JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Enhancement of Antifungal Activity of Juglone (5-Hydroxy-1,4-naphthoquinone) Using a Poly(D,L-lactic-*co*-glycolic acid) (PLGA) Nanoparticle System

Tulin Arasoglu,[†] Banu Mansuroglu,[†] Serap Derman,[*,‡] Busra Gumus,[†] Busra Kocyigit,[†] Tayfun Acar,[‡] and Ismail Kocacaliskan[†]

[†]Science and Letters Faculty, Molecular Biology and Genetics Department, and [‡]Chemical and Metallurgy Faculty, Bioengineering Department, Yildiz Technical University, 34220 Istanbul, Turkey

ABSTRACT: This study aimed to synthesize and characterize juglone-entrapped poly(D,L-lactic-*co*-glycolic acid) (PLGA) nanoparticles and compare the antifungal properties of free juglone with its PLGA nanoparticle formulation for the first time. The juglone-loaded nanoparticles prepared using the oil-in-water (o/w) single-emulsion solvent evaporation method were characterized by the reaction yield (RY), encapsulation efficiency (EE), polydispersity index (PDI), particle size, zeta potential (ZP), FT-IR, and in vitro release properties and evaluated for their morphological features using SEM. The nanoparticle formulation had size, RY, ZP, EE, and PDI values of 212 nm, 66.91 ± 2.4%, −16.3 ± 0.7 mV, 70.66 ± 3.1%, and 0.083 ± 0.024, respectively. In vitro release showed a triphasic pattern with initial burst followed by sustained release and dormant phase over the study period, releasing about 72.8% in total after 42 days. The antifungal studies against *Aspergillus flavus*, *Candida albicans*, and *Fusarium* spp. using agar dilution and top agar dilution methods indicated that the juglone-encapsulated nanoparticle was more effective than free juglone. This study showed that the top agar method, which was applied for the first time on antifungal activity, is more suitable for the nanoparticular system based on sustained release. Therefore, PLGA nanoparticle formulations may be an important tool for application in many areas for the effective and beneficial use of hydrophobic compounds such as juglone.

KEYWORDS: *5-hydroxy-1,4-naphthoquinone, juglone, nanoparticle, antifungal activity*

INTRODUCTION

Allelopathy is a direct or indirect, harmful or beneficial, effect of a plant on other plants, insects, and microbes due to chemical compounds synthesized and secreted into the environment. These compounds are called allelochemicals, and they can usually be found in every tissue of a plant, especially in leaves and roots. These compounds are released into the environment through volatilization, water contact, exudation from root, and plant residues. Allelochemicals can have effects on cell division, respiration, photosynthesis, membrane permeability, protein synthesis, and seed germination.[1,2]

Juglone is an allelochemical that is located in the naphthoquinone subgroup within the quinones and a secondary metabolite produced by the walnut tree (Juglandaceae).[3] Juglone is selectively toxic toward some animals, microorganisms, and plants and has been used for many other purposes throughout history.[4−6] Although it was thought that juglone affects only other organisms but not the plant itself, Duroux et al. reported that this compound is also necessary for plant development.[7] Besides, it was reported that juglone has anticancer,[8,9] cytotoxic and genotoxic,[10,11] antioxidant,[9,12−15] antiviral,[16] antibacterial,[8,9,14,17−20] and antifungal[20−24] effects. However, its application and bioavailability are limited because of its poor dissolution property due to its hydrophobic character.

To provide sustained release of active substances, polymers that convert into nontoxic metabolites are usually preferred.[25] Biodegradable polymers such as polylactic acid (PLA), polyglycolic acid (PGA), and a copolymer of these, poly(D,L-lactic-*co*-glycolic acid) (PLGA), are most commonly used to synthesize active substance encapsulated nanoparticle systems to provide sustained release.[26−30] PLGA, one of the FDA-approved biopolymers used frequently for drug delivery systems, was an appropriate carrier to prepare nanoparticle systems with substances soluble in water or oil.[31] In recent years, the use of nanoparticle systems produced by encapsulating antimicrobial agents into PLGA biopolymer has become very prevalent. Thus, it was stated that pharmacokinetics properties, therapeutic indices, and biocompatibilities of these agents increased remarkably in comparison with the free form of the same drug.[32,33] Additionally, nanotechnology studies on developing antifungal agents are still in the early stages, and there is an urgent need for the development of eco-safe fungicides. Bio-nanomaterials are a promising alternative with high efficacy and relevant cost.[34] Due to their high surface to volume ratio and small size, they can surpass physiological barriers, reach the target, and interact well with the pathogens. Although the introduction of nanomaterials to the field is recent, it has already been agreed that the use of bio-nanomaterials is a necessity for defeating resistant pathogenic organisms.[35]

Received: July 25, 2016
Revised: September 1, 2016
Accepted: September 6, 2016
Published: September 7, 2016

ACS Publications © 2016 American Chemical Society

DOI: 10.1021/acs.jafc.6b03309
J. Agric. Food Chem. 2016, 64, 7087−7094

In the literature, research papers about the antifungal activity of juglone compound are few in number; there are, however, a large number of studies published about walnut tree extracts. In our research, we aimed to encapsulate the juglone molecule into a PLGA nanoparticle system for the first time and to evaluate its antifungal activity comparatively with free juglone. It should be stated that this is the first research in the literature about the antifungal activity of juglone-encapsulated PLGA nanoparticles. To assess this antifungal activity, two different methods were used and compared against *Aspergillus flavus*, *Candida albicans*, and *Fusarium* spp.

MATERIALS AND METHODS

Preparation of Nanoparticles. In this study, juglone, PVA, PLGA (lactide:glicolide = 50:50; inherent viscosity 0.45−0.60 dL/g, M_w ∼ 38−54 kDa P50/50), and DCM were purchased from Sigma-Aldrich (St. Louis, MO, USA). The modified single-emulsion solvent evaporation method used for preparation of juglone-loaded PLGA nanoparticles was described by our previous studies.[36,37] Juglone (50 mg/mL) and PLGA (40 mg/mL) were first dissolved in water-immiscible DCM, mixed together, and then suitably stirred to ensure that all materials were dissolved. This mixture was emulsified with aqueous PVA solution (3% w/v), over an ice bath, using a microtip probe sonicator (output power 100 W, power of 80%, and 2 min, Bandelin Sonopuls, Germany). The obtained oil-in-water (o/w) single emulsion was then diluted in 35 mL of PVA solution (0.1% w/v) and left on magnetic stirring overnight at room temperature. The nanoparticles were collected by centrifugation at 12000 rpm for 40 min (Beckman Coulter, Allegra-X-30R) and washed three times with ultrapure water, and then the obtained suspensions were lyophilized. The free nanoparticles were prepared according to a similar method without using juglone, and lyophilized nanoparticles were stored at −80 °C until used.

Characterization of Nanoparticles. The reaction yield and encapsulation efficiency of nanoparticles were determined in triplicate. The reaction yield was calculated gravimetrically as follows:[36]

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

The indirect quantification method was used to analyze the encapsulation efficiency of nanoparticles using UV−vis spectroscopy at 424 nm in triplicate. The juglone concentration in the supernatant was determined by using a previously constructed standard calibration curve. The encapsulation efficiency of juglone was calculated as follows:

$$\mathrm{EE\%} = \frac{\text{amount of juglone encapsulated in NPs}}{\text{initial juglone added}} \times 100 \quad (2)$$

Both zeta potential (ZP) and mean diameter of particles were analyzed using a Zetasizer (Zetasizer Nano ZS, Malvern, UK) instrument with electrophoretic light scattering (ELS) and dynamic light scattering (DLS) techniques, respectively.[38]

Morphology and FT-IR Study of Nanoparticles. Scanning electron microscopy (SEM, A JSM-7001FA, Jeol, Japan) was used to observe the shape and surface morphology of produced nanoparticles as previously described in our studies.[36] Lyophilized nanoparticles were fixed on metallic studs using adhesive tapes and then coated with gold under vacuum. SEM photomicrographs were taken at an acceleration voltage of 10−30 kV.

Fourier transform infrared spectroscopy (FT-IR) measurements were carried out by using an IR-Prestige 21 FTIR spectrophotometer (Shimadzu, Japan) in ATR mode.[36] The FT-IR spectra ranging between 600 and 4000 cm^{-1} were obtained with resolution of 4 cm^{-1}.

Release Study. The in vitro drug release study was carried out in triplicate using a modified dissolution method.[36] Juglone-PLGA-NPs (5 mg) were suspended in 2 mL of 150 mM phosphate buffer saline (PBS) and incubated 37 °C in a shaking incubator (150 rpm) at pH 7.4. At specified time intervals, the samples were centrifuged at 9000 rpm for 20 min, then the supernatant was collected, and the precipitates were suspended with 2 mL of fresh PBS. The juglone concentration in the supernatant was determined with UV−vis spectroscopy at 424 nm by comparing the concentration to a previously constructed standard calibration curve.

Determination of Antifungal Activities of Free Juglone and Synthesized Nanoparticles. The antifungal activity of free juglone and juglone-PLGA-NPs was assessed by the agar dilution method, according to the recommendations of the National Committee for Clinical Laboratory Standards,[39] and the top agar dilution method, which was applied for the first time in this study. Two methods were performed separately for *C. albicans* (ATCC 10231), *A. flavus* (ATCC 204304), and *Fusarium* spp. (ATCC 20883) (provided by Bacteriological Laboratory, Faculty of Science, at Ataturk University, Erzurum, Turkey).

Three different fungi isolates were subcultured at 27 °C on Sabouraud dextrose agar with 2% dextrose (SDA, from Difco, BD Diagnostic Systems, USA) until good growth was obtained. Spores were harvested in 5 mL of Sabouraud dextrose broth (SDB, from Difco, BD Diagnostic Systems, USA), and the tubes were incubated at 27 °C for 3 days. After the incubation period, the tubes were centrifuged at 5000 rpm for 8 min. The pellet was washed three times in 0.01 M sodium phosphate buffer (pH 7.4) and resuspended in PBS. The spore suspensions in buffer were then adjusted spectrophotometrically to 78−82% transmittance for *C. albicans* and 68−70% transmittance for *Fusarium* spp. and *A. flavus* at a wavelength of 600 nm.[40] These suspensions were used in the top agar method.

Agar Dilution Method. This method involves the incorporation of various desired concentrations of the antifungal agent into a molten agar medium, followed by the inoculation of fungal cultures onto the agar plate surface. The minimum inhibition concentration (MIC) value was determined as the lowest concentration of antifungal agent that inhibits growth after incubation time. The minimum fungicidal concentration (MFC) was regarded as the lowest concentration that does not yield any fungal growth on the solid medium used. Briefly, appropriate amounts of the free juglone and the juglone-PLGA-NPs were dissolved in sterile distilled water and added aseptically into sterile molted SDA medium, ending with a concentration range of 15.33−500 μg/mL. The resulting SDA media were immediately poured into Petri plates after vortexing. Small blocks of agar (2 × 2 mm) from growing fungal cultures were cut and placed on the SDA plates. The inoculated plates were incubated at 27 °C for 7 days. At the end of the incubation period, the plates were evaluated for the presence or absence of fungal growth. In this study, two control plates were used, the first one containing only fungal culture and the latter containing empty nanoparticles and fungal culture. Each test was repeated three times.

Top Agar Dilution Method. The top agar dilution method has been developed by modeling the Ames method[41,42] (*Salmonella*/microsome assay) for this study. In this method, the fungal spores were directly exposed to the test chemical for a certain time in a sterile tube containing buffered medium. Then, they was spread over the surface of SDA after the molten top agar had been added into the same tube. The most important point in the use of this method is the direct interaction between the test substance and the tested organism for a short period or pre-incubation time. The antifungal activity was examined according to the fungal growth on the plates.

In the top agar method, the stock solutions were prepared by dissolving the free juglone and the juglone-PLGA-NPs in sterile distilled water (1.38 mg/mL for the free juglone; 5 mg/mL for the juglone-PLGA-NPs, containing the same amount of active ingredient with free juglone). Six different concentrations for each material were prepared (500, 250, 125, 62.5, 31.25, and 15.33 μg/mL) and distributed into the sterile tubes. After that, 100 μL of the fungal cultures (prepared as mentioned above) was added into the tubes, and the tubes were pre-incubated for 30 min. Finally, the tubes were shaken gently by adding 2 mL of molten top agar containing 0.5 mM NaCl, and the mixtures were poured and spread quickly onto the plates containing SDA. After incubation at room temperature for 7

DOI: 10.1021/acs.jafc.6b03309
J. Agric. Food Chem. 2016, 64, 7087−7094

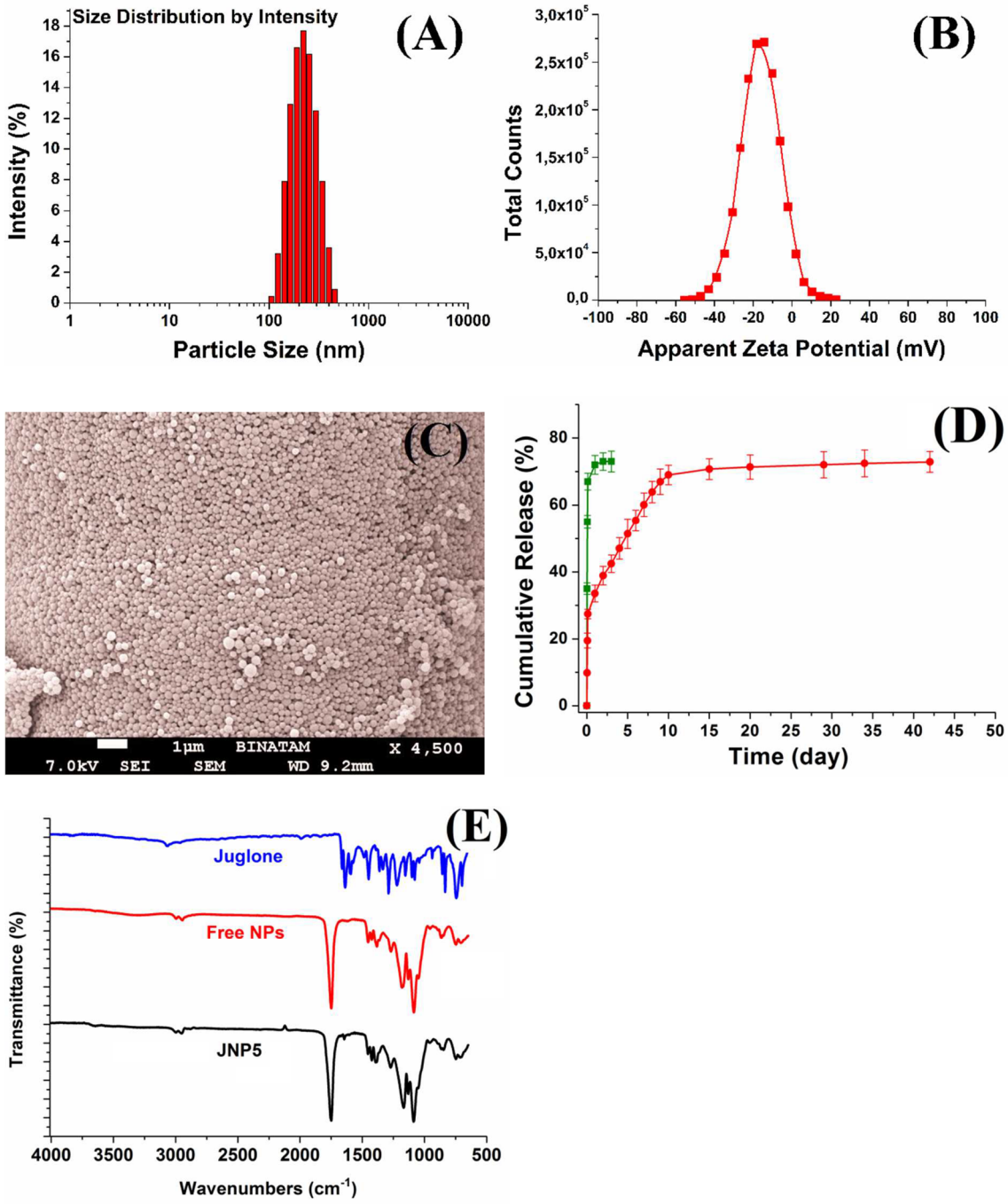


Figure 1. (A) Particle size distribution of JNP5, (B) zeta-potential distribution of JNP5, (C) SEM image of JNP5, (D) time-dependent release pattern of free juglone (squares) and JNP5 (circles), and (E) FT-IR spectra of juglone, free NP, and JNP5.

days, the antifungal activity was evaluated on the basis of the presence or absence of fungal growth. Two control plates were included in each test run. One control plate contained only fungal culture, and the other one contained the free PLGA nanoparticles dissolved in sterile distilled water, in addition to the molten top agar. Each test was repeated three times.

RESULTS

Nanoparticle Preparation and Characterization. The o/w single-emulsion solvent evaporation method was used for the preparation of free and juglone-loaded nanoparticles. The juglone-PLGA-NPs were analyzed for RY, EE, *Z*-av, PDI, and ZP.

After weighing of nanoparticles, the reaction yields were calculated to 50.47 ± 2.6 and 66.91 ± 2.4% for free nanoparticles and juglone-PLGA-NPs, respectively. The encapsulation efficiency of juglone was calculated as 70.66 ± 3.1% using the indirect method.

Particle Size, Zeta Potential, and Polydispersity of Nanoparticles. The particle size and ZP of nanoparticles were measured using dynamic and electrophoretic light scattering, respectively. Figure 1 shows particle size (A) and zeta potential (B) distribution of juglone-PLGA-NPs. The *Z*-average values of nanoparticles were measured as 183.1 ± 1.35 and 212.2 ± 4.65 nm for free nanoparticles and juglone-PLGA-NPs, respectively. It was seen that the mean diameter of juglone-PLGA-NPs

DOI: 10.1021/acs.jafc.6b03309
J. Agric. Food Chem. 2016, 64, 7087−7094

Table 1. RY, EE, Z-Av, PDI, and Zeta Potential (ZP) of Nanoparticles (JNP5)

name	RY%	EE%	Z-av (nm)	PDI	ZP (mV)
free NPs	50.47 ± 2.6		183.1 ± 1.35	0.092 ± 0.020	−23.6 ± 1.6
juglone NPs	66.91 ± 2.4	70.66 ± 3.1	212.2 ± 4.65	0.083 ± 0.024	−16.3 ± 0.7

Table 2. MFC and MIC Values According to Agar Dilution Method

	MFC values (μg/mL)			MIC values (μg/mL)		
test sample	*A. flavus*	*C. albicans*	*Fusarium* spp.	*A. flavus*	*C. albicans*	*Fusarium* spp.
juglone NPs	500	62.5	125	250	31.25	62.5
free juglone	500	250	250	250	62.5	125

increased to 212.2 ± 4.65 nm by encapsulation of juglone. Both *Z*-av values of the nanoparticles were measured as <220 nm in diameter with monodisperse size distribution. As is known, the smaller particle size (100−250 nm) is required for the high cellular uptake of nanoparticles.[43] All formulations were part of a single population (100% intensity) and had low values of polydispersity (0.092 ± 0.020 and 0.083 ± 0.024), which indicates the uniformity of particle size.

Results of the ZP measurement of nanoparticles are shown in Table 1. Free nanoparticles and juglone-PLGA-NPs showed negative ZP values, which indicate the stable state of nanoparticles owing to electrostatic repulsion.[44] In the present study, free juglone and juglone-PLGA-NPs had −23.6 ± 1.6 mV and −16.3 ± 0.7 mV, respectively. due to the use of nonionic emulsifier PVA[45] and the terminal carboxylic group of polymer.[46] This negative zeta potential causes the nanoparticle suspensions to be stable by preventing the agglomeration of nanoparticles.[36,47]

Morphology and FT-IR Study of Nanoparticles. The image of juglone-PLGA-NPs was observed using SEM as shown in Figure 1C. The nanoparticles had smooth surface morphology, spherical topography, and uniform size distributions without any agglomeration. The SEM image is in good agreement with the result measured by DLS, indicating clearly that the produced nanoparticles had a narrow size distribution.

The FT-IR spectra of juglone, free nanoparticles, and juglone-loaded PLGA nanoparticles are demonstrated in Figure 1E. In the FT-IR spectra, absorption peaks are assigned to specific functional groups. Pure juglone showed the main characteristic peak in the region of 1665−1630 cm^{-1} due to carbonyl groups (C═O), and the other important peaks are C═C stretching, which is observed at 1592 cm^{-1}, and C—O stretching, which is observed at 1288 cm^{-1}. It is understood from the spectra of free NPs and JNP5 that the 1750 cm^{-1} peak corresponds to the C═O group and that the peak between 1250 and 1100 cm^{-1} is due to the C—O stretch of the PLGA polymer. When the spectra of free NPs and JNP5 are compared, it is seen that specific functional groups in the surface of JNP5 have almost the same chemical characteristics of the free nanoparticles. The FT-IR spectra show that juglone was successfully encapsulated to PLGA nanoparticles without having any physical or chemical interaction.

Release Study. On the other hand, the release study was performed in 150 mM PBS (pH 7.4). The results presented in Figure 1D show that free juglone was dissolved in the media within 48 h, whereas juglone-loaded PLGA nanoparticles showed sustained triphasic release pattern up to 42 days. The in vitro release profile of juglone-PLGA-NPs over 40 days shows that after 4 h, approximately 30% of juglone had been released from the nanoparticles, which is indicative of initial burst release. Then, in the intermediate phase, the released juglone amount had increased to 69.9% within the following 10 days. Next, the release became very slow, and the release curve slope reaches a plateau corresponding to a maximum of 72.8% at the end of 42 days.

Antifungal Activity of Free Juglone and Synthesized Nanoparticles. In the agar dilution method, the antifungal activities of free juglone and juglone-PLGA-NPs were evaluated on the basis of fungal growth. The MIC and MFC values are shown in Table 2. It was observed that the PLGA nanoparticle systems caused a remarkable decrease in MIC values against *C. albicans* and *Fusarium* spp. Among the three fungal species, the lowest MIC value of the juglone-encapsulated PLGA nanoparticles was 31.25 μg/mL, whereas the lowest MIC value of the free juglone was 62.5 μg/mL against *C. albicans*. The MIC value of the free juglone was determined as 125 μg/mL for *Fusarium* spp., whereas it was 2-fold higher for the juglone-PLGA-NPs. According to the MFC values, the free juglone was 4 times higher for *C. albicans* and 2 times higher for *Fusarium* spp. than for its nanoparticle formulations. In the case of *A. flavus*, the MIC and MFC values for the juglone-encapsulated PLGA nanoparticles were obtained as 250 μg/mL, as for the free juglone.

Figure 2 shows the Petri images of the growth inhibition ratios between juglone-PLGA-NPs, free juglone, and control groups according to the MIC values detected for JNP5 by the agar dilution method against all strains. As shown in this figure, the growth difference between juglone-PLGA-NPs and the free juglone for *C. albicans* and *Fusarium* spp. was slightly observed. Also, this difference appears to be much higher for both groups compared with the control.

The top agar dilution method was performed by adding the molten top agar on fungal spores being exposed directly to juglone and its nanoparticle formulation in the buffered medium for a short time. In this method, the decrease in visible fungal growth was interpreted as antifungal activity or fungicidal activity. According to our results, the MIC and MFC values of free juglone were 4 times higher for *C. albicans*, 4 times higher for *Fusarium* spp., and 2 times higher for *A. flavus*, when compared to the nanoparticle formulations (Table 3). The empty NPs evaluated as the control group showed no effect in both studies (data not shown).

In Figure 3, strong visual evidence of a growth inhibitiory effect was observed in the juglone-PLGA-NPs exposed to Petri plates compared with both free juglone and control groups in the top agar dilution method.

DISCUSSION

Fungal organisms cause a significant problem in the health sector by developing antifungal resistance mechanisms recently

DOI: 10.1021/acs.jafc.6b03309
J. Agric. Food Chem. 2016, 64, 7087−7094

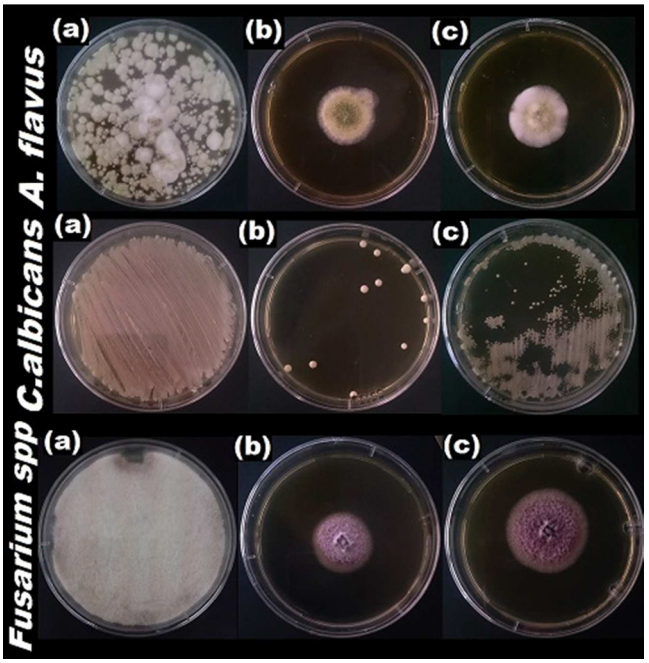


Figure 2. According to agar dilution method, the Petri images of growth inhibition effects of juglone PLGA nanoparticles and free juglone due to MIC values of JNP5 (250, 31.25, and 62.5 μg/mL for *A. flavus*, *C. albicans*, and *Fusarium* spp., respectively) in the agar dilution method: (a) control; (b) JNP5; (c) free juglone.

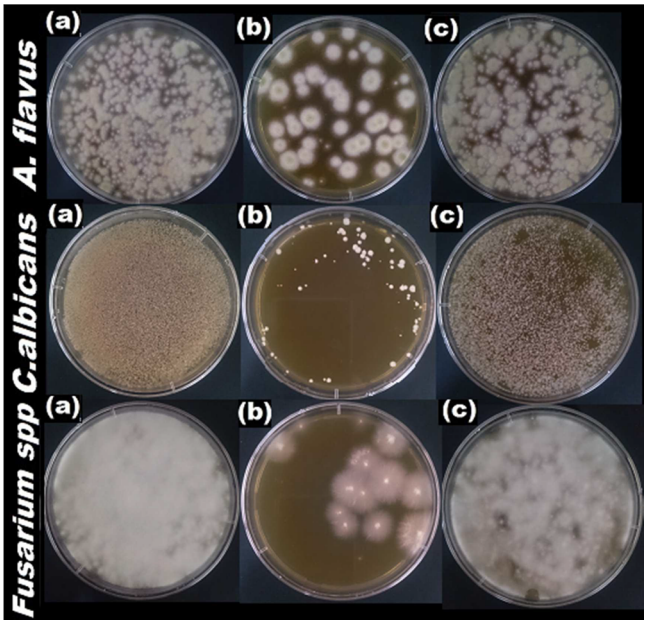


Figure 3. Petri images of growth inhibition effects of juglone PLGA nanoparticles and free juglone due to MIC values of JNP5 (125, 62.5, and 31.25 μg/mL for *A. flavus*, *C. albicans*, and *Fusarium* spp., respectively) in the top agar dilution method: (a) control; (b) JNP5; (c) free juglone.

and in the food industry by decreasing food quality, quantity, and storage time.[3] Therefore, there is an urgent need for exploring new antifungal agents that can be used in both sectors. When the harmful effects of synthetically produced chemical agents in human health are considered, the demand for natural antifungal agents obtained from plants increases gradually.[48] Also, plants are excellent resources for the supply of new antifungal agents.[49] PLGA nanoparticle systems are widely used to improve the efficacy and the impact time of natural bioactive compounds. Considering this situation, PLGA nanoparticle systems have been widely used to improve the efficacy and the impact time of natural bioactive compounds in recent years.[50] Applications of PLGA-NPs are broad, for example, gene therapy, targeted drug delivery, and the effective usage of active agents such as seconder metabolites and pharmaceutical drugs. In antifungal treatments, a major concern with currently available antifungal drugs is the low systemic bioavailability arising from low aqueous solubility and early drug degradation. Keeping this in perspective, the present study aimed to successfully synthesize juglone-loaded PLGA nanoparticle systems using the oil-in-water single-emulsion solvent evaporation method, characterize the system in detail, and evaluate its antifungal activity in comparison with free juglone. The antifungal activity of the synthesized JNP5 was also evaluated by two different methods against *A. flavus*, *C. albicans*, and *Fusarium* spp. for the first time.

Juglone is a naphthoquinone synthesized in walnut tree, and the antifungal properties of this compound have been known for many years.[3] When the literature was reviewed, it was observed that antifungal activity research about naphthoquinone derivatives is predominant,[51−53] but there are only a few studies about the juglone molecule.[20] Although it was reported in these research studies that juglone has an apparent antifungal activity, differences were found between the MIC values, which were thought to be caused by reasons such as methods selected, solvents, juglone source, and fungal strains.

Furthermore, on the basis of our knowledge, there were no reported data about the synthesis and characterization of the juglone-loaded PLGA nanoparticles and the evaluation of the antifungal activity of the juglone-encapsulated nanoparticles, as well. Therefore, in our study, the antifungal activity of the juglone-PLGA NPs was evaluated for the first time.

According to the our results, the juglone-loaded PLGA nanoparticles proved to be more effective in inhibiting fungal growth when compared to free juglone by both methods used. In former studies, it has also been shown that amphotericin b-,[54] voriconazole-,[55] and ITZ-entrapped[56,57] PLGA nanoparticles showed more potent antifungal efficacy than their free formulations congruently. In our study, one of the main reasons behind the enhancement in antifungal activity may be the solubility increase of the hydrophobic juglone molecule, which provided encapsulation into the nanoparticle system.

Another important reason may be existence of chitin, which is the main component of the fungal cell wall structure. β-1,4-

Table 3. MFC and MIC Values According to the Top Agar Dilution Method

	MFC values (μg/mL)			MIC values (μg/mL)		
test sample	*A. flavus*	*C. albicans*	*Fusarium* spp.	*A. flavus*	*C. albicans*	*Fusarium* spp.
juglone NPs	250	125	62.5	125	62.5	31.25
free juglone	>500	500	250	250	250	125

DOI: 10.1021/acs.jafc.6b03309
J. Agric. Food Chem. 2016, 64, 7087−7094

Linked *N*-acetylglucosamine units of the chitin provide a strong positive charge to fungal cells. PLGA nanoparticles, due to their negative charge, form an electrostatic interaction with the fungal cell walls. This situation provides access of the nanoparticles to the cell surfaces, their accumulation and, later on, the entrance of the released active substances or the nanoparticles into the cells and, therefore, increases the antifungal activity by forming an electrostatic attraction between the organisms and the PLGA-NPs.[58] Besides, the hydrophobins secreted by fungal organisms form an amphipathic membrane structure and so provide contact between the hydrophilic medium and hydrophobic agents such as juglone due to their hydrophobic and hydrophilic regions.[57] Still, this interaction is limited, and the drug activity is low. In contrast to that, because of their hydrophilic structure, the juglone-loaded PLGA nanoparticles interact well with the fungal surfaces and reveal a stronger growth inhibitory effect.

As reported in previous research, it is easier for small-sized particles to enter the cells, and the size of the juglone-PLGA nanoparticles synthesized in this study (212.2 ± 4.65 nm) is within the range described as "small-sized nanoparticle" in the literature.[43,57,59] In addition to these, despite the fact that the initial dose of the free juglone provides growth inhibition, drug concentration decreases on the basis of the consumption or degradation by the spores as time progresses. On the contrary, nanoparticle systems burst in the first 4 h (the moment when most of the active substance is released) and then continue to release active material over time. This continuous exposure prevents the regrowth of fungal spores and in that way provides a longer effect.[57]

In the present study, we examined the antifungal activity of the free juglone and its nanoparticle formulation by using two different methods: agar dilution and top agar dilution. The effect of the time-dependent release of the active material on the fungal organisms was observed more specifically in the top agar dilution method due to the interaction occurring between the fungal organisms and nanoparticles in the liquid medium for a while (30 min—preincubation time). Moreover, it was thought that the effect of the pH change—caused by this interaction—on the surface charges has an effect again on the interaction itself as well.[60] It was also thought that the interaction time may be important for the method's efficacy. Because of that, further research will be performed to evaluate the correlation between the antifungal activity and the interaction time in detail. Because the top agar dilution method allows using far less material than the agar dilution method, it is planned to perform validation studies to improve the methodology and provide the efficient usage of the method in release-based nanoparticle systems.

In conclusion, the improved antifungal activity of juglone entrapped in the PLGA nanoparticle system has been shown visually in Petri plates of agar dilution and top agar dilution methods (Figures 2 and 3). Moreover, in this study, more effective results were found in the top agar dilution method, which was used to detect the antifungal activity for the first time when compared to the agar dilution method. A stronger inhibition effect on the fungal growth was observed clearly for the juglone-PLGA-NPs than for the free juglone molecules by the top agar method. This suggests increased sensitivity for the top agar dilution method in the antimicrobial and antifungal activity studies of the PLGA nanoparticle systems, particularly. Additionally, to provide a more detailed explanation of the enhanced antifungal effect detected in this study, we suggest performing further in vivo and in vitro studies on the cellular uptake mechanisms and the interactions between the juglone-PLGA-NPs and fungal species.

AUTHOR INFORMATION

Corresponding Author

*(S.D.) Phone: +090 212 483 4643. E-mail: serapacar5@gmail.com.

Funding

We express our thanks to TUBITAK for supplying financial support (Project 114Z093).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We are grateful to Prof. Dr. Medine Güllüce and Assistant Professor Dr. Zeynep Mustafaeva Akdeste for providing laboratory facilities.

REFERENCES

(1) Seigler, D. S. Chemistry and mechanisms of allelopathic interactions. *Agron. J.* **1996**, *88*, 876−885.

(2) Halbrendt, J. Allelopathy in the management of plant-parasitic nematodes. *J. Nematol.* **1996**, *28*, 8.

(3) Strugstad, M.; Despotovski, S. A summary of extraction, synthesis, properties, and potential uses of juglone: a literature review. *J. Ecosyst. Manage.* **2013**, *13*, 1−16.

(4) Kocacaliskan, I.; Terzi, I. Allelopathic effects of walnut leaf extracts and juglone on seed germination and seedling growth. *J. Hortic. Sci. Biotechnol.* **2001**, *76*, 436−440.

(5) Terzi, I.; Kocaçalişkan, I.; Benlioğlu, O.; Solak, K. Effects of juglone on growth of cucumber seedlings with respect to physiological and anatomical parameters. *Acta Physiol. Plant.* **2003**, *25*, 353−356.

(6) Topal, S.; Kocacaliskan, I.; Arslan, O.; Tel, A. Z. Herbicidal effects of juglone as an allelochemical. *Phyton* **2007**, *46*, 259−269.

(7) Duroux, L.; Delmotte, F. M.; Lancelin, J.-M.; Keravis, G.; Christian, J.-A. Insight into naphthoquinone metabolism: β-glucosidase-catalysed hydrolysis of hydrojuglone β-D-glucopyranoside. *Biochem. J.* **1998**, *333*, 275−283.

(8) Xu, H.; Yu, X.; Qu, S.; Sui, D. Juglone, isolated from *Juglans mandshurica* Maxim, induces apoptosis via down-regulation of AR expression in human prostate cancer LNCaP cells. *Bioorg. Med. Chem. Lett.* **2013**, *23*, 3631−3634.

(9) Zakavi, F. Antibacterial effect of *Juglans regia* bark against oral pathologic bacteria. *Int. J. Dent.* **2013**, *2013*, 1−5.

(10) Kiran Aithal, B.; Sunil Kumar, M.; Nageshwar Rao, B.; Udupa, N.; Satish Rao, B. Juglone, a naphthoquinone from walnut, exerts cytotoxic and genotoxic effects against cultured melanoma tumor cells. *Cell Biol. Int.* **2009**, *33*, 1039−1049.

(11) Paulsen, M. T.; Ljungman, M. The natural toxin juglone causes degradation of p53 and induces rapid H2AX phosphorylation and cell death in human fibroblasts. *Toxicol. Appl. Pharmacol.* **2005**, *209*, 1−9.

(12) Chobot, V.; Hadacek, F. Milieu-dependent pro-and antioxidant activity of juglone may explain linear and nonlinear effects on seedling development. *J. Chem. Ecol.* **2009**, *35*, 383−390.

(13) Yiğit, D.; Yiğit, N.; Aktaş, E.; Özgen, U. Ceviz (*Juglans regia* L.) in antimikrobiyal aktivitesi. *Türk. Mikrobiyol. Cem. Derg.* **1993**, *39*, 7−11.

(14) Tan, D. T. C.; Osman, H.; Mohamad, S.; Kamaruddin, A. H. Synthesis and antibacterial activity of juglone derivatives. *J. Chem. Chem. Eng.* **2012**, *6*, 84−89.

(15) Altikat, S.; Terzi, I.; Kuru, H.; Kocacaliskan, I. Allelopathıc effects of juglone on growth of cucumber and muskmelon seedlings wıth respect to antioxidant enzyme activities and lipid peroxidatıon. *J. Environ. Prot. Ecol.* **2013**, *14*, 1244−1253.

DOI: 10.1021/acs.jafc.6b03309
J. Agric. Food Chem. 2016, 64, 7087−7094

(16) Montenegro, R. C.; Araújo, A. J.; Molina, M. T.; Rocha, D. D.; Lopéz-Montero, E.; Goulart, M. O.; Bento, E.; Alves, A. P. N. N.; Pessoa, C.; de Moraes, M. O. Cytotoxic activity of naphthoquinones with special emphasis on juglone and its 5-methyl derivative. *Chem.−Biol. Interact.* **2010**, *184*, 439−448.
(17) Dama, L.; Poul, B.; Jadhav, B. Antimicrobial activity of naphthoquinonic compounds. *J. Ecotoxicol. Environ. Monit.* **1998**, *8*, 213−215.
(18) Albayrak, A. *Ceviz Yaprak Özütleri ve Juglonun Bazı Mikroorganizmalar Üzerine Etkileri. Yüksek Lisans Tezi*. Dumlupınar Üniversitesi, Kütahya, 2006.
(19) Oliveira, I.; Sousa, A.; Ferreira, I. C.; Bento, A.; Estevinho, L.; Pereira, J. A. Total phenols, antioxidant potential and antimicrobial activity of walnut (*Juglans regia* L.) green husks. *Food Chem. Toxicol.* **2008**, *46*, 2326−2331.
(20) Clark, A. M.; Jurgens, T. M.; Hufford, C. D. Antimicrobial activity of juglone. *Phytother. Res.* **1990**, *4*, 11−14.
(21) Bonjar, G. S.; Aghighi, S.; Nik, A. K. Antibacterial and antifungal survey in plants used in indigenous herbal-medicine of south east regions of Iran. *J. Biol. Sci.* **2004**, *4*, 405−412.
(22) Bonjar, G. S. Anti yeast activity of some plants used in traditional herbal medicine of Iran. *J. Biol. Sci.* **2004**, *4*, 212−215.
(23) Nahrstedt, A.; Vetter, U.; Hammerschmidt, F. Composition of the steam distillation product from leaves of *Juglans regia* [walnuts]. *Planta Med.* **1981**, *42*, 313−332.
(24) Sharma, N.; Ghosh, P.; Sharma, U. K.; Sood, S.; Sinha, A. K.; Gulati, A. Microwave-assisted efficient extraction and stability of juglone in different solvents from *Juglans regia*: quantification of six phenolic constituents by validated RP-HPLC and evaluation of antimicrobial activity. *Anal. Lett.* **2009**, *42*, 2592−2609.
(25) Hans, M.; Lowman, A. Biodegradable nanoparticles for drug delivery and targeting. *Curr. Opin. Solid State Mater. Sci.* **2002**, *6*, 319−327.
(26) Lemoine, D.; Francois, C.; Kedzierewicz, F.; Preat, V.; Hoffman, M.; Maincent, P. Stability study of nanoparticles of poly(ε-caprolactone), poly(D,L-lactide) and poly(D,L-lactide-*co*-glycolide). *Biomaterials* **1996**, *17*, 2191−2197.
(27) Akagi, T.; Baba, M.; Akashi, M. Biodegradable nanoparticles as vaccine adjuvants and delivery systems: regulation of immune responses by nanoparticle-based vaccine. *Adv. Polym. Sci.* **2011**, *247*, 31−64.
(28) Jiang, W.; Gupta, R. K.; Deshpande, M. C.; Schwendeman, S. P. Biodegradable poly(lactic-*co*-glycolic acid) microparticles for injectable delivery of vaccine antigens. *Adv. Drug Delivery Rev.* **2005**, *57*, 391−410.
(29) Mohamed, F.; Van Der Walle, C. F. Engineering biodegradable polyester particles with specific drug targeting and drug release properties. *J. Pharm. Sci.* **2008**, *97*, 71−87.
(30) Kumari, A.; Yadav, S. K.; Yadav, S. C. Biodegradable polymeric nanoparticles based drug delivery systems. *Colloids Surf., B* **2010**, *75*, 1−18.
(31) Çırpanlı, Y. *Kamptotesin İçeren Polimerik Ve Oligosakkarit Bazlı Nanopartiküler Formülasyonların Geliştirilmesi Ve İn Vitro-İn Vivo Değerlendirilmesi*. Hacettepe Üniversitesi, Ankara, 2009.
(32) Soppimath, K. S.; Aminabhavi, T. M.; Kulkarni, A. R.; Rudzinski, W. E. Biodegradable polymeric nanoparticles as drug delivery devices. *J. Controlled Release* **2001**, *70*, 1−20.
(33) Desai, M. P.; Labhasetwar, V.; Amidon, G. L.; Levy, R. J. Gastrointestinal uptake of biodegradable microparticles: effect of particle size. *Pharm. Res.* **1996**, *13*, 1838−1845.
(34) Abd-Elsalam, K.; Alghuthaymi, M. Nanobiofungicides: are they the next-generation of fungicides? *J. Nanotechnol. Mater. Sci.* **2015**, *2*, 1−3.
(35) Blecher, K.; Nasir, A.; Friedman, A. The growing role of nanotechnology in combating infectious disease. *Virulence* **2011**, *2*, 395−401.
(36) Arasoglu, T.; Derman, S.; Mansuroglu, B. Comparative evaluation of antibacterial activity of caffeic acid phenethyl ester and PLGA nanoparticle formulation by different methods. *Nanotechnology* **2016**, *27*, 025103.
(37) Derman, S.; Akdeste, Z. M. Sentetik peptid-protein konjugatlarının parçacık boyutu ve zeta potensiyel analizi [Particle size and zeta potential investigation of synthetic peptide-protein conjugates]. *Biyokim. Derg.* **2015**, *40*, 282−289.
(38) Derman, S.; Mustafaeva, Z. A.; Abamor, E. S.; Bagirova, M.; Allahverdiyev, A. Preparation, characterization and immunological evaluation: canine parvovirus synthetic peptide loaded PLGA nanoparticles. *J. Biomed. Sci.* **2015**, *22*, 1−12.
(39) CLSI. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard*, 9th ed.; 2012; M07-A9, 32, 2.
(40) Al-Hatmi, A. M.; Meletiadis, J.; Curfs-Breuker, I.; Bonifaz, A.; Meis, J. F.; De Hoog, G. S. In vitro combinations of natamycin with voriconazole, itraconazole and micafungin against clinical *Fusarium* strains causing keratitis. *J. Antimicrob. Chemother.* **2016**, *71*, 953.
(41) Ozbek, T.; Gulluce, M.; Agar, G.; Adiguzel, A.; Ozkan, H.; Baris, O.; Sahin, F. Antimutagenic activities of methanol extracts of some endemic *Astragalus* species evaluated by Ames *Salmonella*/microsomal test. *Asian J. Chem.* **2009**, *21*, 451−458.
(42) Mortelmans, K.; Zeiger, E. The Ames Salmonella/microsome mutagenicity assay. *Mutat. Res., Fundam. Mol. Mech. Mutagen.* **2000**, *455*, 29−60.
(43) Win, K. Y.; Feng, S.-S. Effects of particle size and surface coating on cellular uptake of polymeric nanoparticles for oral delivery of anticancer drugs. *Biomaterials* **2005**, *26*, 2713−2722.
(44) Gaur, P. K.; Mishra, S.; Bajpai, M.; Mishra, A. Enhanced oral bioavailability of efavirenz by solid lipid nanoparticles: in vitro drug release and pharmacokinetics studies. *BioMed Res. Int.* **2014**, *2014*, 1−9.
(45) Zigoneanu, I. G.; Astete, C. E.; Sabliov, C. M. Nanoparticles with entrapped α-tocopherol: synthesis, characterization, and controlled release. *Nanotechnology* **2008**, *19*, 105606.
(46) Turk, C. T. S.; Oz, U. C.; Serim, T. M.; Hascicek, C. Formulation and optimization of nonionic surfactants emulsified nimesulide-loaded PLGA-based nanoparticles by design of experiments. *AAPS PharmSciTech* **2014**, *15*, 161−176.
(47) Derman, S. Caffeic acid phenethyl ester loaded PLGA nanoparticles: effect of various process parameters on reaction yield, encapsulation efficiency, and particle size. *J. Nanomater.* **2015**, *2015*, 1−6.
(48) Das, K.; Tiwari, R.; Shrivastava, D. Techniques for evaluation of medicinal plant products as antimicrobial agents: current methods and future trends. *J. Med. Plants Res.* **2010**, *4*, 104−111.
(49) Sardi, J.; Scorzoni, L.; Bernardi, T.; Fusco-Almeida, A.; Giannini, M. M. Candida species: current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. *J. Med. Microbiol.* **2013**, *62*, 10−24.
(50) Li, Z.; Jiang, H.; Xu, C.; Gu, L. A review: Using nanoparticles to enhance absorption and bioavailability of phenolic phytochemicals. *Food Hydrocolloids* **2015**, *43*, 153−164.
(51) Sasaki, K.; Abe, H.; Yoshizaki, F. In vitro antifungal activity of naphthoquinone derivatives. *Biol. Pharm. Bull.* **2002**, *25*, 669−670.
(52) Errante, G.; La Motta, G.; Lagana, C.; Wittebolle, V.; Sarciron, M.-É.; Barret, R. Synthesis and evaluation of antifungal activity of naphthoquinone derivatives. *Eur. J. Med. Chem.* **2006**, *41*, 773−778.
(53) Tandon, V. K.; Singh, R. V.; Yadav, D. B. Synthesis and evaluation of novel 1,4-naphthoquinone derivatives as antiviral, antifungal and anticancer agents. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 2901−2904.
(54) Jung, S. H.; Lim, D. H.; Jung, S. H.; Lee, J. E.; Jeong, K.-S.; Seong, H.; Shin, B. C. Amphotericin B-entrapping lipid nanoparticles and their in vitro and in vivo characteristics. *Eur. J. Pharm. Sci.* **2009**, *37*, 313−320.
(55) Peng, H.-s.; Liu, X.-j.; Lv, G.-x.; Sun, B.; Kong, Q.-f.; Zhai, D.-x.; Wang, Q.; Zhao, W.; Wang, G.-y.; Wang, D.-d. Voriconazole into PLGA nanoparticles: improving agglomeration and antifungal efficacy. *Int. J. Pharm.* **2008**, *352*, 29−35.

DOI: 10.1021/acs.jafc.6b03309
J. Agric. Food Chem. 2016, 64, 7087−7094

(56) Cunha-Azevedo, E. P.; Silva, J. R.; Martins, O. P.; Siqueira-Moura, M. P.; Bocca, A. L.; Felipe, M. S. S.; Tedesco, A. C.; Azevedo, R. B. In vitro antifungal activity and toxicity of itraconazole in DMSA-PLGA nanoparticles. *J. Nanosci. Nanotechnol.* **2011**, *11*, 2308−2314.
(57) Patel, N. R.; Damann, K.; Leonardi, C.; Sabliov, C. M. Itraconazole-loaded poly(lactic-*co*-glycolic) acid nanoparticles for improved antifungal activity. *Nanomedicine* **2010**, *5*, 1037−1050.
(58) Patel, N. R.; Damann, K.; Leonardi, C.; Sabliov, C. M. Size dependency of PLGA-nanoparticle uptake and antifungal activity against *Aspergillus flavus*. *Nanomedicine* **2011**, *6*, 1381−1395.
(59) Rejman, J.; Oberle, V.; Zuhorn, I. S.; Hoekstra, D. Size-dependent internalization of particles via the pathways of clathrin-and caveolae-mediated endocytosis. *Biochem. J.* **2004**, *377*, 159−169.
(60) Dimkpa, C. O.; McLean, J. E.; Britt, D. W.; Anderson, A. J. Antifungal activity of ZnO nanoparticles and their interactive effect with a biocontrol bacterium on growth antagonism of the plant pathogen *Fusarium graminearum*. *BioMetals* **2013**, *26*, 913−924.

DOI: 10.1021/acs.jafc.6b03309
J. Agric. Food Chem. 2016, 64, 7087−7094