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Biosynthesis of silver nanoparticles by using of the marine alga Gracilaria parvispora and its antagonistic efficacy against some common skin infecting pathogens

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ABSTRACT

Biosynthesis of nanoparticles (NPs) is a growing field, the intersection of nanotechnology and biotechnology has attracted a lot of attention due to the growing need to develop environmentally safe and effective technologies in material synthesis. Synthesis of silver nanoparticles (AgNPs) using the marine alga *Gracilaria parvispora* extract is clearly shown here. Transmission electron microscopy (TEM) revealed that the prepared AgNPs are spherical shaped with size range of 12–30 nm. Fourier Transform Infrared Spectroscopy (FTIR) showed that AgNPs were capped and stabilized in solution with algal bioactive compounds. Moreover, the antimicrobial activity of the algal extract alone and with the produced AgNPs has been addressed with certain bacteria and fungi to reveal the benefit of produced AgNPs for making antibacterial and antifungal pharmaceutical topical in future.

INTRODUCTION

The synthesis of metallic NPs are generally performed by physical and chemical processes demanding high costs and may result in significant toxicity (Vieira *et al*., 2016). These problems are mostly solved by nano-bio-technology which is one of the most promising areas in modern nanoscience. This emerging area of research correlates various disciplines of science such as biology, chemistry, physics and materials science (Abdel-Raouf, *et al*., 2013; Iravani *et al*., 2014). The reduction of a material's dimensions results in significant variations in the physical and chemical properties as compared to the same bulk material. In addition, NPs are considered as the fundamental building blocks of nanotechnology (Jena *et al*., 2013).

The most common approach for synthesis of NPs is chemical reduction by organic and inorganic reducing agents However, in most of chemical methods, toxic chemicals were used in the synthesis protocol which restricted the use of such NPs for biological and medical applications. Therefore, there is a growing need to establish an eco-friendly green process for NPs synthesis that does not use toxic chemical (Iravani *et al*., 2014).

It's important to note that the selection of solvent medium and eco-friendly reducing and stabilizing agents are the most important conditions which must be considered in green synthesis of NPs (Iravani *et al*., 2014). It is well known that, extracts from bio-organisms act both as reducing and capping agents in the AgNPs synthesis and metallic NPs in general. Thus, NPs preparation with desirable morphology and size based on green chemistry methods became a major focus of researchers. Several studies used microorganisms and biological

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approaches to prepare NPs (El-Rafie *et al*., 2013). These methods are clean, reliable, biocompatible and benign processes enlighten the way for wide varieties of biological applications (Govindaraju *et al*., 2010, Shenashen *et al*., 2014). These metallic NPs also have significant inhibitory effects against microbial pathogens, so used widely as antimicrobial agents in a diverse range of products (Iravani *et al*., 2014; Kathiraven *et al*., 2015).

About 5000 years ago, Egyptians used specifically silver metal to store food products as they notice its activity in preserving various food types for relatively long times. Until the discovery of antibiotics by Alexzander Fleming, Silver was commonly used as antimicrobial agent. (Srikar *et al*., 2016). Furthermore, AgNPs have attracted considerable interest in almost every field due to its unique properties like easy synthesis, high thermal conductivity, and high resistance to oxidation, anti-fungal and antibacterial activity (Srikar *et al*., 2016).

In the present study, the extract of the marine alga *Gracilaria parvispora* found in nature in abundant mass is used for the biosynthesis of stable AgNPs without any need for toxic chemicals or high-energy consumption methods. Then, the antibacterial activity of the produced AgNPs is assayed to be used afterwards safely in biological or pharmaceutical applications.

MATERIALS AND METHODS

Study area:

Four locations of investigated area were extends along Safaga coast on Red Sea shore, Egypt, specifically, at a (latitude of 26.77 and longitude of 33.94).

Fig. 1: Shows the sites of collection of the marine algae (study area on the coast of Red sea in Egypt.

Collection and preparation of alga sample:

Fresh and healthy marine alga *Gracilaria parvispora* (Fig.2) were collected from the inter-tidal region between (0.2–2.5 m depths) along the Red sea coast of Safaga, Egypt, during the period from April to June 2015. Collected sample was immediately brought to the laboratory in new plastic bags containing Sea-Water to prevent drying of samples. The algal material was washed thoroughly with tap water to remove extraneous materials and shade-dried for 5 days and oven dried at 60^{o}C until constant weight was obtained, then was grind into a fine powder using an electric mixer and stored at 0.0^{o}C for future use. Algal species were identified according to Aleem, (1993) and Coppejans, *et al*., (2009).

Fig. 2: Gracilaria parvispora alga morphology

Synthesis of silver NPs:

The synthesis of AgNPs by *Gracilaria parvispora* was carried out according to Singaravelu *et al*., (2007).

Characterization of AgNPs:

Visual observation:

The color change in the reaction mixture of $AgNO_3$ solution + algal extract was recorded through visual observation on timely manner.

UV–Visible spectroscopy analysis:

The bioreduction of silver ions in aqueous solution was monitored by periodic sampling of aliquots 3 ml and measuring UV–Vis spectra of the solution at 200–800 nm using a 3–5 mm quartz cuvette. UV–Vis spectra of these aliquots were monitored on a UV–Vis Shimadzu UV-2600 spectrophotometer. All the measurements were carried out at controlled room temperature (27 ^{o}C).

Transmission Electron Microcopy (TEM):

The morphological analysis of the AgNPs was done with transmission electron microcopy (TEM). A drop of aqueous AgNPs sample was loaded on a carbon-coated copper grid and it was allowed to dry completely for an hour at controlled room temperature (27 ^{o}C). The TEM micrograph images were recorded on a JEOL 2100 instrument on a carbon coated copper grids with an accelerating voltage of 200 kV. The clear microscopic views were observed and documented in different ranges of magnifications.

Fourier Transforms Infrared Spectroscopy (FTIR):

FTIR was used to identify the possible biomolecules responsible for the reduction of the silver ions and synthesis of the AgNPs by *Gracilaria parvispora* plus the determination of the functional groups involved in the synthesis of AgNPs through the use of (BRUKER VERDEX 70) device.

Anti-microbial activity evaluation via Agar well-diffusion assay:

Staphylococcus aureus, *Pseudomonas aeruginosa* and *Candida Albicans* were sub-cultured using cotton swab of microorganism suspension on Sabouraud Dextrose Agar (SDA) for fungi and Nutrient Agar (NA) for bacteria and identified using the standard protocol. The antimicrobial activity was carried out using agar well-diffusion method. Petri plates were prepared with 20 ml each of sterile NA and SDA for bacteria and fungi respectively. Wells were made using sterile cork borer under aseptic condition. The algal extract and its based NPs with various volumes of the extract (25 µl, 50 µl) were added to the wells. The Petri plates were incubated for 48 hours. The zone of inhibition was measured using a ruler and expressed in mm.

RESULTS AND DISCUSSION

It was recorded that the color of the 10^{-3} M aqueous $AgNO_3$ (Sigma–Aldrich) solution is colorless. In the beginning of the procedure after the addition of (99 ml) of $AgNO_3$ to the *Gracilaria parvispora* extract (concentrated 1ml), the AgNPs formed and visually confirmed by the color change to from colorless to dark brown color followed by gradual darkening in color to the dark brown indicating the formation of AgNPs after 15 min of reaction at controlled room temperature (27°C) (see Figure 3. Such a color transition is often indicative of alterations in the silver oxidation state from ions to NPs (Govindaraju *et al*., 2010).

Fig. 3: (A) The colorless solution of $AgNO_3$, **(B)** The solution of AgNPs prepared from methanolic extract of *Gracilaria parvispora* after 15 minutes, **(C)** The solution of AgNPs after 90 minutes.

In this case, silver ions were reduced to AgNPs by certain bioactive molecules in *Gracilaria parvispora*. It is well known that colloidal AgNPs solution are varying in color from light yellow to dark brown and widely reported by previous studies. Such variation in the color of the solution is due to the macromolecules responsible for the catalytic formation of the AgNPs, the NPs shapes or the capping agents stabilizing them. Proteins mainly were the capping and stabilizing molecules in most biosynthesis processes (Lateef *et al*., 2015; Netala *et al*., 2016). It's worth adding that, AgNPs exhibit a yellow to yellowish-brown color in aqueous solution due to the excitation of surface Plasmon vibrations of AgNPs in solution (Merin *et al*., 2010).

The formation of AgNPs was confirmed by UV–Visible spectrophotometer analysis. The UV–Visible spectrophotometer has proved to be a very useful technique for the analysis of some metallic NPs. The UV–Visible spectra indicate a strong plasma resonance that is located at 412 nm for AgNPs formed by *Gracilaria parvispora* extract shown in Figure 4. The presence of this strong broad Plasmon peak has been well documented for AgNPs (Merin, *et al*., 2010) with sizes ranging from 2 to 100 nm.

The structures and sizes of the biosynthesized AgNPs were studied by transmission electron microscopy (TEM). A typical TEM image showing the size and morphology of the silver NPs is given in Figure 5. It was found that the particles were spherical in shape. The size of AgNPs averaged from 12–30 nm. Furthermore, FTIR was performed to determine the functional groups of *Gracilaria parvispora* extract to predict their role in the synthesis of AgNPs. The control spectra for the extract (untreated with $AgNO_3$) showed a number of peaks thus reflecting the complex nature of *Gracilaria parvispora* methanolic extract.

The FTIR spectrum of the AgNPs synthesized from *Gracilaria parvispora* extract is shown in Figure 6. The presence of three bands at about 3329.26, 2114.51 and 1636.54 cm^{-1} most probably assigned to amide bands of proteins and arises due to carbonyl stretch and free N–H stretch vibrations in the amide linkages of the proteins respectively and it has been previously reported that the band at 1630 is characteristic for amide II band (Xie *et al*., 2007). In addition to that, The FTIR spectroscopic studies has confirmed that the carbonyl group forms amino acid residues and peptides of proteins have the stronger ability to bind to AgNPs, so that the proteins could most possibly form a coat covering the AgNPs (capping of AgNPs) to prevent agglomeration of the particles and stabilizing the NPs in the medium (Abdel-Raouf *et al*., 2013; Lateef *et al*., 2015). So, the biological active molecules could possibly perform both the formation and the stabilizing of the AgNPs in aqueous medium. On the other hand, no significant displacement was observed in the FTIR spectrum, since it overlaps with other bands, the (-OH) stretching band became sharper and the displacement of the infrared bands

corresponding to the carboxyl groups was expected (Abdel-Raouf *et al*., 2013). The band 3329.26 is associated with N-H bond of amines, while 1636 is indicative of C=C stretch of alkenes or C=O stretch of carbonyl in amides respectively (Emeka *et al*., 2014) and both are important in the bio-reduction of silver ions to AgNPs and subsequent capping and stabilization. The AgNPs were spherical in shape and poly-dispersed in nature. This shape and these sizes are in agreement with those reported previously (Kannan *et al*., 2013).

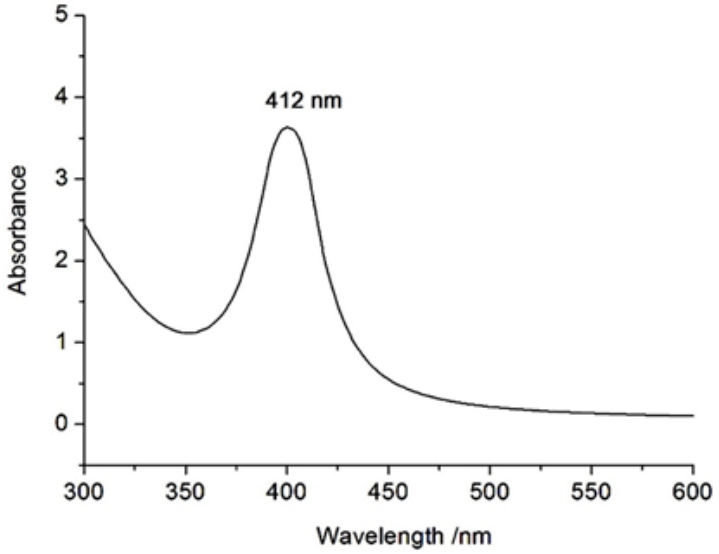

Fig. 4: UV–Visible spectrum of silver NPs synthesized by *Gracilaria parvispora* extract.

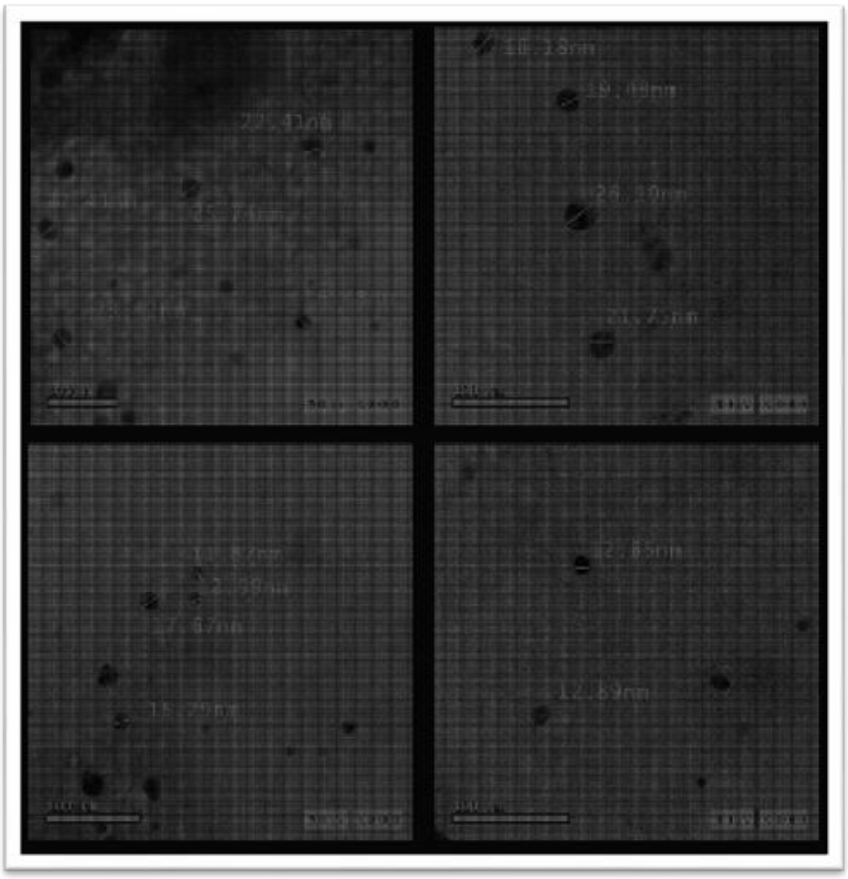

Fig. 5: Representative TEM micrograph of AgNPs synthesized by *Gracilaria parvispora extract*.

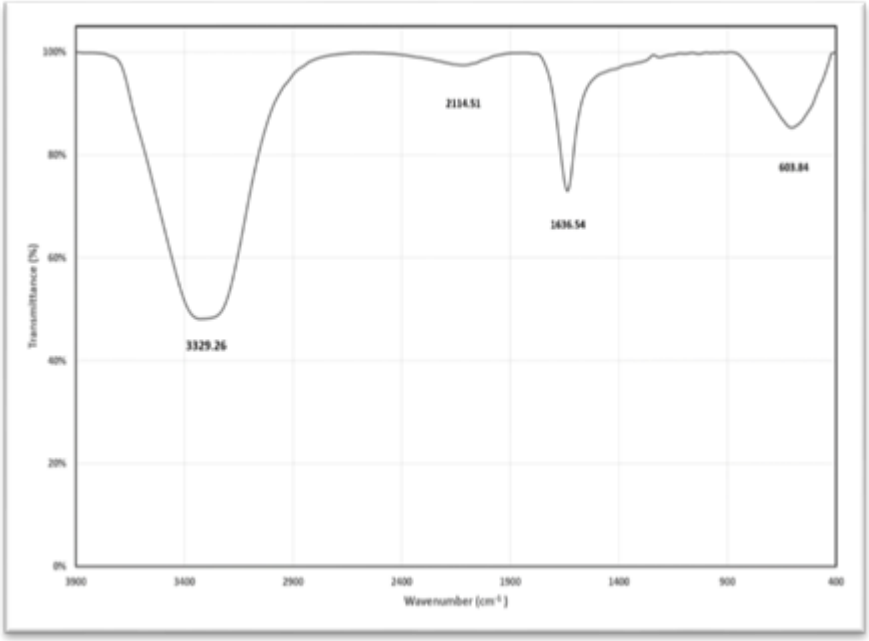

Fig. 6: FTIR Analysis of AgNPs synthesized by *Gracilaria parvispora* extract.

The particles were well dispersed, an indication of good stability in solution for long time. The biosynthesized AgNPs showed maximum absorbance at wavelengths of 412 nm (Figure 4), which falls within the range of (410-450) nm reported previously for AgNPs. It's worth adding that, the sulfate polysaccharide, polypeptides, proteins and polyol groups in the studied algal extract may be involved in the reduction and stabilization of silver ions to AgNPs. In previous studies, it was reported that, hydroxyl and/or carboxyl groups in the amino acid residues were identified as the most active functional groups for silver ions reduction and for directing the anisotropic growth of AgNPs (Vijayaraghavan and Nalini 2010). Furthermore, It was confirmed in many scientific reports that carbonyl groups, terpenoids, phenolics, flavones, amines, amino amides, proteins, pigments, alkaloids and other reducing agents are present in the marine algal extracts and may impact the reduction process or stability of the formed AgNPs (Asmathunisha and Kathiresan 2013).

Our results reported that the AgNPs had potential antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Such antibacterial activity increases with the increment in concentration used. Presently, there are three main explanations that have been proposed to describe the antibacterial activity: (1) The direct interaction of AgNPs with the bacterial cell membrane, causing subsequent membrane damage and complexation with components located inside cells (Sondi and Salopek-Sondi 2004), (2) The interaction with thiol (−SH) groups and production of reactive oxygen species (ROS) (Banerjee *et al*., 2010), (3) The release of silver ions that inhibit respiratory enzymes and generate ROS .

It's well documented that the increased oxidative stress in presence of silver is one aspect of the antibacterial action of the metal, but it is not the only one. Measurement of the intracellular concentration of ROS should be perceived as monitoring one partial effect of Ag^+ action that correlates but cannot be substituted with a quantification of the effective antibacterial action (Le Ouay and Stellacci 2015). However, it was suggested that NPs release silver ions into the bacterial cell, resulting in bactericidal activity. In addition, the mechanism of silver action is linked with its interaction with thiol group compounds found in the respiratory enzyme of bacterial cells as silver ions may deactivate cellular enzymes and DNA by reacting with electron-donating groups such as thiol (-SH) groups and generate ROS (Sambhy *et al*., 2006). In more researches, it was demonstrated that AgNPs attach to the surface of cell membrane, affecting membrane permeability, dissipation of the ATP pool and Proton Motive Force (PMF) and finally caused cell death (Chiu and Che 2006).

The presence of AgNPs could also provide some advantages and improve the potency of the antimicrobial activity since AgNPs could act as silver ions reservoir, and provide continuously high enough concentration of silver antibacterial species in their surroundings to maintain the activity for several days (Le Ouay and Stellacci 2015). In accordance to our results, (Bonde *et al.*, 2012) proved potent efficacy of AgNPs against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. According to Figure 7, the AgNPs have higher antibacterial activity against *Pseudomonas aeruginosa* as a gram negative bacteria (which has an outer membrane) than against *S. aureus*. This observation supports the high efficacy of combining these particles with other antimicrobial agents to prevent the incidence of resistance reported by (Rai *et al*., 2014). Similarly, Ingle *et al*.,(2008) reported a significant inhibition of the growth of these two pathogens and concluded that the

antibacterial activities of AgNPs and such antibacterial activity can be modified by controlling the size of NPs because the activity of AgNPs decreases with the increase in the particle size.

Rai *et al*., (2014) study concluded that the disturbances in membrane penetration account for the antibacterial activity of AgNPs leading to internalization of NPs and subsequent intracellular effects including ROS generation, interaction with -SH groups, inhibition of protein synthesis and interaction with phosphorus-containing molecules, such as DNA. But, in the present study the results showed that AgNPs did not have antifungal activity against *Candida Albicans*.

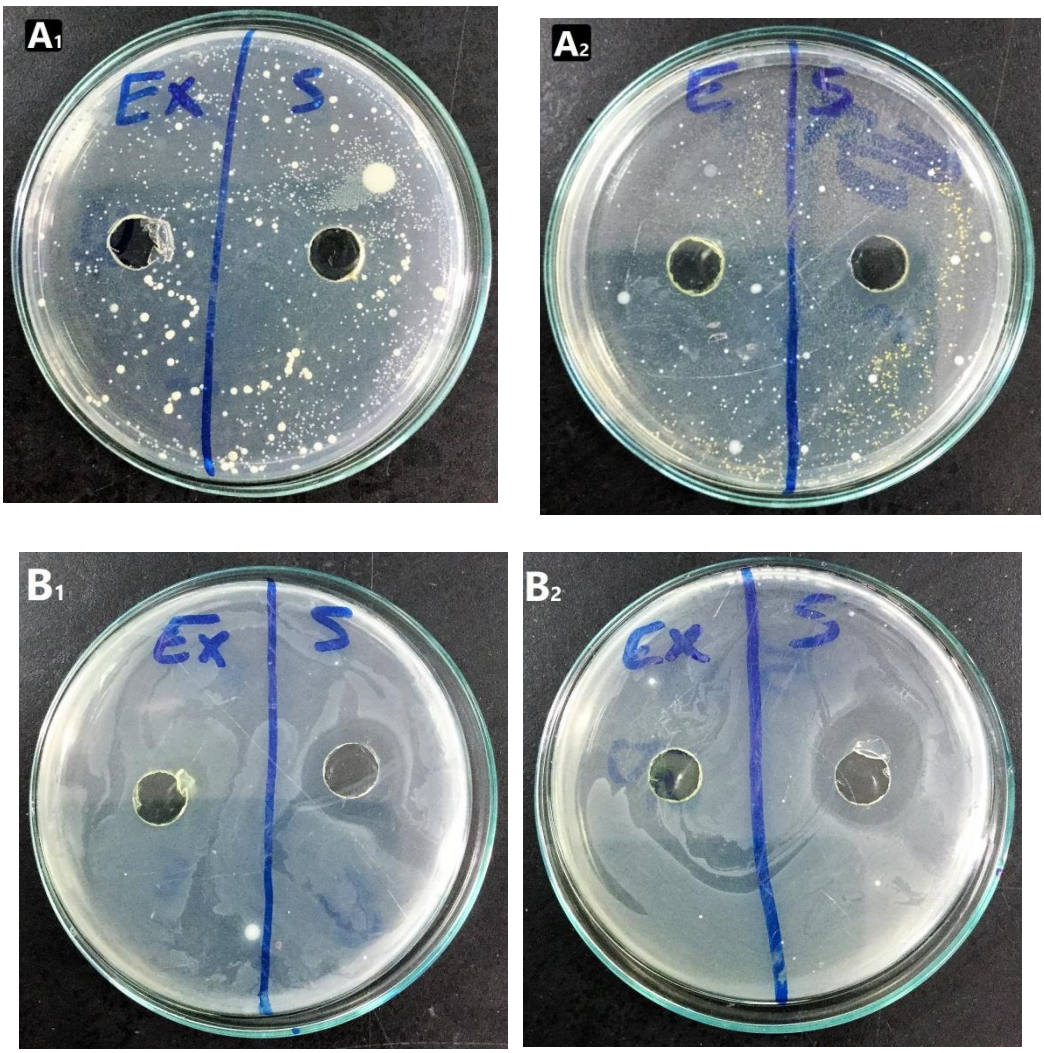

Fig. 7: Antimicrobial activity of Gracilaria parvispora extract and its AgNPs (Ex: Extract, S: AgNPs).

A: the antibacterial activity of the algal extract and its AgNPs against *Staphylococcus aureus* at two different volumes 25 and 50µl for **A_1** and **A_2** respectively.

B: the antibacterial activity of the algal extract and its AgNPs against *Pseudomonas aeruginosa* at two different volumes 25 and 50µl for **B_1** and **B_2** respectively.

Conclusion:

The present study has established the relevance of *Gracilaria parvispora* for biosynthesis of AgNPs that are spherical in shape with a diameter range of 12–30nm. . In addition, an important benefit of the described method of synthesis of AgNPs is their stability in solution over other techniques currently in use. The results revealed the potent antibacterial activity of AgNPs of *Gracilaria parvispora* extract against *S. aureus* and *Pseudomonas aeruginosa*. Finally, this report represents the first reference to the *Gracilaria parvispora* marine alga in the biosynthesis of AgNPs effectively and the resulted AgNPs can be used afterwards in various medicinal and clinical applications.

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