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GREEN SYNTHESIS OF SILVER NANOPARTICLES USING SEAWEED EXTRACTS OF *SARGASSUM WIGHTII* (SW) AND THEIR BACTERICIDAL ACTIVITY AGAINST PATHOGENIC BACTERIA

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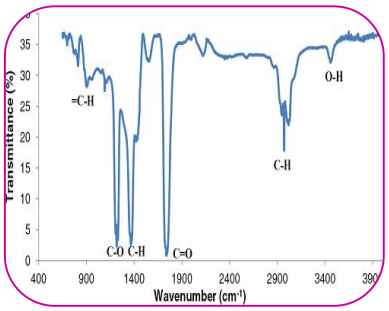
ABSTRACT

Biological reduction agents are being explored worldwide to minimize the effects of toxic chemicals used in nanoparticle fabrication. Eco-friendly synthesis of silver nanoparticles (Ag-NPs) prepared by Sargassum wightii (SW) were evaluated for antibacterial efficacies against Gram-positive and negative MDRs. The resultant nanopowder was characterized using various analytical techniques, such as UV-vis spectroscopy, Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), Scanning electron microscopy (SEM) equipped with Energy dispersive spectroscopy (EDS) and Field-emission scanning electron microscopy (Fe-SEM). Antibacterial effective against Gram-negative Pathogenic bacterial isolates with a maximum inhibition (18 mm) of Ampicillin-resistant E. coli and a minimal (14 mm) of P. aeruginosa, whereas, Gram-positive a maximum inhibition (12 mm) of Methicillin-resistant S. aureus and a minimal (10 mm) of B. subtilis. The suggested procedure was effective a maximum growth inhibition zone of 18 mm were observed in Ampicillin-resistant Escherichia coli (NCIM 2920) and a minimum of 10 mm in Methicillin-resistant Bacillus subtilis (NCIM 5021) due to extracellular polymeric substance (EPS) secretion in Gram-positive bacteria. Thus Sargassum wightii (SW) extract and powder are a good bio-resource/biomaterial for the synthesis of Ag nanoparticles with antibacterial activity.

KEYWORDS: *Green Nanoparticles, Pathogenic Bacteria, Sargassum Wightii, Antibacterial Activity.*

INTRODUCTION

Even though pharmacological industries have produced a number of new antibiotics in the last three decades, resistance to these drugs by pathogenic bacteria has increased that present a major challenge for public health in preventing its spread and impact on human health. Recently, there was an enormous increase of pathogenic bacterial infections which were spreading around the globe, ranging from tropical and subtropical to temperate climates. These were causing substantial human and economic losses worldwide [1-3]. Currently, over 70% of bacterial nosocomial infections in the United States were resistant to one or more antibiotics that are traditionally used to eliminate them. In 2002, the United States (U.S) center for disease control and prevention (CDCP) estimated that, nearly 90,000 deaths per year occurred due to bacterial infection. More than half of them were caused by MDRs at least one commonly used antibiotic [4, 5]. People who become infected with drug-resistant microorganisms usually spend more time in hospital and require a form of treatment that uses two or three different antibiotics in combination which are more effective but also more expensive [6]. The inability of current antibiotics to limit MDR infections coupled with the slow approval rate of new antibiotics necessitate the search for unconventional biocidals. There are several new strategies that have been employed to control microbial infections and are



increasingly recognized as a useful outcome source of potential use in research and health related applications [7]. Hence, there is an urgent need to develop a sustainable path for an environmentally less harmful non-toxic antibiotic against MDRs.

Nanotechnology is a rapidly emerging field with applications in Science and Technology for the purpose of manufacturing new materials at nanoscale level [8]. Nanoparticles are being synthesized globally owing to various exciting and unique properties, which facilitate their exploitation in completely unrelated fields, such as, nano-diagnostics, nano-medicine and antimicrobials on one hand and luminescence photocatalytic potential and photodiode response on the other. Eco-friendly technologies for the synthesis of silver nanoparticles (Ag-NPs) are believed to be nontoxic, biosafe, and biocompatible and have been used as drug carriers, cosmetics, and fillings in medical materials [9-11]. The use of nanoparticles (NPs) as antibacterial agents have been the subject of several studies and Ag-NPs possesses natural antibacterial properties that are strengthened at nanoscale. Although physical and chemical methods are more popular in the synthesis of NPs, the use of toxic chemicals greatly limits their environmental applications [12]. The developments of reliable, non-toxic methods require extensive labour, and time in the synthesis of NPs. Furthermore, large quantities of secondary waste are generated, resulting from the addition of chemical agents for precipitation and reduction in these processes. An eco-friendly synthetic method employing plant extracts have drawing attention as a simple and viable alternative to chemical and physical methods. These advantages include lower cost; ease of synthesis, white appearance NPs based antimicrobial formulations could be used as efficient bactericidal materials in modern medicine [13]

In the present study, an extract of brown seaweed *Sargassum wightii* (SW) was used for the eco-friendly synthesis of silver nanoparticles (Ag-NPs) obtained by using seaweed as both a reducing and stabilizing agent [14]. *S. wightii* (SW) is available throughout all seasons in large abundances at the Mandapam coastal region (latitude 78° 8’ east and longitude 9° 17’ north) in the Gulf of Mannar at the Bay of Bengal. This would allow a large scale production of SWAg-NPs. Biological approaches appear to be a cost-effective alternatives to conventional physical and chemical methods of synthesis. The obtained SWAg-NPs were characterized by UV-vis spectroscopic analysis followed by, Fourier Transform infrared spectroscopy (FTIR), Scanning electron microscopy (SEM) equipped with energy dispersive spectroscopy (EDS), and Field-emission scanning electron microscopy (Fe-SEM). SWAg-NPs demonstrated significant antibacterial activity against Gram-positive and -negative strains such as methicillin-resistant *Bacillus subtilis*; *Staphylococcus aureus* and negative ampicillin-resistant *Escherichia coli*; *Vibrio cholera* was studied.

EXPERIMENTAL METHODS

Pathogenic bacteria for testing

Pathogenic bacteria Gram-positive and negative strains such as Methicillin-resistant *Bacillus subtilis* (NCIM 2920); *Staphylococcus aureus* (NCIM 5021), Ampicillin-resistant *Escherichia coli* (NCIM 2931); *Vibrio cholera* (MTCC 3906) and multidrug-resistant *Pseudomonas aeruginosa* (NCIM 5029) the drug-susceptible cultures were obtained from the Council of Scientific and Industrial Research - National Chemical Industrial Microorganisms (CSIR-NCIM), Pune, India and the Council of Scientific and Industrial Research - Microbial Type of Culture Collection and Gene Bank (CSIR-MTCC), Chandigarh, India.

Collection and extraction of seaweed

Fresh specimens of brown seaweed *S. wightii* (SW) were collected from the Mandapam coastal region (latitude 78° 8’ east and longitude 9° 17’ north) in the Gulf of Mannar at the Bay of Bengal by using sterile polyethylene bags. Then the collected samples were cleaned thoroughly with seawater followed by tap water and distilled water to remove adhering debris, associated epifauna/ epiphytes [15]. After cleaning, seaweed was dried in the shade at room temperature (28 $\pm$ 2 ^{0}C) for a week. The shade dried SW was macerated as to make a coarse powder using mortar and pestle. From that sample 20 g of SW powder were mixed with 200 ml of Milli-Q water (Millipore, USA) and kept in a boiling water bath at 120 ^{0}C for 15 min.

After cooling, the crude seaweed extract [*S. wightii* (SW); SE] was filtered through a Whatman No.1 filter and was stored in a refrigerator at 4 ^{0}C until further study [16].

Synthesis and characterization of silver nanoparticles (Ag-NPs)

An aqueous solution of 1 mM silver nitrate ($AgNO_3$) (analytical grade - Merck, India) was used for the synthesis of silver nanoparticles (Ag-NPs). The reaction mixture was prepared by adding 5 ml of SE and 95 ml of 1 mM $AgNO_3$ solution in a 250 ml Erlenmeyer flask and kept in a boiling water bath at 70 ^{0}C until the color changed to dark brown [17]. The formation of dark brown color indicates an eco-friendly synthesis of *S. wightii* (SW) mediated silver nanoparticles (SWAg-NPs).

These were confirmed by UV-vis spectroscopy (Shimadzu 1700, America Varian Cary 5000 spectrophotometer), the size and morphology were elucidated by scanning electron microscopy (SEM; HITACHI, S-3000H). Further confirmation was obtained by X-ray diffraction (Nicolet Model: 6700) and a field-emission scanning electron microscopy (ZEISS, Fe-SEM), equipped with energy dispersive spectroscopy (EDS), measured at 20kV accelerating voltage. Composition and functional groups were studied by fourier transform infrared spectroscopy (FTIR). Analysis was taken place using the KBr pellet technique at a range of 4000 cm^{-1} to 400 cm^{-1} (Made spectrum RX 1, Male Perkin Elmer) at the CSIR-CECRI laboratory in Karaikudi, Tamil Nadu, India. The UV-visible (UV-vis) diffuse reflectance spectra were obtained by an America Varian Cary 5000 spectrophotometer.

In-vitro antimicrobial efficacy against Pathogenic bacteria

All the media, standard disks, sterile swabs and HiAntibiotic ZoneScale - C were purchased from Hi-Media (Mumbai, India). Isolates of MDRs were grown in nutrient agar medium, and then followed by frequent sub-culturing into fresh nutrient broth medium incubated at 37 ± 1 ^{0}C for 24 - 48 hrs for an antimicrobial efficacy test. *In-vitro* antimicrobial sensitivity assays were carried out using a well diffusion assay to test samples SWAg-NPs against certain MDRs plated on a Muller Hinton Agar (MHA) medium. Sterile cotton swabs were used to inoculate standardized bacterial suspensions (test culture suspensions prepared in sterile 0.85% saline matching an optical density of 0.5 McFarland standards corresponding to 10^8 CFU/mL) on the surface of agar plates for homogeneous growth [18-21].

Lyophilized SWAg-NPs were dissolved in Milli-Q water (Millipore, USA) and sonicated in order to prevent the agglomeration of particles. Four wells each of 6 mm diameter were made on each plate with different concentrations of eco-friendly synthesized SWAg-NPs solutions (20, 40, 60 and 80 µg ml^{-1}) were loaded into each well. These plates were incubated at 37 ± 1 ^{0}C for 24 - 48 hrs after incubation. Zones of inhibition were measured by a ruler/HiAntibiotic ZoneScale-C. Assays were performed in triplicate and average values were recorded [22].

RESULTS AND DISCUSSION:

Several natural and engineered NPs have demonstrated strong antimicrobial properties through diverse mechanisms including the photocatalytic production of reactive oxygen species that damage cell components and viruses, compromising the bacterial cell envelope, interrupting energy transduction, and inhibiting enzyme activity and DNA synthesis [23]. Muthukumar and co-workers [17] reported that seaweeds are extremely different from terrestrial plants with the ability to reduce silver to silver ions (Ag^+). However, when 1 mM $AgNO_3$ solution was added to aqueous *S. wightii* (SW) seaweed extract (SE), no reaction occurred. Instead, after 48 hrs of incubation at room temperature, the color of the solution intensified to dark brown indicating the formation of Ag nanoparticles as shown in Fig. 1(a). This characteristic color change may be due to the excitation of surface plasmon resonance (SPR) and reduction of biosynthesized SWAg-NPs. The $AgNO_3$ solution control remained as such without any change in color [24]. This suggests that the color intensity of the biosynthesized SWAg-NPs studied by UV–visible spectroscopy is a convenient tool for measuring the reduction of metal ions based on optical properties called SPR [25]. The reaction mixture

has an absorption maximum of 430 nm through an increase of color extinction with time and the colorless product indicates a cessation of the reduction reaction shown in Fig. 1(a).

Intense FTIR analysis bands were observed at 3429 cm^{-1}, 1637 cm^{-1} and 685 cm^{-1}, which indicate the presence of molecular functional groups that are responsible for the reduction of biomolecules for capping and stabilization of SWAg-NPs in Fig. 1(b). FTIR peaks unveil the presence of phenolic compounds (3429 cm^{-1}) with a hydroxyl group (OH) bonded directly to an aromatic hydrocarbon group (685 cm^{-1}). The band peak at 685 cm^{-1} could be assigned to the stretching vibration of aromatic rings that may be attached to a free OH-group reported as silver ions (Ag^{+}), which possibly bind to phenolic compounds with one or more aromatic rings resulting in the formation of Ag-NPs [26]. XRD data showed four strong peaks at 32.5^0, 46.4^0, 57.4^0 and 76.3^0. These values were comparative with the original XRD pattern of $AgNO_3$ crystals and pure silver (Ag) [that was published by the Joint Committee on Powder Diffraction Standards (JCPDS - file no. 84-0713 and 04-0783)]. Intense peaks at 38.1^0, 44.3^0, 64.4^0, and 77.3^0 were indexed with the 111, 200, 220 and 311 planes of Ag, respectively (Fig. 2a). The XRD spectra indicated that particles were of acceptable crystallinity with the cubic structure form of Ag-NPs and aggregations formed due to the action of stabilizing agents in the algal extract [27]. The diffraction angles of Ag-NPs were quite close to Ag crystals.

The morphological characteristics of SWAg-NPs size and shape were characterized through SEM analysis and showed spherical particles of a size less than 75 nm (20–50nm) (Fig. 2b). This was also confirmed by Fe-SEM analysis that demonstrated the spherical, crystalline and poly-dispersed SWAg-NPs of 22 nm sizes with minimal agglomeration due to the presence of organics of *S. wightii* (SW) as stabilizing agents (Fig. 2c). An earlier study by Morones and co-workers [28] showed bactericidal effects of Ag-NPs of 1-100 nm size, proving broad spectrum bactericidal activity. The EDS results also showed an elemental silver (Ag) signal peak at higher than 92 % (Fig. 2b).

Antibacterial activities of biosynthesized SWAg-NPs samples were found to reveal considerable activity against most of the pathogenic bacteria. That concentration and species-specific characteristics play an important role in antimicrobial screening clearly depicts the potent nature of the synthesized SWAg-NPs. A considerable difference in the diameter of inhibition zone was observed with different concentrations of SWAg-NPs as displayed in Table 1. Antibiotics are among the most successful drugs used in human therapy. However, since they can challenge microbial populations, they must be considered as crucial pollutants as well. Besides being used for human therapy, antibiotics are extensively used for both animal farming and other agricultural purposes. Residues from the human environment and farms may contain antibiotics and antibiotic resistance genes that can pollute natural environments [29].

In addition, higher SWAg-NPs concentrations than 80 µg ml^{-1} showed a greater sensitivity than lower concentrations (20 µg ml^{-1}) of all tested microorganisms. That concentration and species-specific characteristics play an important role in the antimicrobial screening clearly depicts the potent nature of the synthesized SWAg-NPs. A maximum growth inhibition zone of 18 mm was observed in Ampicillin-resistant *Escherichia coli* (NCIM 2931) and a minimum of 12 mm in Methicillin-resistant *Staphylococcus aureus* (NCIM 5021) due to extracellular polymeric substance (EPS) secretion in Gram-positive bacteria. Amro and co-workers [30] as well as Panacek and co-workers [31] reporting on the mechanisms of Ag-NPs toxicity suggested that the attachment of particles to the surface of cell membranes caused a disturbance of permeability and respiration. SWAg-NPs are known to bind with thiol groups of DNA and RNA affecting the protein biosynthesis of bacteria [26]. Studies have demonstrated that silver ions (Ag^{+}) interact with sulfhydryl (SH) groups of proteins as well as the bases of DNA, leading either to respiratory inhibition or to the unwinding of DNA [32]. The bactericidal activity was dependent on the shape and size of the nanostructures and their concentrations. Besides, SWAg-NPs react with the thiol groups of proteins and interfere with DNA replication leading to bacterial inactivation [33,34]. These biosynthesized SWAg-NPs showed a good stability and novel broad-spectrum antibacterial materials for multidrug-resistant strains in near future.

CONCLUSION

We were able to biosynthesize Ag-NPs from *S. wightii* (SW) at room temperature within 48 hrs of incubation. The formation of SW-AgNPs was confirmed by UV-spectra, FT-IR, SEM, EDS and Fe-SEM measurement studies. The suggested procedure was effective against Gram-negative pathogen isolates with a maximum inhibition (18 mm) of Ampicillin-resistant *E. coli* and a minimal (14 mm) of *P. aeruginosa*, whereas, Gram-positive a maximum inhibition (12 mm) of Methicillin-resistant *S. aureus* and a minimal (10 mm) of *B. subtilis* by rupturing the membrane of the Gram-positive and negative bacteria cell walls as well as extracellular polymeric substance (EPS) secretion from binding to intracellular material. Moreover, biosynthesized SWAg-NPs exhibited an excellent stability and long-term usability. The results demonstrated that green synthesized SWAg-NPs could cause significant impact on the efficiency of bacterial inactivation.

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Table 1: *In-vitro* antibacterial and photocatalytic efficacy of green synthesized SWAg-NPs against multi-drug resistant strains (MDRs).

Table 1: *In vitro* antibacterial activity of green synthesized SWAg-NPs against pathogenic bacteria

S.No.	pathogenic bacteria	Inhibition zones of green synthesized SWAg-NPs (mm)			
		20 µg ml^{-1}	**40 µg ml^{-1}**	**60 µg ml^{-1}**	**80 µg ml^{-1}**
Gram-positive					
1	*Bacillus subtilis*	--	10	10	10
2	*Staphylococcus aureus*	--	--	10	12
Gram-negative					
3	*Escherichia coli*	10	10	14	18
4	*Vibrio cholera*	10	10	12	16
5	*Pseudomonas aeruginosa*	--	10	10	14

Figure 1: (a) Visible color change and UV–Vis spectroscopy absorbance; (b) FTIR spectra of seaweed extract (alone) and green synthesized SWAg-NPs from Sargassum wightii (SW) seaweed extract; (c) XRD pattern mixed phase of face-centred cubic (fcc) structures; (d) SEM images of green synthesized SWAg-NPs

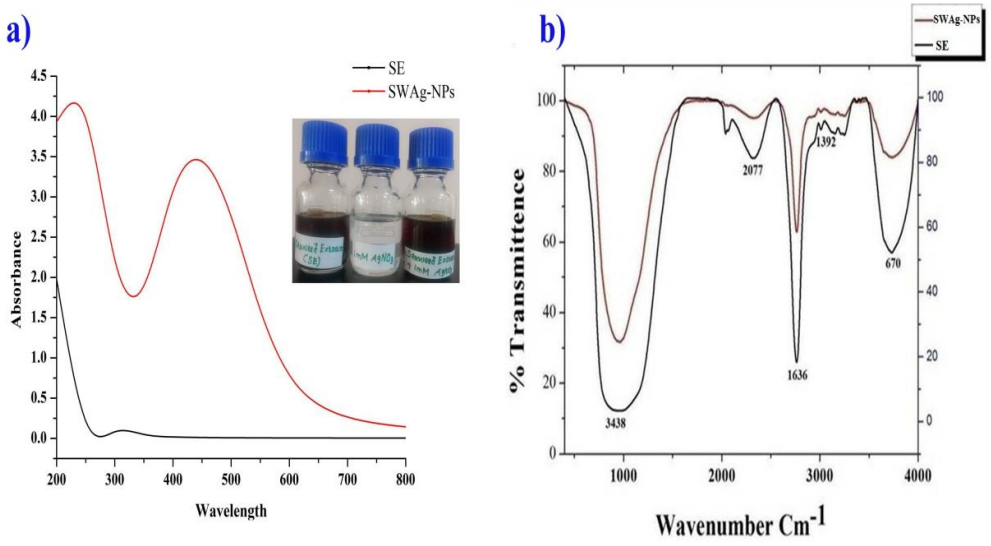

Figure 2: (a) Fe-SEM and SADE images of randomly selected SWAg-NPs observation; (b) EDS image of green synthesized SWAg-NPs by S. wightii (SW) seaweed extract