHA plates.

The second selected method, time kill studies, were conducted according to ASTM standard (ASTM 2013). For this study, the test samples in 50 ml MHB were added 0·5 ml of bacterial suspensions (*c.* 10^5 CFU per ml) and incubated at 37°C in a shaking incubator. At specific intervals (0, 6, 24, 48, 72 h), 100 μl of samples were spread on MHA and after overnight incubation, viable colonies were quantified. The results were evaluated in two ways; the growth-inhibiting efficiency (Eqn 7) and the bactericidal activity (Eqn 8):

$$\text{Growth-Inhibiting efficiency, } \%\left(\frac{\text{CFU}}{\text{ml}}\right) = \frac{B-A}{B} \times 100 \quad (7)$$

$$\text{Bactericidal activity, } \%\left(\frac{\text{CFU}}{\text{ml}}\right) = \frac{C-A}{C} \times 100 \quad (8)$$

Here, A is the viable colony number in the flask containing the test substance after the predetermined time, B is the viable colony number in the negative control flask at that time point and C is the initial number of colonies in the negative control flask.

Results

Particle characterization

The o/w single emulsion solvent evaporation method was used for production of juglone loaded PLGA nanoparticles. The effect of four different process parameters on reaction yield (RY), encapsulation efficiency (EE), mean particle size (Z-Ave), polydispersity index (PDI) and zeta potential was examined. The basic characteristics change with the modification of these process parameters were given in Table 2 and illustrated in Fig. 1.

As shown in Fig. 1a, increase of the initial amount of Juglone has a significant rise on reaction yield and entrapment efficiency while the particle sizes vary between 183·1 and 329·2 nm. Increase of the reaction yield and entrapment efficiency might be due to the augmentation of the juglone in the organic phase and more interaction between juglone and PLGA molecules. The increase in particle size with rising of the initial juglone amount can be explained by the viscous property of the organic phase that makes the mutual dispersion of both phases more difficult and it results in the creation of larger particles (Song *et al.* 2008; Chourasiya *et al.* 2015).

It can be observed in Fig. 1b that increasing the PLGA amount has no significant effect on reaction yield whereas it decreases encapsulation efficiency. Also, the size of nanoparticles increases with the increased amount of PLGA. Diffusion of the organic phase into the water phase becomes difficult with the increased amount of polymer which leads to aggregation and large particle formation (Ghasemian *et al.* 2013).

Table 2 Physicochemical properties of different nanoparticle formulations

Particle no	Reaction yield (%)	Encapsulation efficiency (%)	Particle size Z-Ave (nm)	Polydispersity Index (PDI)	Zeta potential (mV)
F1	72·90	33·27	265·7	0·205	−23·65
F2	76·08	55·23	329·2	0·180	−21·00
F3	76·61	66·69	286·9	0·168	−15·85
F4	77·78	75·29	281·8	0·217	−19·55
F5	75·83	58·69	382·5	0·184	−23·05
F6	66·50	42·43	417·0	0·265	−17·90
F7	64·58	39·09	338·2	0·137	−19·85
F8	63·83	41·45	300·1	0·161	−20·50
F9	41·25	32·65	218·7	0·133	−16·45
F10	28·41	27·12	213·0	0·127	−11·34
F11	29·16	30·10	208·3	0·085	−15·95
F12	36·75	10·95	222·1	0·077	−13·40
F13	76·45	45·27	292·6	0·216	−19·10
F14	82·53	35·10	371·6	0·094	−20·32
F15	87·30	41·83	395·4	0·123	−20·65
F16	50·47	–	183·1	0·092	−23·60

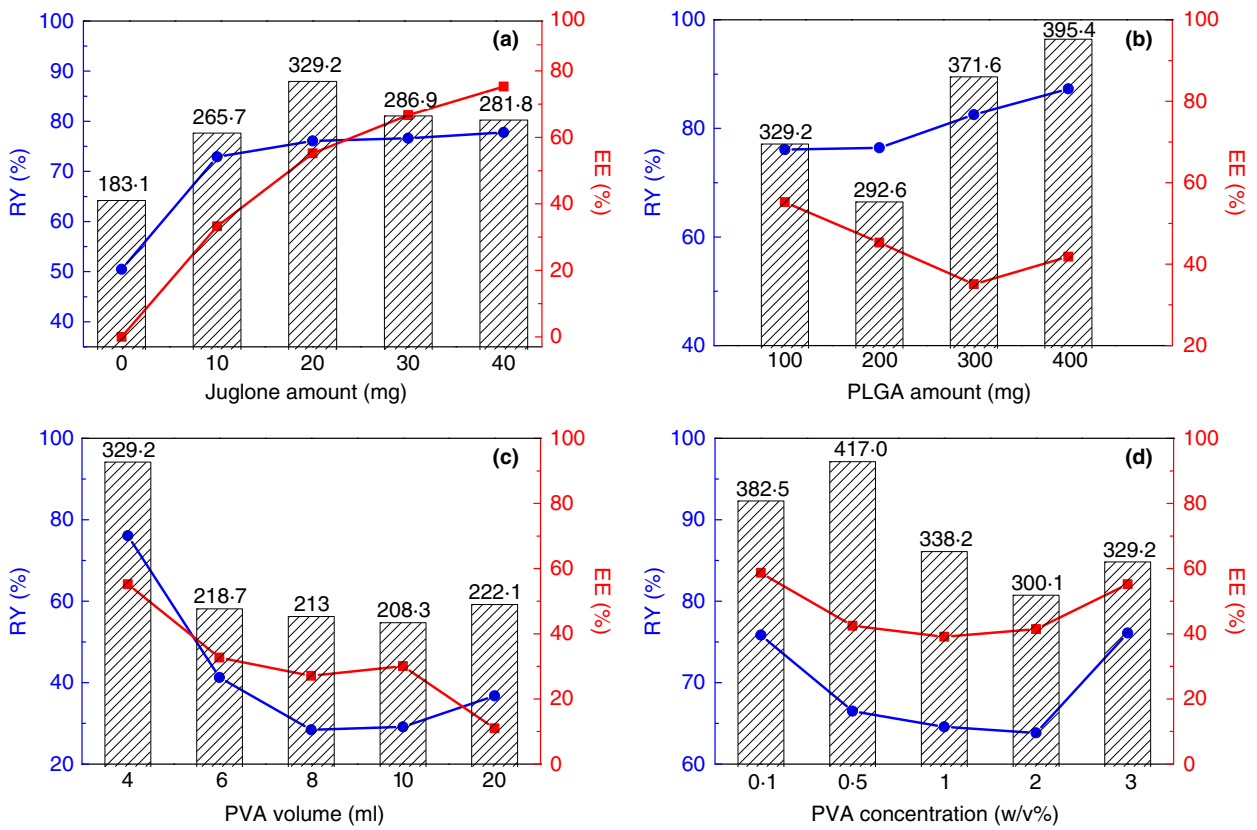


Figure 1 Effect of various process parameters on reaction yield (RY, blue and circle), encapsulation efficiency (EE, red and square). Vertical bars show mean particle sizes. The initial juglone amount (a), initial PLGA amount (b), PVA volume (c) and PVA concentration (d). (—●—) RY; (—■—) EE; (▨) Z-Ave. [Colour figure can be viewed at wileyonlinelibrary.com]

As PVA volume increases, reaction yield and encapsulation efficiency decreases. The particle size initially decreased, but subsequent increases in PVA volume (at 20 ml) re-raised the particle size. Here, we also investigate the effect of aqueous phase volume on the particle size of juglone loaded PLGA nanoparticles produced by single

emulsion solvent evaporation method. With augmenting the percentage volume of PVA 0·1–3 by keeping fixed amount of organic phase (3·5 ml), the size of nanoparticles extends from 300·1 to 417 nm (Fig. 1c).

Both reaction yield and encapsulation efficiency decreases by varying the % PVA concentration from 0·1 to 2% (w/v) but it is understood that they increase at 3% (w/v) PVA concentration. Depending on the variation of the PVA concentration, high encapsulation efficiency was obtained at 3% PVA concentration with 329·2 nm particle size, 76·08% RY, and 55·23% EE (Fig. 1d), while particle size decreased.

It was observed that the initial PLGA amount is the most significant factor on particle size while the increase of initial juglone amount significantly increases the encapsulation efficiency. As PVA volume increased, reaction yield and encapsulation efficiency decreased, the particle size initially decreased and then increased. Depending on the variation of the PVA concentration, it was observed that while particle size decreased, high encapsulation efficiency was obtained at 3% PVA concentration with 329·2 nm particle size, 76·08% RY, and 55·23% EE (Fig. 1d, F4 formulation). Therefore, all detailed characterization, release kinetic study and antimicrobial activity study were carried out for this formulation.

Detailed characterization of F4 nanoparticles

After the detailed characterization of nanoparticles, the reaction yield was obtained 50·47 ± 2·6% (F16) and 77·78 ± 4·2% (F4), additionally, the encapsulation efficiency for F4 was calculated 75·29 ± 3·4% by using an indirect method. Otherwise, as can be seen in Table 1, F16 and F4 nanoparticles were displayed a mean size of 183·1 ± 1·35 nm and 281·8 ± 0·63 nm, respectively, with low PDI values indicating a quite narrow particle population (Fig. 2a). Additionally, the average zeta potential was measured −23·60 ± 1·6 mV and −19·55 ± 0·9 mV, for F16 and F4, respectively (Fig. 2b). Negative zeta potentials indicate that repulsive forces are stronger than attractive forces, therefore, it makes the solutions stable by enhancing the dispersion of the nanoparticles.

Morphology of nanoparticles

Figure 3a illustrates the SEM image of the F4. In Fig. 3b, nanoparticles size distribution of the related sample is depicted. Nanoparticles appear in a quite in spherical form and closely aligned to each other. An average nanoparticle size of 308 nm ± 29 nm is calculated through Gaussian fit that is obtained from more than 850 particles.

DSC analysis of nanoparticles

The thermal properties of Juglone loaded nanoparticles (F4), PLGA, free juglone, and the physical mixture of PLGA-Juglone, were determined using a DSC (Fig. 4a). The thermogram curves gave information on the thermal transition in the nanoparticles and the state of the juglone after encapsulation process. Juglone has a melting point (T_m) at about 105°C, resulting in an endothermic peak in the DSC curve which represents the crystalline structure of Juglone. On the other hand, the glass transition temperature (T_g) of the PLGA and F4 were about 50 and 53°C, respectively. The results were obtained in agreement with the reported T_g of PLGA 50 : 50 in the literatures (between 40 and 60°C) (Amjadi *et al.* 2013). The little shift of T_g in the F4 thermogram from 50 to 53°C indicates molecular dispersion of the juglone to the polymer matrix. Additionally, the physical mixture of juglone and PLGA showed nearly the same thermal behaviour as the individual components. The absence of juglone T_m peak in the thermogram of F4 suggests that there was no interaction with juglone and PLGA in a solid state and juglone was totally entrapped inside the polymer without any surface adsorption.

In vitro release of nanoparticles

The *in vitro* release of juglone from different nanoparticle formulations was studied by using a modified dissolution method in PBS at pH 7·4 (Arasoglu *et al.* 2015). The data of cumulative drug release from different formulations were shown in Fig. 4b. Formulations have similar release profiles but different release quantity, depending on the amount of the juglone used in nanoparticle formulations and encapsulation efficiency.

On the whole, all nanoparticle formulations have biphasic release pattern which was characterized by an initial burst release and a subsequent sustained release. In the release study, the cumulative release of different formulations was noticed that the amount of initial burst release increases by increasing of the juglone content. The highest amount of juglone release was obtained for F4.

Juglone loaded PLGA nanoparticles (F4 formulations) exhibited a burst effect of close to 12·7% within the first three hours followed by a slow release. During the release study, nearly 33·55 and 72·04% of juglone was released in 1st and 30th days, respectively.

Obtained drug release data were fitted into different release kinetic models such as zero order, first order, Higuchi and Kormeyer–Peppas. All release kinetic graphs were shown in Fig. 5a–d and acquired values were presented in Table 3. On the most appropriate basis of

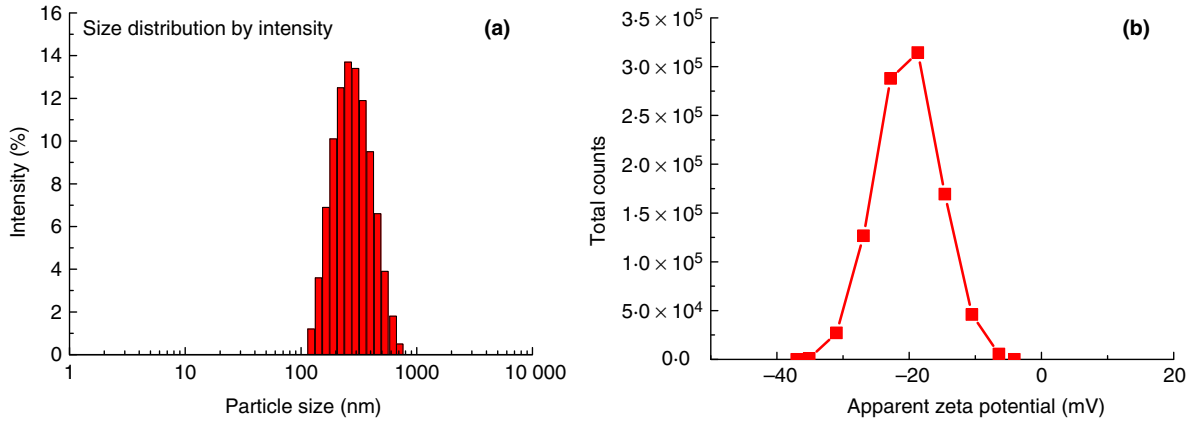


Figure 2 Particle size distribution (a) and zeta potential (b) of F4. [Colour figure can be viewed at wileyonlinelibrary.com]

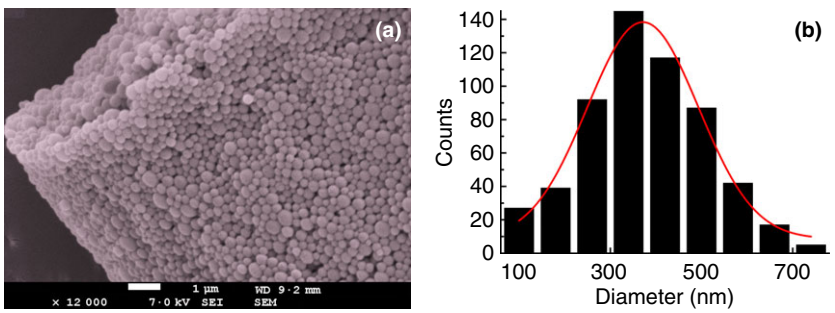


Figure 3 SEM image (a) and particle-size distribution with Gaussian fit (b) of F4 (■) counts; (—) Gauss fit counts. [Colour figure can be viewed at wileyonlinelibrary.com]

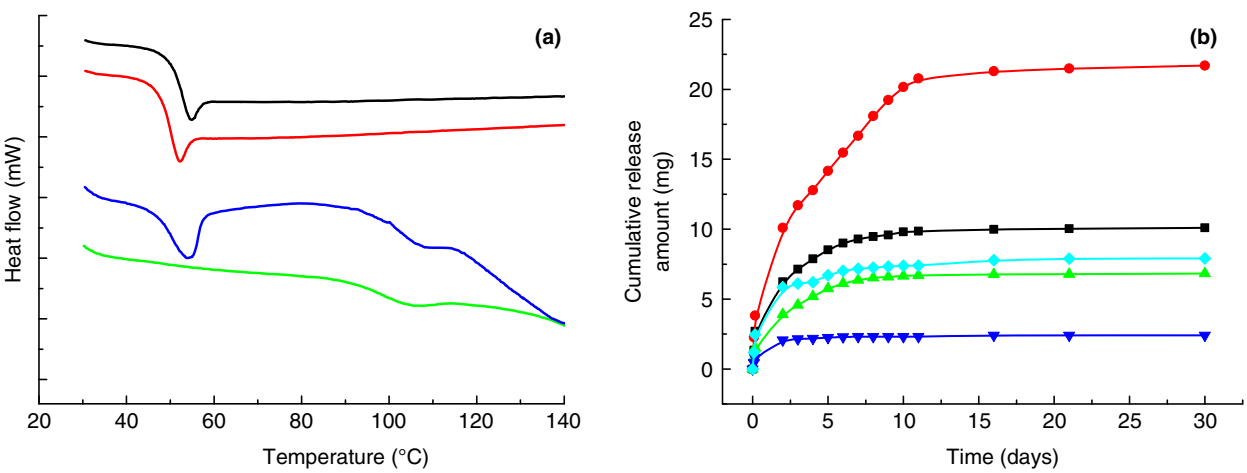


Figure 4 Comparative DSC thermogram (a) (—) F4; (—) PLGA; (—) Juglone; (—) Physical mixture., and release profile of different nanoparticle formulations (b) (—) F2; (—) F4; (—■—) F5; (—●—) F11; (—▲—) F15. [Colour figure can be viewed at wileyonlinelibrary.com]

fitting with the highest correlation (R^2) value, it is indicated that the release of juglone from PLGA nanoparticle formulations, follows the Korsmeyer–Peppas model with release exponent value of 0·42.

For Korsmeyer–Peppas, k is also the rate constant and n is the release exponent characterizing the diffusion mechanism. For $n = 0{\cdot}5$ or less, the Korsmeyer–Peppas model becomes identical with the Higuchi model.

Table 3 Determination coefficient (R^2), rate constant (k) and diffusion exponent (n) after fitting the release data to kinetic models

Model	R^2	K	n
Zero order	0·5997	$1{\cdot}6 \times 10^{-3}$ min^{-1}	–
First order	0·3250	2×10^{-5} min^{-1}	–
Higuchi	0·8802	0·3861 min$^{-1/2}$	–
Korsmeyer–Peppas	0·9980	1·015 min^{-n}	0·434

According to the literature data (Mello and Ricci-Júnior 2011; Mouzam *et al.* 2011), it was observed that the release of Juglone from F4 formulation is controlled by Fickian diffusion in our study.

Assessment of antibacterial activity of juglone and its nanoparticle formulations

Qualitative analyses

In the disc diffusion studies, both juglone loaded nanoparticles and the free juglone did not display any antibacterial activity at applied concentrations (data not shown).

On the contrary, the results of agar well diffusion method (Fig. 6 and Table 4) showed that both F4 and free juglone have moderate antibacterial activity against *S. aureus*. Also, *B. cereus* was found sensitive to F4 and free juglone, but it is thought that the antibacterial effect is weak and not important on the basis of the limits prescribed in international standards.

Differences in the results of the qualitative methods were thought to be caused by applied substance amount (10 μl for disc diffusion and 100 μl for agar well diffusion). Moreover, it was known that results of qualitative methods, especially for nanoparticle systems, affected by the size, structure, diffusion ratio, solubility and types of nanoparticle system (Arasoglu *et al.* 2015). Therefore, we recommend the use of qualitative and quantitative methods together in the antimicrobial studies.

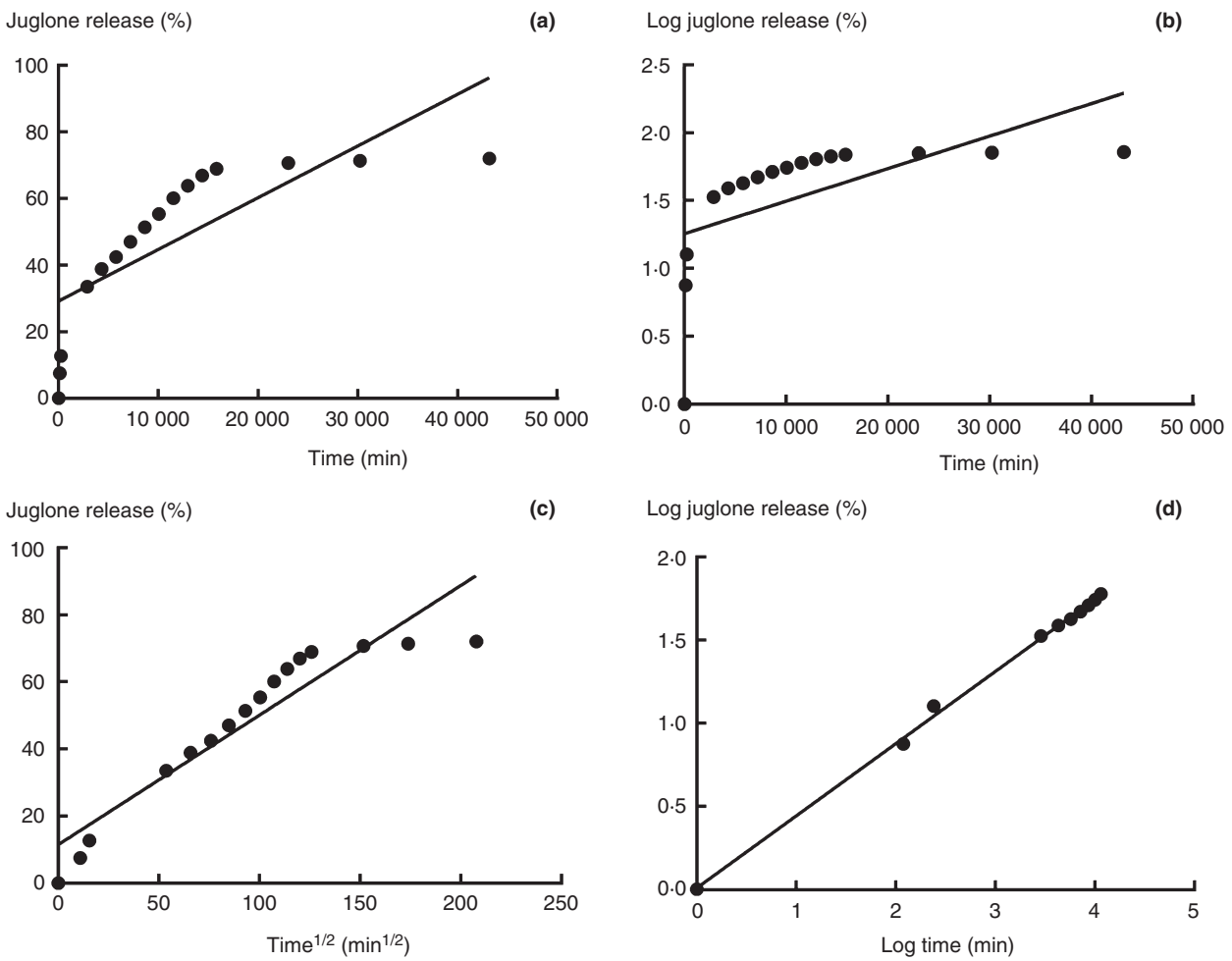


Figure 5 Juglone release kinetic plots of F4 formulation, zero order (a), first order (b), Higuchi (c) and Korsmeyer–Peppas (d).

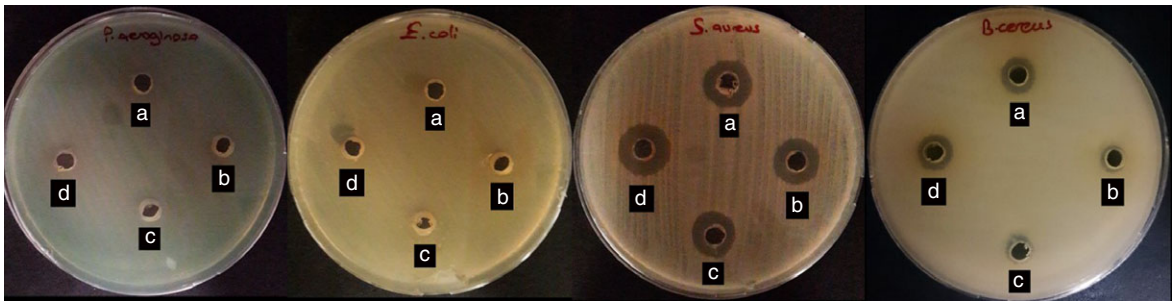


Figure 6 Agar well diffusion inhibition zone of (a) F4 (200 μg/100 μl), (b) Free juglone (27·7 μg/100 μl), (c) F4 (100 μg/100 μl), (d) Free juglone (55·5 μg/100 μl) on *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus* and *Bacillus cereus*, respectively. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 4 Inhibition zone results of agar well diffusion method

	Inhibition zone diameter (mm)			
Test samples	*Escherichia coli*	*Pseudomonas aeroginosa*	*Staphylococcus aureus*	*Bacillus cereus*
F4 (200 μg/100 μl)	–	–	15	9
F4 (100 μg/100 μl)	–	–	13	7
Free Juglone (55·5 μg/100 μl)	–	–	16	9
Free Juglone (27·7 μg/100 μl)	–	–	14	7

Quantitative analyses

With broth microdilution, the MIC and MBC values were determined and presented in Table 5. The results showed that there is no significant difference between the free juglone and its nanoparticle formulation (F4) in terms of antibacterial activity, thus indicated that the PLGA nanoparticle systems do not cause a remarkable decrease in the MIC values. As in the agar well diffusion assay, *S. aureus* was found to be the most susceptible bacterium to juglone and F4 nanoparticles among others (MIC and MBC: 17·5 μg ml^{-1}). Broth microdilution analysis showed that juglone and F4 have also antimicrobial activity against *B. cereus* (MIC: 17·5 μg ml^{-1} and MBC: 31·25 μg ml^{-1}).

Following the broth microdilution, time kill studies were performed based on the determined MIC concentrations. According to the results, both the free juglone and F4 nanoparticles strongly inhibited the growth of *S. aureus* and *B. cereus*, and showed a 99% decrease in number of colony compared to growth (negative) controls after 6 h. This growth inhibition effect lasted for 3 days (Fig. 7c,d), while the bactericidal activity of free juglone continued through 3 days (99·50, 94·50, 90·58%, respectively) against *S. aureus* and 2 days (95·51 and 63·37%) against *B. cereus*. In case of F4, the bactericidal activity was observed only for 1 day (93·58% for *S. cereus*; Fig. 7c,d).

Figure 7b shows that F4 nanoparticles did not kill *P. aeruginosa* bacteria, there was an initial decrease in the viable colony count at the end of the 6th hours (98·28%) and a subsequent regrowth at the end of 24th hours (8·51%). However, the growth inhibition effect of the free juglone lasted for 24 h and then, a regrowth was observed. Additionally, the bactericidal activity against *P. aeruginosa* was not detected in the specified time interval.

When it comes to *E. coli*, the bacterial growth was inhibited for 48 h by juglone nanoparticles as well as free juglone and then the inhibitory effect decreased (71·29% for juglone nanoparticles and 64·84% for free juglone after 48 h) (Fig. 7a).

Figure 7c shows that F4 nanoparticles showed weaker growth inhibitory activity than the free juglone against all strains for the first 24 h. Nevertheless, especially for *E. coli* and *P. aeroginosa*, this effect of nanoparticle system improved partially after 24 h and reached to the highest level after 72 h when compared to the free juglone.

When the literature is reviewed to compare the results, it was observed that antibacterial activity researches related to juglone usually performed by using different parts of walnut tree and its extracts, rather than the juglone molecule itself. According to the results of these studies, walnut extracts were generally found to be more effective against Gram positive bacteria (Oliveira *et al.* 2008; Pereira *et al.* 2008; Yigit *et al.* 2009). In a few researches about juglone molecule, the juglone, its synthetic derivatives and chemically modified formulations

Table 5 Determination of minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) values of test samples

	MIC values (μg ml^{-1})				MBC values (μg ml^{-1})			
	Microorganisms				Microorganisms			
Test samples	*Escherichia coli*	*Staphylococcus aureus*	*Bacillus cereus*	*Pseudomonas aeroginosa*	*Escherichia coli*	*Staphylococcus aureus*	*Bacillus cereus*	*Pseudomona aeroginosa*
F4	62·5	17·5	17·5	>500	125	31·5	31·5	>500
Free Juglone	62·5	17·5	17·5	>500	125	17·5	31·5	>500

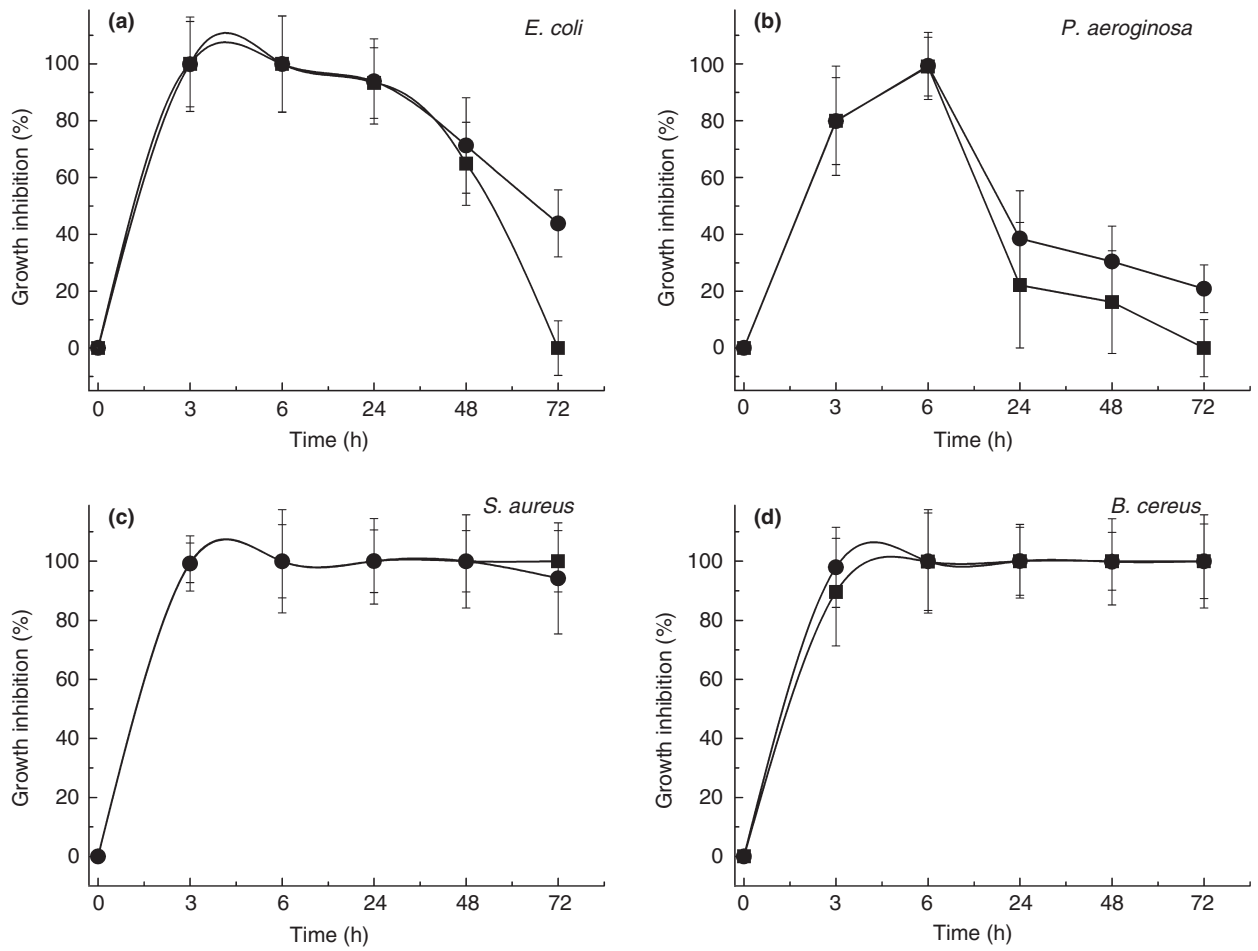


Figure 7 The growth inhibiton effect of Juglone nanoparticle (circle) and the free juglone (square) on *Escherchia coli* (a), *Pseudomonas aeruginosa* (b), *Staphylococcus aureus* (c) and *Bacillus cereus* (d) during 72 h exposure.

were investigated in terms of antimicrobial activity. Chuan Tan *et al.* (2012) evaluated the antibacterial activity of commercial juglone and the produced synthetic derivatives against *B. cereus*, *S. aureus*, *E. coli*, *Klebsiella pneumonia*. According to the results, MIC values were found as 0·72 mmol l^{-1} for *S. aureus* and 0·36 mmol l^{-1} for the others (Chuan Tan *et al.* 2012). In an another research conducted by Clark *et al.*, Gram positive bacteria was found to be sensitive to commercially obtained juglone whereas no effect was detected against Gram negative bacteria (Clark *et al.* 1990). In the antibacterial activity study of juglone performed by Mi Youn Lim, it was detected that juglone has no effect against *E. coli* but hydroxyl groups in its structure created a growth inhibition on *C. perfringens* (Lim *et al.* 2007).

In view of all literature about juglone and walnut tree extracts, it was observed that results of our research related to free juglone were consistent with these studies, especially for *S. aureus*. Nevertheless, it was thought that MIC differences between these researches and ours caused by reasons such as methods chosen, solvents, juglone source, and bacterial strains. Furthermore, it should be stated that there is no research published about antibacterial activity of juglone loaded PLGA nanoparticles.

Discussion

Walnut, one of the oldest known plants that has considerable allelopathic effect, is part of our daily diet and an important protective agent against infections due to its antimicrobial activity (Yigit *et al.* 2009). There are 35 phenolic compounds that are identified in walnut and most of them show allelopathic symptoms against plants and antimicrobial activity against both aerobic and anaerobic microorganisms (Riffel *et al.* 2002; Coder 2011). The compound that is mainly responsible from the biological activity of this plant is juglone, because it is one of the most toxic compounds and its concentration is relatively high among the others (>20 times) (Clark *et al.* 1990; Coder 2011).

In this work we were aimed to investigate the antibacterial activity of juglone encapsulated PLGA nanoparticles in our laboratories and study for the first time in literature. For this purpose, different process parameters were evaluated for obtaining maximum encapsulation efficiency with a minimum particle size. It was observed that the initial PLGA amount is the most significant factor on particle size, while the increase of initial juglone amount significantly increases the encapsulation efficiency. As PVA volume increases, reaction yield and encapsulation efficiency decreases, and the particle size initially decreases and then increases. Depending on the variation of the PVA concentration, it was observed that high encapsulation efficiency was obtained at 3% PVA concentration with 329·2 nm particle size, 76·08% RY and 55·23% EE (Fig. 1d, F4 formulation), while particle size decreases. For this reason, the release kinetic study and antimicrobial activity study were carried out with the F4 formulation which has the highest encapsulation efficiency. The results of the release study showed that release of the juglone from the nanoparticular system was controlled by the Fickian diffusion. In conjunction with the highest encapsulation rate, the highest amount of juglone release was obtained for F4. Therefore, all detailed characterization, release kinetic study, and antimicrobial activity study were carried out for F4 comparatively with free juglone for the first time by using four methods and four bacterial strains.

However, we observed differences between results of each method. Differences in results of qualitative methods (agar well and disc diffusion methods) were thought to be caused by applied the substance amount (10 μl for disc diffusion and 100 μl for agar well diffusion). Moreover, it was known that results of qualitative methods, especially for nanoparticle systems, affected by the size, structure, diffusion ratio, solubility, and types of nanoparticle system (Arasoglu *et al.* 2015). So, we recommend the use of qualitative and quantitative methods together for the antimicrobial studies. In our study, the time kill study was conducted based on MIC values detected in broth microdilution method and the growth inhibition ratios of this study were consistent with MIC values for all strains. Therefore, results obtained from quantitative methods (broth dilution and time kill methods) completed each other.

In the literature, it was observed that juglone was chemically modified to improve its existing antimicrobial activity (Chuan Tan *et al.* 2012). Alternatively, we aimed to increase the antibacterial activity by encapsulating juglone into PLGA nanoparticle systems. For the antimicrobial activity studies were conducted with equal amount of juglone in free and F4 formulation. As a result of 24 h MIC study, the free juglone and nanoparticle formulation showed similar antimicrobial activity. Considering to the amount of released juglone from nanoparticle formulation (only 33·55% of the juglone was released from the F4 nanoparticle during the first 24 h), it was found that the antibacterial activity of the juglone nanoparticles is higher and longer than the free juglone for time kill study.

A further research is needed to clarify the correlation between the antibacterial activity and the toxicity of juglone molecule. Additionally, it was observed that the PLGA encapsulated juglone nanoparticles still continued to exhibit the growth inhibition effect compared with the regrowth of bacteria number for the free juglone together with the extension of time, especially for 72 h (Fig. 7). Based on this result, we shouldproposed that usage of PLGA nanoparticle systems have more advantageous for longtime applications, as treatment and support process in healthy sector, product protection, and storage process in food industry. According to our results, we suppose that the use of nanoparticle formulations of juglone in biological systems and applications could be more beneficial rather than its free form due to its toxicity. Additionally, evaluating this activity with in vivo mouse models will be a right approach for future researches.

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Conflict of Interest

The authors declare no competing financial interest.

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