In Vitro and *in Vivo* Antibacterial Activities of Cyanidinum Chloride-loaded Liposomes against a Resistant Strain of *Pseudomonas aeruginosa*

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Abstract

Pseudomonas aeruginosa remains a common cause of wound infections. Different studies have shown that the entrapment of plant-derived materials in liposomes could increase their antibacterial activity against *Pseudomonas aeruginosa*. The aim of this study was to prepare cyanidinum chloride-loaded liposomes and evaluate their *in vitro* and *in vivo* efficacy against a resistant strain of *Pseudomonas aeruginosa* ATCC 15692. Cyanidinum chloride-loaded liposomes were prepared by extrusion method. The minimum inhibitory concentrations of cyanidinum chloride in the free and liposomal forms against *Pseudomonas aeruginosa* ATCC 15692 were determined *in vitro* by broth dilution method. The *in vitro* killing rates for free and liposomal cyanidinum chloride were analyzed. Ultimately, the *in vivo* therapeutic efficacy of the prepared liposomes in mice skin infected by ATCC 15692 was investigated. The minimum inhibitory concentrations of the free and liposomal forms of cyanidinum chloride against ATCC 15692 were 1.5×10^{-3} and 7.7×10^{-4} M, respectively. *In vivo* treatment with the free and cyanidinum chloride-loaded liposomes resulted in almost 40 and 100% survival rates, respectively. Our results showed that cyanidinum chloride-loaded liposomes would be a good choice for the treatment of wound infection caused by *Pseudomonas aeruginosa* because of their high effectiveness.

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Introduction

Pseudomonas aeruginosa is an opportunistic pathogen that causes serious wound infections [1]. Since *P. aeruginosa* can quickly colonize and infect wound sites and rapidly disseminate from wounds into the bloodstream, the clinical outcome in these patients can lead to sepsis, which is often fatal [2]. One of the major problems associated with *P. aeruginosa* infection is resistance to most conventional antibiotics [1]. Therefore, there is a compelling need to develop novel agents, strategies, and methods to overcome this resistance [3].

Cyanidinum ion (**Fig. 1**), a hydrolysis product from cyanidin salts and a flavonoid occurring in many red berries, possesses a range of biological and medicinal properties, including antioxidant, anticancer, antiobesity, and antiviral activities [4, 5]. Other physiological functions of cyanidinum ion include anti-inflammatory activity and reduction in memory impairment effects [6, 7]. It has been shown that plant extracts that contain a wide range of polyphenolic compounds such as cyanidinum ion have antimicrobial activity against drug-resistant bacteria [8, 9]. Cyanidinum ion has a major antibacterial and antifungal activity effect on *Bacillus subtilis* and yeast, respectively [10]. Also, this compound has antituberculosis activity [11].

Liposomes are spherical and colloidal vesicles that range from a few nanometers to several micrometers in diameter [12, 13]. These carriers are composed of natural phospholipids and other lipids, such as cholesterol, and can be used as a vehicle for the administration of nutrients and pharmaceutical drugs [14–16].

Previous studies showed that the encapsulation of plant-derived materials into liposomes markedly alter their pharmacokinetics, increasing their half-lives and effectiveness [17, 18]. While some of these compounds, such as usnic acid, have relative antibacterial effectiveness in the liposomal form, others, such as oleic acid, have been

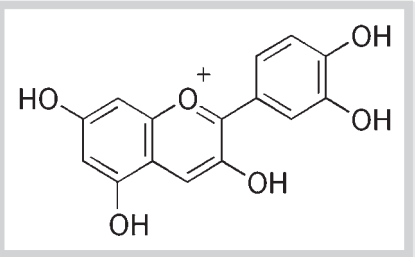

Fig. 1 Cyanidinum ion structure.

more effective in this formulation [19,20]. The anti-*P. aeruginosa* effectiveness of cyanidinum chloride-loaded liposomes has not yet been studied. The primary aim of this study was to prepare cyanidinum chloride-loaded liposomes and evaluate their *in vitro* antibacterial activity against the resistant strain of *P. aeruginosa* ATCC 15692. A secondary aim was to investigate the therapeutic efficacy of prepared liposomes using a mouse model of skin wounds infected with this bacterium.

Materials and Methods

Chemicals

Cyanidinum chloride (purity ≥ 95%), cholesterol, egg lecithin, and amikacin hydrate (an aminoglycoside antibiotic, as the positive control, purity ≥ 78%) were purchased from the Sigma Chemical Company. Chloroform, HPLC grade methanol, and Mueller-Hinton broth were purchased from Merck.

Animals

Fifty male BALB/c mice (18–20 g) were obtained from the National Institute of Pasture, Iran. The mice were handled according to the national guidelines for laboratory animals, received food and water *ad libitum* and were housed in separate and pathogen-free cages [21]. Animal care and protocols were performed and approved by the Institutional Animals Ethics Committee of Tarbiat Modares University (Number: 145, Tehran, Iran, 3/3/2011).

Microorganism

P. aeruginosa ATCC 15692 was purchased from the American Type Culture Collection. This strain was inoculated onto blood agar plates and then incubated for 24 h at 37 °C and used for experiments.

Preparation of liposomes

The cyanidinum chloride-loaded liposomes were prepared by the extrusion method, as described previously [17]. Briefly, the egg lecithin and cholesterol in the molar ratio of 4 : 1 were dissolved in chloroform and dried to a lipid film with a rotary evaporator (Brinkman) under vacuum and N_2 flow at 30 °C. The dried lipids were dispersed by agitation in 6 mL of an aqueous solution of cyanidinum chloride (150 mg/mL in PBS, pH 7.4) and sonicated at 4 °C in ultrasonic bath (Braun-sonic 2000). Finally, cyanidinum chloride-loaded liposomes were obtained by extruding the respective suspension through a polycarbonate membrane with 100 nm-sized pores 12 times, and separating the excess free drug and larger lipid aggregation by ultracentrifugation (100 000 g, 30 min). The control liposomes were prepared similarly, but PBS (pH 7.4) was used instead of the cyanidinum chloride solution.

Determination of encapsulation efficacy

The content of the cyanidinum chloride in the liposomes was determined by HPLC as previously described [22], and the percentage of cyanidin loading was then calculated as: amount of cyanidinum chloride in liposome × total volume tested × 100/total sample volume × initial amount of cyanidinum chloride. This experiment was done in triplicate.

Particle size, zeta-potential, size distribution, and polydispersity index determination

The mean particle size, zeta-potential, size distribution, and polydispersity index of the liposomes were evaluated using a Malvern zetasizer (Malvern instrument) apparatus, as reported previously [23,24]. Each experiment was done in triplicate.

Antimicrobial susceptibility testing

The minimum inhibitory concentrations (MICs) of the free and cyanidinum chloride-loaded liposomes and amikacin for *P. aeruginosa* ATCC 15692 were determined by the broth dilution technique as recommended by CLSI (formerly NCCLS) [25]. A bacterial suspension of ~ 5×10^5 cells/mL was diluted in the Mueller-Hinton broth and dispensed (100 µL) into a microtiter tray containing serial twofold dilutions of cyanidinum chloride. The tray was then incubated for 24 h at 37 °C. The MICs were recorded as the lowest concentrations of cyanidinum chloride in the free and liposomal forms that prevented visible bacterial growth and were expressed in µg/mL. All experiments were done in triplicate.

Time-kill studies

Time-kill studies were performed in triplicate in 10 mL tubes containing 2 mL of Mueller-Hinton broth as previously described [26]. Briefly, 100 µL of the bacterial suspension were resuspended in 10 mL of the Mueller-Hinton broth, incubated overnight at 37 °C and adjusted to a McFarland standard of 0.5. Then, 100 µL of this standardized inoculum were added to separate culture tubes containing 1 mL of Mueller-Hinton broth with 1 mL cyanidinum chloride solutions in the free and liposomal forms at 1, 2, and 4 times the MIC and incubated at 37 °C. Subsequently, colony counts were performed at 0, 2, 4, 6, and 24 h, and the results were expressed as log colony forming unit per milliliter (CFU/mL). All experiments were performed in triplicate.

Animal studies

The *in vivo* therapeutic efficacy of cyanidinum chloride-loaded liposomes in a mouse skin suture-wound model was tested according to a described method [27], with some modifications. Briefly, sterile silk sutures were cut and threaded onto sterile surgical needles and soaked for 45 min in undiluted broth cultures of the *P. aeruginosa* ATCC 15692 that had been incubated at 35 °C for 8 h. The mice were anesthetized with a ketamine-xylazine mixture (50 mg/kg each, given intramuscularly). Subsequently, the fur on the back and flanks was clipped, and the skin was swabbed with 70% ethanol. A 1-cm length of inoculated suture was inserted under the skin of the mid-back and secured by knotting the other side of the suture. Then the infected mice were divided into 5 groups. Prior to the treatment starting, the free and liposomal forms of cyanidinum chloride, empty liposomes, amikacin, and physiological saline solutions were prepared. Subsequently, the gel forms of these treatments were prepared according to a previously described method [28]. Then, all groups were treated topically as follows: group 1 received cyanidinum chloride-loaded liposomes gel (contained 250 mg cyanidinum chloride/

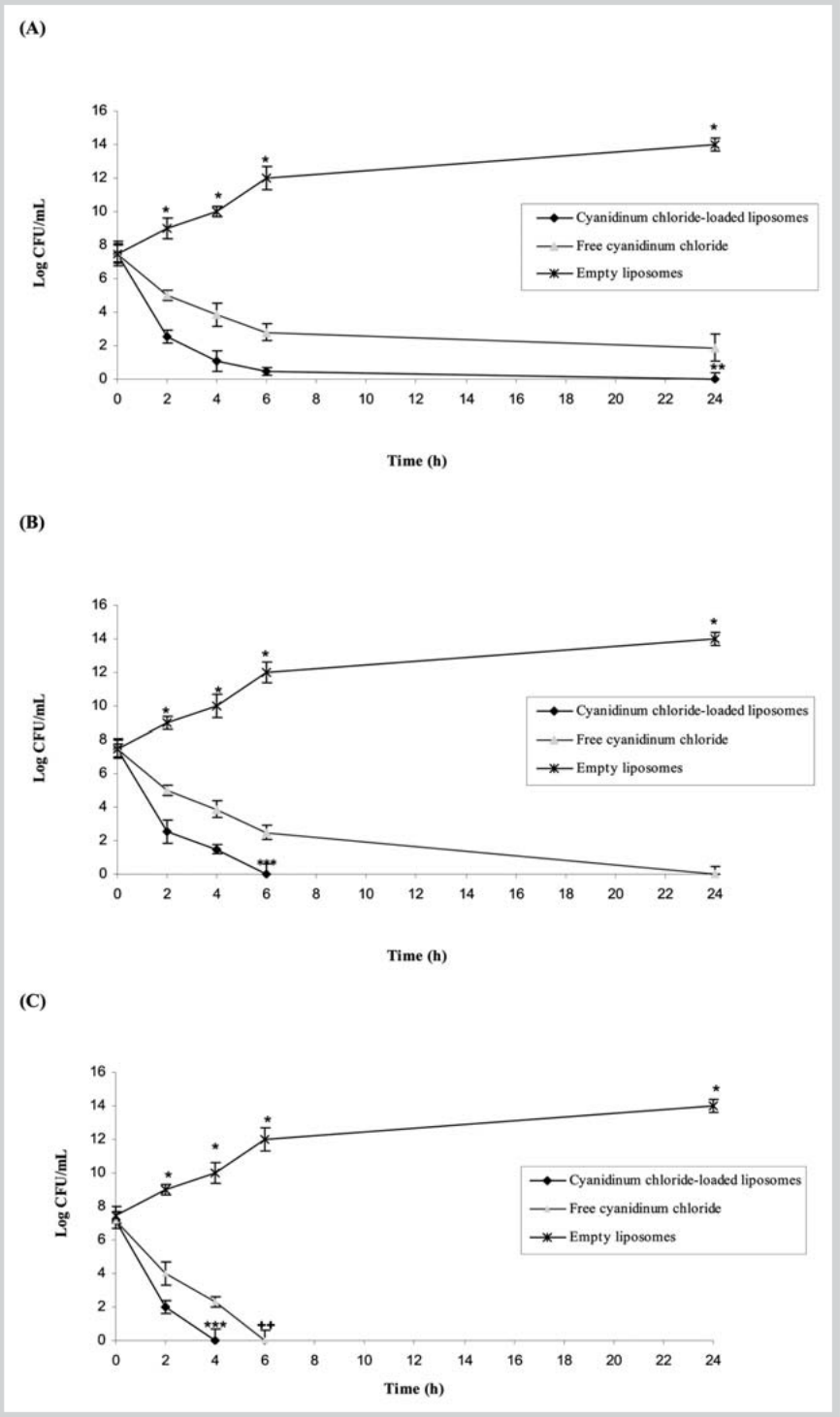

Fig. 2 Killing curves for *P. aeruginosa* ATCC 15692 when exposed to various concentrations (**A** = 1 × MIC, **B** = 2 × MIC, and **C** = 4 × MIC) of cyanidinum chloride in the free and liposomal forms. All experiments were done in triplicate.* Significant difference between the killing rate of the empty liposomes versus free and cyanidinum chloride-loaded liposomes ($p < 0.01$); ** significant difference between the killing rate of the cyanidinum chloride-loaded liposomes versus free cyanidinum chloride ($p < 0.05$); *** significant difference between the killing rate of the cyanidinum chloride-loaded liposomes versus free cyanidinum chloride ($p < 0.01$); + significant difference between the killing rate of the free cyanidinum chloride and the empty liposomes ($p < 0.05$); ++ significant difference between the killing rate of the free cyanidinum chloride and the empty liposomes ($p < 0.01$).

kg/24 h); group 2 received free cyanidinum chloride gel (250 mg/kg/24 h); group 3 received empty liposomes gel (250 mg/kg/24 h); group 4 received physiological saline gel (1 mL/kg/24 h); and group 5, as the positive control group, received amikacin gel (250 mg/kg/24 h); for 8 days starting from the 3rd day postinfection. Two days after the last dose, the surviving animals were anesthetized and sacrificed by cervical dislocation, and the skin, liver, and spleen of each animal was removed under sterile conditions and homogenized for 5–10 min in PBS (2 mL/g). The homogenates were serially diluted and plated for growth in the soybean-casein digest agar medium and incubated at 35 °C for 24 h, and then the colony forming unit (CFU) was counted. The colony counts were performed in triplicate.

Data analysis

The results are expressed as means ± standard errors for all of the experimental measurements. The data from the killing rate study were statistically evaluated by paired Student's t-test, and a p value < 0.05 was considered significant. The results of the survival rates of the control and treated animals were determined by using the chi-squared test with Yates correction and by Fisher's exact test.

Table 1 Survival rate of infected mice and colony-forming units (CFUs) of *P. aeruginosa* ATCC 15692 in different organs.

Treatment	Tissue/Organ	Log CFU/Gram tissue	Percentage of survival mice (n = 10)
Control without drug administration (received physiological saline, topically)	Skin	3.213 ± 0.200	none survived
	Spleen	3.871 ± 0.400	
	Liver	3.182 ± 0.500	
Empty liposomes (250 mg · kg^{-1}, topically)	Skin	3.316 ± 0.500	none survived
	Spleen	3.766 ± 1.100	
	Liver	3.210 ± 0.500	
Free cyanidinum chloride (250 mg · kg^{-1}, topically)	Skin	2.122 ± 0.300	40
	Spleen	2.795 ± 1.100	
	Liver	2.547 ± 0.120	
Cyanidinum chloride-loaded liposomes (250 mg · kg^{-1}, topically)	Skin	Nil*	100
	Spleen	0.623 ± 0.100**	
	Liver	Nil*	
Amikacin (250 mg · kg^{-1}, topically)	Skin	0.120 ± 0.200**	100
	Spleen	0.843 ± 0.500**	
	Liver	0.355 ± 0.300**	

The results are expressed as mean ± standard error of mean. The analysis of variance of one-way classification between the treatment means was heterogeneous, and the t-test values (two-tailed) were significant. * $p < 0.001$ and ** $p < 0.05$

Supporting information

The particle size, zeta-potential, and polydispersity index of the empty and cyanidinum chloride-loaded liposomes as well as the size distribution pattern of cyanidinum chloride-loaded liposomes are shown in the Supporting Information.

Results and Discussion

The use of plant-derived compounds to eliminate harmful and resistant bacterial infections has been widely investigated [8,9]. However, the main problems associated with the application of these materials are insufficient quantities in the target site and *in vivo* instability [9]. To overcome these problems, the investigators focused on the encapsulation of plant-derived compounds in carriers such as liposomes [29,30]. However, the preparation of plant-derived liposomes with high encapsulation efficacy may not be easy because the variable interactions between these materials and bilayer lipids can occur [31]. In this study, we evaluated the potential of incorporating cyanidinum chloride into liposomes. The results showed that cyanidinum chloride can be encapsulated into the prepared liposomes with high entrapment efficacy (85.00% ± 0.15). It has been shown that positive interaction between lipids and loaded components could enhance entrapment efficacy [16,17], and probably some of the various known types of weak links make the increase of cyanidinum chloride encapsulation efficacy.

The homogeneity of the mean particle size of the empty and loaded liposomes suggested that the cyanidinum chloride was entrapped in the lipid bilayer, according to previous studies [17, 23]. The zeta-potential of the liposomes revealed that the prepared cyanidinum chloride-loaded nanoparticles have appropriate stability in aqueous dispersion [31,32]. The size distribution study showed a monomodal distribution with a mean diameter of 92.5 ± 0.2 nm.

The MIC values of cyanidinum chloride in both of the free and liposomal forms and amikacin for *P. aeruginosa* ATCC 15692 were 1.5×10^{-3}, 7.7×10^{-4}, and 6.8×10^{-6} M, respectively. The difference between the MICs of the free and liposomal cyanidinum chloride was significant ($p < 0.05$). Our results suggest that the entrapped cyanidinum chloride in the liposomes enhanced its antibacterial activity against *P. aeruginosa* when compared to the free cyanidinum chloride. The killing curves of the cyanidinum chloride in the free and encapsulated forms at 1, 2, and 4 times the MICs are shown in **Fig. 2**. In all of the conditions, the encapsulated cyanidinum chloride was more effective on reducing the bacterial counts when compared to the free cyanidinum chloride (**Fig. 2**). At one time MIC only, the cyanidinum chloride-loaded liposomes could eliminate *P. aeruginosa* ATCC 15692 after 24 h (**Fig. 2A**). At two times MIC, both the free and encapsulated forms of cyanidinum chloride eradicated the bacteria after 6 and 24 h, respectively (**Fig. 2B**). At four times MIC, the cyanidinum chloride-loaded liposomes eliminated the bacteria after 4 h (**Fig. 2C**).

It has been reported that encapsulating methyl-N-methylanthranilate and alcohol α-bisabolol (organic compounds from *Zanthoxylum tingoassuiba*) in liposomes could eliminate the resistant strain of *P. aeruginosa* as well [33]. Some hypotheses, including increasing the penetration of plant-derived materials into bacteria cells and their non-sensitivity to degradation bacterial enzymes, may explain the mechanism of the enhanced antimicrobial activities of these liposomal formulations [8,33]. The time-kill assays confirmed that the potency of cyanidinum chloride-loaded liposomes was higher than free cyanidinum chloride. As reported previously [20], we hypothesized that the electrostatic interaction between the outer membrane lipopolysaccharides of *P. aeruginosa* and the liposomes could enhance the mechanism of cyanidinum chloride entry into this microorganism's cell. Wounds and other exposed tissues are particularly susceptible to bacterial contamination and infections [34,35]. Compared to the untreated animals, the treatment of the wound-infected mice with cyanidinum chloride-loaded liposomes showed a significant reduction in CFU values in the evaluated organs, especially in the skin and liver (**Table 1**). It was found that the mortality of the mice without the administration of cyanidinum chloride was 100% after 8 days, whereas the animals treated with cyanidinum chloride in free and encapsulated liposomes showed an increase in the survival rate of 40 and 100%, respectively. Also, the viability of the amikacin-treated mice (as positive control) was 100%.

It has been proven that the potential mortality from wound infections, even with aggressive antibiotic therapy, may approach or exceed 50% [36]. The treatment of mice with cyanidinum chloride-loaded liposomes resulted in a 100% survival rate and in an almost complete eradication of the bacteria from the skin and liv-

er of each of the infected animals. This data may be due to optimal antibacterial delivery, which has been reported by several investigators [19,37]. It has also been reported that oleic acid-loaded liposomes (LipoOA) could eliminate methicillin-resistant *Staphylococcus aureus* (MRSA) *in vitro* and *in vivo* [20]. Also, the encapsulation of usnic acid (a dibenzofuran originally isolated from lichens) improved the burn healing process in rats [19]. When liposomes containing compounds are applied topically, they can interact with the cell membranes of exposed tissues, and therefore protect the wound tissues from further bacterial infections [19,20,37].

In conclusion, the *in vitro* and *in vivo* testing showed that cyanidinum chloride-loaded liposomes have a strong protective effect of wounds infected by *P. aeruginosa* ATCC 15692 and may be a good choice for the treatment of patients with such infections.

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Conflict of Interest

The authors report no conflict of interest.

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