

Antibacterial Screening of Amoxicillin/Clavulanic acid functionalized silver nanoparticles synthesized by the microalgae *Bryopsis pennata* and *Caulerpa taxifolia*

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Abstract: The loss of antibiotics efficacy over common infections has raised concerns and resulted in significant research efforts to the search for new antibiotics or chemically altering existing ones for a better control of infectious diseases. In this study, the aqueous extracts of *Bryopsis pennata* and *Caulerpa taxifolia* were used to synthesize silver nanoparticles. These nanoparticles were functionalized with Amoxicillin/Clavulanic acid (amoxiclav). UV/visible spectroscopy was used to monitor the synthesis of the silver nanoparticles. The organic surface groups responsible for the capping and stabilization of the nanoparticles were analyzed using Fourier transmission infrared spectroscopy (FTIR). Scanning Electron Microscopic (SEM) studies showed that the silver nanoparticles formed had sizes in the range of 7 nm to 65 nm. The aqueous extracts of *Bryopsis pennata* and *Caulerpa taxifolia* showed very low activity against *Escherichia coli*, *Klebsiella oxytoca* and *Bacillus mycoides*. *Salmonella enterica*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* with values for the zone of growth inhibition ranging from 7 mm to 15 mm for both extracts at the maximum concentration of 500 µg/mL. Silver nanoparticles exhibited much higher activity than their respective extracts as the zone of growth inhibition values ranged between 23 mm to 26 mm at the maximum concentration. The activity of Amoxicillin/Clavulanic acid was improved when conjugated with silver nanoparticles with very high values for the zone of growth inhibition ranging from 30 mm to 34 mm at the maximum concentration of 500 µg/mL. Therefore, the functionalization of silver nanoparticles with antibiotics is medically important and can be used to improve the activity of existing antibiotics.

Keywords: Antibiotic resistance; Silver nanoparticles; Microalgae; *Bryopsis pennata*; *Caulerpa taxifolia*; Amoxiclav Functionalization.

1. Introduction

The use of nanoscale materials in different forms and distributions in various biological applications have gained significance in recent years [1]. Conventional physical and chemical preparation methods used in producing nanomaterials are generally expensive, time-consuming and potentially hazardous to the environment [2]. Cost-efficient, environmentally benign procedures are essential with regards to applications of metal nanoparticles. Biosynthetic processes mediated by micro-organisms such as fungi, bacteria, yeasts and actinomycetes or macroorganisms such as plants and algae are considered as novel approaches for producing metal nanoparticles [3]. Plant mediated synthesis of metal nanoparticles has been shown to be a facile and cost-efficient procedure in that, plants are readily available and the phytochemicals are less toxic and may not require high temperatures for synthesis [4].

Silver nanoparticles are widely recognized in nanoscience as one of the materials with remarkable physical, chemical and antimicrobial properties. Many studies have investigated the activity and engrossing application of silver nanoparticles in diverse fields such

as water treatment, textiles, food packaging, cosmetics, ointments, photocatalysis and drug development to tackle antimicrobial resistance [5]. The increase in antibiotic resistance due to inefficacy of commonly known antimicrobials has driven researchers to develop functionalized materials with strong and efficacious antimicrobial properties [6].

The rapidly growing problem of antibiotic resistance has inspired the development of novel and more effective antimicrobial strategies. Recent studies have showed that the combination of antibiotics with silver nanoparticles increases the effectiveness of antibacterial therapy [7]. The combination of kanamycin with silver nanoparticles shows improved antibacterial activity against *Pseudomonas aeruginosa* [8]. Silver nanoparticles functionalized with ampicillin have been showed to exhibit increased activity against drug resistant *P. aeruginosa* and *E. aerogenes* [9].

Recent studies on biologically synthesized silver nanoparticles show that the antibacterial activity of bare silver nanoparticles increase significantly when they are functionalized with antibiotics, such as tetracycline, ampicillin, kanamycin, gentamicin, streptomycin and

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vancomycin [10]. This study involves the synthesis of silver nanoparticles using the aqueous extract of the microalgae, *Bryopsis pennata* and *Caulerpa taxifolia*. The silver nanoparticles were functionalized with amoxicillin/clavulanic acid and comparative evaluation of the antimicrobial activity of the as-synthesized and antibiotic functionalized silver nanoparticles was performed to determine the extent to which the silver nanoparticles enhance the efficacy of amoxicillin/clavulanic acid against antibiotic resistant bacteria.

2. Material and methods

2.1. Collection of algae

The species of algae were collected by detaching them from rock surfaces using a knife from Paradise beach (5° 46' 32.97", 0° 38' 50.85") and Sakumono beach (5° 36' 59.9", 0° 02' 60.0"). The samples were immediately kept in an ice box and transported to the laboratory where it was sorted out and washed thoroughly. The samples were properly identified and authenticated by a marine botanist at the University of Ghana and they were kept frozen until the extraction period.

2.2. Preparation of aqueous extract

The algal samples, *Bryopsis pennata* and *Caulerpa taxifolia* were thoroughly washed and crushed using a mortar and a pestle. A wet mass of 70.13 g of *Bryopsis pennata* was subjected to maceration in 300 mL of dichloromethane: methanol (2:1) and 147.7 g of *Caulerpa taxifolia* was subjected to maceration in 600 mL of dichloromethane: methanol (2:1) in respective conical flasks and was left in a water bath overnight at 37 °C for 24 hours to obtain a solution and allowed to cool. The extract was passed through a muslin cloth and then poured into a separating funnel. The aqueous extract of the respective algae was obtained and filtered. The filtrate was collected and were stored at -4 °C until ready for use.

2.3. Phytosynthesis of Ag NPs

Ag⁺ ions were separately reduced by addition of 2.5 mL of either *Bryopsis pennata* or *Caulerpa taxifolia* aqueous extract to 50 mL of 10⁻³ M aqueous AgNO₃ solution in a 100 mL Erlenmeyer flask and kept for incubation at 35 °C for 60 min. The reaction process was carried out in the dark to prevent side photochemical reactions. The color change of the AgNO₃ solution from colorless to dark brown was observed and the obtained silver nanoparticles (AgNPs) was confirmed by Ultraviolet-visible spectroscopy. The obtained Ag NPs were purified through repeated centrifugation at 10000 rpm for 20 min and dispersion of the pellet in deionized water to remove unbound particles and further used for characterization.

2.4. Phytochemical screening

The aqueous extract of *Bryopsis pennata*, *Caulerpa taxifolia* and their respective derived silver nanoparticles were investigated for the presence of phytochemicals viz. alkaloids, carbohydrates, proteins, saponins, phenol, amino acids, tannins, diterpenes and phytosterols by following standard biochemical methods [11].

2.5. Characterization of Ag NPs

Synthesis of Ag NPs was confirmed by Ultraviolet-visible spectroscopic analysis. The absorbance spectra were recorded via Ultraviolet-visible spectroscopy (UV-1800 Shimadzu UV spectrophotometer) at a wavelength range of 300–700 nm. Fourier Transform Infrared Spectroscopy (FTIR) was performed on Thermo scientific Nicolet 50 FTIR Spectrophotometer to detect the possible functional groups in biomolecules attached to the silver nanoparticles. The X-ray diffraction (XRD) measurement was performed on Panalytical Xpert-PRO X-ray diffractometer operated at 30 kV was recorded by CuK α radiation at 1.5406 Å in the 2 θ range of 20°–80°. The morphology and size of the AgNPs were examined using a scanning electron microscope (SEM). The particle size distribution of the AgNPs were determined using particle size analyzer (Zetasizer nano ZS, Malvern at 25 °C, 90° detection angle).

2.6. Functionalization of AgNPs

Amoxicillin/Clavulanic acid (Co-amoxiclav) with trade name AugmentinTM was used as the functionalizing agent. 2 mg/mL aqueous solution of Co-amoxiclav was prepared and 10mL of Co-amoxiclav solution was transferred into a conical flask. Then, 10 mL of silver nanoparticles of concentration 1 mg/mL was added to the Co-amoxiclav solution and incubated for 24 hours. The solutions were centrifuged at 4000 rpm for 5 min and washed with ethanol to remove any loose particles. The process of centrifugation and washing were carried out thrice to achieve a better separation of nanoparticles. The obtained silver nanoparticles were air dried for 3 hours. The silver nanoparticles were further grinded in a crucible and stored at 4 °C in Eppendorf tubes.

2.7. Antimicrobial screening

The antibacterial assays of the phytosynthesized AgNPs was assessed by using the Kirby–Bauer method against human pathogenic bacteria *Bacillus mycoides*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella oxytoca*, *Salmonella Subsp3B* and *Escherichia coli* grown in Mueller-Hinton Agar medium at 37 °C for 24 hours [12].

Freshly cultured bacterial colonies of tested bacteria were taken and 100 μ L of inoculum was spread on each Mueller-Hinton agar plates. Sterile Whatman filter paper disks (6 mm in diameter) were loaded with 500, 250, 125, 62.5 and 31.25 μ g/disk of synthesized AgNPs and Amoxiclav/AgNPs. The algae extract and Amoxiclav were

used as control in each plate and incubated at 37 °C for 24 hours. The plates were examined for presence of zones of inhibition, indicated by clear area around the discs. The diameters of inhibition zones were measured and the mean value for each organism was recorded.

3. Results and discussion

3.1. Phytochemical screening

The results of qualitative screening of phytochemicals in the extract of *Bryopsis pennata*, *Caulerpa taxifolia* and their respective AgNPs are shown in (Table 1). Phytochemical profile of both *Bryopsis pennata* and *Caulerpa taxifolia* revealed the presence of carbohydrates, alkaloids, saponins, proteins, amino acids, phenol, diterpenes, tannins and phytosterols. Synthesized AgNPs from *Bryopsis pennata* and *Caulerpa taxifolia* showed the presence of phytosterols, diterpenes, phenols, and proteins except for the presence of tannins on AgNPs from *Caulerpa taxifolia*. These phytochemicals present on the AgNPs may be responsible for the efficient capping and stabilization of the silver nanoparticles.

3.2. UV/Visible spectroscopic (UV/Vis) analysis of silver nanoparticles

The synthesis of the AgBPs and AgCTs in aqueous solution was monitored by UV/Vis analysis at a

wavelength range of 300-650 nm (Fig.1). It was observed that solution of silver nitrate turned dark brown on addition of algae extracts of *Bryopsis pennata* and *Caulerpa taxifolia* which indicated the formation of AgNPs. In the UV-Vis spectra for AgBP and AgCT, strong and broad surface plasmon resonance (SPR) peak was observed at 420 nm and 417 nm respectively. Over 60 min, the absorbance of the peaks increased, signifying the increase in concentration of silver nanoparticles.

3.3. Fourier Transform Infra-red spectroscopic analysis of silver nanoparticles

As-synthesized silver nanoparticles from *Bryopsis pennata* (AgBP) aqueous extract exhibited a frequency peak at about 3480 cm^{-1} due to O-H stretching (Fig.2). The peaks at 2853 cm^{-1} , 1625 cm^{-1} and 1483 cm^{-1} can be linked to C-H stretching and bending vibrations for CH_2 and CH_3 groups, respectively. The weak and intense bands at 1273 cm^{-1} and 1160 cm^{-1} are most likely due to the stretching vibrations of C-O. For as-synthesized nanoparticles from *Caulerpa taxifolia* (AgCT), the peak at about 3365 cm^{-1} is due to O-H stretching. The peak at 2925 cm^{-1} is due to C-H stretching. The intense peak at 1630 cm^{-1} can be attributed to C=C stretching vibration. The two peaks recorded at 1378 cm^{-1} and 1325 cm^{-1} are most likely due to the presence of CH_2 and CH_3 groups. The peak at 1082 cm^{-1} is due to the stretching vibrations of C-O.

Table 1

Phytochemicals present in the aqueous extract of *Bryopsis pennata* and *Caulerpa taxifolia*.

Phytochemicals	<i>Bryopsis pennata</i>	<i>Caulerpa taxifolia</i>	AgBP	AgCT
Carbohydrates	+	+	-	-
Alkaloids	+	+	+	+
Saponins	+	+	-	-
Proteins	+	+	+	+
Amino acids	+	+	-	-
Phenols	+	+	+	+
Diterpenes	+	+	+	+
Tannins	+	+	-	+
Phytosterols	+	+	+	+

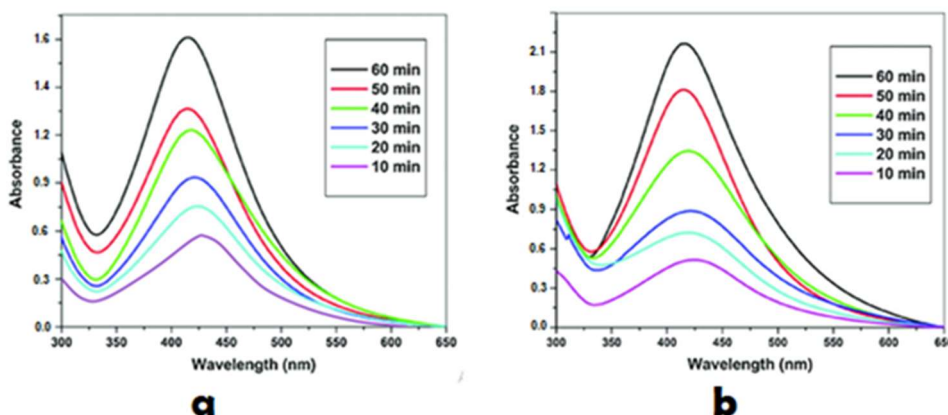


Fig.1. UV-visible spectra for the synthesis of silver nanoparticles from a) *Bryopsis pennata* and b) *Caulerpa taxifolia*.

3.4. Scanning Electron Microscopy analysis of silver nanoparticles

Scanning Electron Microscopy (SEM) studies revealed the irregular nature of particles synthesized from silver metal derived using *Bryopsis pennata* and *Caulerpa taxifolia* (Fig.3). For silver nanoparticles derived using *Bryopsis pennata* had sizes ranging between 15 nm to 65 nm with some showing 3D cuboid morphology while silver nanoparticles derived using *Caulerpa taxifolia* had sizes ranging between 7 nm to 30 nm with indefinite morphology.

3.5. Anti-microbial screening

The antibacterial activities of the aqueous extracts of *Bryopsis pennata* and *Caulerpa taxifolia*, AgBP, AgCT and their respective Amoxiclav functionalized AgNPs were investigated against a range of pathogenic microorganisms using agar disk diffusion method. For the aqueous extracts of *Bryopsis pennata* and *Caulerpa*

taxifolia, the diameter of inhibition zone in millimeter is shown in (Fig.4). The aqueous extracts of *Bryopsis pennata* and *Caulerpa taxifolia* were active against all tested bacteria. The antimicrobial activity of the *Bryopsis pennata* extract was particularly pronounced against *Salmonella enterica*, *Pseudomonas aeruginosa* and *Escherichia coli*. The *Bryopsis pennata* aqueous extract at the maximum concentration of 500 µg/mL had values for the zone of growth inhibition ranging from 7 mm to 12 mm for the six pathogenic resistant bacterial strains. The aqueous extract of *Caulerpa taxifolia* generally exhibited more activity than that of *Bryopsis pennata*. The antimicrobial activity of the *Caulerpa taxifolia* extract was particularly pronounced against *Escherichia coli*, *Salmonella enterica*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The *Caulerpa taxifolia* aqueous extract at the maximum concentration of 500 µg/mL had values for the zone of growth inhibition ranging from 10 mm to 15 mm for the six pathogenic resistant bacterial strains.

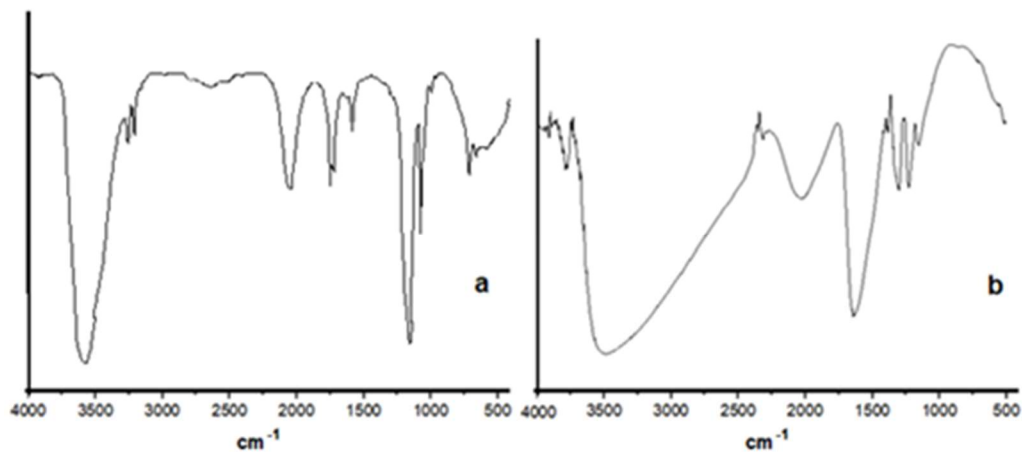


Fig.2. FTIR spectra of silver nanoparticles from a) *Bryopsis pennata* and b) *Caulerpa taxifolia*.

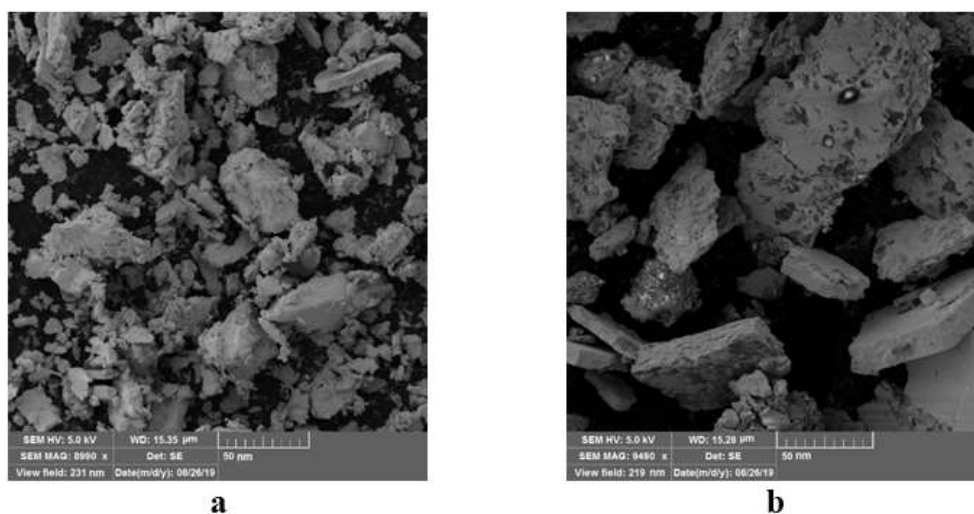


Fig.3. SEM images of silver nanoparticles from a) *Bryopsis pennata* and b) *Caulerpa taxifolia*.

For the silver nanoparticles derived with the aqueous extract of *Bryopsis pennata* and *Caulerpa taxifolia*, the diameter of inhibition zone in millimeter is shown in (Fig.5). Antibacterial activity of the silver nanoparticles synthesized using the algal extracts were studied against six pathogenic resistant bacterial strains with well diffusion assay. Superior antibacterial activity relative to the algal extracts was observed.

The *Bryopsis pennata* and *Caulerpa taxifolia* derived silver nanoparticles were active against all tested bacteria. The antimicrobial activity of the silver nanoparticles derived using *Bryopsis pennata* extract was consistently pronounced against *Salmonella enterica*, *Staphylococcus aureus* and *Klebsiella oxytoca*. The *Bryopsis pennata* derived silver nanoparticles at the maximum concentration of 500 µg/mL had values for the zone of growth inhibition ranging from 23 mm to 26 mm for the six pathogenic resistant bacterial strains. The silver nanoparticles derived from the extract of *Caulerpa taxifolia* generally exhibited slightly greater activity than the silver nanoparticles obtained using *Bryopsis pennata*. The antimicrobial activity of the silver nanoparticles derived using *Caulerpa taxifolia* extract was particularly pronounced against *Escherichia coli*, *Klebsiella oxytoca* and *Bacillus mycoides*. The *Caulerpa taxifolia* derived nanoparticles at the maximum concentration of 500 µg/mL had values for the zone of growth inhibition ranging from 24 mm to 26 mm for the six pathogenic resistant bacterial strains.

For the amoxiclav functionalized silver nanoparticles derived from the aqueous extract of *Bryopsis pennata* and *Caulerpa taxifolia*, the diameter of inhibition zone in millimeter is shown in (Fig.6). Antibacterial activity of amoxiclav and the amoxiclav functionalized silver nanoparticles synthesized using the algal extracts were studied against six pathogenic resistant bacterial strains with well diffusion assay. The amoxiclav functionalized silver nanoparticles showed far more superior activity relative to the algal extract derived silver nanoparticles.

The amoxiclav functionalized silver nanoparticles were active against all tested bacteria. The amoxiclav functionalized silver nanoparticles derived from *Bryopsis pennata* extract consistently showed pronounced activity against *Pseudomonas aeruginosa*, *Klebsiella oxytoca* and *Bacillus mycoides* at all concentrations. These amoxiclav functionalized silver nanoparticles at the maximum concentration of 500 µg/mL had values for the zone of growth inhibition ranging from 30 mm to 33 mm for the six pathogenic resistant bacterial strains. The amoxiclav functionalized silver nanoparticles derived from the extract of *Caulerpa taxifolia* generally exhibited slightly greater activity than the amoxiclav functionalized silver nanoparticles obtained using *Bryopsis pennata*. The amoxiclav functionalized silver nanoparticles derived from *Caulerpa taxifolia* extract consistently showed pronounced activity against *Salmonella enterica*, *Klebsiella oxytoca* and *staphylococcus aureus* at all concentrations. These amoxiclav functionalized silver

nanoparticles at the maximum concentration of 500 µg/mL had values for the zone of growth inhibition ranging from 32 mm to 34 mm for the six pathogenic resistant bacterial strains.

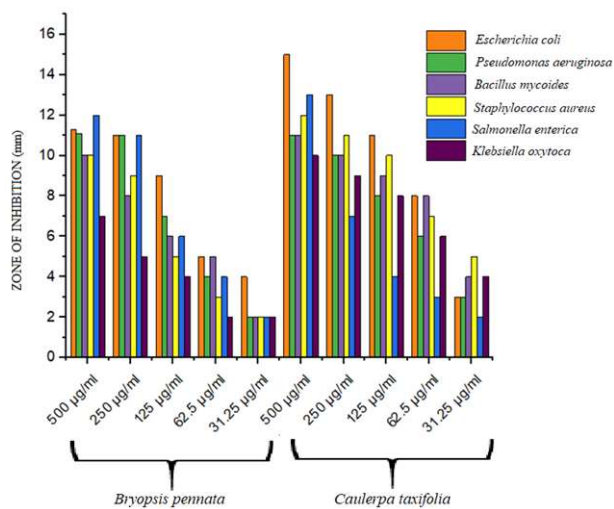


Fig.4. Antibacterial activity of the algal extracts of *Bryopsis pennata* and *Caulerpa taxifolia*.

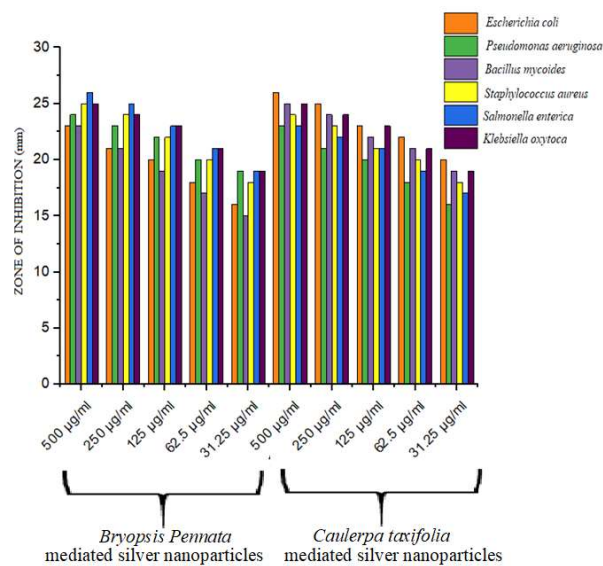


Fig.5. Antibacterial activity of silver nanoparticles derived from the algal extracts of *Bryopsis pennata* and *Caulerpa taxifolia*.

The amoxiclav functionalized silver nanoparticles derived from both algal extracts showed higher activity relative to amoxiclav. The amoxiclav functionalized silver nanoparticles were particularly very active against all tested bacterial strains. At the maximum concentration of 500 µg/mL for the amoxiclav functionalized silver nanoparticles derived from both algal extracts, minimum zone of growth inhibition of 30 mm was observed for the six pathogenic resistant bacterial strains while the amoxiclav at the same concentration had values for the zone of growth inhibition ranging from 24 mm to 30 mm. The superior activity of the amoxiclav functionalized silver nanoparticles relative to activities of amoxiclav and the algal derived nanoparticles showed that the

antibiotic functionalization of silver nanoparticles create a synergistic effect which can be further explored for the advancement of new antimicrobials in the treatment of diseases caused by microbes.

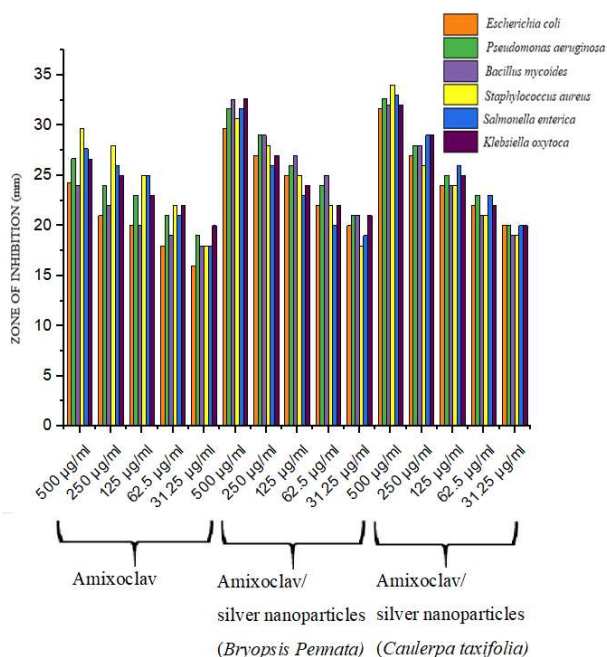


Fig. 6. Antibacterial activity of amoxiclav and amoxiclav functionalized silver nanoparticles derived from the aqueous extracts of *Bryopsis pennata* and *Caulerpa taxifolia*.

4. Conclusion

Silver nanoparticles were successfully synthesized at ambient conditions within 60 min using the aqueous extract of *Bryopsis pennata* and *Caulerpa taxifolia* with particle sizes below 65 and 30 nm respectively. The overall results indicate that the functionalized silver nanoparticles derived from *Bryopsis pennata* and *Caulerpa taxifolia* show superior activity relative to the algal derived nanoparticles while the algal derived nanoparticles showed higher activity when compared to the aqueous algal extract. The superior antimicrobial activity of the antibiotic functionalized silver nanoparticles relative to the bare antibiotic shows that the efficacy of the antibiotic was enhanced by coupling with the algae derived silver nanoparticles. Thus, antibiotics coupling with silver nanoparticles is a medically important process that can be utilized to improve the antimicrobial activity of existing antibiotics and reduce the scourge of antibiotic resistance.

Conflicts of interest

The authors declare that they have no conflict of interest.

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