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Antimicrobial Effect of Silver Nanosilver Particles on Multi-Drug Resistant Isolates of *Kluyvera Cryocrescens* and *Citrobacter Freundii*

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Abstract

Background: Members of the Enterobacteriaceae family, *Kluyvera cryocrescens* and *Citrobacter freundii* are infrequent cause of infection in healthy human and multi-drug resistant (MDR) strains of both species had been isolated from different clinical specimens. The difficulty in treating of MDR biofilm producing bacteria invites the researchers to find a new way to resolve this problem. According to the various studies, using of silver nanoparticles (AgNPs) can be considered as an alternative therapy to eliminate the MDR biofilm forming bacteria. The aim of this study was to investigate the effects of silver nanoparticles on the MDR biofilm-forming strains of *K. cryocrescens* and *C. freundii* isolated from human blood, and urine, and cow's milk specimens.

Materials and methods: The *K. cryocrescens* and *C. freundii* strains were isolated from various samples and their identity and antibiotic susceptibility pattern was determined using VITEK 2 AST system. The biofilm producing property was investigated using Congo red agar method. The antimicrobial activity of AgNPs was evaluated by agar well diffusion method.

Results: The results revealed the average inhibition zone of 29 ± 0.64 for 22 *K. cryocrescens* isolates and 14 ± 0.4 mm for 11 *C. freundii* isolates.

Conclusions: Silver nanoparticles could be used as a potential alternative therapy for treatment of infections caused by *K. cryocrescens* and *C. freundii*. Further *in vivo* studies are required to confirm the effects of AgNPs.

Keywords: AgNPs, Biofilm, *Citrobacter freundii*, *Kluyvera cryocrescens*, Multi-drug resistant

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Introduction

Kluyvera and *Citrobacter* sp. are Gram-negative bacteria, less Commonly Encountered Enterobacteriaceae[1, 2]. They are opportunistic pathogens and predominantly colonizes the gastrointestinal, respiratory and urinary tracts[1, 3] and they widely distributed in different environmental sources such as hospital sinks, water, soil, milk, and cows but it rarely cause infection[4-6]. Little cases were reported as adult bacteremia[5]. *K. Cryocrescens* was reported as a second species commonly isolated from environment and rarely isolated from clinical specimens[6]. *K. cryocrescens* reported as a virulent pathogens because of it resistance activity to the ampicillin and 1st,2ed generation of cephalosporin's [7]. While *Citrobacter* bacteria known as low virulence but lately reported as multi- drug resistant (MDR) bacteria [8, 9]. Some MDR bacteria associated with biofilm producer ability. Bacterial biofilm is an aggregation of bacteria on biotic and a biotic surface and encased in a expolysaccharied matrix and extracellular DNA characterized by ability to antibiotics, disinfectant, phagocytosis resistant [10].

Difficulty treating of biofilm bacteria invites to find a new way to resolve the problem. Silver nanoparticles (AgNPs) were recommended to eliminate the MDR biofilm forming bacteria as alternative medicine.

Nanoparticles is a small particles that have size range from 1-100 nm (nm=10^{-9} m) [11]. It has a novel or unusual properties than their bulk minerals counterparts and act as union part [12, 13] and have special and superior properties to make it suitable candidate for various applications such as medicine and pharmaceutical industry[14].

In present day a novel way to solve most antibiotic resistance problematic was a silver nanoparticles solution use, without development resistant mutation, in addition to its effect to prevent biofilm bacteria [15]. Silver nanoparticles was reported to be more effective against gram-positive and negative bacteria [16], because it have ability to puncture the bacterial cell membrane due to nanoparticles size instead of micrometer cell membrane and harmless to large host cell [17].

The current study aimed to estimate the antibacterial activity of AgNPs on MDR *K. cryocrescens* and *C. freundii* isolated from different sources.

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Materials and methods:

Samples were presented to the Al-Qasim green university (microbiology Lab) for investigation mentioned in table I. Specimens were cultured on different type of media (nutrient agar, MacConkey agar and blood agar) by streaking method for single isolated colony. After 24 hours (incubation period) growth was noticeable on all types of media. Gram stain done (standard method used). Fresh culture 24hr done for all samples were used for identification by Biomerieux Vitek 2 System.

Table I: samples information details

Source	No.	No growth	Others bacteria	*K. cryocrescens*	*C. freundii*
Human Blood	22	9	7	3	3
Cow's Milk	64	32	15	12	5
Human Urine	12	0	2	7	3

Three isolates of each *K. cryocrescens* and *C. freundii* from each source were chosen to complete the study.

Biofilm detection: To detect biofilm-forming bacteria by **Congo red agar method** according to[18] by prepared a Congo red stain as stock solution, autoclaved at 121°C for 20 min. then added to autoclaved brain heart infusion broth with agar agar and 5% sucrose at 55°C. [19]. The bacterial strains were inoculated and incubated at 37 °C for 24 to 48 hrs. then read the result as following: if the bacteria formed black colonies with a dry crystalline consistency that was mean it biofilm producer isolates while if it formed red colonies that was mean the non-biofilm producer isolates[20].

Antibiotics susceptibility tests: VITEK 2 AST system to determine the Minimum inhibitory concentration (MIC) to many tested antibiotics. All the following steps were done according to the manufacturer's instructions as VITEK 2 AST system supplemented with antimicrobial susceptibility testing cards Enterobacteriaceae contains more than 15 antibiotics (Table II). The results read also digitally on monitor connected to VITEK2 system apparatus.

Table II: Antibiotics provide by VITEK AST card for Enterobacteriaceae with MIC breakpoints according to M100 [21]:

Antibiotic	MIC Breakpoints (µg/mL)	Antibiotic	MIC Breakpoints (µg/mL)

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	S	I	R		S	I	R
Ticarcillin	≤8	16	≥32	Cefepime	≤2	-	≥16
Ticarcillin / clavulanic acid	≤16/2	32/2-64/2	≥128/2	Trimethoprim /Sulfamethoxazole	≤2/38	-	≥4/76
piperacillin	≤16	32-64	≥128	Gentamicin	≤4	8	≥16
Piperacillin-tazobactam	≤16/4	32/4-64/4	≥128/4	Tobramycin	≤4	8	≥16
Aztroenam	≤4	8	≥16	Ciprofloxacin	≤1	2	≥4
Meropenem	≤1	2	≥4	Amikacin	≤16	32	≥64
Ceftazidime	≤4	8	≥16	Minocycline	≤4	8	**≥16**
Imipenem	≤1	2	≥4				

+=Deduced drug *= AES modified **= user modified

Silver nanoparticles synthesis: it was synthesis by chemical synthesis method: as follow

Twenty drops of 0.1M $AgNO_3$ was added dropwise (1 drop per sec.) to 50ml of 0.001M $NaBH_4$ in beaker (250ml) on magnetic stirrer at (400 for 30 min in dark condition and in room temp.) then the change in color was noted. The reaction mixture was stirred vigorously on a magnetic stirrer[22, 23] according to equation below:

$AgNO_3 + NaBH_4 \rightarrow Ag + H_2 + B_2H_6 + NaNO_3$

Optimize silver nanoparticles characterization:

The silver nanoparticles were characterized by UV. Spectrophotometer and Size analyzer[24]. All these analyses were carried out at pharmacy and Science College, Kufa and veterinary college of Al-Qasim green university.

UV–visible spectrophotometer analysis:

The Surface Plasmon Resonance of silver nanoparticles was measured by UV–visible spectrophotometer at wave length ranging from 300-500 nm. By sampling 1ml of AgNPs solution to different wave length were measured every ten degree at resolution of 1nm [25].

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Size analyzer:

Laser diffraction particle size analyzers, which measure light scattering and assume an index of refraction to calculate the particle size distribution[26]. Silver nanoparticles sample was examination in size analyzer after incubated in sonicator water bath at 35C for 30 min. Emulsion diluted sample with deionized water were put in grove of apparatus and the size were measured during 5 min. by using laser beam scattering in beta sizer apparatus. The results were monitoring on computer's screen.

Antimicrobial activity assay of AgNPs:

The antimicrobial activity of AgNPs were evaluated by agar well diffusion method by using Muller Hinton plate inoculated with tested biofilm-forming bacteria at inoculum 1.5 x 10^8 CFU/ml by streaking method and waited 10 min. to dry then made well by cork borer in the center of inoculated plate and fill the well with 100 µl of filtered AgNPs, incubated at 37C to 24hrs.at dark condition. After that, the inhibition zone diameter were measured by ruler and compared to the nearest whole millimeter.

The Results

In our study, *K. cryocrescens* and *C. freundii* produce biofilm which isolated from urine, blood and milk specimens were indicated by Congo red method (Fig. 1)

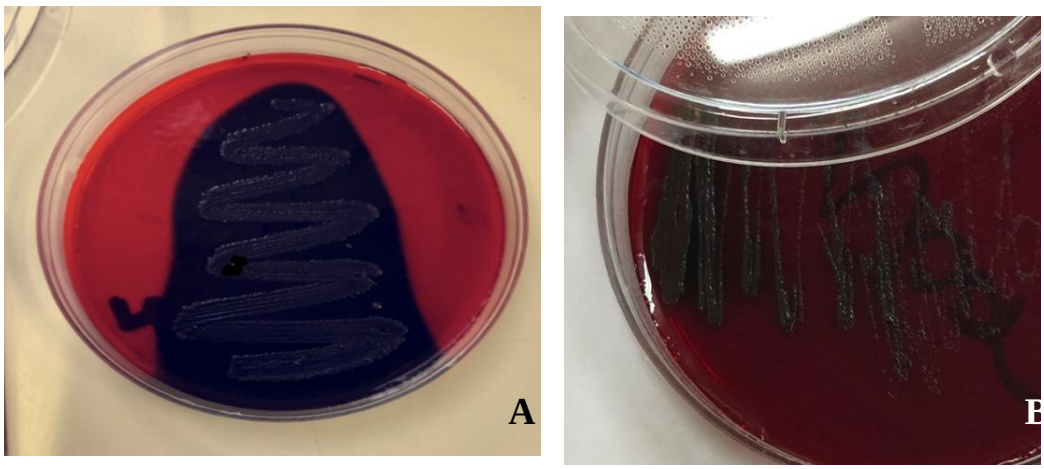

Figure 1: Congo red plate indicates biofilm formation A= *K. cryocrescens*, B= *C. freundii*.

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Antibiotics sensitivity profile:

The results revealed that *K. cryocrescens* and *C. freundii* isolates were resisted most tested antibiotics (table III).

	K. cryocrescens		***C. freundii***			***K. cryocrescens***		***C. freundii***	
Antibiotic	MIC (µg/ml)	Interp.	MIC (µg/ml)	Interp.	Antibiotic	MIC (µg/ml)	Interp.	MIC (µg/ml)	Interp.
Ticarcillin	≥12	R	≥12	R	Cefepime	≥32	R	≤8	S
Ticarcillin / clavulanic acid	≥8	R	≥8	R	Trimethoprim /Sulfamethoxazole	≥320	R	≥320	R
piperacillin	≥128	R	≥128	R	Gentamicin	≥16	R	≥16	R
Piperacillin-tazobactam	≥4	R	≥8	R	Tobramycin	≥16	R	≥16	R
Aztroenam	≥1	R	≥7	R	Ciprofloxacin	≤0.25	S	≤0.31	S
Meropenem	≤0.25	S	≤0.25	S	Amikacin	≤2	S	≥8	R
Ceftazidime	≥64	R	≥32	R	Minocycline	≤1	S	≤1	S
Imipenem	≤0,25	S	≤1	S					

Table III: Vitek AST results of biofilm forming *K. cryocrescens* and *C. freundii* isolates.

R= resistant, S= sensitive, MIC= minimum inhibitory concentration

Characterization of synthesis silver nanoparticles (AgNPs):

Color change indicator:

During AgNPs synthesis, the color of mixture solution of $AgNO_3$ and $NaBH_4$ converted from colorless to dark brown color (Fig. 2)

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UV. Spectrophotometer analysis:

The optical density of AgNPs production were revealed beak at 390 nm (Fig. 3) in UV-spectrophotometer.

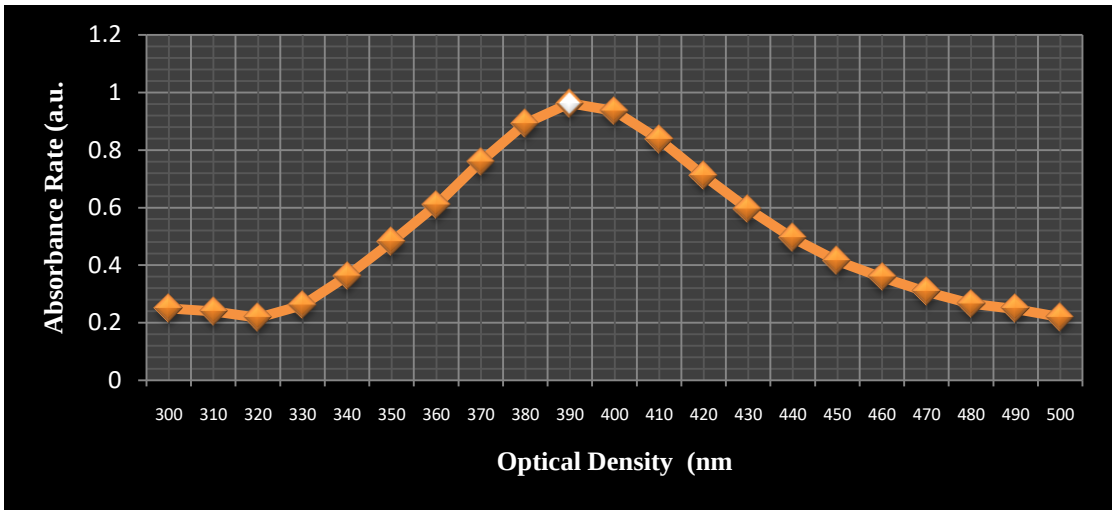

Figure 3: UV-Vis spectrophotometer analysis of silver nanoparticles synthesis.

The size of synthetic AgNPs was determined by dynamic light scattering by nano laser particle size analyzer. The figure below showed the size of AgNPs at 5 nm.

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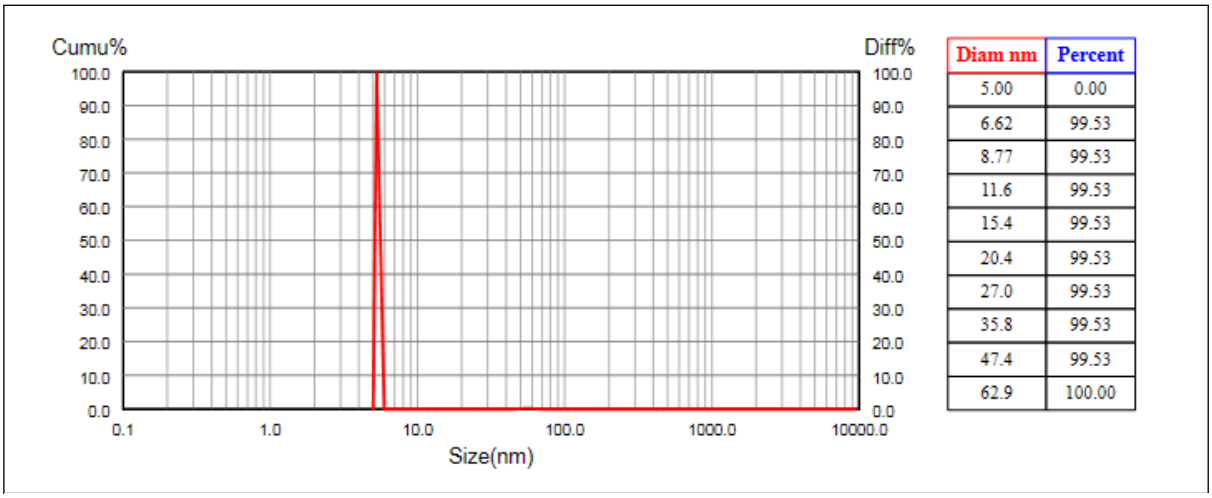

Diam nm	Percent
5.00	0.00
6.62	99.53
8.77	99.53
11.6	99.53
15.4	99.53
20.4	99.53
27.0	99.53
35.8	99.53
47.4	99.53
62.9	100.00

Figure 4: Size distribution analysis of synthetic AgNPs particle size was approximately 5 nm.

The antibacterial activity of synthetic AgNPs:

The antibacterial activity of AgNPs was evaluated by agar well diffusion method. The results revealed that inhibition zone of biofilm formation *K. cryocrescens* growth at average 29 mm to all isolates from different source (Fig. 5A) with significant differences among *K. cryocrescens* isolates at *p* value = 0.008 (table 4).

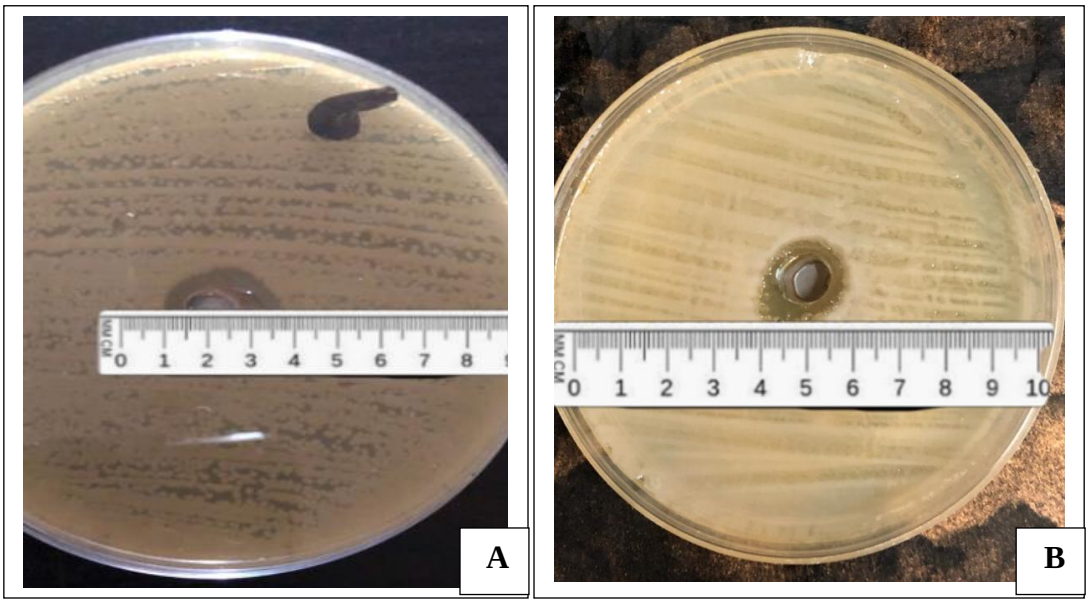

Figure 5: Inhibition zone of A= biofilm formation *K. cryocrescens* growth and B= *C. freundii* isolated growth as a result of antibacterial activity of synthetic AgNPs.

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While antibacterial activity of AgNPs on biofilm formation *C. freundii* as inhibitor growth was indicated by inhibition zone appear around AgNPs well at average 14 mm to all isolates from different sources (Fig. 5B). The statistical analysis revealed there is no significant differences among *C. freundii* isolates at *p* value = 0.12 (table IV).

Table IV: statistical analysis of antibacterial effect of AgNPs on biofilm formation *K. cryocrescens* and *C. freundii*.

Bacteria	N.	IZ Mean ± SE	*P* value
K. cryocrescens	9	29 ± 0.64	0.008
C. freundii	9	14 ± 0.4	0.12

IZ= inhibition zone, SE= standard error.

The antibacterial effect of AgNPs against biofilm formation *K. cryocrescens* and *C. freundii* was variable (fig. 6) which showed a highly antibacterial effect on *K. cryocrescens* than *C. freundii* and that variability between theses bacteria have a significantly differences at *p* value > 0.001 (table V).

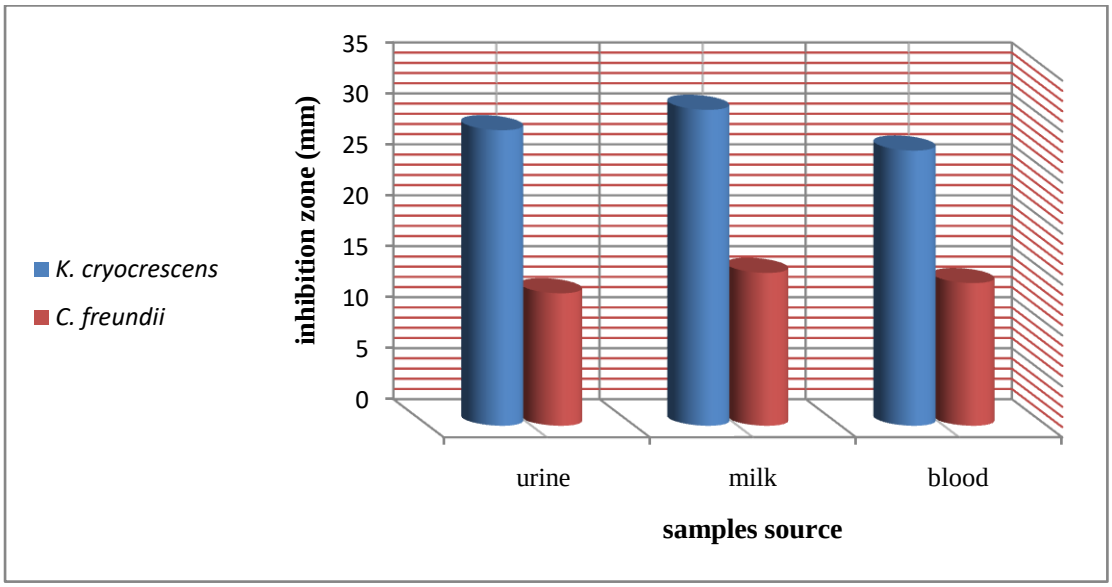

Figure 6: Inhibition zone of biofilm formation *K. cryocrescens* and *C. freundii* growth isolated from (urine, milk, blood) as a result of antibacterial activity of synthetic AgNPs.

Table V: statistical analysis (t test) of antibacterial effect of AgNPs between biofilm formation *K. cryocrescens* and *C. freundii*

Bacteria	N.	IZ Mean ± SE	P value

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K. cryocrescens	9	29 ± 0.64	> 0.001
C. freundii	9	14 ± 0.4	

IZ= inhibition zone, SE= standard error.

The discussion:

Kluyvera and *Citrobacter* sp. are uncommon gram-negative bacteria produce biofilm in clinical specimen, there is no study about their biofilm formation. Little and new studies pointed that *Citrobacter freundii* as opportunistic and life threating bacteria when detect as sepsis causative agents [27] and urinary tract infection [28] the most important causes for morbidity and mortality because the bacteria have multi drug resistant ability.

In (fig.1), the black color colony indicate biofilm formation of bacteria due to stain exopolysaccharide matrix producing during biofilm process by Congo red stain[29].

Isolation sources:

According to the National Institutes of Health (NIH) very complicated matter when we found biofilm in the urothelium as it consider serious problem for urinary tract cause it is one of the main areas that predisposing to the biofilm growth other complicated things could be prostate stones and implanted foreign bodies [30]. Therefore more, Bloodstream infection of *K. cryocrescens* and *C. freundii* in adults can disseminate from the abdominal cavity or the urinary tract, lungs, or intravascular catheters [2, 8, 9].

C. freundii blood stream infection are commonly multidrug resistance especially, in those patients who are received antibiotics treatments and associated with highly mortality rate [8].
Always, sepsis because of microbial colonization to the bloodstream as it is serious health problem associated with high mortality rates. Heavy colonization of bacteria in the bloodstream like *K. cryocrescens* and *C. freundii* will be able in causing a life-threatening infection leading to sepsis [7, 27].
Some research pointed that bacteria can biofilm former in milk may relate to adhesion of bacteria to their container stainless steel [31] the ability of *K. cryocrescens* and *C. freundii* to adhesion to stainless steel was strain-dependent in comparison with other biofilm bacteria found that, it is the most adhesion over 106 adherent cells per cm^2[32, 33].

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Antibiotics sensitivity profile:

The resistant ability of *K. cryocrescens* and *C. freundii* (table III) were related to have an ability to produce biofilm. The biofilm formation incorporated with antibiotic resistant to be multi drug resistant bacteria (MDR)[34, 35]. Many mechanism of antibiotic resistant were have to biofilm forming bacteria such as, biofilm act as barrier by decreases diffusion of antimicrobial proteins, lysozymes, and defenses, decreases diffusion of antibiotics and shields bacteria from phagocytes[36]. In addition the biofilm concentrates bacterial enzymes that degrade antibiotics (lactamases), Altered growth of bacteria in the biofilm (due to limited nutrients and oxygen and accumulation of metabolic wastes)[37]. Expression of resistance genes by bacteria in the biofilm [38].

Characterization of synthesis silver nanoparticles (AgNPs):

Color change indicator:

The dark brown color of mixture ($AgNO_3$ and $NaBH_4$) (Fig. 2) which indicate AgNPs production and that related to excitation of AgNPs surface Plasmon vibration [39].

UV. Spectrophotometer analysis:

The absorption degree of AgNPs depend on Plasmon resonance rate which represent the ratio of silver ion to silver zero valent[24].

Size analyzer test:

The size of synthetic nanoparticles is very important and effective because the antibacterial activity of nanoparticles depend upon its size, the small particles more effective as antibacterial than large one [39] especially when the nanoparticle size at 5 nm like current study.

The antibacterial activity of synthetic AgNPs:

Many studies pointed that AgNPs had antibacterial activity on different planktonic bacterial isolates [40, 41] but the challenge is their activity on biofilm forming bacteria because the biofilm bacteria have ability to resist the antibiotics, disinfectant or any antibacterial materials and moreover when AgNPs tested against less common biofilm bacterial like *K. cryocrescens* and *C. freundii*.

Recent search showed a good antibacterial activity of AgNPs against gram negative biofilm forming bacteria especially those who isolated from urine [35].

The antibacterial activity of AgNPs against biofilm forming bacteria studies is limited except littles studies [39, 42, 43] and no study about their effect against biofilm forming *Kluyvera* sp. or *Citrobacter* sp.

The inhibition zone of biofilm formation *Kluyvera* against silver nanoparticles at 29 mm were highest in contrast to other closely studies [43] and although that *Kluyvera* and *Citrobacter* have resistance result to the antibiotics in contrast the susceptible result indicated with AgNPs antimicrobial activity and that pointed a significantly antibacterial activity of AgNPs.

AgNPs mentioned previously as an antimicrobial effect which is approved in this study as it reveal an inhibition zone with the resistance bacterial suggestions it as a good source in treating multidrug resistance bacteria

The antibacterial activity of AgNPs suppose related to many mechanisms but it still unknown, AgNPs may interact with the bacterial cell membrane lead to disturb the permeability and functions of respiration [39] and may penetrate the bacteria cell [42].Many researchers also proposed that Ag+ ions interact with the thiol groups in bacteria proteins, affecting the replication of DNA[44].

Conclusion

K. cryocrescens and *C. freundii* showed highly resistant rate to most tested antibiotics in Vitek2 AST and highly sensitivity to sliver nanoparticles showed antibacterial activity against biofilm formation bacteria isolated from different sources. In current study the Silver nanoparticles consider alternative drug to eliminate multi drug resistant bacteria and resolve world problem.

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