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ANTIMICROBIAL ACTIVITIES OF SILVER NANOPARTICLES BIO-SYNTHESIZED FROM DIATOM *AMPHORA* SP.

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Abstract: A biological method was employed to synthesize silver nanoparticles through marine diatom *Amphora* sp. Antimicrobial efficacy test against different pathogenic bacteria were performed through synthesized silver nanoparticles. The physio-chemical properties of synthesized silver nanoparticles were studied using analytical techniques such as UV-Vis spectrophotometer, Field Emission Scanning Electron Microscopy (FESEM), Energy-Dispersive X-ray Spectroscopy (EDX) and Fourier Transform Infrared Spectroscopy (FTIR) and X-ray Powder Diffraction (XRD). UV-Vis color intensity study and higher magnification of the Field Emission Scanning Electron Microscopy image showed the synthesized silver nanoparticles were rod shaped with a size range from 42 nm to 46 nm. The synthesized nanoparticles exhibited antibacterial activities in varying magnitudes. About 10 mg/ml of silver nanoparticles were able to inhibit the growth of gram-negative bacteria while gram-positive bacteria were resistant towards similar concentrations of silver nanoparticles.

Key words: *Biosynthesis, Silver nanoparticles, Antimicrobial, Diatoms*

INTRODUCTION

Nanotechnology is no more a peculiar field in the current world of advanced science and technology. Eventually, the capability of nanotechnology to modulate the metals into nanoscale materials results in the change of chemical, physical and optical properties of metals. Furthermore, a significant role could be played by nanotechnology in healthcare, cosmetics, food and beverage, biomedical sciences, environmental health and drug delivery biological based product industries. Researches and roles played by nanotechnology similar to the aforementioned industries are often referred to as nano-biotechnology application. Although a vast field of applications was identified, many of the existing metal nanoparticles synthesis methods are anguished by drawbacks due to hazardous chemicals in use, eco-destruction and incompatibility with medicinal applications due to the presence of toxic capping agents [1]. Therefore, identification and formulation of sustainable synthesis of nanoparticles must be identified. Such environmental and non-hazardous nanoparticle synthesis methods will benefit society and the ecosystem. Algae, fungi, bacteria; as well as biomolecules such as proteins, amino acids, carbohydrates and sugars could be used to biosynthesis nanoparticles [2].

Many centuries ago silver particles were used as an antimicrobial substance. Oligodynamic effects of silver enables the binding of silver ions to the reactive sites of the bacterial cells, resulting in precipitation and inactivation of the organisms [3]. As a result, nanosilver particles based antiseptics are mainly in use nowadays to induce microbial resistance than antibiotics [4]. In addition, toxicity of silver nanoparticles (AgNPs) towards mammal cells is quite low [5]. Therefore, silver nanoparticles are widely used in creating hygienic products like the lining of washing machines, refrigerators, dishwashers and toilet seats [6]. Although, silver nanoparticles are not directly harmful to mammal cells, the production of silver nanoparticles usually consumes harmful chemical substances. Therefore, environmental friendly approaches that are free of

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harmful chemicals, as well as cost effective processes are under investigation in various parts of world. One of the most promising approach is green synthesis of silver nanoparticles using microalgae and microalgae derived materials [2,7]. There are a number of researches reported on the biosynthesis of silver nanoparticles thorough a diverse species of macro- and microalgae.

For instance, diatom is a type of microalgae which belongs to phylum *Bacillariophycae* with approximately 100,000 known species. In this study, a marine diatom was used to reduce silver nitrate into nano-sized silver particles. The biosynthesized silver nanoparticles have been characterized through UV-Vis spectroscopy, Field Emission Scanning Electron Microscopy (FESEM), Fourier Transform Infrared Spectroscopy (FT-IR), EDX and XRD. Furthermore, biosynthesized silver nanoparticles (AgNPs) were then tested for antimicrobial activity using gram-positive and gram-negative bacteria.

METHODS

Preparation of microalgae

Marine diatom *Amphora* sp. maintained at Bioprocess Laboratory, Faculty of Industrial Sciences & Technology, Universiti Malaysia Pahang were used in this research. The microalgae were cultured in a F2 medium and left for 25 days under an illumination of 20 µml $m^{-2}s^{-1}$ light at 20 ±2°C added with a sterile aeration.

Biosynthesis of silver nanoparticles

Twenty-five day old microalgae culture was used for the biosynthesis of silver nanoparticles. About 20 ml of *Amphora* sp. extract mixed with freshly prepared 1 mM silver nitrate ($AgNO_3$). After briskly stirring the mixture, it was left in room temperature for 24 hours. Diatom culture which was not mixed with $AgNO_3$, was used as a control in this experiment. A color change in the mixture indicated that reduction was occurring. After complete reduction, the synthesized medium was centrifuged at 12,000 rpm for 30 minutes. The pallet was collected and dried in an oven at 45 °C. The biosynthesis reaction was achieved without using any catalytic chemicals and polymers as a stabilizing and capping agent [8].

Characterization of silver nanoparticles

The UV-Vis spectra of silver nanoparticles were recorded by spectrophotometer (Genesys 10S) at a resolution of 1.0 nm at 300 nm to 800 nm. The structure and composition of nanoparticles were studied using the field emission scanning electron microscope (JEOL, JSM-7800F, Japan) attached with energy dispersive X-ray diffraction. X-ray diffractometer-Ringku mini flux II, Japan with Cu Kα radiation source (λ = 1.5415 Å) at 30 kV, 15 mA with scanning speed at 1°/ min was used to study the crystalline nature of biosynthesized silver nanoparticles. Further synthesized silver nanoparticles were prepared in thin pellets using potassium bromide (KBr) for FTIR analysis. IR spectra were obtained from PERKIN Elmer model at the resolution of 1 cm^{-1} in the range of 4000 to 400 cm^{-1}.

Antibacterial activity of silver nanoparticles

Antibacterial activity of silver nanoparticles synthesized from marine diatom was carried out by disc diffusion method. The gram-negative bacteria, *Escherichia coli* and *Proteus vulgaris*, and the gram-positive bacteria *Enterococcus faecalis* and *Bacillus subtilis* were used for the antibacterial test. Deionized water was used as the negative control. Bacterial cultures were spread equally onto the agar plate. The prepared sterile paper discs containing four different concentrations of silver nanoparticles (10 mg/ml, 20 mg/ml, 40 mg/ml and 80 mg/ml) were placed onto the bacterial cultured agar plate. The zone of inhibition was measured after 24 h of incubation. Meanwhile, Gentamicin and Ampicilin for gram-negative and gram-positive bacteria were used respectively as positive controls.

RESULTS AND DISCUSSION

In this study $AgNO_3$ and *Amphora* sp. (Figure 1) mixture were used to synthesize silver nanoparticles. Colorless solution of silver nitrate turned into purplish brown in an hour after the addition of diatom culture. The intensity of the color change increases with time. The change of color happens due to the formation of silver nanoparticles over time. The ability of enzymes responsible for the synthesis of silver nanoparticles increases under alkaline conditions [9]. Similar to this study, the formation of silver nanoparticles was indicated by the color change from a clear to brownish solution by [10–12].

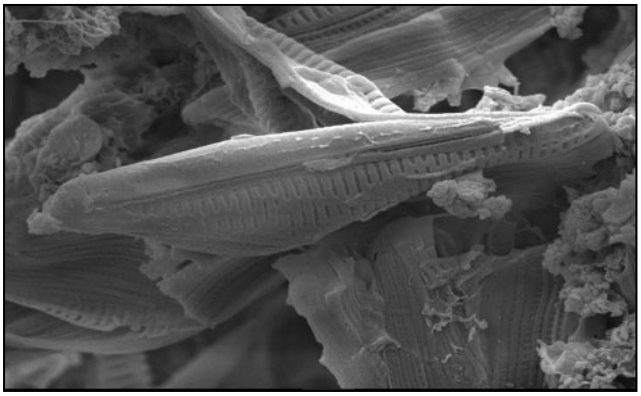

Figure 1 FESEM image of *Amphora* sp. which was used in this study.

The color change resulted from excitation of surface plasmon resonance (SPR) in the metal nanoparticles [13]. Figure 2 shows the color change of silver nanoparticles from clear to brown as an evident of AgNPs synthesized via *Amphora* sp. Other than color change the UV-spectra provides strong evidence of nanoparticle formation. Light wavelength of 200 – 800 nm at UV-visible spectroscopy is commonly used to characterize various sizes of metal nanoparticles [14]. Figure 3 shows the absorbance spectrum at λ_{max} = 427 nm, which indicates the formation of AgNPs in the solution.

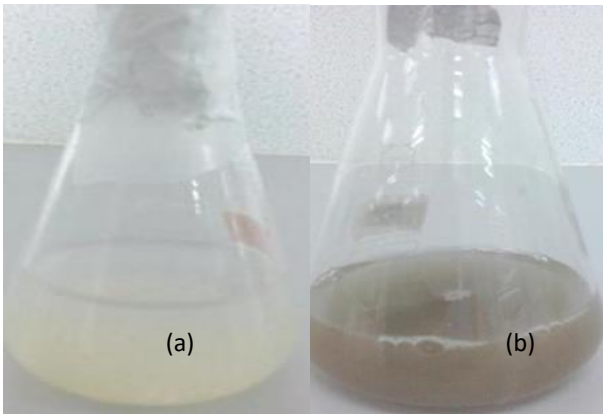

Figure 2 (a) Silver nitrate ($AgNO_3$) solution; (b) Brown color formation after the reaction with $AgNO_3$ solution with *Amphora* sp.

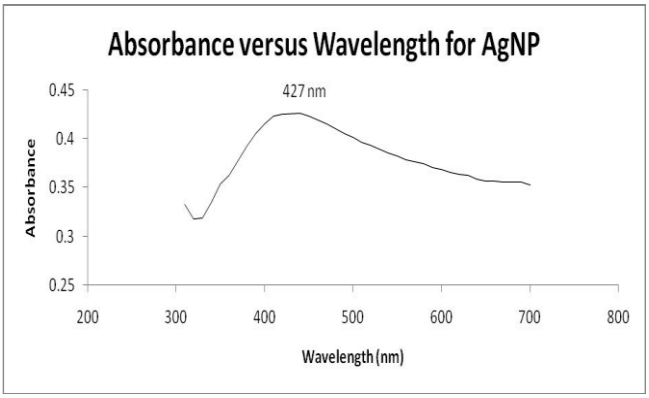

Figure 3 UV-Visible spectra of silver nanoparticles synthesized by addition of fresh marine diatom culture into $AgNO_3$ solution.

Previous studies suggested that a usual silver nanoparticles SPR pattern is present at wavelength in the range of 400 – 480 nm [15]. For example [16], observed that band occurs at 400 nm and 402 nm for *Spirulina platensis* and *Nostoc* sp. Silver reduction initiated through metabolites is present in algae culture medium. The plasma bands are broad with an absorption tail in the longer wave length, which could be in principle due to size distribution of nanoparticles. The reduction of silver ions occur through electron shuttle or through reducing agents released into solution by algal culture. Different wavelength readings indicate that AgNPs are formed in various particle sizes, shapes and surface properties. Therefore, such dissimilar physical formation of AgNPs is widely used as antimicrobial and antifungal agents in healthcare, food industry, textile coatings and electronic devices.

Figure 4 represents the size and morphology of synthesized AgNPs that were determined through FESEM micrograph. The FESEM images clearly indicate that non spherical nanoparticles with an average size ranging from 42 nm to 50 nm are produced. At the same time, dispersion of nanoparticles without any direct contacts is the indication of stable nanoparticles through capping agents. Such bio-capped nanoparticles (NPs) will help to prevent agglomeration of NPs and at the same time enhances the antimicrobial activity [15].

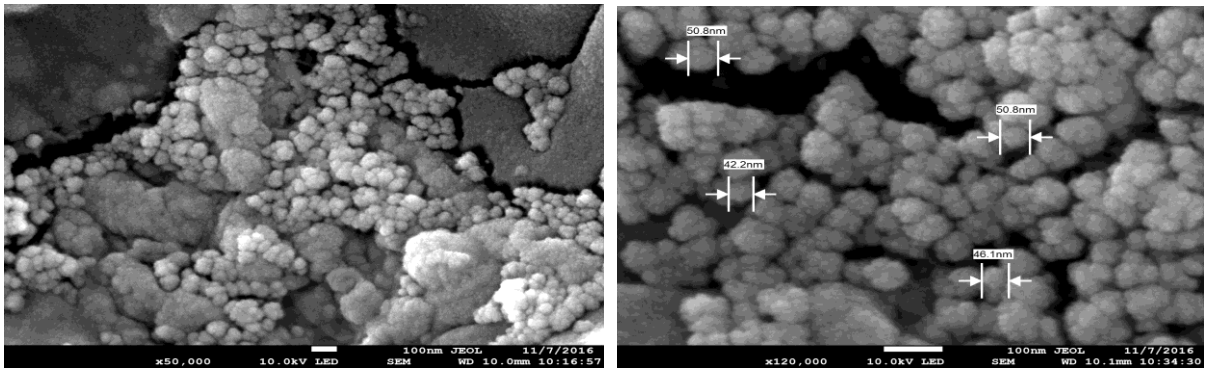

Figure 4 Image of silver nanoparticles (AgNPs).

Additionally, Figure 5 shows EDX spectrum of synthesized silver nanoparticles. The EDX spectrum has confirmed the presence of silver in the sample. The optical absorption band peaks were found in the range of 3-4 keV, which is common for pure metallic silver nanocrystals absorption peaks. This result correlates with the findings from [14,17]. Additional peaks for Cl, O and Mg were observed. These are dominant compounds of enzymes, proteins and secondary metabolites which were present in the cell wall of diatoms [18]. Meanwhile other peaks might indicate the presence of cell biomass that trapped with AgNPs [19].

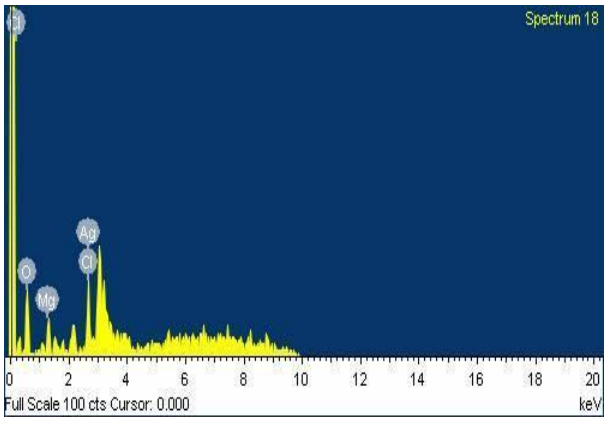

Figure 5 EDX spectra of silver nanoparticles solution

The powder XRD pattern of the AgNPs is shown in Figure 6. The data shows peaks at 2θ values ranging from 30 to 80 which can be indexed (111), (200), (220), (311) and (222). The results indicate that the AgNPs

synthesized from *Amphora* sp. has planes of face centered cubic silver. The XRD pattern suggests that biosynthesized silver nanoparticles were crystalline in nature. On the other hand, the intensive diffraction peak at a value of 38.2 from the (111) lattice plane of face-centered cubic silver clearly indicates that the particles are made of pure silver [20]. It has been suggested that this plane may possess the lowest surface tension [21,22]. In addition to that, [1] had indicated that polysaccharides, proteins, polyols present in the bio-extract are able to act as reducing agents as well as capping layers. Such interaction in between bio-compounds and metal atoms would limit the nanoparticles size within 60 nm. As the size, can be calculated by Scherer's equation by means of determining the width of the (111) Bragg reflection, 5-60 nm is the usual size of crystalline nanoparticles. As per earlier discussion, the nanoparticle size synthesized through *Amphora* sp. is identified from 42 nm to 46 nm.

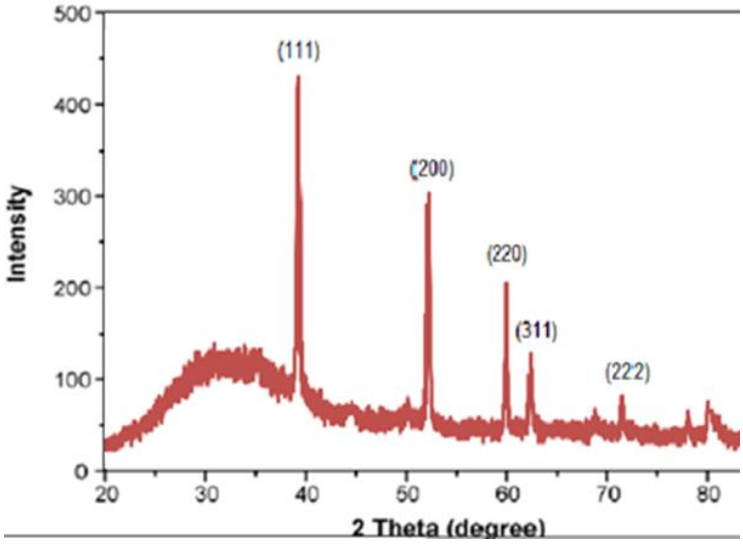

Figure 6 Powder X-ray differection patterns of AgNPs

The identification of biomolecules bound to the silver surface was executed through Fourier transform infrared spectroscopy (FTIR) within the spectral range of 1000 – 4000 cm^{-1}. As per (Figure 7a), the marine diatom culture showed peaks at 1637.33 cm^{-1}, 2123.36 cm^{-1} and 3351.48 cm^{-1}. The results for *Amphora* sp. synthesized silver nanoparticles solution showed peaks at 3351.98 cm^{-1}, 2108.24 cm^{-1} and 1637.65 cm^{-1} as shown in (Figure 7b). The peak and its attributions has summarized in (Table 1). The FTIR results revealed that protein molecules were present in both fresh diatom cultures and synthesized AgNPs. Enzymes consisting of proteins would have contributed for the formation of silver nanoparticles. As per [23], stabilization of AgNPs by protein occurs when protein bind to nanoparticles through the electrostatic attraction of negatively charged carboxylate groups. Above findings revealed that *Amphora* sp. is capable to biosynthesize rod like shaped silver nanoparticles

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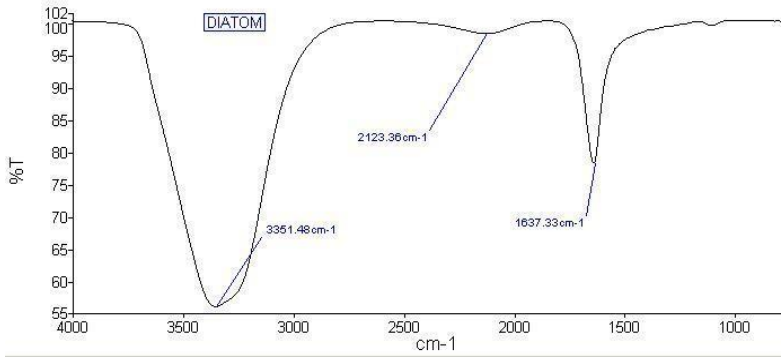

Figure 7a FTIR peaks obtained from fresh *Amphora* sp. culture

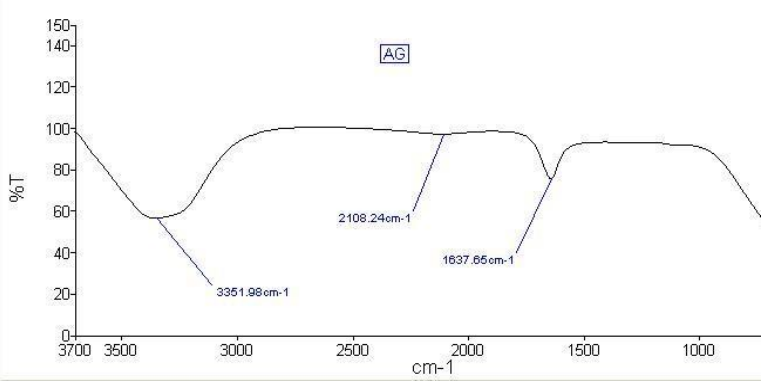

Figure 7b FTIR peaks obtained from AgNPs synthesized using *Amphora* sp.

Table 1 FTIR results obtained from fresh culture and AgNPs.

Fresh Amphora sp. culture		Amphora sp. synthesized AgNPs	
Frequency (cm1)	Bond/ Stretching	Frequency (cm1)	Bond/ Stretching
1637.33	O-H stretching – Primary amines	1637.65	C=N stretching – Amines
2123.36	N-H stretching – Secondary amines	2108.24	C-N stretching
3351.48	O-H stretching – Carboxylic acids	3349.28	O-H stretching – Carboxylic acids

As a final study, antimicrobial potential test was performed. The gram-negative bacteria *Escherichia coli* and *Proteus vulgaris*; gram-positive bacteria *Enterococcus faecalis* and *Bacillus subtilis* were used for antibacterial test. Different concentrations (10 mg/ml, 20 mg/ml, 40 mg/ml and 80 mg/ml) of AgNPs were tested. The antibacterial test revealed that AgNPs synthesized by *Amphora* sp. is capable of inhibiting the growth of gram-positive and gram-negative bacteria respectively. The inhibition zones formed against the bacteria recorded as per (Table 2).

Table 2 Inhibition zone of AgNPs against gram negative and gram positive bacteria

	Inhibition zone (mm)					
Bacteria	10 mg/ml	20 mg/ml	40 mg/ml	80 mg/ml	Positive control	Negative control
Enterococcus faecalis	-	7.7	7.7	9.2	29.5	0.0
Bacillus subtilis	-	6.3	8.3	7.0	7.30	0.0
Escherichia coli	6.3	8.0	8.7	8.0	24.3	0.0
Proteus vulgaris	7.0	8.0	7.7	9.2	23.3	0.0

In summary, no inhibition zone was found at 10 mg/ml of AgNPs concentration for *Enterococcus faecalis* and *Bacillus subtilis*. However, optimal inhibition zone at around 9.2 mm and 8.3 mm was observed for *Enterococcus faecalis* at concentration of 80 mg/ml and *Bacillus subtilis* at concentration of 40 mg/ml respectively. The growth of *Escherichia coli* and *Proteus vulgaris* was inhibited by minimal concentration (10 mg/ml) AgNP's aqueous solution.

The silver nanoparticles exhibited antibacterial activity in varying magnitudes. From the results obtained, it was noticed that the silver nanoparticles were more bactericidal against gram-negative bacteria as compared to gram-positive bacteria, whereby 10 mg/ml of AgNPs were capable to inhibit the growth of gram-negative bacteria. However, the same concentration was unable to produce any noticeable zone of inhibition for gram-positive bacteria. Previous studies confirmed that silver nanoparticles were more bactericidal against gram-negative bacteria [23,24]. Optimal antibacterial effect was observed with gram-negative bacteria since the thin cell wall with peptidoglycan allows for easier permeability when compared with thick cell wall made gram-positive bacteria [25]. In addition, the DNA of bacteria loses its capability for replication and inactivation of cellular proteins occurs upon Ag+ penetration treatment [26]. The silver nanoparticles were able to express its antimicrobial property by penetrating into the bacteria, damaging cell membrane and release of cell contents [27].

On the other hand, silver nitrate ($AgNO_3$) was tested against four selected bacteria for antibacterial efficacy. Although $AgNO_3$ exhibited antibacterial activity, the inhibition was not as vigorous as silver nanoparticles. Silver nitrate at 1 mM was unable to inhibit the growth of *Bacillus subtilis*. Table 3 summarizes the antibacterial activity of silver nitrate directly towards bacteria.

Table 3 Inhibition zone by $AgNO_3$.

	Inhibition zone (mm)		
Bacteria	$AgNO_3$ (1 M)	Positive control	Negative control
Enterococcus faecalis	5.0	29.0	0.0
Bacillus subtilis	-	8.6	0.0
Escherichia coli	7.6	24.0	0.0
Proteus vulgaris	5.0	27.0	0.0

A similar result was obtained by [21] whereby, inhibition of $AgNO_3$ was less efficient compared with AgNPs. It was reported that silver nanoparticles are more toxic towards *E. coli* then $AgNO_3$. In another study [1] explained this scenario by indicating that AgNPs are able to disquiet the permeability and respiratory functions of cells by attaching to the bacterial cell wall. It is also possible for AgNPs to penetrate inside the cell body and further damaging the cell functions. Although the real mechanism of AgNPs bactericidal effects are still under investigation, the positive charge of Ag^+ plays a role in attaching negatively to the charged cell membrane of microorganism. It is hypothesized that electrostatic force is an additional cause for the interaction of the nanoparticles with the bacteria [21,28]. Additionally [29], indicated that Ag^+ is able to catalyze DNA losses its ability to replicates and expression of ribosomal subunit proteins. The Ag^+ was able to restrict expression of key enzymes for ATP production.

CONCLUSIONS

In conclusion, we have demonstrated that diatom *Amphora* sp. was able to reduce silver nitrate into silver nanoparticles. Through EDX, the formation of silver nanoparticles was confirmed and the size range from 42 nm to 46 nm respectively. It is also confirmed that the presence of protein molecules in AgNPs derived from *Amphora* sp. In addition, synthesized silver nanoparticles possess toxic effects towards *Enterococcus faecalis, Bacillus subtilis, Escherichia coli* and *Proteus vulgaris*. Based on the inhibition zones formed, the biosynthesized silver nanoparticles were found to be more bactericidal against gram-negative bacteria compared to gram-positive bacteria.

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