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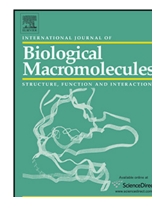

Chitosan nanoparticles enhances the anti-quorum sensing activity of kaempferol

Sedef Ilk[a,*], Necdet Sağlam[b], Mustafa Özgen[c], Feza Korkusuz[d]

[a] Faculty of Ayhan Şahenk Agricultural Sciences and Technologies, Ömer Halisdemir University, 51240 Nigde, Turkey
[b] Department of Nanotechnology and Nanomedicine, The Institute of Science and Engineering, Hacettepe University, Beytepe, 06800 Ankara, Turkey
[c] Department of Plant Production and Technologies, Faculty of Ayhan Şahenk Agricultural Sciences and Technologies, Ömer Halisdemir University, 51240 Nigde, Turkey
[d] Department of Sports Medicine, Faculty of Medicine, Hacettepe University, Beytepe, 06800 Ankara, Turkey

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ABSTRACT

Quorum sensing (QS) is a cell density dependent expression of species in bacteria mediated by compounds called autoinducers (AI). Several processes responsible for successful establishment of bacterial infection are mediated by QS. Inhibition of QS is therefore being considered as a new target for antimicrobial chemotherapy. Flavonoid compounds are strong antioxidant and antimicrobial agents but their applications are limited due to their poor dissolution and bioavailability. Our objective was to investigate the effect of kaempferol loaded chitosan nanoparticles on modulating QS mediated by AI in model bioassay test systems. For this purpose, kaempferol loaded nanoparticles were synthesized and characterized in terms of hydrodynamic diameter, hydrogen bonding, amorphous transformation and antioxidant activity. QS inhibition in time dependent manner of nanoparticles was measured in violacein pigment producing using the biosensor strain *Chromobacterium violaceum CV*026 mediated by AI known as acylated homoserine lactone (AHL). Our results indicated that the average kaempferol loaded chitosan/TPP nanoparticle size and zeta potential were 192.27 ± 13.6 nm and +35 mV, respectively. The loading and encapsulation efficiency of kaempferol into chitosan/TPP nanoparticles presented higher values between 78 and 93%. Kaempferol loaded chitosan/TPP nanoparticle during the 30 storage days significantly inhibited the production of violacein pigment in *Chromobacterium violaceum CV*026. The observation that kaempferol encapsulated chitosan nanoparticles can inhibit QS related processes opens up an exciting new strategy for antimicrobial chemotherapy as stable QS-based anti-biofilm agents.

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1. Introduction

Many bacterial species communicate with each other in a population-dependent mechanism to regulate their physiological functions. This mechanism is called as quorum sensing (QS) which is mediated through the release of diffusible and small signaling molecules called as autoinducers [1]. QS is an important process involved in bacterial survival and infections, recent research has focused on the development of therapeutic agents which prevent or manage bacterial pathogenesis by inhibiting bacterial QS. The inhibition of QS offers an alternative to antibiotic mediated bactericidal approach and reduces the risk for development of resistance. Inhibition of bacterial QS by attenuating the signals can prevent the development of bacterial virulence and successful establishment of infections [2]. Natural compounds have earned more attention as a source of anti-QS compounds for the treatment of biofilms [3]. Many gram negative bacteria, including *Chromobacterium violaceum* (*C. violaceum*), use *N*-acyl homoserine lactones (AHLs), signal molecules to monitor their own population density. *C. violaceum* is commonly found in soil and water, particularly in the tropical and subtropical areas and produces violacein, a purple pigmented compound when *N*-hexanoylhomoserine lactone (C6-HSL) is present [4,5]. *C. violaceum CV*026 is a mini-Tn5 mutant of *C. violaceum* which does not produce violacein unless it is supplied with C6-HSL. *C. violaceum CV*026 lacks the autoinducer synthase CviI and thus requires exogenous C6-HSL for violacein formation, which is QS-mediated [6].

Flavonoid compounds have a significant effect in natural antimicrobial applications. Although kaempferol (KAE) has a wide range of health benefits including anti-inflammatory, anticancer and antioxidant advantages while its biological effects are limited due to low aqueous solubility. Previous studies have reported that lim-

* Corresponding author.
E-mail addresses: ilksedef@hotmail.com, sedefilk@nigde.edu.tr (S. Ilk).

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ited water solubility and poor dissolution of flavonoids effected their chemical stability and bioavailability [7]. The use of nanoparticles can emerge to improve the solubility and bioavailability of KAE in a controlled manner through increased absorption and enhanced stability against free radicals during consumption and storage of food compounds [8]. Moreover, nanoparticles are suitable for efficient and sustainable delivery of active compounds and their large surface area enables rapid penetration of actives. Chitosan is a cationic and linear heteropolysaccharide composed of units of *N*-acetylglucosamine and glucosamine. It is linked by β-1,4 bonds and showed multiple properties such as biocompatibility, biodegradability, bioadhesion, absence of allergenicity and toxicity, anti-hypercholesterolemic, and antimicrobial activity. Therefore, chitosan has been presented as having potential for application in the industries of food, cosmetics, and pharmaceutics [9]. The aim of the present study is to develop a novel nanoparticle system (kaempferol loaded chitosan nanoparticle) to elucidate the anti-QS mechanism against reporter strain *Chromobacterium violaceum CV*026.

2. Materials and methods

2.1. Chemicals

Chitosan (75–85% deacetylated, low molecular weight), kaempferol (KAE, 99% purity), sodium tripolyphosphate (TPP), sodium hydroxide (NaOH), glacial acetic acid, phosphate buffer saline (PBS) tablets (pH: 7.4) and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich. Luria-Bertani (LB) was supplied by LABM. Other chemicals and solvents were of analytical reagent grade.

2.2. Preparation of kaempferol-loaded chitosan/TPP nanoparticles

Kaempferol-loaded chitosan/TPP nanoparticles were prepared according to methods of Calvo et al. [10] and Fan et al. [11] with some modifications, based on the ionic gelation of chitosan with TPP anions. Chitosan (0.2%, *w*/*v*) was dissolved in an aqueous solution of acetic acid (0.1%, *v*/*v*). The pH of the chitosan solution was adjusted to 4.8 using aqueous NaOH solution (20%, *w*/*v*). TPP (0.2%, *w*/*v*) was dissolved in ultrapure water. Kaempferol solutions at concentration of 20, 30, 40, 60, 80 and 100 μg/mL were prepared in DMSO (10%, *v*/*v*). To prepare kaempferol loaded chitosan/TPP nanoparticles; into 10 mL of the preheated chitosan solution (60 °C for 10 min), kaempferol solution was added under magnetic stirring. After that, 3.3 mL of 4 °C TPP solution was injected by dropwise (injection rate: 1 mL/min, syringe internal diameter: 0.38 mm) in chitosan/kaempferol solution at room temperature under magnetic stirring at 1000 rpm for 30 min. Opalescent suspension was formed spontaneously. The nanoparticles suspension was then centrifuged (12,000 rpm, 30 min) and washed with water to remove the unencapsulated compounds. After centrifugation, nanoparticle suspension was sonicated for 10 min to avoid the aggregation of particles. The empty chitosan/TPP nanoparticles were synthesized with above described method using chitosan and TPP solution without kaempferol solution.

2.3. Determination of hydrodynamic diameter and zeta potential of the nanoparticles

The particle size, polydispersity index (PDI) and zeta potential of the kaempferol loaded chitosan nanoparticles were determined by dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments, UK) at 25 °C. The nanoparticle solutions were analyzed in a folded capillary cell. All measurements were carried out in triplicate with three replications. The results were presented as mean ± standard deviation of the obtained values.

2.4. Determination of encapsulation efficiency and loading content

The kaempferol encapsulation efficiency and loading (%) of the synthesized nanoparticles were measured by UV/Vis spectrophotometer (PG Instruments, T60U UV–vis, UK) at wavelength of 373 nm. After nanoparticles were centrifugated for 30 min at 12,000 rpm, non-entrapped kaempferol concentration in recovered supernatant was determined. Supernatant recovered from nanoparticles without kaempferol was used as a blank. Possible kaempferol release was taken into consideration after every washings. Encapsulation efficiency (EE%), and loading content (LC%) were calculated by the following Eqs. (1) and (2), respectively:

$$\mathrm{EE}\ (\%) = \left(\frac{\text{Actual amount of KAE encapsulated in NPs}}{\text{Initial amount of KAE used}}\right) \times 100 \tag{1}$$

$$\mathrm{LC}\ (\%) = \left(\frac{\text{Weight of KAE in NPs}}{\text{Weight of NPs}}\right) \times 100 \tag{2}$$

2.5. Nanoparticle morphology observation

Scanning Electron Microscopy (SEM) (Quanta FEG 250; FEI, North America) was used for the surface morphology of the kaempferol loaded chitosan nanoparticles. Before the examination, one drop of diluted nanoparticle suspension was mounted onto aluminium pin-type stubs (12 mm in diameter) with carbon tape. NPs were coated with gold to minimize the effect of heat during high-power magnification after drying procedure.

2.6. Evaluation of interactions of kaempferol with chitosan/TPP nanoparticle

The spectra of Fourier transform infrared spectroscopy (FT-IR) and ^{1}H nuclear magnetic resonance (^{1}H NMR) analyses were used to analyze molecular bonding formation between kaempferol and chitosan nanoparticles. The FT-IR spectra of pristine kaempferol and kaempferol loaded chitosan nanoparticles were recorded using Perkin Elmer Nicolet 520 spectrophotometer (Perkin Elmer, Boston, USA). The lyophilized samples were ground with spectroscopic grade potassium bromide (KBr) powder and then, pressed into 1 mm pellet for FTIR measurement in the range of 450–4000 cm^{-1} with 4 cm^{-1} resolution, using 16 scans. In addition, ^{1}H NMR spectra of samples was obtained from Bruker 2300 NMR system (Bruker, Germany). For analysis, each sample (5 mg) was dissolved into 0.8 mL DMSO-*d*.

X-ray diffractometry (XRD) and differential scanning calorimetry (DSC) were used to determine the crystal polymorphism of free kaempferol and kaempferol loaded chitosan nanoparticles. The crystalline patterns of samples were obtained using the XRD (Panalytical, Empyrean) with Ni-filtered Cu Kα radiation. Measurement was performed at a voltage of 40 kV and 25 mA. The scanned angle was set from $2° \leq 2\theta \leq 50°$, and the scan rate was 1° min^{-1}. The thermal transition properties of samples were recorded using Perkin Elmer DSC Q8000 calorimeter (Perkin Elmer Inc., Boston, USA). The thermal measurements were carried out (scanning rate: 10 °C/min, temperature range: 30–500 °C) under Ar purge (50 mL/min). All samples were analyzed and recorded in triplicate.

2.7. Measurement of antioxidant activities assays

2.7.1. Free radical scavenging capacity

The free radical scavenging activity of free kaempferol, empty chitosan nanoparticle and kaempferol loaded chitosan nanoparti-

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