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Evaluation of Antibacterial Activity of PHY-ZnONPs against Certain Multi-Drug Resistant (MDR) Bacteria

Soheila DAVAEIFAR[1], Kamran Akbari NOGHABI[2,5,*], Hossein Shahbani ZAHIRI[2], Mohammad Hossein MODARESI[1], Mehdi MOHAMMADI[2], Morvarid SHAFIEI[3], Hadi MALEKI[4]

[1]Department of Biology, Science and Research branch, Islamic Azad University, Tehran-Iran
[2]National Institute of Genetic Engineering and Biotechnology (NIGEB), P.O. Box 14155-6343 Tehran-Iran
[3]Department of Bacteriology, Microbiology Research Center (MRC), Pasteur Institute of Iran, Tehran-Iran
[4]Department of Microbiology and Microbial Biotechnology, Faculty of Life Sciences and Biotechnology, Shahid Beheshti University, Tehran-Iran
[5]Horticulture Department, Agriculture & Natural Resources College, Abhar branch, Islamic Azad University, Abhar, Iran

*Corresponding Author:
E-mail: akbari@nigeb.ac.ir

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Abstract
In recent years, due to the increasing drug resistance and toxicity of many existing drugs the use of bioactive compound to overcome the pathogens has been highly regarded. Phycocyanin is one of the important biocompatible compounds with the significant antibacterial effects. It is the major light-harvesting pigment in cyanobacteria. On the other hand, nanoparticles are a new area to fight the infections. The biogenic synthesis of ZnO nanoparticles using capping potential of bioactive compounds can be a novel strategy to enhance their antibacterial activities. Considering this goal, the antibacterial and antibiofilm activity of phycocyanin-Zinc Oxide nanoparticles (PHY-ZnONPs) was investigated for the first time. Phycocyanin pigment was isolated from a native cyanobacterial strain, *Limnothrix* sp. KO05. In the following, the manufacturing functionalized phycocyanin, PHY-ZnONPs were synthesized. The antibacterial effect of PHY-ZnONPs evaluated on selected clinical Gram negative ESBL (extended spectrum beta lactamases)-producing *E. coli*, ESBL *Pseudomonas aeruginosa* and Gram positive methicillin-resistant bacterium *Staphylococcus aureus* (MRSA). The effect of PHY-ZnONPs on extracellular polysaccharides (EPS) production by tested clinical isolates showed a definite order of inhibitory effect as follow: *E. coli* > *S. aureus* > *P. aeruginosa*. PHY-ZnONPs with a high effect against selected clinical MDR isolates can be envisaged as a prospective nanoantibiotic for inhibiting of biofilm formation and bacterial virulence.

Keywords: phycocyanin, nanoparticles, antibacterial activity, natural compounds

INTRODUCTION

Cyanobacteria are the one of the oldest photosynthetic organisms known on earth and the ability of oxygenic photosynthesis has made them the only plant-like photosynthetic organisms. The major light-harvesting pigments in cyanobacteria are called phycobiliproteins (PBPs) and include phycocyanin, phycoerythrin and allophycocyanin. C-phycocyanin (C-PC) is one of the major biliproteins purified from Cyanobacteria. This pigment-protein is an accessory photosynthetic pigment that acts an important role in photosynthesis. It has anti-cancer, immunomodulatory and radical scavenging properties. In addition to the health benefits of C-PC, it has many pharmaceutical applications such as anti-inflammatory and antibacterial activity confirmed previously [1, 2, 3]. Although there are many solutions to fight with infections but the problem of infectious diseases is still one of the main problems in the world and drug resistance problems especially in the case of biofilm producing bacteria are the major threats to health [4]. The development of drug resistance can cause to increasing of prescription of antibiotics that can cause fatal toxicities. Therefore the Healthcare Infection Control Practices Advisory Committee (HICPAC) is seeking ways to treat the infections caused by multidrug-resistant bacteria [5]. Today, scientists are trying to use modern medicines to combat diseases, especially in the fight against those microorganisms that are become resistant to several drugs. Therefore, the use of nano-medicines or drugs that transported by nanoparticles are under special attention [6]. The use of nano drugs not only can cause to achieve the appropriate levels of drug to the target tissue but also can prevent the development of drug resistance in many cases due to the higher dose of the drug administrations [7]. Also, the use of natural compounds produced by microorganisms along with nanoparticles due to less intervention in the natural environment has been well regarded [8, 9]. In the current study, the evaluation of antibacterial activity of PHY-ZnONPs was carried out in order to examine its antibiofilm activity against multidrug resistant bacteria.

MATERIALS and METHODS

Material

Zinc acetate dehydrate (≥ 98% $Zn(CH_3COO)_2.\ 2H_2O$) were purchased from Sigma-Aldrich (Australia). Clinical isolates were obtained from Microbial Culture Collection of Iran University of Medical Sciences.

Methods

Limnothrix sp. KO05 was earlier isolated from swamps near the Azerbaijan province in the northwest of Iran [10].

Phycocyanin purification

An axenic culture of this strain was used for phycocyanin isolation and cell extraction was done by sequential freeze & thaw for 3 times. The cell extract was purified by a precise complex procedure. Briefly, the 45% ammonium sulfate was used as a step in purification procedure followed by 12% activated carbon to eliminate undesired proteins plus concentrate the sample. Dialysis was performed against 10 mM PBS at pH 7.5 overnight. Finally, anion exchange chromatography was used to achieve the highest purity. The purity index reported by the measurement of $OD_{620\ nm}/OD_{280\ nm}$ [11, 12].

PHY-ZnONPs synthesis

Purified phycocyanin was added to zinc acetate dehydrate ($Zn[CH_3COO]_2$, $2H_2O$, 0.02M) through constant stirring using a magnetic stirrer. 0.1M NaOH was added to the solution until a pale white solution emerged (color of reaction mixture change to white at this time). After this time, centrifugation was done at 5,500 rpm for 15 min, and pellets were washed with Milli Q water and dried in vacuum oven for 3 h. Dried powder material was kept in an airtight container at room temperature.

MIC and MBC determination

Macrodilution broth method was used to determine MIC and MBC as recommended by CLSI [13]. Briefly, two-fold serial dilutions of phycocyanin, ZnONPs and PHY-ZnONPs from the ranges of 250 µg/ml to 7000 µg/ml were prepared in Mueller-Hinton Broth (MHB). Bacterial strains were added in prepared media to achieve final inoculums of 8 log CFU/ml, kept at 37°C for 24 h. Positive and negative controls were included. The minimum concentration of samples resulted with no visible growth, defined as MIC. A 100-µl aliquot of all tubes with no visible bacterial growth was seeded on MHA plates. Lowest concentration of samples that kills the initial bacterial inoculum is defined as MBC.

Inhibitory effect of PHY-ZnONPs on biofilm formation

Biofilm formation assay was performed by microtitre plate according to the method that S. Dwivedi *et al*. described [14]. Three dilutions of phycocyanin, ZnONPs and PHY-ZnONPs (500 µg/ml, 1000 µg/ml and 1500 µg/ml) prepared in 2 ml MHB. The bacterial strains were added in prepared media and wells were then treated with 0.1% (v/v) crystal violet solution. Subsequently, the absorbance was read in 570 nm. The percentage of biofilm inhibition expressed by the following formula: [1 – ($OD_{570\ nm}$ of samples treated with PHY-ZnONPs / $OD_{570\ nm}$ of non-treated control samples)] [15]. Positive and negative controls were included.

The effect of PHY-ZnONPs on extracellular polysaccharides (EPS) production

The EPS production was evalated according to the method by Khan et al. with minor modification [16]. To this end, 2 ml Mueller-Hinton broth (MHB) medium was inoculated with each bacterial strain to obtain final counts (8 log CFU/ml). Three dilutions of phycocyanin, ZnONPs and PHY-ZnONPs (500, 1000 and 1500 µg/ml) were prepared and added to bacterial suspensions. Bacterial cells were harvested (after 24 hours at 37ºC) by centrifugation at 10,000 rpm for 10 min. EPS was precipitated with 3 volumes of chilled ethanol (95%) and dissolved in 500 µl Mili-Q water. Finally, the EPS concentration was determined by the phenol-sulfuric acid method.

Statistical analysis

Data analysis was expressed as mean ± standard deviations (SD). Values obtained from three independent experiments. P-values less than 0.05 were considered as statistical significance.

RESULTS

Phycocyanin purification

A multi-step procedure, including anion exchange chromatography, was employed to achieve the highest purity of phycocyanin (Fig. 1).

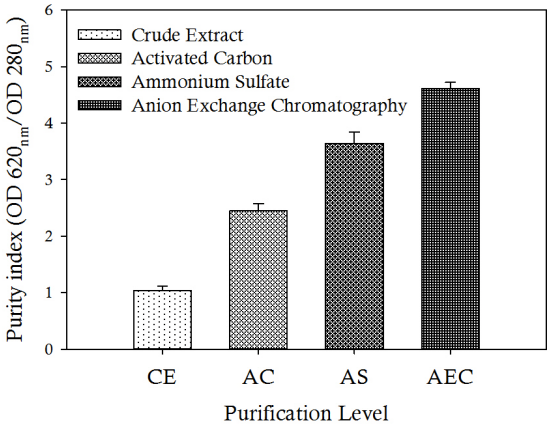

Figure 1. Phycocyanin purification procedure and its related purity index. Crude extract (CE), activated carbon (AC), ammonium sulfate (AS), anion exchange chromatography (AEC).

MIC and MBC determination

The MIC and MBC values of PHY-ZnONPs was estimated and compared with ZnONPs and phycocyanin (Table 1).

Table 1. MIC and MBC values against tested bacterial strains according to CLSI protocol

Strains	ZnONPs		Phycocyanin		PHY-ZnONPs	
	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)
ESBL *E. coli*	4500	4750	5250	6000	2000	2250
MRSA *S. aureus*	5250	5750	6000	6500	2500	2500
ESBL *P. aeruginosa*	5500	5500	6750	7000	2000	2750

MIC defined as minimum inhibitory concentration, MBC defined as minimum bactericidal concentration.

Biofilm assay

The biofilm inhibition appeared to occur with increasing the concentration of PHY-ZnONPs up to 1500 µg/ml. The rate of biofilm inhibition for PHY-ZnONPs concentrations of 500, 1000 and 1500 µg/ml was as 40.94%, 57.82%, 67.83% in ESBL *E. coli*, 38.35%, 51.86%, 65.93% in MRSA *S.*

aureus and 18.94%, 28.56%, 46.08% in ESBL *P. aeruginosa*, which was higher than ZnONPs and phycocyanin (Fig. 2).

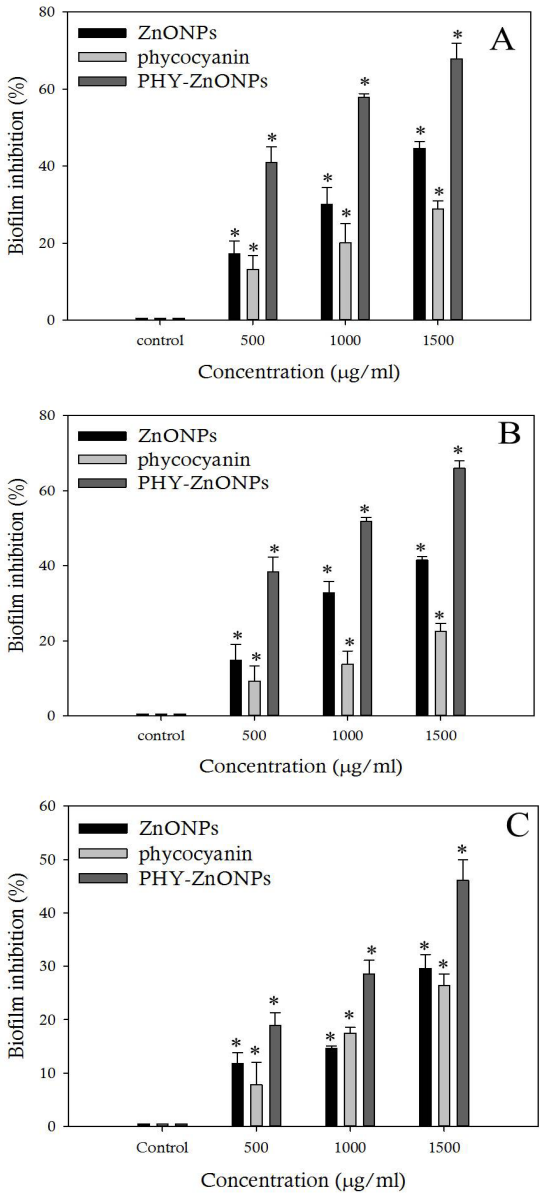

Figure 2. The biofilm inhibition effect of ZnONPs, phycocyanin and PHY-ZnONPs on ESBL E. coli (A), MRSA S. aureus (B) and ESBL P. aeruginosa (C). * P < 0.05; Bars represent the mean of values and error bars represent mean ± SD.

EPS assay

PHY-ZnONPs can inhibit production of EPS up to 72%, 78% and 90% in ESBL *P. aeruginosa*, MRSA *S. aureus* and ESBL *E. coli*, respectively. Overall effect of PHY-ZnONPs on extracellular polysaccharides (EPS) production by tested clinical isolates showed a definite order of inhibitory effect as follow: ESBL *E. coli* > MRSA *S. aureus* > ESBL *P. aeruginosa*, exhibiting higher inhibitory effect than both ZnONPs and phycocyanin (Fig. 3). The results demonstrated that EPS production yield was significantly decreased in the presence of PHY-ZnONPs.

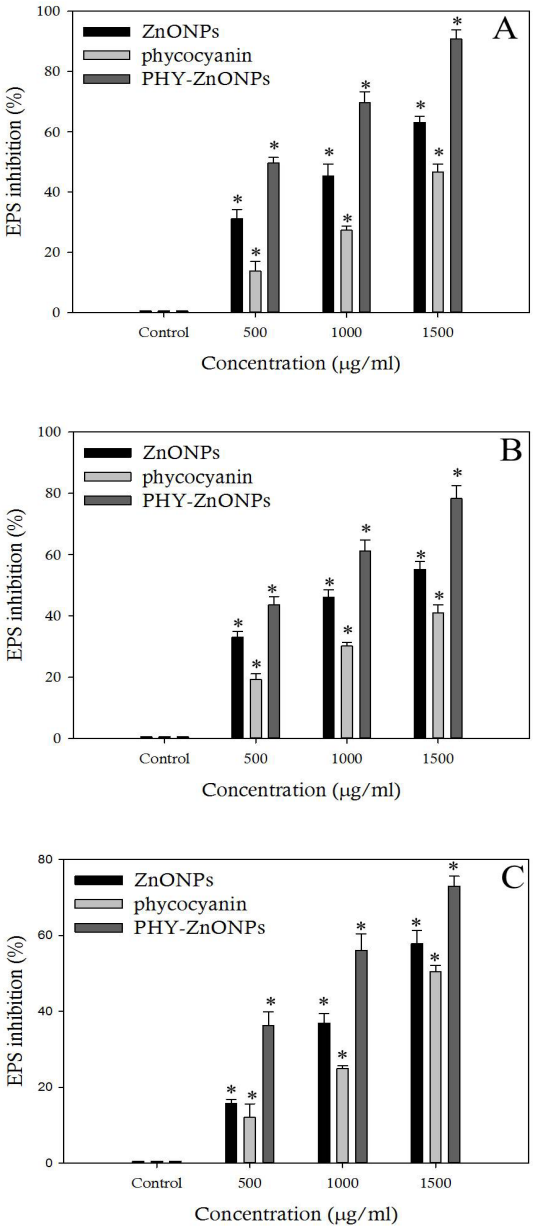

Figure 3. Inhibitory effect of ZnONPs, phycocyanin and PHY-ZnONPs on EPS production by ESBL E. coli (A), MRSA S. aureus (B) and ESBL P. aeruginosa (C). * P < 0.05; Bars represent the mean of values and error bars represent mean±SD.

CONCLUSION

Clinical management of biofilm producing bacteria especially in relation to chronic diseases is a very challenging issue. ESBL producing *P. aeruginosa* and *E. coli* are the most important biofilm producing strains among other clinical isolates. Due to the high prevalence of these strains, the control and novel strategies for elimination of biofilm production is of great importance in public health. Biofilm resident bacteria can decrease their cellular metabolism or membrane permeability. They can also change their gene expression pattern and subsequently become resistant to host immune response system or other body defense systems like phagocytosis [17, 18]. Penetration of nanoparticles into biofilms and the antibacterial effect of the bioactive compounds along with them may have important influence

on the treatment [19]. Phycocyanin is a water soluble protein pigments in cyanobacteria with antibacterial effects. There are several reports of ZnO due to its antibacterial activity, which is attributed to the generation of reactive oxygen species (ROS) on the surface of this oxide [20]. The benefit of using these inorganic oxides as antimicrobial agents is that they comprise mineral elements considered essential to the humans when applied in a small amount. The ability of PHY-ZnONPs, generated from bioactive and biocompatible phycocyanin pigment can have a particular impact on its use in the medical sectors. Surface features, small size and the potency of PHY-ZnONPs to penetrate into the biological barriers is a way one would use as an anti-biofilm and antimicrobial agent. Bhande et al. examined the synergistic effects of zinc oxide nanoparticles with β-lactam antibiotics against some clinically isolated extended spectrum β-lactamase (ESBL) producer strains including *E. coli*, *K. pneumoniae*, *S. paucimobilis*, and *P. aeruginosa* [21]. They found that the utilization of ZnONPs can potentiate the bactericidal activity of β-lactam antibiotics against all isolates tested. Another study revealed that the antibacterial activity of ciprofloxacin against *S. aureus* and *E. coli* is increased in the presence of ZnO nanoparticles in both test strains [22]. Staining with crystal violet showed that the ability of biofilm formation was decreased with increasing PHY-ZnONPs concentration in all three strains and the maximum inhibition of biofilm formation was achieved at an inhibitor concentration of 1500 µg/ml. The maximum effect of PHY-ZnONPs on the inhibition of biofilm formation was attained in ESBL *E. coli* (67.83%), MRSA *S. aureus* (65.93%) and ESBL *P. aeruginosa* (46.08%), respectively. The bactericidal and biofilm inhibition ability of ZnO-NPs (in a concentration dependent manner) was shown in a study that conducted by S. Dwivedi et al [14]. The results from this present study also indicated that the biofilm producing Gram-positive bacteria are more sensitive to PHY-ZnONPs compared to the Gram-negative bacteria. Earlier, K. Alia et al. conducted a study in which ZnO nanoparticles (ZnONPs) were synthesized through exploitation of the capping potential of *Aloe barbadensis Miller* (*A. vera*) leaf extract (ALE). The study revealed a significant inhibitory effect for bacterial growth (ESBL *E. coli and MRSA),* exopolysaccharides and biofilm formation of ALE-ZnONPs, and the results were consistent with our findings [8]. In conclusion, the combination of phycocyanin (as a bioactive compound) and ZnONPs with significant biocidal features seems to be a good candidate to act against the infections caused by the Gram-positive/ Gram-negative bacteria especially ESBL-producing clinical isolates. By our knowledge, this the first report and results obtained here suggest that the PHY-ZnONPs with cumulative therapeutic features can be used in combination therapy and provide a useful insight for the development of novel antimicrobial agents with lower toxicity.

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