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Antibiofilm Activity of Silver Nanoparticles Synthesized from Seed Extract of *Garcinia Kola*

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Abstract

Silver nanoparticles from plant extracts are novel compounds with potential antimicrobial properties. Studies on antibiofilm activity of Ag-NPs synthesized from seed extracts of *Garcinnia kola* (G. kola) were carried out. *Garcinnia kola* seed were obtained from Keffi market, Nigeria. Green synthesis of Ag-NPs from the seed was carried using 2.0mm silver-nitrate by use of standard method. The Ag-NPs synthesized from the seed were characterized using former transmission infrared (FITR) spectroscopy and scanning election microscope. The antimicrobial activity of the Ag-NPs against *Klebsiella pneumonia* (Kp) isolates were carried out using agar dilution method. The biofilm formation by the isolates as well as the inhibition and dissolution by Ag-NPs were evaluated using microplate method. The functional groups detected in the Ag-NPs were N-H, C-O, N-O, and CΞC with peaks 906.5cm^{-1},1282.2cm^{-2}, 13344cm^{-1}, 1550.6cm^{-1} and 217.1cm^{-1} respectively. The size of the particles ranges from 179-296nm. The minimum inhibiting concentration (MICs) of the particles and meropenem against the isolates were 250µg/l and

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4.0μg/l. The functional inhibiting concentrates of the particles were 1.0. The optical clarity of biofilm formed by the isolates was 2.073 and 2.049. the percentage biofilm inhibiting effects of the particles was highest apart. KpC (*K. Pneumoniae* ATCC BAA 1075) with percentage inhibit ranges from 27.28-21.67% at 80-12.5% of the MICs. The percentage inhibiting effect of Ag-NPs in with meropenem was highest at MICs but low in MIC 12.5 with percentage inhibition 28.26% and 27.18%. The Ag-NPs alone and antibacterial activity and biofilm inhibiting effect while Ag-NPs in with meropenem had effect but against isolate but with potential antibiofilm activity.

Keywords: Biofilm, Nanoparticle, *Garcinnia kola*, Ag-NPs, *Klebsiella pneumonia*

INTRODUCTION

Biofilms are complex surface attached communities of microorganisms held together by self-produced polymer matrixes mainly composed of polysaccharides, secreted proteins, and extracellular DNAs (Tremblay *et al.,* 2013). A biofilm can consist of a single microbial species or a combination of different species of bacteria, protozoa, archaea, algae, filamentous fungi, and yeast that strongly attach to each other and to biotic or abiotic surfaces (Bogino *et al.,* 2013; Silva *et al.,* 2014; Costa-Orlandi *et al.,* 2017; Raghupathi *et al.,* 2017). They are key contributors to the growing antibiotic resistance crisis and account for two-thirds of all infections (An AY *et al.,* 2021). Biofilms are also a major medical issue which cause 60–80% of microbial infections and present a unique challenge in regards to disease diagnosis and treatment (Hall-Stoodley *et al.,* 2012). They are also known to contribute to bacterial virulence by causing persistent and recurrent infections, highly resistant to antibiotics and host defence mechanism (Grant *et al.,* 2013).The antibacterial resistance in biofilm producing Multi-drug resistance (MDR) bacterial pathogens are due to several reasons such as; expression of multi-drug drug efflux pump, restricted diffusion of antibiotics into the biofilm matrix, decrease permeability and action of modified or inactivation enzymes (Eduardo *et al.,* 2018).

Plants have been reported as alternatives for treatment of infections caused by biofilm resistant bacteria aside from their antibacterial effects (Eduardo *et al.,* 2016; Ijewereme *et al.*, 2018). Historically, plants extract and their bioactive compounds have been a valuable source of natural product which play a vital role in the prevention and treatment of Drug resistant bacteria species, helping to maintain human health (Eduardo *et al.,* 2016). They are

also widely accepted due to the fact that they are safe and have long history of use as medicine to cure diseases and illness since ancient times (Rasamiravaka *et al.,* 2015).

The importance of *Garcinia Kola* for treatment of microbial diseases and other illness have been reported (Crellin *et al.,* 1989; Ademola and Eloff, 2011; Onyekwelu *et al.,* 2015) but studies on the antibiofilm properties of the plant have not been established. However, antibiofilm properties of other plants such as *Citrullus lanatus* have been reported in previous studies conducted by Ijewereme *et al.* (2018). Therefore, this research work aimed at evaluating antibiofilm activity of silver nanoparticles synthesized from seed extract of *Garcinia kola.*

METHODS

Collection and Preparation of *Garcinia kola*

The leaf of *Garcinia kola* was source from some selected farms in Keffi metropolis and air-dried at room temperature. The dried seeds were then grinded into fine powder with a mortar and pestle and stored in a plastic container for further use in accordance with the method described by Masfufatun *et al*., 2019.

Extraction of the *Garcinia kola* Extracts

The crude aqueous extracts of *Garcinia kola* were prepared following a method described by Sanchez *et al.* (2010). One hundred grams (100.0 g) of dried and powdered *Garcinia kola* were soaked in 500 ml of distilled water for 24 h at room temperature after which the extracts were filtered with Whatman filter paper No. 1 and then air-dried using water bath at 45^{o}C.

Synthesis of Silver Nanoparticles

The Ag-NPs were synthesized from *Garcinia kola* as follows; 10.0 g of the seed powder was added to 100 ml of sterilized deionized water and heated at 60^{o}C for 20 minutes. It was allowed to cool and then, filtered through Whatman filter paper No. 1. Fifty milliliter (50 ml) of the filtrate was added to 450 ml of 1 mM $AgNO_3$ solution and incubated at room temperature in the dark for 24 hours. After 24 hours incubation, the solution with amber or brown color, which indicates the presence of Ag-NPs was centrifuged at 4000 rpm for 20 minutes and then filtered through Whatman's filter paper No. 1. The resulted residue was dried at 60^{o}C for 5 hours and stored at 4^{o}C for further use.

Characterization of Silver Nanoparticles

The synthesized Ag-NPs were characterized using Fourier-transform infrared spectroscopy (FT-IR). Spectra of silver nanoparticles were measured using FTIR spectrometer with a KBr bullet to investigate the chemical composition of the nanoparticles. Furthermore, the size of the nanoparticles was evaluated by the Debye-Scherrer formula (Ajitha, *et al.,* 2014) using the scanning electron microscope (SEM).

Determination of Antibacterial Activity of Synthesized Silver Nanoparticles (Ag-NPs) and Meropenem The antibacterial activity of synthesized silver nanoparticles (Ag-NPs) and meropenem against the carbapenemase resistant isolates were carried out using agar dilution method as described by Irith *et al,* (2008). Different concentrations of Ag-NPs usually 500µg - 31.25 µg and meropenem usually 512 µg - 0.5 µg were prepared in MHA plates and 10 µl of (10^5cfu). The test organism adjusted to the turbidity equivalent to McFarland standard 0.5 standards were inoculated into each plate and the plates were incubated at 37^0C for 24h. The minimum that inhibited the growth of test organism were read as the MICs. The McFarland standard was prepared as follows, 0.5 µl of 1.172 ($^w/_v$) $BaCl_2$ $2H_2O$ was added to 99.5 ml of 18 H_2SO_4.

Evaluation of Antibacterial Activity of Combination of Silver Nanoparticles and Meropenem

The antibacterial activity of combination of Ag-NPs and meropenem against the test organism were carried out using agar dilution method, different concentration of combine MICs of Ag-NPs and meropenem usually 2xMIC, 1xMIC, ½ x MICs $^1/_8$xMICs and $^1/_{16}$ MICs were prepared in MHA plates and 10µl (CFU) of the test organism were inoculated into each of the plates and the fractional inhibitory concentration (FICs) was determined. Synergistic, antagonistic and indifference effect of combination were interpreted as follows; 0-0.5 synergistic effect, 1.0-2.0 antagonistic effect while >2.0 indifference effect

Assessment of Biofilm Formation

The assessment of biofilm formation was done using the micro-titer plate method. 0.1mL of bacterial culture obtained by adjusting turbidity to 0.5 McFarland standards was transferred to micro titer wells containing 10mL Brain Heart Infusion agar with 2%

sucrose, which were incubated at 37°C for 24 hours. The medium was then removed and the wells were washed three times with distilled water, air-dried and biofilm formation was assayed by crystal violet (Pour *et al.,* 2011). The optical density (OD) of each was measured at 570 nm. Results were interpreted according to the followings criteria; OD <0.500 (-), OD 0.500-1.500 (+), OD >1.500 (++).

Anti-biofilm activity of Synthesized Silver Nanoparticles (Ag-NPs) from *Garcinia kola* Extract

The effect of the crude Ag-NPs from *Garcinia kola* on biofilm formation was examined following the method of Yarwood *et al.*(2004) using the micro-titer plate assay with a little modification. The appropriate concentration of plant extracts usually 80%, 50%, 25% and 12.5% MICs of Ag-NPs from *Garcinia kola* were prepared in 96 well micro-titer plates containing double strength Brain Heart Infusion agar supplemented with 2% sucrose and 5 µl of the standardized test organism was inoculated into each well and incubated for 24 hours at 37°C. After incubation, the growth medium was discarded, and the wells were washed thrice with sterile physiological saline (0.85% NaCl). The adhered cells were further stained with 0.1% crystal violet for 10 minutes. The excessive stain was removed by washing twice with 0.85% NaCl and allowed to dry. The wells were distained using 200µl absolute ethanol and the absorbance measured at 578 nm using a micro-titer plate reader.

Evaluation of Synergistic Activity of Silver Nanoparticles with Carbapenem Antibiotic Agent

The synergistic activity of synthesized crude aqueous Ag-NPs with Meropenem was tested following agar dilution method as described by Irith *et al.,* 2008. Different concentration of the Ag-NPs such as 5000µg/ml, 2500µg/ml, 1250µg/ml, 625µg/ml and 312.5µg/ml was dispensed into sterile water by serial dilution. Different concentrations of the meropenem such as 32.0µg/ml, 16.0µg/ml, 8.0µg/ml, 4.0µg/ml and 2.0µg/ml were also dispensed in sterile water by serial dilution. Each concentration (i.e. 10^{-1} dilution of both Ag-NPs and meropenem) was mixed with a double-strength Mueller-Hinton agar, poured in petri dishes and allowed to set. A sterile loop was used to transfer 10 µl of the bacterial suspension onto the surface of the agar and incubated at 37°C for 24 h. After which the agar plates were checked for presence of bacteria growth. The minimum concentration of the synthesized Ag-NPs and meropenem that inhibits the visible growth of the test organism

was read as the MICs. 80%, 50%, 25% and 12.5% of the MIC were further tested using the micro titer plate method and further measured using a micro-titer plate reader.

RESULTS

Table 1: The cultural, morphological and biochemical characteristics of *Klebsiella pneumoniae*

Cultural characteristics	Morphological characteristics		Biochemical characteristics				Inference
	Gram Staining	Morphology	IN	MR	VP	CT	
Pinkish Mucoid Colonies on EMB Agar	-Ve	Rod shape	-	+	+	-	*Klebsiella pneumoniae*

EMB = Eosin Methylene Blue, IN = Indole, MR = Methyl Red, VP = Voges Proskauer, CT = Citrate, - = Negative, + = Positive.

Table 2: The Minimum Inhibitory Concentrations (MICs) of silver nanoparticles synthesized from leaf extracts of *G. kola* and meropenem against Carbapenemase producing *K. pneumoniae* isolates.

ISOLATES	**Crude Ag-NPs MICs (µg/ml)**	**Aqueous Ag-NPs MICs (µg/ml)**	**Meropenem MICs (µg/ml)**
K. pneumoniae ATCC BAA 1705	500	250	4.0
*K. pneumoniae*1 (Kp1)	500	250	4.0

Table 3: The Fractional Inhibitory Concentrations (FICs) of combination of Crude Ag-NPs and meropenem against carbapenemase producing isolates

ISOLATES	**FICs (µg/ml)**	**INFERENCE**
K. pneumoniae ATCC BAA 1705	1.0	Antagonistic effect
*K. pneumoniae*1 (Kp1)	1.0	Antagonistic effect

Table 4: The Fractional Inhibitory Concentrations (FICs) of combination of Aqueous Ag-NPs and meropenem against carbapenemase producing isolates

ISOLATES	FICs (µg/ml)	INFERENCE
K. pneumoniae ATCC BAA 1705	1.0	Antagonistic effect
*K. pneumoniae*1 (Kp1)	1.0	Antagonistic effect

Evaluation of Biofilm Formation

The formation of biofilm by the carbapenemase producing *K. pneumoniae* is as shown in Figure 1. The carbapenemase producers form strong biofilm with optical density from 2.049 ± 0.071 to 2.073± 0.16.*K. pneumoniae* ATCC BAA 1705 had the lowest optical density of 2.049 ± 0.071 as shown in Figure 1.

Inhibition of Biofilm Formation

The percentage inhibition of biofilm by silver nanoparticles from crude extract of *G. kola* and combination of the silver nanoparticles with meropenem at different concentrations of MIC_{80}, MIC_{50}, MIC_{25}, and $MIC_{12.5}$ is as shown in Figure 4.3 and 4.4 respectively. The percentage inhibition of the silver nanoparticles was highest at 80% MIC ranging from 42.35-24.2% while the lowest was at 12.5% MIC with percentage inhibition ranging from 21.13-14.52% as shown in Figure 4.3.For combinations of crude Ag-NPs and meropenem, the percentage inhibition was highest at 80% MIC against *K. pneumoniae* ATCC BAA 1705 at 28.79% as shown in Figure 3.

The percentage inhibition of biofilm by silver nanoparticles from aqueous leaf extract of *G. kola* and combination of the silver nanoparticles with meropenem at different concentrations of MIC_{80}, MIC_{50}, MIC_{25}, and $MIC_{12.5}$ is as shown in Figure 4 and 5 respectively. The percentage inhibition of the silver nanoparticles was highest at 80% MIC ranging from 27.28-23.35% while the lowest was at 12.5% MIC with percentage inhibition ranging from 21.67-11.86% as shown in Figure 4.5.For combinations of aqueous Ag-NPs and meropenem, the percentage inhibition was highest at 80% MIC against *K. pneumoniae* ATCC BAA 1705 at 28.64% and highest at 80% MIC against *K. pneumoniae*1 at 29.32% as shown in Figure 5.

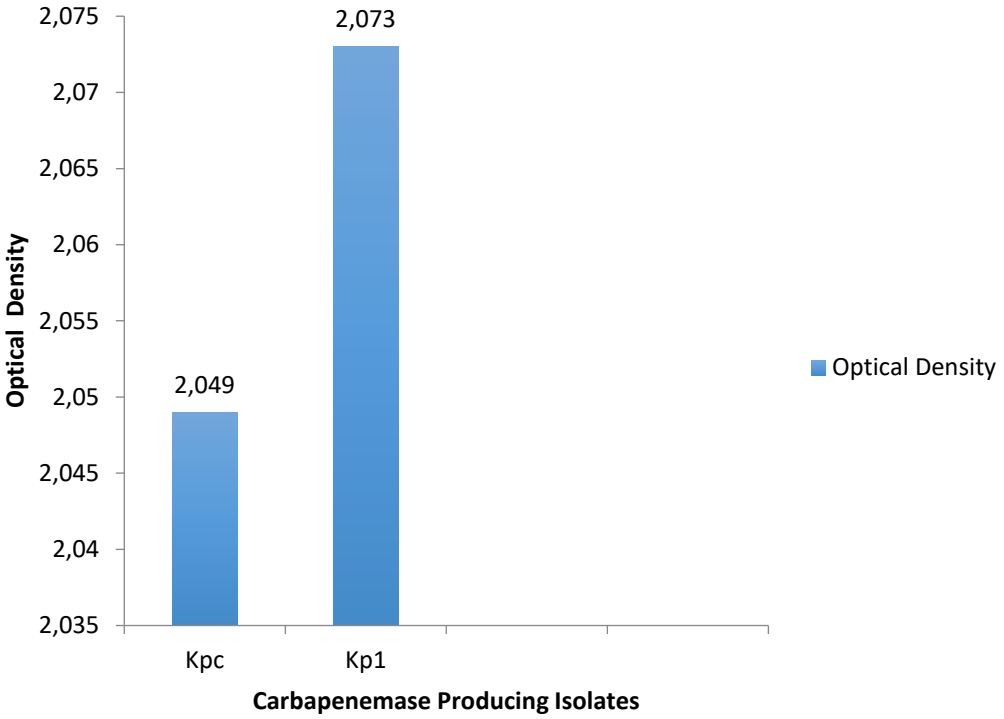


Figure 1: The optical density of the biofilm formed by Carbapenemase producing *K. pneumoniae*

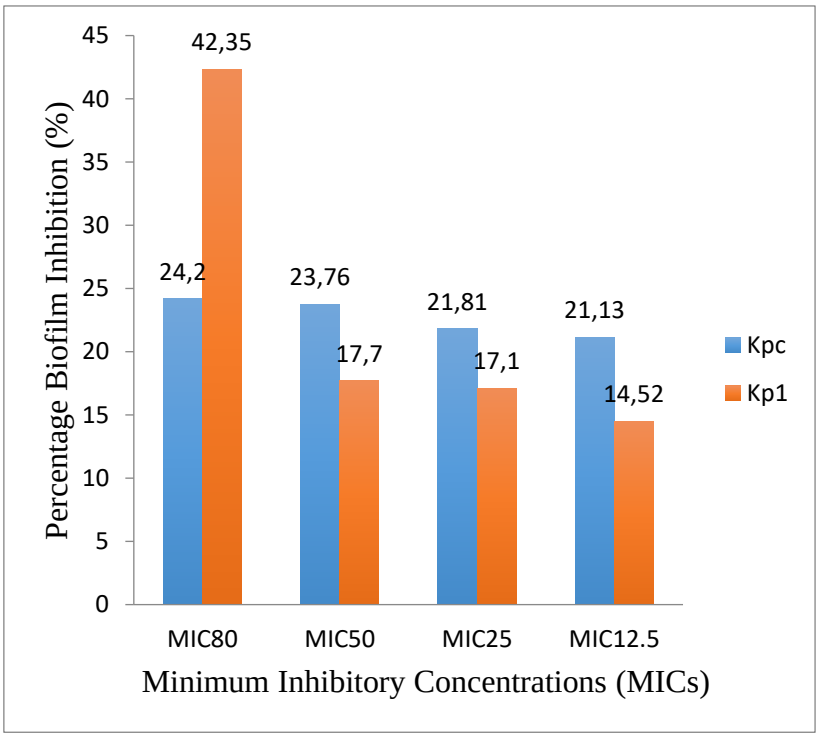


Figure 2: The percentage biofilm inhibition by 80%, 50%, 25% and 12.5% of the MIC of silver nanoparticles from crude extract against the carbapenemase producing isolates

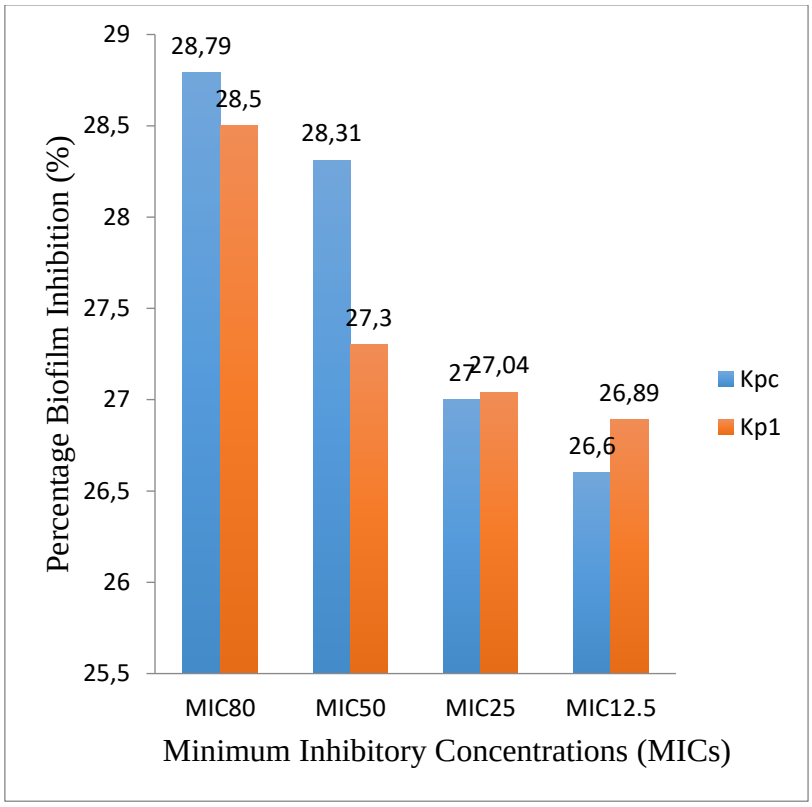


Figure 3: The percentage biofilm inhibition of the MIC of silver nanoparticles from crude extract in combination with meropenem against the carbapenemase producing isolates

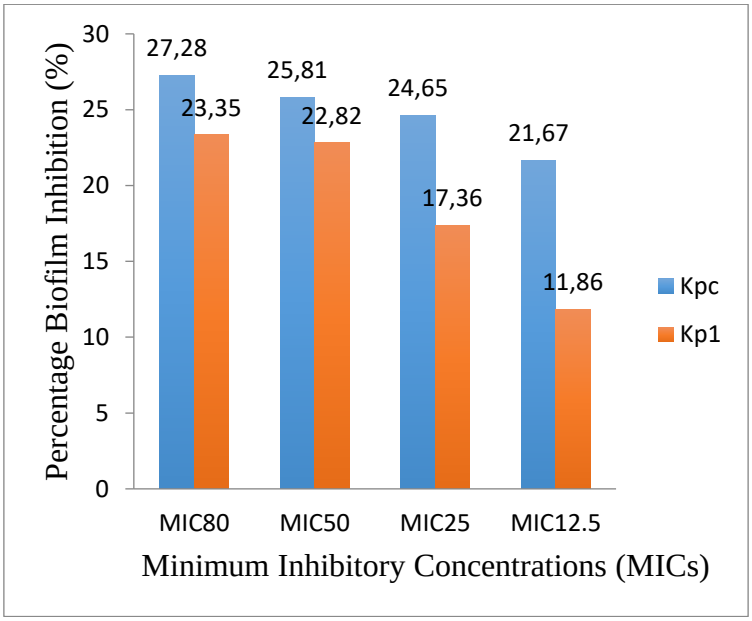


Figure 4: The percentage biofilm inhibition by 80%, 50%, 25% and 12.5% of the MIC of silver nanoparticles from aqueous extract against the carbapenemase producing isolates

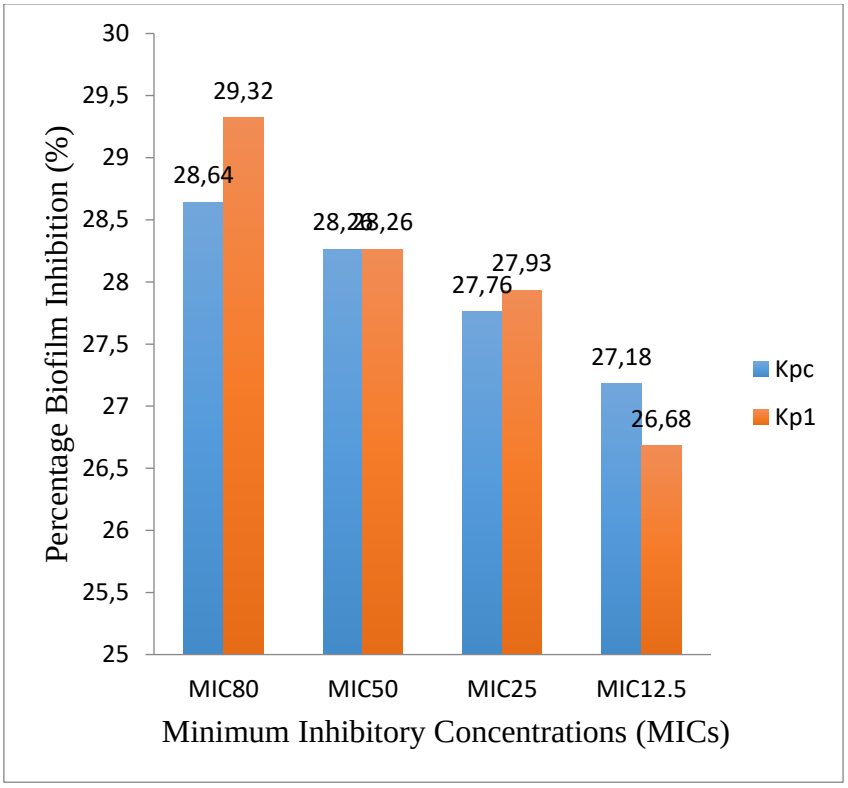


Figure 5: The percentage biofilm inhibition of the MIC of silver nanoparticles from aqueous extract in combination with meropenem against the carbapenemase producing isolates.

DISCUSSION

The growing occurrence of antibacterial drug resistance by most bacteria has triggered a public health concern (Bennani *et al.,* 2020, Buchy *et al.,* 2020) hence; extensive research has been dedicated to searching for an alternative remedy to bacterial infections (Nawab, *et al.,* 2020). In this study, crude and aqueous seed extracts of *G. kola* were synthesized into silver nanoparticles using the cold maceration method. Successful synthesis of AgNPs was confirmed by visual observation of a color change of the solution, in which the pale-yellow color of the mixture of extract and $AgNO_3$ turned to a deep brown color.

According to Ajitha, *et al.,* (2014), FTIR analysis was carried out on the silver nanoparticles to test for the functional groups present and the AgNPs from crude extract has peaks ranging from 723.1-3362.1cm^{-1} with dominant functional group to be amines (N-H) with stretching vibrations while for aqueous AgNPs, the peaks range from 906.5-3190.6cm^{-1} with the functional groups to be amines, ester, alkanes, carboxylic acid and alkanes.

Structural mechanism reveals that free amine groups in the saponins and phenolics present in the extracts have the ability to bind to the AgNPs and stabilize them through the surface-binding of a variety of plant compounds (Saranyaadevi *et al.,* 2014). Using scanning electron microscope (SEM), the mean size of the synthesized Ag-NPs of the crude and aqueous extracts based on the scanning electron microscopy was 224nm, which could have contributed to the efficacy of the silver nanoparticles.

The antibacterial assay of extracts of the plants was performed individually by agar dilution method according to Maragathavalli *et al.* (2012). The fractional inhibitory concentrations of the combination of the AgNPs with meropenem to combat carbapenemase producing isolates had antagonistic effects on the isolates, hence, the AgNPs synthesized by the plant extracts alone were more promising than the combination with over the counter antibiotics. Multiple reports support the use of AgNPs as antibacterial agents (Abbasi *et al.*, 2016, Nayak *et al.*, 2016) and several mechanisms have been proposed regarding the antibacterial activity of AgNPs. Earlier studies by Sondi and Salopek-Sondi (2004) focused on the interaction of AgNPs with *E.coli* and confirmed that at the first stage of interaction, AgNPs attach to the bacterial cell wall. After stable adherence, AgNPs penetrate the bacterium and induce cell death by rupturing the cell membrane. AgNPs acting as oxidizing agents on the surface of proteins present on the plasma membrane and cellular homeostasis have also been suggested as the mechanism underlying AgNP antibacterial activity.

Biofilm dissolution by the extracts and synthesized silver nanoparticles was evaluated as described by Bazargani and Rohloff (2016).Treatment of *K. pneumoniae* for 24 h with AgNPs (100 µg/ml) synthesized using *G. kola* extracts, reduced biofilm formation by <50%.Limited research has been conducted on the anti-biofilm activity of AgNPs. In the antibacterial activity of the silver nanoparticles, it showed a greater potential effect against the carbapenem resistant isolates but showed reduced activities in the biofilm inhibition and the synergistic application with meropenem. However, there are many synergetic applications of the AgNPs along with current commercial antibiotics with different concentrations that show more antibacterial activities (Hussain *et al.,* 2019, Rolim *et al.,* 2019). The upcoming application of nanoparticles is highly promising as the uncontrolled spread of microbial contaminations is now a great threat worldwide.

CONCLUSION

In this study, plant extracts derived from *Garcinia kola* were used to synthesize AgNPs. The use of the plant extracts has an advantage over chemical or physical synthesis of AgNPs due to their ability to stabilize AgNPs, their own antibacterial properties, their high level of efficacy, and their low toxicity. The plant-derived AgNPs exhibited strong antibacterial and inhibition of biofilm activity against clinically important human pathogens. Hence, it is a safer and more advantageous method to combat antibiotics resistance isolates and this line of research should be more greatly explored in the biomedical sciences.

Recommendations

i. Further exploration and research on the use of plant extracts to synthesize metal nanoparticles for antibacterial and antibiofilm purposes.

ii. Synergistic combination of silver nanoparticles and carbapenem drugs should not be administered for treatment without testing the sensitivity of the isolates on the combined drugs.

iii. Lastly, G. kola is a very remarkable medicinal plant with a variety of traditional usage that has been documented since antiquity; it contains nutritionally and pharmacologically essential compounds. Research into the mechanisms behind the bioactivity of the constituent chemical components is required.

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