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ORIGINAL RESEARCH ARTICLE

# Enhancement of anticancer effect of azurin using polymeric nanoparticles

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## ABSTRACT

According to the World Health Organization (WHO), breast cancer is among the most common cancers worldwide. Most of the anticancer agents have been showing a variety of side effects. Recently, bacterial proteins have been investigated as promising anticancer agents. Azurin is a bacterial cupredoxin protein secreted from *Pseudomonas aeruginosa* and has been reported as a potent multi-targeting anticancer agent, which makes it an appropriate candidate for drug delivery. Azurin may be delivered to cancer cells using different carriers like polymeric micro and nanoparticles. In the present study, azurin was extracted from the bacterial host and loaded into chitosan particles. Then its effect on MCF-7 cell line was investigated. Chitosan-azurin particles were made using the ion gelation method. Results showed that chitosan-azurin particles are about 200 nm, and the loading of the protein in particles did not affect its integrity. The MTT assay showed a significant reduction in cell viability in azurin and chitosan-azurin-treated cells. The toxicity level after 5 days was 63.78% and 82.53% for free azurin and chitosan-azurin-treated cells, respectively. It seems using an appropriate carrier system for anticancer proteins like azurin is a promising tool for developing low side effect anticancer agents.

***Keywords:*** Chitosan Nanoparticles; Azurin; Anticancer Activity; Breast Cancer; Bacterial Protein

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## 1. Introduction

Cancer is the overproduction and malfunction of the body's cells that could invade different tissues. Cells become cancerous because of the buildup of defects or mutations of their DNA. Inherited genetic defects, infections, environmental elements like air pollutants, and unhealthy lifestyle alternatives, along with smoking and heavy alcohol use, also can harm DNA and result in cancer. According to the World Health Organization (WHO), cancer is a leading cause of death worldwide, accounting for an estimated 9.6 million deaths in 2018. The most common cancers are lung (2.09 million cases), breast (2.09 million cases), colorectal (1.80 million cases), prostate (1.28 million cases), skin cancer (1.04 million cases), and stomach (1.03 million cases). Today, the main challenge in cancer therapy is the secondary effects caused by standard treatments like ionizing radiation, chemotherapy, immunotherapy, and tumor cell resistance. Therefore, new therapeutics and novel strategies for drug delivery with fewer side effects are essential. It has been proved that some bacterial-purified products, especially those that target and

specifically kill cancerous cells, can be used as anticancer agents[1].

*Pseudomonas aeruginosa* is a gram-negative opportunistic pathogenic bacterium. Depending on its culture medium, it secrets different pigments, such as pyocyanin, pyoverdine, and pyorubin[2]. Azurin (Az) is a member of the cupredoxin protein family, secreted by several bacteria, especially *P. aeruginosa*. Az is a small blue-colored protein that consists of 128 amino acids with a molecular weight of about 14 kDa. It comprises one α-helix and two β-sheets, creating a β-barrel motif[3]. Az is effective in the electron transfer cycle of the bacterial respiration system. This small protein is one of the bacterial derivatives efficient as an anticancer factor against different cancerous cell lines like breast cancer cell line MCF-7; also, its antiparasitic and anti-HIV properties have been proven[4].

Az has been used in various kinds of research because of its anticancer abilities. The first intention for investigating Az anticancer properties on MCF-7 cell line showed it can block proliferation and induce apoptosis significantly in vitro and in vivo[5]. Az can enter human melanoma (UI-SO-MEI-2) in nude mice and make significant regression in this cancerous cell line[6]. The record has shown it also induces apoptosis in human osteosarcoma U2OS[7]. Az and its peptide, Laz, effectively treat leukemia K562 and HL60 cells[8]. Also, Laz was useful in attacking brain tumors and disrupting the entry barrier of this highly protected organ[9]. Azurin's strong anticancer effect was exhibited in the human colon carcinoma cell line (HCT116)[10] and simultaneously with anticancer drugs in oral squamous carcinoma cells (YD-9)[11]. Lastly, the p28 amino acid of Az was quite effective in treating advanced solid tumors at a clinical trial level[12].

It seems that Az forms a compound with p53 and raises its intracellular level, leading the increased p53 to provoke apoptosis in cells through amplifying Bax formation and releasing mitochondrial cytochrome c in the cytosol[13]. Az is reportedly the first bacterial protein to make a compound with the protein p53. Az easily enters human cancerous cells, whereas it is insufficient in entering normal cells. The p28 amino acid of this protein is the main factor for preferential entry to cancerous cells[14].

Chitosan (CS) is a polymer obtained from the deacetylation of chitin. It is one of the popular drug carriers because of its unique characteristics, such as biocompatibility, biodegradability and antibacterial properties. This polymer also has a stunning affinity to some proteins[15]. Based on previous studies on azurin's anticancer and apoptosis induction proficiency, it is predictable to use nanotechnology targeting and delivery systems to eliminate cancerous cells using Az. By using convenient nanoparticles (NPs) for carrying Az, we might be able to terminate cancer cells effectively. Amongst different nanocarriers for drug delivery, polymeric NPs are the most efficient[16].

Nano drug delivery system offers many advantages like increasing absorption, reducing the dose and side effects. It also increases the drug concentration at the treatment site[17,18]. CS was the subject of many studies, used to deliver gene, protein and anticancer chemical drugs. In 1994 CS NPs were used for delivering 5-fluorouracil (an anticancer drug) for the first time[19], later it was used for delivering other anticancer agents such as doxorubicin[20] and paclitaxel[21]. This polymer was efficient in delivering a variety of proteins in treating cancers. It was used to encapsulate anti-β-catenin siRNA against colon cancer cells to reduce β-catenin and thereby minimize tumor progression[22]. Furthermore, recombinant neutrophil-activating protein derived from helicobacter pylori, encapsulated with CS, effectively treated breast cancer[23]. Moreover, in a study, curcumin was encapsulated in alginate-CS-pluronic composite NPs against HELA cells, where inhibition was more efficient in encapsulated curcumin than the free curcumin[24].

CS NPs can be prepared in different ways, such as emulsion cross-linking, reverse micellar method, ionic gelation[25], precipitation[26], microfluidics[27] and spray drying[28]. In the present work, CS-Az NPs were prepared and characterized. Then particles' toxic effect on the breast cancer cell line was investigated.

## 2. Material and methods

### 2.1 Materials

*P. aeruginosa* ATCC 27853 was prepared by the Microorganism's Bank of I.A.U Science and Research Branch University, Iran. CS was purchased from Sigma Aldrich, Lysogenia Broth (LB) medium, ammonium sulfate, Coomassie brilliant blue, penta-sodium triphosphate, phosphoric acid, sodium dodecyl sulfate (SDS), MTT, trypan blue, and 2-mercaptoethanol were all ordered from Merck. MCF-7 cell line, DMEM, FBS, and phosphate buffered saline (PBS) were purchased from Gibco.

### 2.2 Azrin extraction and characterization

*P. aeruginosa* was cultured in LB medium at 37 °C for 21 h[29]. Copper sulfate (1 µg/mL–5 µg/mL) and potassium nitrate (5 µg/L–20 µg/L) were introduced both together and separately to the medium. After centrifugation at 13,200 g for 15–20 min, the bacterial suspension's pellet was suspended in 0.02 M, pH 7.0 potassium phosphate buffer, sonicated, and then centrifuged at 10,000 g for 20 min. The resulting supernatant was collected, treated with 45% ammonium sulfate, and left overnight at 4 °C. Subsequent centrifugation at 20,000 g and 23,000 g concentrated the supernatant containing Az, dissolved in 0.02 M, pH 7.0 potassium phosphate buffer[2,30].

The molecular weight of the extracted Az was determined using SDS-PAGE following established procedures[2]. Gel preparation used deionized water, tris, ammonium persulfate, SDS, acrylamide, and TEMED. Extracted Az and bromophenol blue were applied to the gel and subjected to electrophoresis at 100 V for 100 min. Coomassie brilliant blue staining, destaining, and lamp-based examination followed[2,31,32].

Protein concentration was evaluated using the Bradford assay. The Bradford reagent was formulated by mixing 100% ethanol with Coomassie brilliant blue G-250, adding 85% phosphoric acid, and adjusting the volume with DI water. This mixture was filtered, and a standard curve was created using serial dilutions of 1 mg/mL BSA in PBS buffer. Various amounts of extracted Az were mixed with the Bradford reagent incubated in darkness for 5 min, and their absorbance was measured at 595 nm using a spectrophotometer.

### 2.3 CS-Az nanoparticle synthesis

The ion gelation technique was used for the preparation of CS-Az NPs. CS solution (solution 1) was prepared by adding 3% w/v of medium molecular weight CS to DI containing 1% w/v acetic acid under stir mood. The pH of solution 1 was adjusted to 5.5, and a 0.45 µm filter filtered the solution to discard unsolved components. Another solution (solution 2) that contains 3% w/v of penta-sodium triphosphate was prepared, and its pH was adjusted to 9.5[33,34]. Az was added to solution 2 in the desired concentration. 1 mL of solution 2 was added dropwise to 3 mL of solution 1 under stirring in an ice bucket at 1,000 rpm for 30 min. The slight turbidity of the final solution was considered as particle formation.

### 2.4 CS-Az particles characterization

#### 2.4.1 SEM and SDS-PAGE

CS-Az particles morphology, size and dispersity were investigated by particle size analyzer (Malvern, U.K.) and scanning electron microscopy (LEO 440i, U.K.)[35]. The particles were used without any filtration and dilution for size analysis.

#### 2.4.2 Encapsulation efficiency

The amount of encapsulated Az in CS NPs was measured by spectrophotometry (PG Instruments). Prepared NPs were centrifuged at 10,000 g for 20 min. After mixing crude with Coomassie brilliant blue reagent, it was left for 5 min and measured at 595 nm wavelength, and efficiency was calculated due to the standard curve.

#### 2.4.3 Protein integrity

SDS-PAG assay (Bio-Rad) was used to ensure the encapsulation of Az protein. Prepared CS-Az NPs was centrifuged at 10,000 g for 20 min, and the crude mixed with bromophenol was loaded in lanes with 100 W for 100 min for profiling integrity.

### 2.5 CS-Az particles anticancer effect assay

#### 2.5.1 MCF-7 cell culturing

MCF-7 cell line was cultured in the medium

which contains commercial DMEM with 10% FBS and 1% penicillin/streptomycin and incubated at 37 °C under 5% $CO_2$[13,36,37]. After 24 h, cells were counted using a hemocytometer and trypan blue and cell numbers were calculated by Equation (1). Here DF is the dilution factor.

$$\text{Cell number} = \text{number of cell in 1 mm}^2 \times 10{,}000 \times \text{DF} \quad (1)$$

### 2.5.2 MTT assay

MCF-7 cells were seeded in 96-well microplates at a density of 104 cells and incubated overnight at 37 °C in 5% $CO_2$[38]. Then the supernatant was discarded, and 100 µL of samples, i.e., extracted Az and CS-Az NPs, were added to each well. After 24 h and 72 h, the solution was replaced with 100 µL of MTT reagent and incubated for 3 h at 37 °C. Finally, 100 µL of DMSO was added to each well for dissolving formazan crystals, and samples OD were measured at 570 nm by a microplate reader (ELx808, Bio Tek, U.S.). Untreated cells were considered as control during the MTT assay[14,39].

## 3. Results

### 3.1 Extracted protein analysis

For Az extraction, the optical density of grown bacteria (**Figure 1**) was measured at 265 nm and adjusted according to clinical and laboratory standards 2018 (1.5 × 108 CFU/mL).

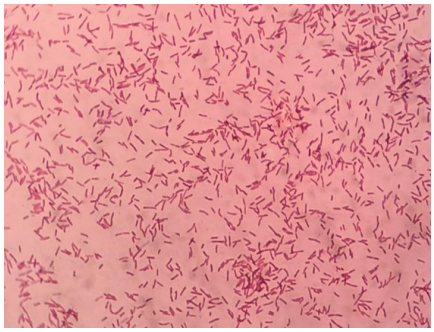

**Figure 1.** Gram staining assay, the red color of bacteria and rod shape morphology unique gram-negative *P. aeruginosa.*

All samples, i.e., the Az extracted with sonication with different doses of $CuSO_4$ and $KNO_3$ in their culture, were all characterized using SDS-PAGE where results showed extracted substances contain 14 kD Az protein (**Figure 2**). The Az was further purified with dialysis in its buffer to discard extra salt and smaller proteins.

Extracted protein concentrations were assayed using Bradford assay (**Table 1**). As shown in the table concentration of extracted Az by sonication in the absence of $CuSO_4$, and $KNO_3$ was about 10.73 µg/mL. Az concentration in the simultaneous addition of $CuSO_4$ and $KNO_3$ in the culture medium was 2.35 µg/mL. Besides, in addition to $KNO_3$ and $CuSO_4$ separately, the concentration was about 6.97 and 5.24 µg/mL, respectively.

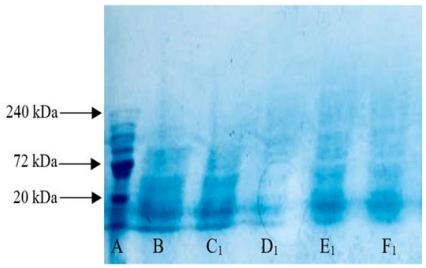


**Figure 2.** Molecular weight determination of extracted protein. The protein that extracted by sonication, showing 14 kDa bond of Az. **(A)** protein ladder.; **(B)** and **($C_1$)** Az in absence of $CuSO_4$ and $KNO_3$ in the culture medium; **($D_1$)** Az in presence of $CuSO_4$ and $KNO_3$ in the culture medium; **($E_1$)** Az in presence of $KNO_3$ in the culture medium; **($F_1$)** Az in presence of $CuSO_4$ in the culture medium.

**Table 1.** Concentration and optical density of extracted proteins at 595 nm.

| Samples | B | $C_1$ | $D_1$ | $E_1$ | $F_1$ |
|---|---|---|---|---|---|
| OD | 0.17 | 0.18 | 0.03 | 0.11 | 0.08 |
| Concentration (µg/mL) | 10.44 | 11.02 | 2.35 | 6.97 | 5.24 |

### 3.2 CS-Az NPs characterization

As mentioned, Az was encapsulated in CS using the ionic gelation method. The particle size and zeta potential were assayed using SEM and DLS (**Figure 3**). SEM result (**Figure 3a**) showed that the particles have a spherical shape and their size is about 200 nm. CS-Az particles' hydrodynamic size, PDI, and zeta potential were about 265 nm, 0.26 and +27 mV, respectively (**Figure 3b,c**).

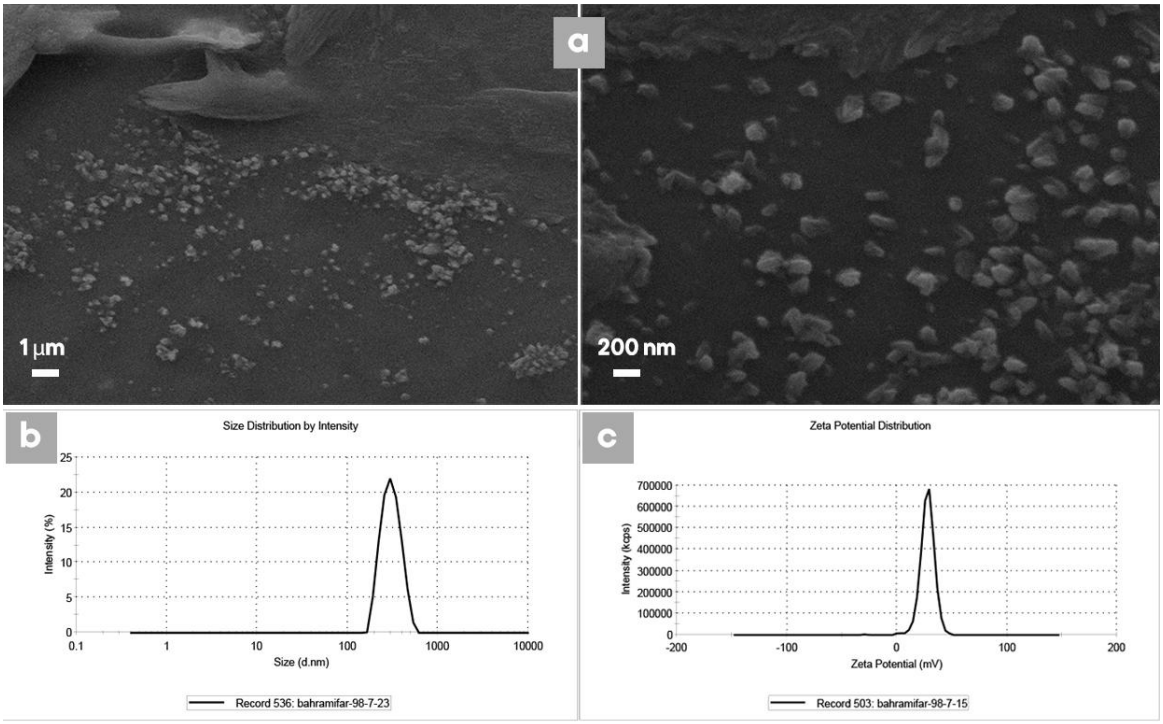


**Figure 3. (a)** SEM of CS-Az NPs in two different scales; **(b)** NPs hydrodynamic size, PDI; and **(c)** zeta potential.

## 3.3 Encapsulated protein analysis

### 3.3.1 Concentration and integrity

Protein concentration was calculated by its absorbance at 595 nm using the standard curve. The results showed 1.48 µg Az was loaded in each mL of NPs. The integrity of the protein that led in NPs was assayed by SDS-PAGE. As detailed in **Figure 4**, the free and encapsulated Az band was seen in an acrylamide gel.

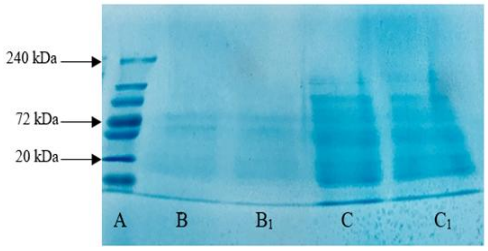


**Figure 4.** Analysis of integrity and presence of Az in NPs profiled by SDS-PAGE. **(A)** protein ladder; **(B)** CS-Az NPs; **(C)** free Az; **($B_1$)** and **($C_1$)** are repeat of previous specimens respectively.

### 3.3.2 Anticancer efficiency

MTT assay was used to monitor the anticancer effect of CS-Az NPs on the MCF-7 cell line. MCF-7 cells were treated with free Az and CS-Az NPs in 1, 3 and 5 days, considering untreated cells as control. In comparison, after 24 h, free Az had more inhibition effect than NPs. Further outcomes from MTT assay after 72 h define CS-Az NPs had better toxic efficiency than free Az. Obtained information from the free protein treatment clarifies 74% toxicity in one day and 67% after 5 days. For CS-Az NPs, it was 63% and 82% after one day and five days, respectively. Mann-Whitney U test showed during 5 days, the efficiency of CS-Az NPs was increased significantly ($P$ value $< 0.05$) while the free Az partially lost its efficiency (**Figure 5**).

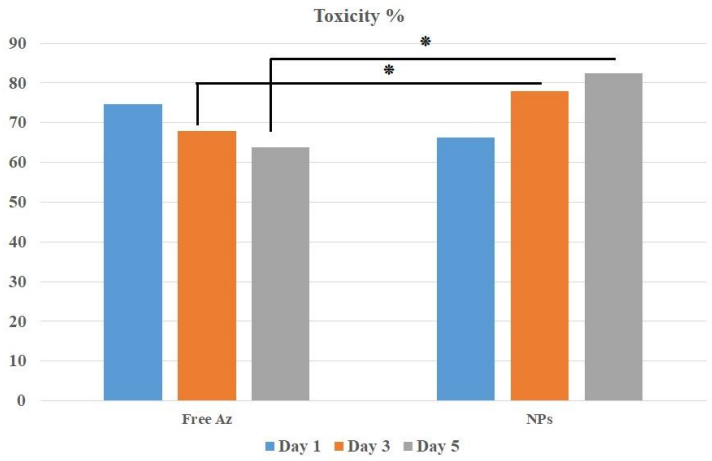


**Figure 5.** Toxicity (%) of free Az, and CS-Az NPs on MCF-7 cells.
* $p < 0.05$ using Mann-Whitney U test.

## 4. Discussion

The present study evaluated the anticancer effect of Az combined with CS nanocarriers on the MCF-7 cell line. Cupredoxin protein Az was extracted from *P. aeruginosa* (ATCC 27853), while the production of this protein by this microorganism was promised[2,29]. Az was extracted using sonication. Visualized bonds confirmed the presence of 14 kD Az[2,30,31] in SDS-PAGE and the concentration was calculated using a spectrophotometer. Ramachandran reported that $CuSO_4$ and $KNO_3$ in a culture medium can increase the amount of Az[2,30]. At the same time, this wasn't clarified in this intention, and another research by Sutherland declared the amount of Az increased in a small range by adding $CuSO_4$ to the medium yet failed to rise more by adding more copper (up to 10–17 µg/mL)[40]. Still, the comparison between SDS-PAGE bonds shows that they might be effective in the purity of producing Az and reducing the unnecessary secreted proteins.

Amongst different bacterial derivatives, there was much attention on Az derived from *P. aeruginosa* because of its anticancer details and multi-targeting ability. It can enter cancerous cells without any toxic effect on normal cells. Its anticancer effect and the inhibitory property have been proved on various cancerous cell lines such as melanoma[6], bone[7], leukemia[8], malignant brain tumor[9], and in vivo apoptosis properties of Az on MCF-7 cell line were reported by Punj *et al.*[5].

In this study, CS-Az nanoparticle was produced using the ion gelation method. CS polymer was used considering its remarkable characteristics and beneficial advantages in drug delivery, like reducing the possibility of embolism and easy injection properties[41]. This polymer has good stability and less toxicity, making it a good choice for making NPs[42]. CS can improve the stability of NPs in biological solutions, blood circulation, and while entering tissues and cells[43,44]. Various reagents and conditions, such as heat, organic compounds and pH changes, can cause protein denaturation. The ion gelation method is one of the easiest and most common ways of drug delivery; it does not require heat and organic solvent, which makes it a suitable technique for encapsulating proteins[26]. The process accomplishes through inter and intramolecular cross-linkage, cooperating with anionic molecules. The size characterization and zeta potential of CS-Az NPs were measured by dynamic light scattering. The assessment showed hydrodynamic size, PDI and zeta potential were about 265 nm, 0.26 mV and 27 mV, respectively, and NPs were stable[25]. CS NPs were used in combination with the Az gene and mammaglobin agencies. 98.8% of these NPs had a size of 111.7 nm. The size difference of NPs can be related to production procedures or differences in encapsulated drugs[26].

Based on results from the MTT assay, free Az and CS-Az NPs were both functional and showed extraordinary anticancer effects. CS-Az NPs showed decisive inhibitory action over time in comparison to free protein. This change demonstrates that encapsulation of Az with CS has made it more stable, so protein structure remains sustained during the time and as exposed to the environment. Based on the results of Punj *et al.*[5], after 72 h of treating MCF-7 cells with 53 µM pure Az, cell viability was 29%. In another study, Osman *et al.*[31] reported near 34% cell viability for MCF-7 cells treated with Az 5 µg/mL. Comparing these results, CS-Az NPs showed better inhibition results. Toxicity results also define 74%, and 66% toxicity after treatment on the first day with free and NPs loaded Az, respectively. Still, after five days, the toxicity of CS-Az NPs increased to 82%, whereas for free Az, it decreased to 63%. High toxicity is critical in producing anticancer drugs; although toxicity results in this research were satisfying, it takes much effort to put these valuable results into use at clinical levels. Encapsulating Az with CS can protect it from the attack of the immune system and can help to pass through main drug-resistant mechanisms[1,45,46]; in addition, it helps Az to remain in the targeted areas longer than free protein and extends drug releasing time. One of the problems we face in common cancer treatments is that in treatments like chemotherapy, there is no border in attacking healthy and cancerous cells, but by using nanocarriers, we can reduce the harmful attacks on healthy cells. Also, the need for more injections makes financial difficulties for patients while slow drug re-

lease of CS-Az NPs can decrease numerous injections, so considering the ability of CS-Az NPs, this can be an inflection point in breast cancer treatment with fewer side effects and lower price.

## 5. Conclusion

Overall properties of Az in former studies prove its apoptotic qualification on MCF-7 human breast adenocarcinoma cell line. Based on this and CS characteristics in drug delivery, we analyzed the anticancer efficiency of CS-Az NPs, successfully synthesized by ionic gelation method on the MCF-7 cell line. Results from the MTT assay imply significant inhibition of cells treated with our NPs compared with ones treated with free Az. As the size of NPs can be effective in their capability to enter cells, different studies can focus on CS-Az NPs with different sizes and dosages. Lastly, regarding azurin's potential to inhibit diverse cancers, CS-Az NPs can be tested on other cell lines. Also, further investigations can be done on CS-Az NPs in vivo levels.

## Author contributions

Conceptualization, HB and PM; methodology, SB; software, HB; validation, SB, HB and PM; formal analysis, SB; investigation, SB; resources, SB; data curation, HB; writing—original draft preparation, SB; writing—review and editing, HB; visualization, PM; supervision, HB; project administration, PM; funding acquisition, SB. All authors have read and agreed to the published version of the manuscript.

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## Conflict of interest

The authors declare no conflict of interest.

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