

Antibacterial and Anti-biofilm Activity of Three Phenolic Plant Extracts and Silver Nanoparticles on *Staphylococcus aureus* and *Klebsiella pneumoniae*

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Abstract Background: Multi-drug resistance is a growing problem in the treatment of infectious diseases and the widespread use of broad-spectrum antibiotics has produced antibiotic resistance for many human bacterial pathogens. Advances in nanotechnology have opened new horizons in Nano medicine, allowing the synthesis of nanoparticles that can be assembled into complex architectures. **Method:** Novel studies were carried out to assess the antibacterial effect of phenolic plants extracts and silver nanoparticles on the development of biofilm formation of bacterial species (*Klebsiella pneumoniae*) and (*Staphylococcus aureus*) using fourteen isolates from both clinical and environmental samples. Also, the experiments were conducted to study the antibacterial effect by determining the minimum inhibitory concentrations (MICs), and susceptibility test of these strains for 8 antibiotics. Moreover, the study used silver nanoparticles Ag-NPs which indicated a size range of 101.77 nm as antimicrobial agent. Although the *Zingiber officinale* Roscoe, *Thymus vulgaris* L. and *Cinnamomum zeylanicum* phenolic plant extracts concentrations (50, 25, 12.5, 6.25, 3.125 and 1.625 µg/ml) (W/V) were used through antibiotic and biofilm inhibition assay. **Result:** The result showed that the isolates behaved differently in their sensitivity to the different extracts added to their growth medium. Among the different plant phenolic extracts tested, *Z. officinale* showed antibiofilm efficacy against *k. pneumoniae* and *S. aureus* followed by *T. vulgaris* and *C. zeylanicum* extracts exhibited. **Conclusion:** The silver nanoparticles with either the plants extract compound (phenolic compound) may provide a safe and highly effective alternative to commonly used antibiotics, which are ineffective towards the antibiotic-resistant *S. aureus* and *K. pneumoniae*. Also, The silver nanoparticles presented strong antimicrobial and antibiofilm activity against and *K. pneumoniae* and *S. aureus* in an indication that represents a new potential candidate for alternative antibiofilm.

Keywords: *Zingiber officinale*, *Thymus vulgaris*, *Cinnamomum zeylanicum*, antibacterial activity, biofilm, phenolic extracts

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1. Introduction

For years, many antimicrobial agents have been used to control or eliminate bacteria from households, industry and for the treatment of common bacterial infections of public human health. However, the irrational use of these antibacterial agents has produced strains of multiple antimicrobial resistant bacteria in households and industry where the cleaning products are no more effective against the microorganisms than good personal and household hygiene. Green synthesis of metallic silver nanoparticles has attracted nowadays and alternative to physical and chemical approaches. Furthermore these green synthesized AgNPs were found to show higher antimicrobial efficacy against multi drug resistant clinical isolates [1]. Green synthesis of nanoparticles with low range of toxicity and

conjugation to antibiotics has become an attractive area of research for several biomedical applications. Nano conjugates exhibited notable increase in biological activity compared to free antibiotic molecules [2]. Among the factors contributing to microbial resistance is the ability of the microbes to exist in biofilm forms that allow them to withstand harsh environmental conditions and antimicrobial agents. *Staphylococcus aureus* is the most common infectious agent involved in the development of Skin infections that are associated with antibiotic resistance, such as burn wounds. Also, *Klebsiella pneumoniae* is the second most common cause of gram-negative bacteremia after *Escherichia coli*. *K. pneumoniae* bacteremia causes significant morbidity and mortality in general populations [3,4].

The biofilms have been implicated in a wide range of hospital infections and food spoilages thus posing a serious concern in both the food and medical industries.

By definition, a biofilm is a complex community of cells attached to either a biotic or abiotic surface enclosed in an exopolysaccharide matrix [5]. This matrix is composed of extracellular polymeric substances (EPS), particulates from different origins and microbial lyses products [6]. When bacteria form biofilms, they become more resistant to many effective environmental factors such as fluctuation of nutrients and oxygen, alteration of pH, and antibiotics effects [7].

Silver nanoparticles (Ag-NPs) are three-dimensional structures up to 100 nm in diameter formed from silver ions reduced to Ag⁰ clusters which are then stabilized by coating ligands. The antimicrobial activity of Ag-NPs has already been demonstrated towards multiple fungi and bacteria species, regardless of their susceptibility or resistance to common drugs [8]. The antimicrobial properties of Ag-NPs are attributed to the direct effect that these particles have on the bacterial cell as well as to the activity of silver ions continuously released from their surface [9]. Antimicrobial activity of silver nanostructures is based on their ability to affect cellular components such as the cell wall, membrane, proteins and nucleic acids [8]. An interesting evolution of using nanoparticles against *S. aureus* isolates is represented by Hamed and Al Shahwany [10], they suggesting that combining AgNPs with phenolic plant extracts could be a possible alternative therapeutic strategy against bacterial infectious diseases. While biofilms of bacteria are more resistant to antimicrobial agents. Most works with plant-based antimicrobial studies have been focused on the planktonic forms [11]. Therefore, in the present study an attempt has been made to screen different plant extracts against microbial biofilms. Thus, the goal of this study was to evaluate the antimicrobial properties of the compunction effects of some plant extracts with silver nanoparticles on *S. aureus* and *K. pneumoniae* isolates growth and biofilm formation.

2. Material & Method

2.1. Collection of Plant Samples

Plant samples included *Zingiber officinale* dry rhizomes dry leaves of *Thymus vulgaris* and dry bark of *Cinnamomum zeylanicum* were obtained from local herbarium market in Baghdad city. After the plants were air derided and powdered, it kept at 4°C until further investigations.

2.2. Preparation of Phenolic Plant Extracts

Phenols were extracted according to previous studies [12] 200g of plant powder were divided into two equal portions, one was mixed with 300 ml of distilled water and the other one was mixed with 300 ml of 1% hydrochloric acid. Then, samples were homogenized in electric shaker for 5 min. The two mixtures were transferred to boiled water bath for 30-40 min, there after cooled and filtered through muslin cloth and centrifuged with speed of 3000 rpm for 10 min. The two supernatants were mixed. Equal quantity of N-propanol was added to the mixture prior to sodium chloride was added until the solution was separated into two layers. The lower layer

extracted in separating funnel with ethyl acetate, and concentrated by using rotary evaporator at 40°C for 1 to 2 h. The upper layer was dried by rotary evaporator at 40°C for 1 to 2 h. The dried material of both layers were mixed and dissolved in 5 ml of 96% ethanol, after words, left in oven until it turned into powder and kept in refrigerator until use.

2.3. Preparation of Different Concentrations of Plant Extracts

Stock solutions were prepared by mixing 2 g from the dried extract with 20 mL Ethylene glycol, and then it was sterilized with membrane filter (0.22 µm). Subsequently, different concentrations (1, 5, 10 mg. mL⁻¹) were prepared as diluents.

2.4. Selection of the Isolates

The bacterial strains used in this study were include 14 isolates from each *Staphylococcus aureus* and *Klebsiella pneumoniae*, isolated from urine, blood, wound, ear water, sewage, soil and water . Those isolates showed increase resistance to commonly available antibiotics by using Kirby- Bauer disc diffusion method. The bacteria identified by standard microbiological procedures (Gram staining, colonial morphology, catalase test, cytochrome oxidase reaction, motility, and other biochemical tests) which carried out depending on Bergey's manual of systematic Bacteriology [13], also by analytical profile index (API) 20 E system and vitek 2 system [13].

Antibiotic Susceptibility Test [14].

From an overnight culture plate, 4-5 colonies of bacterial isolates were picked up and suspended in 5ml of sterile normal saline until the turbidity is a proximately equivalent to that of the McFarland No. 0.5 turbidity standard. Ten minutes later, by a sterile forceps the antimicrobial disc Amoxicillin, Ampicillin, Cefotaxime, Cephalothin, Clarithromycin, Methicillin , Nitrofurantoin and Tetracyclin were picked up and placed on the surface of Mueller Hinton plates. The plates were incubated at 37°C for 18-24 hours. After incubation, the plates were examined for the presence of inhibition zone of bacterial growth around the antimicrobial discs. The diameter of the zone of inhibition was measured by millimeters using a metric ruler and compared to standard inhibition zone according to Clinical and Laboratory Standards Institute [15].

Determination of minimum inhibitory concentration (MIC)

MIC of plant extracts was determined by microdilution method in sterile 96-wells microliter plates according to the protocol described previously [16]. Different plant extracts concentrations (50, 25, 12.5, 6.25, 3.125 and 1.625 µg/ml) (W/V) were prepared containing bacterial cells comparable to McFarland standard no. 0.5 in a final volume of 200 µl. Sterile distilled water, broth and plant extracts was used as a negative control while sterile distilled water, broth and bacteria was used as positive control. After 24 h at 37°C, the MIC of each sample was determined. The MIC is considered the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after 24 h incubation [17].

Detection of biofilm formation

The method described by [18] was followed with some modifications. Briefly, overnight cultures were diluted (1:100) with trypton soya broth supplemented with 1% (w/v) glucose. Aliquots (200µl) from the culture were then transferred to the wells of a 96-well polystyrene microliter plate and incubated overnight at 37°C. After incubation, supernatants were removed from each well and biofilms were gently washed twice with normal saline, then dried and fixed at 65°C for 1 hr. Subsequently, the plates were stained with 0.1% (w/v) crystal violet for 10 min, gently washed twice and the quantitative analysis of biofilm production was performed by adding 200 µl of 95% ethanol for 10 min. Finally, the absorbance of the methylene blue present in the de-staining solution (ethanol) was measured at 630 nm by microplate reader.

Silver nanoparticles (Ag-NPs): The Silver nanoparticles (Ag-NP) was brought from the Department of Medical Engineer, College of Engineering, Al-Nahrain University, Baghdad, Iraq. The average diameters of silver nanoparticles found equal to 101.77nm

2.5. Statistical Analysis

Complete Randomized Design (CRD) was used as experimental design. Data were analyzed using statistical

analysis system- SAS (2003) to study the effect of different plant extracts and the nanoparticles on some bacterial isolates. Least significant difference (LSD) was used to compare the significant difference between means at differences were considered significant when $P \leq 0.05$ [19].

3. Results

In this study, the bacterial isolates were chosen because the importance of these isolates in the hospital environment and their outbreak in the community. Multidrug resistance and biofilm have been recognized as virulence factors of great scale in clinical infections. Because of the increase in intricacy of the majority microbial infections and the resistance to straight treatment, researchers have been prompted to identify alternatives for the action of infections. Plant extracts biologically active compounds isolated from plants have gained extensive attention in this look upon as they have been known to cure diseases and sickness since very old times. Silver nanoparticles are also able to assess human healthy through a variety of commercial products. Studies have shown that silver nanoparticles cause toxicity to germ line stem cells through reduction in mitochondrial function and induction of membrane leakage and apoptosis [20].

Table 1. Antibiotic susceptibility of *S. aureus* isolates according to CLSI [21]

S. aureus isolates	Amoxicillin	Ampicillin	Cefotaxime	Cephalothin	Clarithromycin	Methicillin	Nitrofurantoin	Tetracycline
1	R	R	S	R	R	R	R	R
2	R	R	I	R	R	R	R	R
3	R	R	S	R	R	R	R	R
4	R	R	S	R	R	R	R	R
5	R	R	I	R	R	R	R	R
6	R	R	S	R	R	R	R	R
7	R	R	I	R	R	R	R	R
8	R	R	I	R	R	R	R	R
9	R	R	S	R	R	R	R	R
10	R	R	I	R	R	R	R	R
11	R	R	I	R	R	R	R	R
12	R	R	I	R	R	R	R	R
13	R	R	I	R	R	R	R	R
14	R	R	I	R	R	R	R	R

R=Resistant, I=Intermediate, S=Sensitive.

Table 2. Antibiotic susceptibility of inhibition zones for *K. pneumoniae* isolates according to CLSI [21]

K. pneumoniae isolates	Amoxicillin	Ampicillin	Cefotaxime	Cephalothin	Clarithromycin	Methicillin	Nitrofurantoin	Tetracycline
1	R	R	R	R	R	R	R	R
2	R	R	R	R	R	R	R	R
3	R	R	R	R	R	R	R	R
4	R	R	R	R	R	R	R	R
5	S	I	R	R	R	S	R	R
6	S	S	R	R	R	S	R	R
7	I	S	R	R	R	I	R	R
8	R	R	R	R	R	R	R	R
9	I	S	R	R	R	I	R	R
10	R	R	R	R	R	R	R	R
11	S	I	R	R	R	I	R	R
12	I	I	R	R	R	S	R	R
13	R	R	R	R	R	R	R	R
14	R	R	R	R	R	R	R	R

R=Resistant, I=Intermediate, S=Sensitive.

Antibiotic Susceptibility of *Staphylococcus aureus* and *K. pneumoniae* isolates.

In a biofilm form, bacteria are more resistant to various antimicrobial treatments. Susceptibility tests were summarized in Table 1 and Table 2 for *S. aureus* and *K. pneumoniae* isolates to eight different antibiotics by disc diffusion method recommended by CLSI [21] guidelines. These antibiotics were used in this study due to their mode of action inhibiting cell wall synthesis which cause the release of the bacterial cell DNA into the surroundings [22].

Table 1 and Table 2 indicate that all isolates were resistance to Ampicillin, Amoxicillin. Many studies focused on that Methicillin resistance strains should be considered to be resistant to all Cephalosporins, and other β -lactams, such as Ampicillin-sulbactam, Amoxicillin-clavulanic acid, Ticarcillin-clavulanic acid, Piperacillin-tazobactam,

and the Carbapenems, regardless of the in vitro test results obtained with those agents [23,24].

Beside, all *S. aureus* isolates were ranging from intermediate and sensitive to Cefatoxime antibiotic.

Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration (MIC) value is important to determine efficacy of antibacterial agent. Low MIC value may be an indication of high efficacy or that microorganism has no potential to develop resistance towards the bioactive compound. Figure 1 and Figure 2 show effects of MIC values of Ag-NPs with different concentrations on *K. pneumoniae* and *S. aureus*. Obviously, MIC ranged value was in concentration between 6.25 to 12.5% for both *K. pneumoniae* and *S. aureus* isolates. Moreover, results indicated that *Z. officinale* and *T. vulgaris* MIC reached 6.25 -25 % for most isolates. However, it formed 25- 50 % for *C. zeylanicum* for both bacteria isolates.

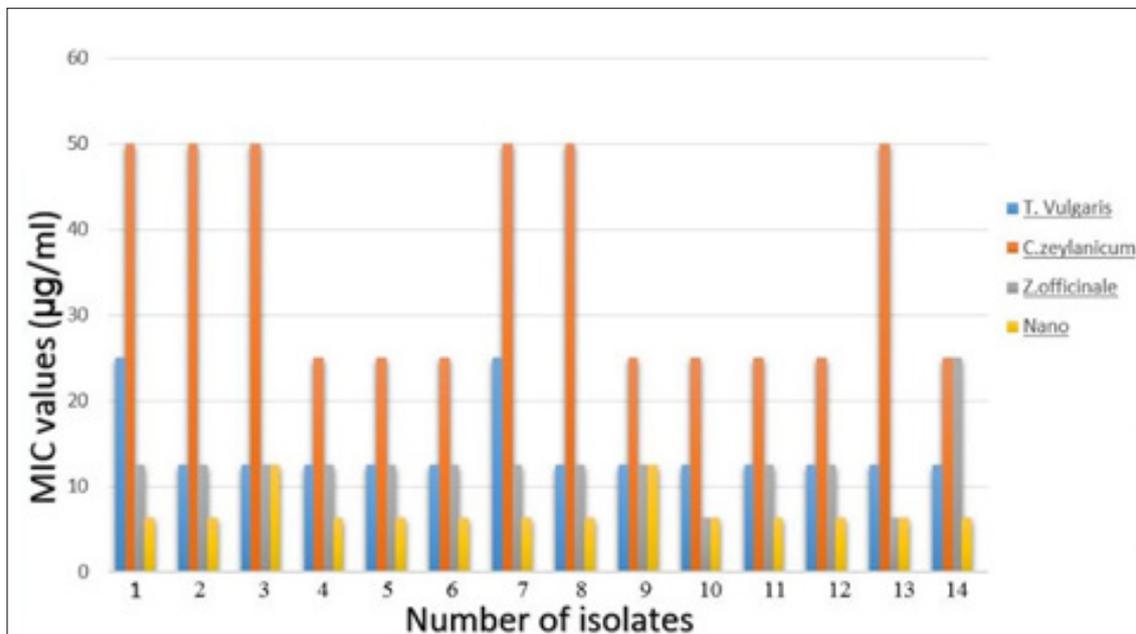


Figure 1. Minimum inhibitory concentration (MIC) value *K. pneumoniae*

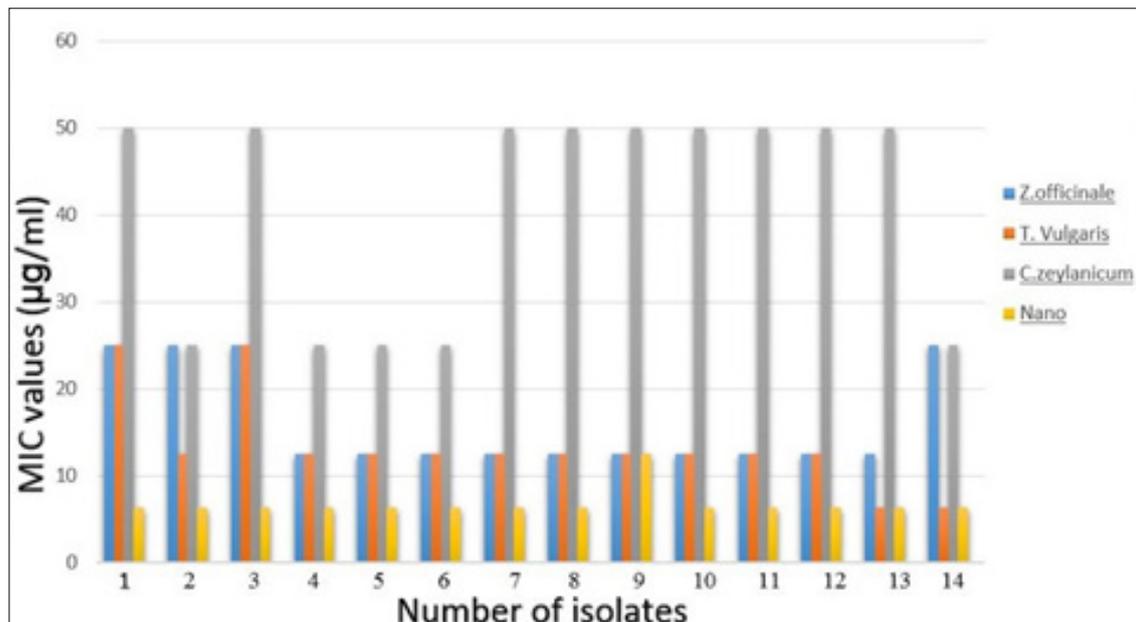


Figure 2. Minimum inhibitory concentration (MIC) value for *S. aureus*

Gao and Yang [25] reported the antibacterial activity of *Z. officinale* extract against various microorganisms was due to their polysaccharide, flavonoid compounds. The antimicrobial activity of ginger may be attributed to the fact that it contains antimicrobial substances such as zingiberol, zingiberine and bisabolene [26]. The rhizome of ginger contains pungent vanillyl ketones including gingerol and paradole, etc. [27].

Antibiofilm activity:

The results of *in vitro* biofilms activity of three phenolic plants extracts were presented in Figure 3 and Figure 4. For comparison, the results of the activity of Ag-NPs was also listed in Figure 3 and Figure 4. Furthermore, biofilm reduction assay results showed that Ag-NPs at 0.0596 $\mu\text{g/mL}$ could inhibit 100% of *K. pneumoniae* biofilm and at 0.053 $\mu\text{g/mL}$ decrease 50 % of biofilm formation.

Moreover, The biofilm reduction assay results showed that Ag-NPs at 0.0683 $\mu\text{g/mL}$ inhibited 100% the formation of *S. aureus* biofilm and at 0.050 $\mu\text{g/mL}$ decrease 50 % of the biofilm formation. It is noteworthy that the best plant extracts effective concentrations was 0.106 $\mu\text{g/mL}$ for *Z. officinale* extract on the growth of *K. pneumoniae* cells. Additionally, *Z. officinale* extract at concentration 0.150 $\mu\text{g/mL}$ was able to inhibit biofilm formation of *S. aureus* as depicted in Figure 4.

The difference in biofilm thickness results from different reasons such as differences in isolates capacity to form biofilm, perhaps the primary number of cells that succeeded in adherence and the differences of quality and quantity of autoinducers (Quorum sensing signaling molecules) that produced from each isolate and play an essential as well as important role in biofilm formation [28].

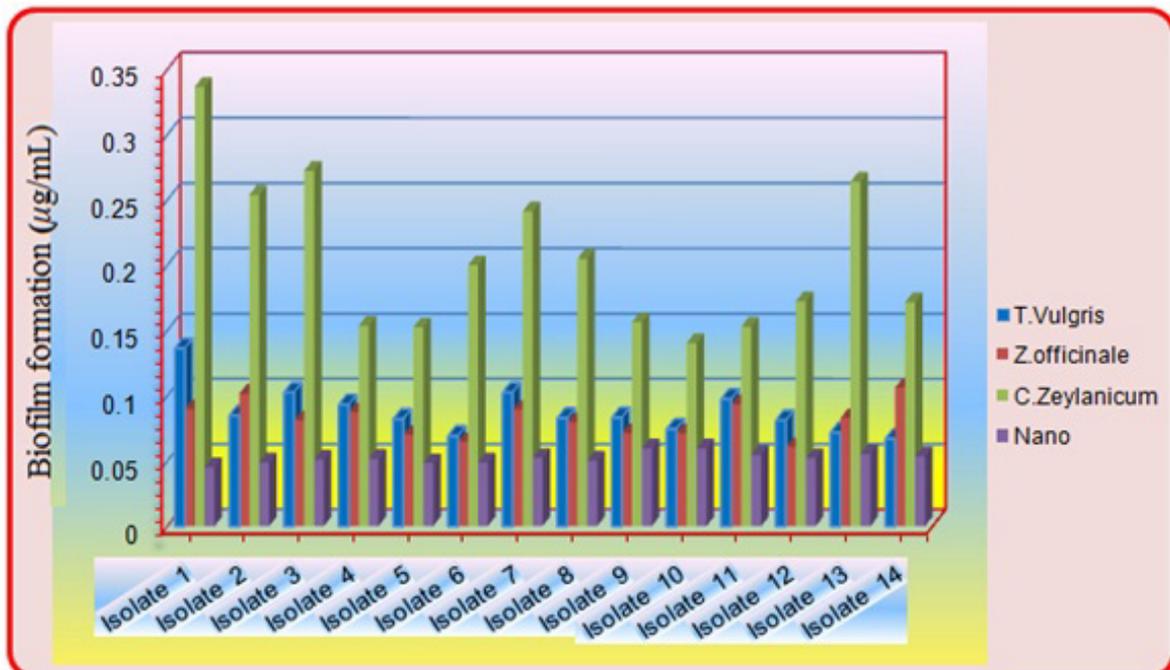


Figure 3. *Klebsiella pneumoniae* biofilm formation assays

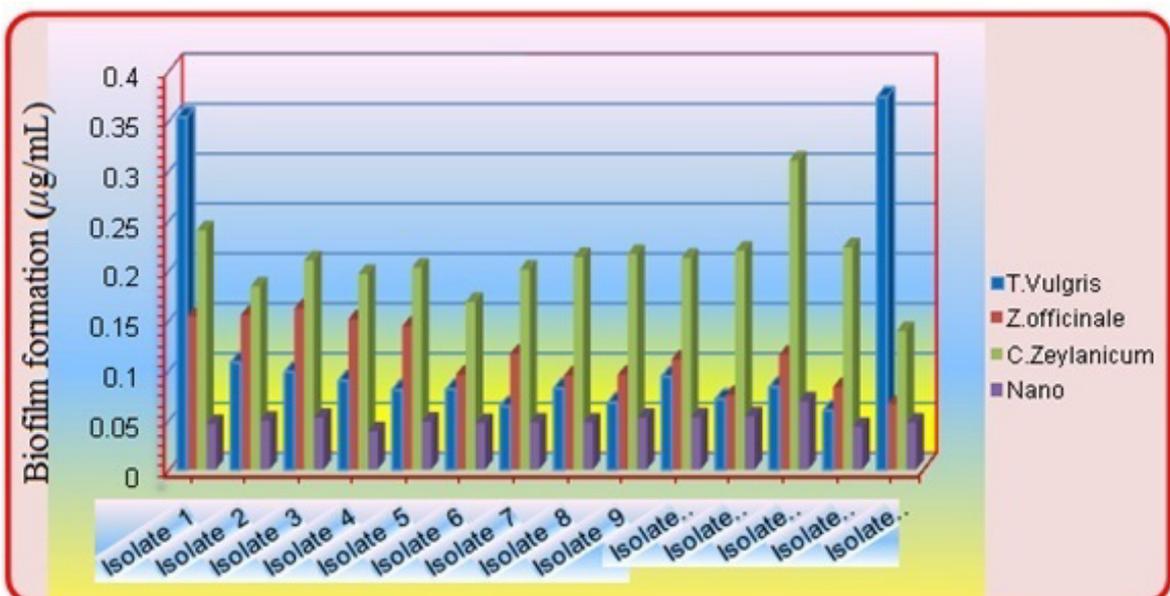


Figure 4. *Staphylococcus aureus* biofilm formation assays

4. Discussion

The Ag-NPs with plants extracts had shown to have significant effects on all examined bacterial isolates in terms of biofilm formation via bacterial growth. Many studies have reported that AgNPs can damage cell membranes leading to structural changes, which render bacteria more permeable. This effect is highly influenced by the nanoparticles' size, shape and concentration and a study using Gram-negative bacteria confirmed that AgNPs accumulation on the membrane cell creates gaps in the integrity of the bilayer which predisposes it to a permeability increase and finally bacterial cell death [24].

Further studies is required to make any further conclusion. It is possible that silver nanoparticles act similarly to the antimicrobial agents used for the treatment of bacterial infections. Those agents show four different mechanisms of action: (1) interference with cell wall synthesis, (2) inhibition of protein synthesis, (3) interference with nucleic acid synthesis and (4) inhibition of a metabolic pathway. Finally, the antimicrobial susceptibility of silver nanoparticles synthesized was investigated. The presence of nanoparticles at a certain level inhibited bacterial growth by more than 99 %.

The Ginger *Zingiber officinale* rhizome is rich in the secondary metabolites such as phenolic compounds (gingerol, paradol and shogaol), volatile sesquiterpenes (zingiberene and bisabolene) and monoterpenoids (curcumene and citral) [29]. Previous studies have demonstrated that plant extracts and isolated compounds from *Z. officinale* possess strong antioxidant [30].

Yahya *et al.* [31] mentioned that *Z. officinale* ethanolic extract able to inhibit *P. aeruginosa* biofilm formation under both aerobic and anerobic conditions. With respect to extracellular biofilm DNA. Also, they found that *Z. officinale* extract has affected the DNA release by *P. aeruginosa* biofilm. They believed that the anti-biofilm activity was due to the activity of zingiberene, the major compound in *Z. officinale* ethanolic extract.

Alves-Silva *et al* [32] revealed that thymol and carvacrol are the main constituents and responsible for their disinfection potential through a variety of inhibition and killing mechanisms, which target multiple sites of the bacterial cell will preferentially partition from an aqueous phase into membrane structures. This result in membrane expansion, increased membrane fluidity and inhibition of a membrane-embedded enzyme. Also, Nadia *et al.* [33] found many bioactive compounds such as phenolics, flavonoids, thymol, carvacrol, biphenyl's and aliphatic phenols in thymus species. They inducted that Phenolic acids are present in thymus species which contribute to their therapeutic potential. While Aghsaghali *et al.* [34] notes that Caffeic acid is present in *T. vulgaris* which has antibacterial, antiviral and antifungal activity. Also, the *C. zeylanicum* antimicrobial abilities was mainly attributable to the presence of phenolic components in their extract [35].

5. Conclusions

In conclusion we can say that, silver nanoparticles was capable of microbial inhibition on a commendable scale and this may hopefully be an indication of its potential

future as part of antimicrobial strategy. Investigation on large amount of clinical, environmental and commensally strains is required to understand the survival strategies biofilm producers. The silver nanoparticles with either the plants extract compound (phenolic compound) may provide a safe and highly effective alternative to commonly used antibiotics, which are ineffective towards the antibiotic-resistant *S. aureus* and *K. pneumoniae*. Thus, further studies are required to test their activity against other pathogenic bacteria and fungi and study the possibility of using these active components by drugs companies. Finally, further studies using *in vivo* models are needed to study the impact of nanoparticles on health.

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