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The Role of Biofilm to Isolate the *Serratia Marcescens* in Its Resistance to Antibiotics and Study the Effect of Gold Nanoparticles in Inhibiting this Resistance

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Abstract

The biofilm of *Serratia marcescens* are synthesized when it is linking to the surface within the aqueous environment. Some strains of *S. marcescens* are known to be problematic to the nosocomial infections because of its resistances to the drug. This paper aims to evaluate the antibiofilm efficacy of gold nanoparticles toward the clinical isolates of *Serratia marcescens* isolates. The anti-biofilm activity of AuNPs with subMIC concentration of (18.71) µg/ml was investigated using the microtiter plate method. The results exhibited that gold nanoparticles reduced the ability of *Serratia marcescens* to produce the biofilm by 51.6 – 80.4%.

Keywords: Biofilm, Antibiofilm**,** Gold nanoparticles, *Serratia marcescens*

Introduction

Serratia marcescens, is one of the gram negative microbes, plays critical role as opportunistic human pathogens. Its growth is causing the severe nosocomial infection [1]. However, *S. marcescens* is a critical microbe leads to occur the epidemic and endemic nosocomial infections because its resistance to different anti-microbial agents and maybe lead for severing complications, like bacteremia of *Serratia*. Also, *S. marcescens* is implicated in the range of the ocular infection like keratoconjunctivitis, endophthalmitis, keratitis, and conjunctivitis. Environmentally, this bacterium also causes Cucurbit yellow vine diseases in cucurbit crop like *Cucurbita pepo* (pumpkin) and *Cucurbita maxima* (squash), resulting in a heavy economic loss [2][3]. The biofilm is a complex mixture of the microbe which is predominantly bonded to the hard surface, it is often enclosed using a thick polysaccharides layer which make it resistant to the antibiotic and therefore very hard for eliminating[4]. It is now well documented that the biofilm is exceptionally difficult for eradicating and is often resistant to the systemic antibiotic therapy and removal of infected devices become necessary [5]. The biofilms is progressively recognized as a main growth form in life cycles of the bacterium in nature. It is a matrix-encased, high-density population linked to the surface. The biofilm is more resistant to the eco-stress (for example, oxidative and nutritional stress) and to the host-assisted response (for example, the complement protein and the phagocyte). Due to the increased resistance, the biofilm causes a problem in medical and industrial settings. Thus, it is increasingly significant to understand how the biofilm detach and form, involving external and internal factors which control on these processes [6]. However, some strains of *S. marcescens* makes problems to nosocomial infections because of their resistance to the drug [7]. The capability of *S. marcescens* for form and adhere biofilms was indicated on abiotic and biotic surfaces, plays the significant

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role in producing nosocomial infections [8]. Bacteria in a biofilm that survive antibiotic exposure can cause recurrence of infection once antibiotic treatment is stopped, the *S. marcescens* biofilm cells isolates showed resistance patterns to different class of antibiotics, the presence of biofilms decrease penetration of antibiotics, that leads to develop the resistance of drugs [9]. Currently, most of the employed antibiotics are becoming in-efficient toward the biofilm-related multi-drug resistant microbes. Thus, it has become important to seek for alternative methods for mitigating biofilms related problems [10]. Recently, several inorganic and organic nanoparticles (NPs) were explored for inhibition of biofilms [11]. The nanoparticle exhibits anti-microbial characteristics because of its unique properties, like particular shapes, ultrafine sizes, finding biochemical moieties on its surfaces (surfaces coatings or the functional group), and high ratio of surface area to the volume. However, these parameters define the adverse or therapeutic impacts of NPs [12]. The AuNPs exhibit bacteriostatic or bactericidal activity to microbial cells, AuNPs were have strong anti-microbial efficacies of various concentrations of the solutions involving gold nanoparticles demonstrated that Gram negative and positive multi-drug resistant (MDR) human pathogen bacteria were inhibited [13].

Materials and Methods

Preparation of gold nanoparticles solution

The approach of [14] has been adopted for preparing the colloidal gold nanoparticles at the concentration 74.87 μg/ml and concentrations have been obtained (56.15, 37.43, 18.71 and 9.35) μg/ml as follow: Firstly, the solution of HAuCl4.4H2O with the concentration 0.49 mol/ has been achieved using dissolution 605.0 mg of HAuCl4.4H2O in three milliliters 10% HCl. Next, the diluted 0.2 mM of the solution of HAuCl4.4H2O has been prepared by adding 40 μL (19.6 μmol) of solution of HAuCl4.4H2O in 100 ml DW as for producing the solution A. Secondly, 559 mg Tri-sodium Citrate has been dissolved with 50 ml DW for making the solution B. The solution concentration has been controlled at 38.8 mmol/L. Solution A has been obtained to a rolling boil at 150 °C with stirring vigorously as for getting the homogenous size of colloidal gold nanoparticles. However, 10 mL of 38.8 mmol/L of sodium citrate has been added quickly on the vortex. The color of mixture changed from pale yellow to red color. Stirring and boiling were continued for 10 minutes. then, the heating was stopped and the stirring was continued for a new time of 15 minutes. After cooling the solution to the room temperature, it has been filtered by the membrane filter (0.8 μm). The achieved mixture has been stored at 4 ºC and measured by UV-Visible at wavelengths from 400 to 800 nm. Also, the analyses of AFM and TEM were achieved.

Isolation and identification of *Serratia marcescens*

A total of 80 clinical specimens collected from wounds, burns, sputum and urinary tract infections. These specimens were obtained from patients referring Ramadi Educational Hospital.

Specimens have been cultured on MacConkey and agar blood agar then incubated for 24 hr at 37 °C. After the bacterial growth took place, developed colonies have been identified with Vitek2 compact system (bioMérieux, France) by the card of GN (Gram Negative) depending to instructions the manufacturer.

Calculation of MICs of gold NPs

MIC (Minimum inhibitory concentration) has been defined for completing growth inhibition of bacteria without turbidity using AuNPs action at lowest concentrations.

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Calculation of MIC of gold NPs toward *Serratia marcescens* followed from CLSI (Clinical and Laboratory Standards Institute) guidelines, 2016 [15].However, the MIC of AuNPs has been determined by microdilution broth method. Briefly, serial concentrations of AuNPs including (9.35, 18.71, 37.43, 56.15, and 74.87, µg/ml) were prepared with tryptic soy broth. However, the lowest concentrations inhibit the bacterial growth of considered MIC. The minimum inhibitory concentration has been calculated using optical density parameters of the growth of bacteria at 630 nm after 24 hours of the incubation. Table 1 clearly exhibits a significant inhibition of growth of *Serratia marcescens* by 74. 87,56.15, 37.43, 18.71and 9.35 µg/ml of AuNPs. 18.71 µg/ml of AuNPs considered minimum inhibitory concentration (MIC).

Tabie 1 significant inhibition of *Serratia marcescens*

AuNPs concentration (µg/ml)	OD630
74.87	0.022
56.15	0.046
37.43	0.075
18.71	0.194
9.35	0.296
Negative Control	0.025

Biofilm formation

The capability of *Serratia marcescens* to form biofilms by microtiter plate method described by [16]. In brief; an aliquot (20 µl) of bacterial suspension obtained from an overnight culture (comparable to McFarland standard No. 0.5) was used to inoculate microtiter wells containing 180 µl of Tryptone soya broth. Control wells contained 200 µl of sterile Tryptone soya broth. The microtiter plate was covered with its lid during incubation at 37°C for 24 hr. Un attached bacterial cells have been eliminated through wash wells three times by phosphate buffered saline (pH 7.2); thereafter, dishes have been dried at the room temperature. However, the volume (20 µl) of methanol has been added to wells for 15 min; afterward, dishes have been dried at the room temperature. An amount 200 µl crystal violet (0.1%) has been added to the wells for 15 minutes. After that, the solution of crystal violet was removed and washing three times with phosphate buffered saline (pH 7.2) for removing the unbounded dye. Drying the wells was accomplished at room temperature for 15 minutes. About 200 microliters of the glacial acetic acid (33%) has been added and the absorbance of all wells has been calculated at 630 nm by the microplate reader.

Effect of gold nanoparticles on formation of Biofilm

The described approach by [17] was employed to estimate biofilm formation. In brief; An overnight bacterial culture (in trypton soya broth) was adjusted to be compatible to McFarland standard No. 0.5. Tryptone soya broth that contain sub-MIC of GNPs was inoculated with previously prepared bacterial suspension and incubated at 37°C for 24 h. An amount of 200 µl of the culture, prepared in step a, was transferred into polystyrene microtiter plate well in triplicate in the vertical rows of the plate for each isolate served as control. A volume of 200 µl of culture that containing the sub-MIC concentration of the GNPs was transferred into another three wells. All dishes have been incubated at 37°C for 24 hours. Then, the formation of biofilm protocol was followed as reported earlier(Fig.1). Percentages of inhibition of biofilm have been calculated following the equation:

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%of inhibition of biofilms formation=1–(O.D of treatments/O.D of controls)×100 …(1)

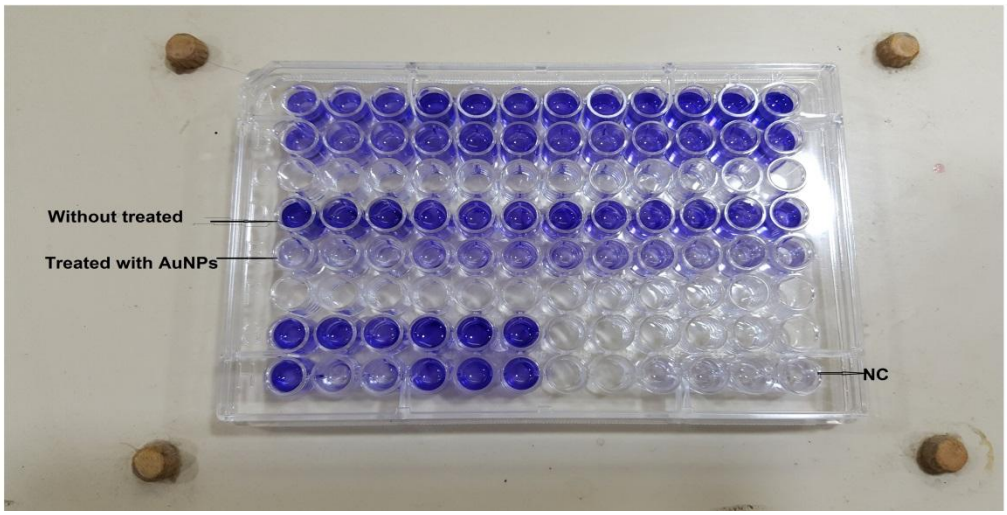

Figure 1. Anti-biofilm activities of Au-NPs against *Serratia marcescens* by micro-titer plate.

Results and Discussion

Identification of *Serratia marcescens* isolates

Out of 80 clinical specimens, 30 isolates were found to be *Serratia marcescens*; The microscopic examination showed Gram negative rods and characterized as nitrate tests are positive, Catalase production (24h) are positive , indole and oxidase-negative, citrate test positive for citrate utilization, and that appeared commonly smooth colonies and sometimes mucoid colonies on the solid medium, with the color ranged from the red color to pale yellow(Fig.2).

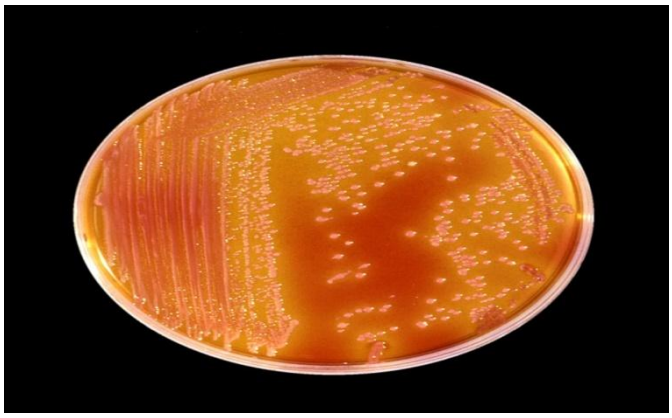

Figure 2. The culture appearance of *Serratia marcescens* colonies on MacConkey agar

Antibiofilm Activity of AuNPs

Biofilms related infections are commonly chronic and recurrent. It's commonly related with failure of treatment due to the higher levels of the resistance of drug. The biofilm can be visually inspected upon the medical device whereas the removing from the body of patients and must be cultured for knowing organism kind colonized so that early onset of treatment can be initiated[18]. The eradication of biofilm and

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inhibition of formation, in addition to the production of other virulence agents using bio-synthesized F-AuNPs, have been generally identified in levels of subMIC. In attempts for selectiing lower pressures for the resistance, targeting the formation of biofilm and expression of genes of other significant virulence agents are considered to be methods with the most potential, and which are generally included in nano-technology applications [19].The present study showed that after 24 hr of treatment with AuNPs nanoparticles inhibited the biofilm formation was observed at sample N7 (80.4%) the result showed that AuNPs were highly effective inhibited the biofilm formation, while other specimens revealed moderate inhibited the biofilm formation ranging between (51.6% - 72.5%) as shown in (Table 2).

Table 2 :Inhibition effect of Au-NPs on *Serratia marcescens* biofilm formation

Isolate code	OD_{630}		T test	Biofilm inhibition(%)
	Control	With AuNPs		
N1	0.417	0.202	2.00E-08	51.6
N2	0.276	0.11	4.80E-05	60.2
N3	0.453	0.19	1.90E-05	58.1
N4	0.241	0.105	7.80E-09	56.5
N5	0.407	0.197	6.60E-07	51.6
N6	0.322	0.122	2.70E-08	62.2
N7	0.464	0.091	2.40E-09	80.4
N8	0.267	0.11	5.40E-05	58.9
N9	0.276	0.11	4.80E-05	60.2
N10	0.271	0.101	3.10E-07	62.8
N11	0.281	0.09	2.70E-05	68
N12	0.246	0.085	1.30E-05	65.5
N13	0.294	0.09	3.20E-05	69.4
N14	0.547	0.199	2.80E-06	63.7
N15	0.565	0.219	1.50E-07	61.3
N16	0.276	0.11	4.80E-05	60.2
N17	0.29	0.08	1.50E-10	72.5
N18	0.306	0.099	3.10E-05	67.7
N19	0.276	0.11	4.80E-05	60.2
N20	0.322	0.122	2.70E-08	62.2
N21	0.436	0.165	4.50E-06	62.2
N22	0.317	0.098	7.00E-06	69.1
N23	0.295	0.083	7.40E-06	71.9
N24	0.517	0.196	1.30E-05	62.1
N25	0.322	0.122	2.70E-08	62.2
N26	0.301	0.094	1.50E-09	68.8
N27	0.294	0.088	6.90E-07	70.1
N28	0.46	0.133	3.10E-06	71.1
N29	0.322	0.122	2.70E-08	62.2
N30	0.317	0.098	7.00E-06	69.1
NC	0.038			

NC=Ngative Control

The presnt results are in agreement with the previously reported anti-biofilm efficacy of AuNPs by Al- Tae *et al.*[14], showed that Au-NPs have a high effectiveness in inhibiting the ability of MRSA biofilm formation, which has been studied using the method of microtiter plate assay that consider the best and most reliable method of detecting biofilm production and the adhesion of bacteria. Studies

have also found that both AgNPs and AuNPs inhibited the formation of biofilms of *Pseudomonas aeruginosa* and *E. coli* at concentrations of sub-MIC of 12.5 mg/ml for the AuNPs and 6.25 mg/ml for AgNPs. Inhibition of biofilms at concentrations of sub-MIC might be due to inhibitory impact on genes expression associated with formation of biofilm and motility or to non-lethal damage [20]. Research of Yu *et al.* [21]showed that the AuNPs exhibiting antibiofilm efficacy on biofilms formation of *Pseudomonas aeruginosa*. Also, the gold nanoparticles exhibited significant inhibitory impacts just when its concentration reached to 10 ppm (IC50 = 68.56–75.01 ppm). Nevertheless, 5 ppm of gold nanoparticles strongly inhibited the formation of biofilm (IC50 = 6.851–6.937 ppm). Thus, gold nanoparticles also strongly attenuated formation of biofilm of these bacteria. Study by[22] the average thickness of biofilms decreased from 19.3 ± 0.29 to 12.1 ± 0.51 μm after treatment by phytol (10 μg/ml). Results shown differences in the inhibitory effect of Au-NPs on the bacterial isolates this is due to the difference in isolation site, environmental conditions that may cause appearance changes in the isolates, as well as the difference in the physiological activity of each isolate due to the difference in their genetic structure, which in turn reflects their different metabolic activities and enzyme activity. Au-NPs greatly reduced the ability of *Serratia marcescens* to biofilm formation by penetrated the bacterial cell and the interaction with the proteins and enzymes responsible for adhesion and quorum sensing, led to reduce the capability of the bacterium to form the biofilm [23]. Furthermore, Au-NPs inhibit biofilm formation by preventing the production of polysaccharides, and small sizes of AuNPs can penetrate biofilm matrix and communication between bacterial cells causing inhibit biofilm formation [24]. Another study by [25] demonstrated that AuNPs exhibited an excellent anti-bacterial activity against *Escherichia coli* (gram-negative bacteria) and Bacillus Calmette-Guerin (gram-positive bacteria). The current research also suggested that the anti-bacterial efficacy could be due to uptake of single AuNPs by the bacterium and re-arrangement of them inside the cytoplasm. The anti-bacterial efficacy of AuNPs toward gram negative and positive microbes is various because of the structure of the bacterial membrane of proposing the need to higher doses of NPs. It was recorded that anti-bacterial efficacy of AuNPs occurred due to the decreased activities of adenosine triphosphate synthase which disrupt the metabolic process and a decline of the sub-unit of the ribosome for t-RNA binding, therefore, resulting in a collapse of biological mechanisms [26]. The high surface area enhanced the direct interaction of NPs with the bacteria. The NP interfered with the cytoplasm and proteins of bacteria, leading to cause death of cells [27].

Conclusion :

Gold nanoparticles' antibacterial activity against ***Serratia marcescens*** can be due to the production of Reactive Oxygen Species (ROS), which induces increased oxidative stress in the form of vacuole formation as an indicator of potent activity.

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