Original Research Paper

Green Synthesized Silver Nanoparticles Prepared from the Antimicrobial Crude Extracts of two Brown Seaweeds Against Plant Pathogens

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Abstract: This study aimed for green synthesis of silver nanoparticles (Ag-NPs) from the antimicrobial crude extracted in chloroform: methanol(1:1 v/v) from the two brown seaweeds *Spatoglossum asperum* and *Hedophyllum sessile* active against two pathogenic bacteria (*Xanthomonas axonopodis* pv. *citri* and *X. oryzae* pv. *oryzae*) and a fungus *Ustilaginoidea virens* cause diseases in plants under *in vitro* assay. Crude extracts exhibit high antibacterial activity and low antifungal activity. Green synthesized Ag-NPs showed very high antimicrobial properties on comparing its crude extracts. Among the crudes, extract of *Spatoglossum asperum* exhibits higher bioactivity than the extract of *Hedophyllum sessile* but Ag-NPs prepared from the extract of *H. sessile* possess very strong bioactivity over Ag-NPs of *Spatoglossum asperum.* Reduction of Ag-NPs was confirmed by UV spectra. FTIR data indicate that active groups related to terpenoids and phenols found in the crude extracts were responsible for the reduction of Ag-NPs. The XRD data showed that the pure three types of crystal silver structure at 2θ values 32.51, 46.50 and 74.62 corresponding to 111, 200 and 220 planes for silver, respectively. This study concludes that chloroform: methanol (1:1 v/v) extracts of *Hedophyllum sessile* and *Spatoglossum asperum* containing active groups related to terpenoids and phenols and they are acting as reducing agents for green synthesis of silver nanoparticles which are potential source for controlling the plant pathogens studied.

Keywords: Brown Seaweeds, Silver Nanoparticles, Antimicrobial Property-Plant Pathogens

Introduction

Nanotechnology is emerging as a rapidly growing field with its application in Science and Technology for the purpose of manufacturing new materials at the nanoscale level (Prabhu *et al*., 2010). The synthesis and characterization of nanoparticles is an important area of research as selection of size and shape of nanoparticles provides an efficient control over many of the physical and chemical properties and their potential application in optoelectronics, recording media, sensing devices, catalysis and medicine (Mazur, 2004). The current chemical methods for synthesizing nanoparticles are energy intensive; employ toxic chemicals which produce hazardous wastes that preclude them for any biomedical application. Conversely, the physical methods of nanoparticle synthesis such as sputter deposition, thin films etc., were often difficult to achieve. Therefore, there is a growing need for the uses of bio-compatible, non-toxic, cost-effective and eco-friendly methods for production of silver nanoparticles (Jae *et al*., 2009). Biological methods are considered safe and ecologically sound for the nanomaterial fabrication and alternative to conventional physical and chemical methods. Biological systems such as yeast, fungi, bacteria, plants etc., considered as biomimetics for the synthesis of nanostructures of biocompatible metal and semiconductors. Among the biological systems, the marine organisms are greatly deserved for the syntheses of different nanoparticles (Klaus *et al*., 1999; Nair and Pradeep, 2002; Konishi and Uruga, 2007) because of their diversity richness and high tolerance under ambient conditions of temperature, pressure and acidity. Innumerable organisms including algae, bacteria and fungi (Vigneshwaran *et al*., 2007) have already been harnessed for the green synthesis of silver nanoparticles. Marine macroalgae(seaweeds) have various unique phytochemicals including heteropolysaccharides, alkaloids, steroids, phenols, saponins and flavonoids flavonoids (Mansuya *et al*., 2010). Studies have assured that biomolecules like protein, phenols, flavonoids and some phytochemicals have the ability to reduce the ions to the nanosize and also play an important role in the capping of the nanoparticles for its stability (Vedpriya, 2010). Silver nanoparticles (Ag-NPs), is gaining greater demand due to the emergence of antibiotic-resistant pathogens (Goldmann *et al*., 1996). Metallic silver in the form of nanoparticles has made a remarkable response as a potential antimicrobial agent (Chastre, 2008). Silver components have been proven as an effective tool for retarding and preventing the microbial infections. In addition, silver is known to exhibit oligo dynamic effect because of its ability to exert inhibitory effect at minute concentrations (Tien *et al*., 2009).

Canker in citrus caused by the bacterium *Xanthomonas axonopodis* pv. *citri* reported to produce up to 70% yield loss (Graham *et al*., 2013). Estimated yield loss of bacterial blight caused by the bacterium *Xanthomonas oryzae* pv. *oryzae* in tropical regions was from 2 to 74% depending on location, season, weather conditions and cultivars (Reddy *et al*., 1979). A fungus *Ustilaginoidea virens* cause false smut disease in paddy and its infection usually low (1-10%) or may increase to 50-60% in years and severe infection causing 50.3-75.4% empty grains (Li *et al*., 1986). Pesticidal agents of biological origin are advantageous one to control the pests as well as safe to environment. In this line, green synthesized Ag-NPs prepared from seaweeds have potential application in the biopesticides production. Works have been attempted on the green synthesis of Ag-NPs using higher plants (Gilaki, 2010; Khalil *et al*., 2014) and microalgae (Patel *et al*., 2015) but limited studies are made using marine macroalgae (seaweeds) (Govindaraju *et al*., 2009; Kumar *et al*., 2013). In this study, crude extracts prepared from the two brown seaweeds showing antimicrobial activity were used for green synthesis of Ag-NPs against plant pathogens.

Materials and Methods

Seaweed Collection and Preparation of Crude Extract

Live and healthy specimens brown seaweeds such as *Hedophyllum sessile* (C. Agardh) Setchell-Puthumadam (9°16’47”N, 79°7’12”E) and *Spatoglossum asperum* J. Agardh-Thiruchendur (8.4833° N, 78.1167° E) were collected along the coast of Gulf of Mannar, India during January 2014. The samples were washed thoroughly in seawater followed by tap water and immediately air-dried under shade at room temperature for 7 days, chopped, pulverized and stored in desiccators till further study.

For the preparing the crude extract, 50 g of pulverized sample of seaweed was soaked in 100 mL of chloroform: methanol (1:1 v/v) using 250 mL conical flask and kept under dark for 10 days at 27°C. The extraction was repeated thrice. Then the extracted crudes were combined and concentrated using rotavapour under reduced pressure at 45°C. The concentrated crude extract was weighed and stored at 0°C till further study.

Green Synthesis of Ag-NPs (Song and Kim, 2008)

A portion of crude extract of each seaweed weighing 0.1 g was dissolved with 3 mL distilled H_2O and a same amount of $AgNO_3$ (1×10^1 M) and stirred the extract well at room temperature (25°C). Then the solution was incubated in dark at 37°C under static condition for observing colour change. A control setup was also maintained without crude extract. The mixture was then centrifuged at 5000 rpm for 20 min and pellets containing crude silver nanoparticles were collected. Aqueous pellet was freeze dried and stored at 0°C till further study.

Antimicrobial Activities of Crude Extracts and Green Synthesized Ag-NPs

Antimicrobial potential of crude extracts and green synthesized Ag-NPs was evaluated using two bacteria and a fungus cause diseases in plants. The pathogenic bacteria, *Xanthomonas axonopodis* pv. *citri* (Hasse) Vauterin *et al*. AGACA 01 cause citrus canker and *Xanthomonas oryzae* pv. *oryzae* (Ishyama) Dye AGACA 02 cause blight in paddy. The fungus *Ustilaginoidea virens* (Cooke) Takah AGACF 01 cause false smut in paddy. The test pathogens available in the Botany Research Laboratory of Alagappa Government Arts Science College, Karaikudi were used for antimicrobial assay. Sterile 5.0 mm diameter Whatman # 1 paper discs loaded with 50 µl of 25, 50 and 100 µg of crude seaweed extracts and green synthesized Ag-NPs were dried aseptically and impregnated on Petri plates for antimicrobial assay.

For antibacterial Assay (Arunkumar and Rengasamy, 2000), agar diffusion technique using 100 mm diameter Petri plates containing Ca. 20 mL of 1.5% nutrient agar medium (g L^1, Peptone -10, Beef extract-10, NaCl- 5, Agar-15, in distilled H_2O, pH -7.0) smeared with 0.05 mL of bacterial culture in exponential phase of 1.0 OD at 590 nm were incubated at 27°C.

For antifungal assay (Suvega and Arunkumar, 2014), Petri plates were seeded ca. 10 mL of 3% Potato Dextrose Agar (PDA) (g L^1, Potato-200, dextrose- 20, agar-30, in distilled water, pH 5.6). A loop full of fungal mycelia mixed evenly with 10 mL of 1.5% PDA was poured over preseeded 3% PDA plates and incubated at 25°C. Antibacterial and antifungal activities were measured after 48 h of incubation.

The diameter of agar clear zone around the disc as result of diffusion of active substances was measured millimeters as antibacterial/antifungal activity. The solvents used for reconstituting the substances loaded on the paper discs were treated as solvent control did not show any bioactivity. Triplicates were maintained in each experiment and mean value expressed.

Characterization of AgNPs

Freeze dried AgNPs prepared from both seaweeds were recorded in UV spectra between 200 to 600 nm (Gajbhiye *et al*., 2009). FT-IR spectroscopy (Spectrum RX I, Perkin-Elmer) was recorded by KBR pellet method in the spectral range of 4000 to 400 cm^{1} to identify the biomolecules responsible for the reduction of the Ag+ ions and capping material of synthesized Ag-NPs. For comparison, crude seaweed extracts were also recorded FT-IR spectroscopy. For XRD diffraction (Chandran *et al*., 2006), Ag-NPs were measured using X-ray diffractometer (PXRD-6000 SCHIMADZU) in the angle range of 10°C-80°C at 2θ, scan axis: 2:1 sym. The size of the Ag-NPs was calculated from the PXRD peak positions using Bragg's law.

Results and Discussion

Antimicrobial Activity of Crude Extracts

Crude extracts prepared from various solvents and methods from different species of seaweeds displayed wide range antimicrobial properties against human and plant pathogens (Arunkumar *et al*., 2010). Extracts prepared in chloroform: methanol (1:1 v/v) from the brown seaweeds posses polar compounds exhibit bioactivity against plant pathogenic bacteria (Arunkumar and Rengasamy, 2000) and the efficacy of crude is not as equal to commercial antibiotic, streptomycin sulphate used to control canker in citrus and blight in paddy (Arunkumar *et al*., 2013). In this present study, crude extracts obtained in chloroform: methanol (1:1 v/v) of two brown seaweeds *Hedophyllum sessile* and *Spatoglossum asperum* exhibit antimicrobial activities which was increased with increasing concentration of extracts (Fig. 1). Studies so far conducted on antimicrobial screening of seaweeds against plant pathogens showed investigation in this two brown seaweeds *Hedophyllum sessile* and *Spatoglossum asperum* are not yet made (Arunkumar *et al*., 2010; 2013). This study found that antibacterial activity of the crude extracts was higher than antifungal activity and crude extracts of *Spatoglossum asperum* exhibits more bioactivity (Fig. 1 and 2) than the extract of *Hedophyllum sessile* (Fig. 1 and 3).

Antimicrobial Activity of Green Synthesized Ag-NPs

Recent works have proved that green synthesized silver nanoparticals exhibit potential broad-spectrum of antimicrobial activities (Gajbhiye *et al*., 2009; Govindaraju *et al*., 2009). Green synthesized silver-nanoparticles prepared from extracts of plants including algae possessing antimicrobial properties tested against human and plant pathogens (Mohandass *et al*., 2013; Kumar *et al*., 2013; Paulkumar *et al*., 2014). Crude extracts showing antimicrobial activity are natural, less expensive and environmental safe hence in this study crude extracts obtained from the *Hedophyllum sessile* and *Spatoglossum asperum* were used as reducing agents to produce silver nanoparticles through green synthesis. Ironically antimicrobial activity of biosynthesized Ag-NPs prepared from the crude extracts of both seaweeds *Hedophyllum sessile* and *Spatoglossum asperum* was remarkably higher than the potential of crude extracts (Fig. 1). It was further observed that the Ag-NPs prepared from *Hedophyllum sessile* showed higher bioactitivty than *Spatoglossum asperum* (Fig. 2-4), however chloroform: methanol (1:1 v/v) extracts both seaweeds *Hedophyllum sessile* and *Spatoglossum asperum* are the potential reducing agents for green synthesis of silver nanoparticles as source for preparing biopesticides to control the plant pathogens.

Characterization of Ag-NPs by UV, FTIR and XRD Study

Maximum absorption at 400 nm in the UV spectroscopy characteristics of Silver nanoparticles (Mohandass *et al*., 2013) was recorded in *Hedophyllum sessile* (Fig. 5) and *Spatoglossum asperum* (Fig. 6). FTIR spectra data is commonly used for identifying biomolecule responsible for the reduction of Ag^{+} ions and the capping of the bioreduced Ag-NPs (Li *et al*., 2007; Song *et al*., 2009; Travan *et al*., 2009; Vanmathiselvi and Sivakumar, 2012). FTIR spectral peaks of Ag-NPs were strong and of crude were weak in *Hedophyllum sessile* (Fig. 7) as well as *Spatoglossum asperum* (Fig. 8). As earlier study (Vanmathiselvi and Sivakumar, 2012), in the FTIR spectra, a strong peak in the crude and a weak peak in the Ag-NPs recorded near 2900 cm^{-1} in *Hedophyllum sessile* (Fig. 7) and 2985 cm^{1} in *Spatoglossum asperum* (Fig. 8) were assigned to N-H stretching of alkaline. A band at 1600 cm^{1} in both spectra corresponds to primary amines (Vanmathiselvi and Sivakumar, 2012; Sahayaraj *et al*., 2012). A peak at 1230 cm^{1} corresponds to C-O stretching of aromatic ethers exclusively present in the Ag-NPs of *Spatoglossum asperum*.

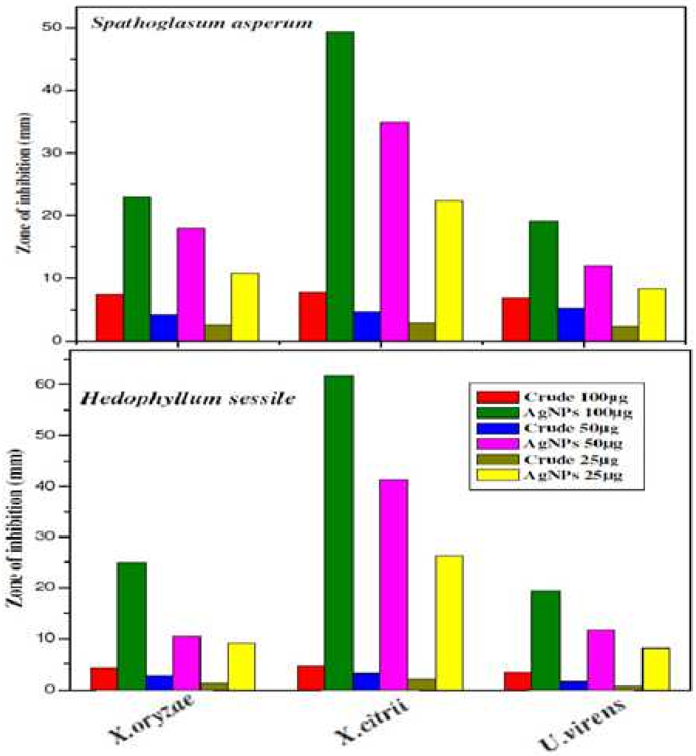

Fig.1. Antibacterial(*X.oryzae* and *X.citri*) and antifungal (*U.virens*) activities of different concentrations of crude extract and green synthesized Ag-Nps prepared from the chloroform: methanol (1:1 v/v) of *Hedophyllum sessile* and *Spathoglossum asperum*

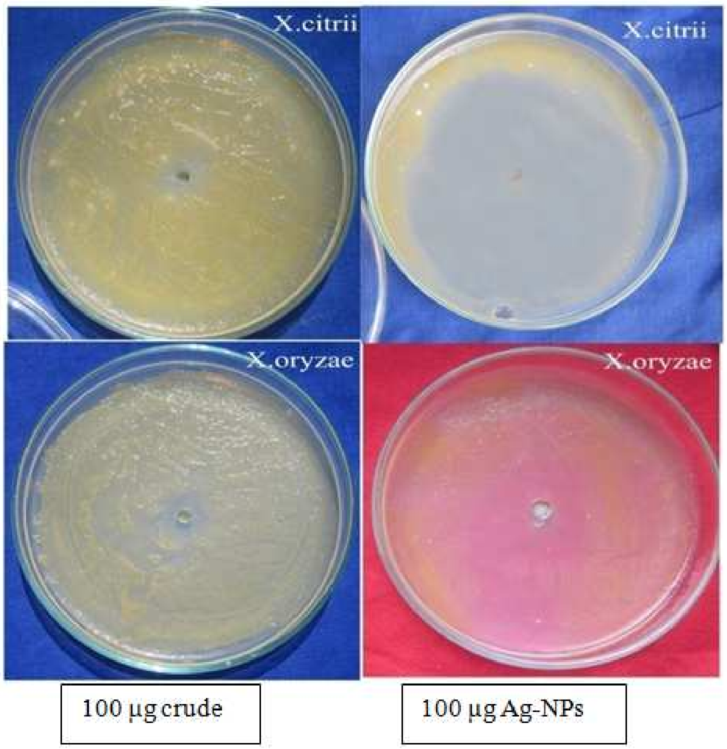

Fig. 2. Antibacterial activity of crude extract and green synthesized Ag-NPs obtained from the *Spathoglossum asperum*

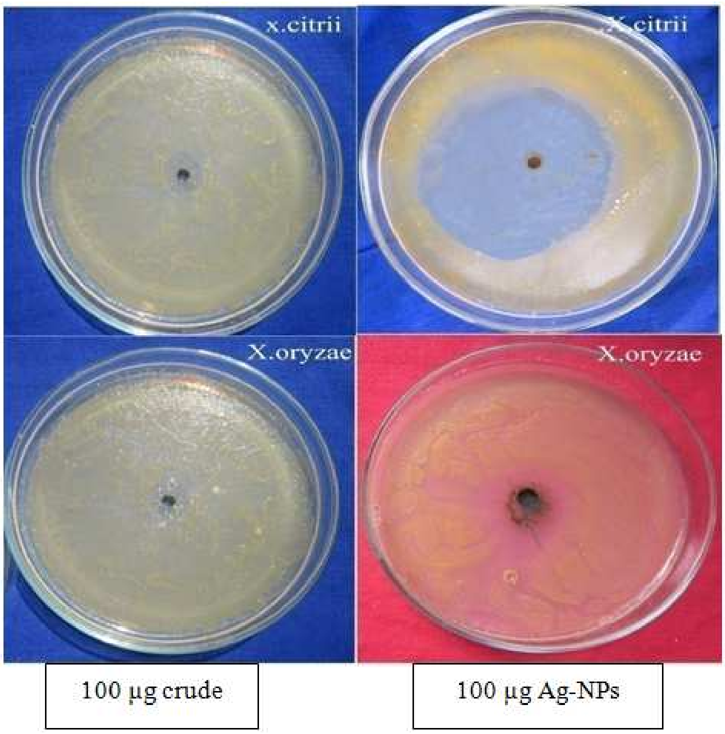

Fig. 3. Antibacterial activity of crude extract and green synthesized Ag-NPs obtained from the *Hedophyllum sessile*

Fig. 4. Antifungal activity of crude extract of *Spathoglossum asperum* (A) green synthesized Ag-NPs of *Spathoglossum asperum* (B) and *Hedophyllum sessile (C)*

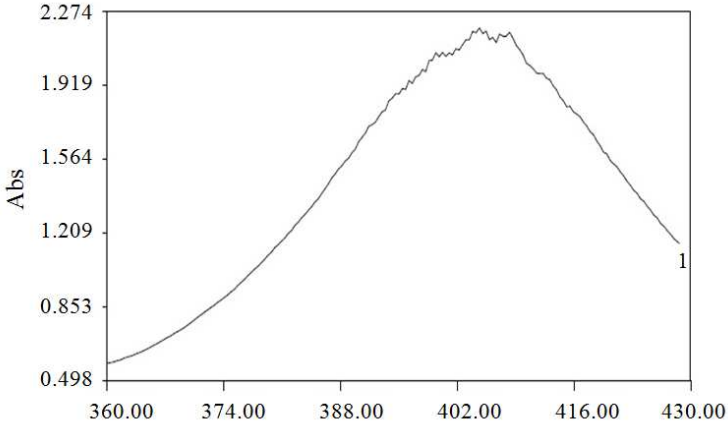

Fig. 5. UV spectrum of green synthesized AgNPs prepared from the chloroform: methanol (1:1 v/v) extract of *Hedophyllum sessile*

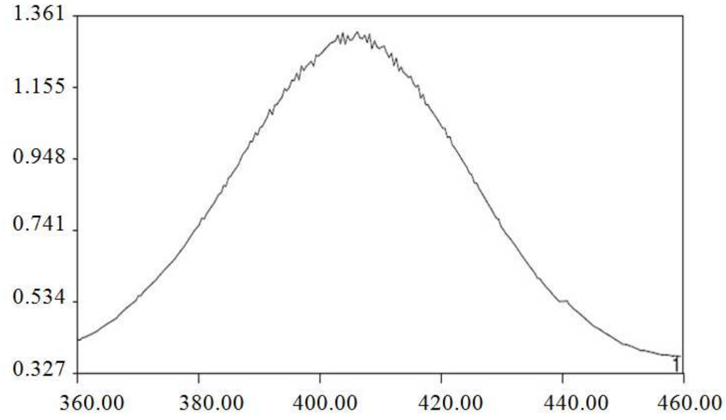

Fig. 6. UV spectrum of green synthesized Ag-NPs prepared from the chloroform: methanol (1:1 v/v) extract of *Spathoglassum asperum*

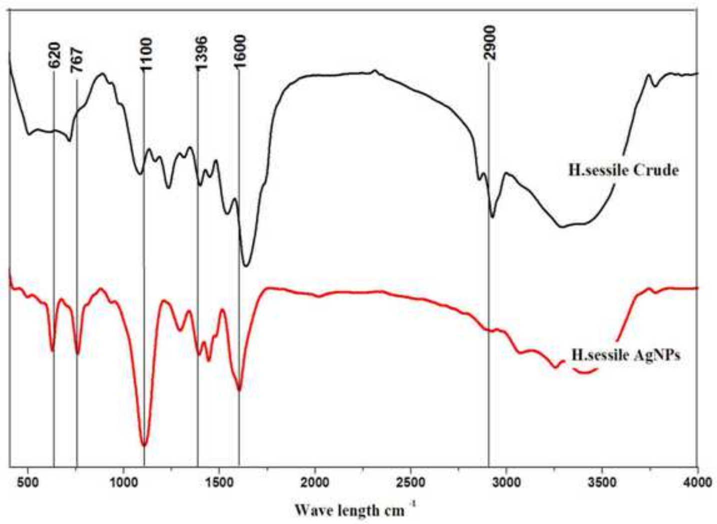

Fig. 7. FTIR spectroscopy of crude and green synthesized Ag-NPs prepared from the *Hedopyllum sessile*

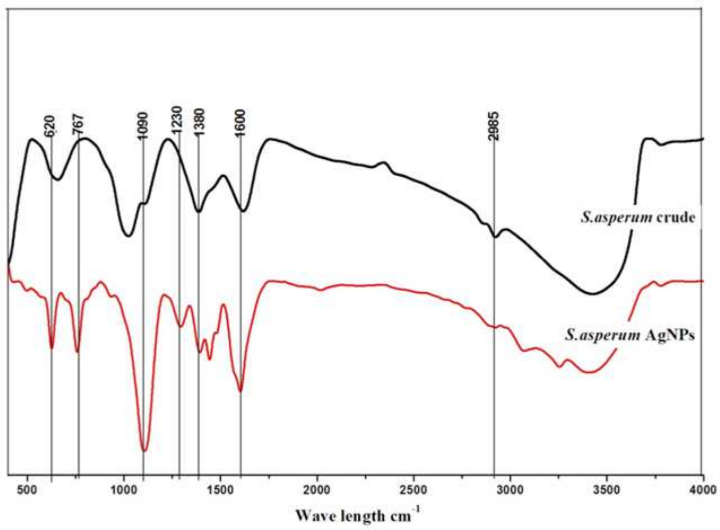

Fig. 8. FTIR spectroscopy of crude and green synthesized Ag-NPs prepared from the *Spathoglossum asperum*

A strong peaks at 1100 cm^{-1} and 1090 cm^{-1} in *Hedopyllum sessile* and *Spatoglossum asperum,* respectively corresponds to C-N stretching of the aliphatic amine, which is commonly found in protein, indicating protein as ligands for Ag-NPs, which increase the stability of nanoparticles synthesized (Shetty *et al*., 2006). Peaks at 620 cm^{-1} and 767 cm^{-1} in Ag-NPs of both seaweeds were assigned to the C-X stretching of either chloroalkanes or bromoalkanes (Anil Kumar *et al*., 2007). These FTIR data indicate functional groups in the secondary metabolites such as phenols and terpenoids found in the crude extracts of *Hedopyllum sessile* and *Spatoglossum asperum* are the source of reducing agents for synthesis of Ag-NPs (Shetty *et al*., 2006).

The biosynthesized Ag-NPs was further structurally characterized through peaks observed in the XRD pattern. The sharp diffraction pattern of the XRD spectra obtained by the annealing at 200°C indicates a pure crystalline silver structure (Fig. 9 and 10). The XRD peaks at 2θ values of 32.51, 46.50 (for both seaweeds) and 64.62 (*Hedopyllum sessile*) corresponding to hkl values of 111, 200 and 220 for planes of silver nano particles, respectively. All diffraction peaks correspond to the characteristic face centered cubic phase of silver nanoparticles (JCPDS No. 04-0783; Anil Kumar *et al*., 2007).

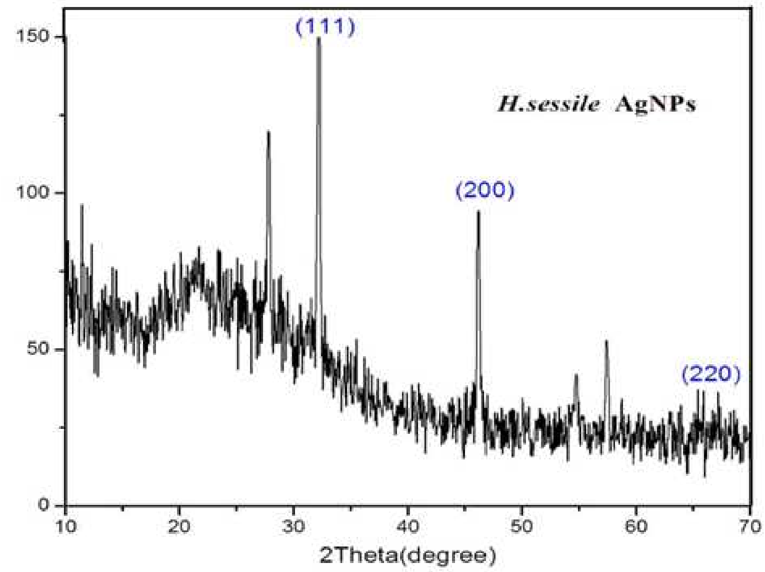

Fig. 9. XRD pattern of green synthesized Ag-NPs prepared from the chloroform: methanol (1:1 v/v) extract of *Hedophyllum sessile*

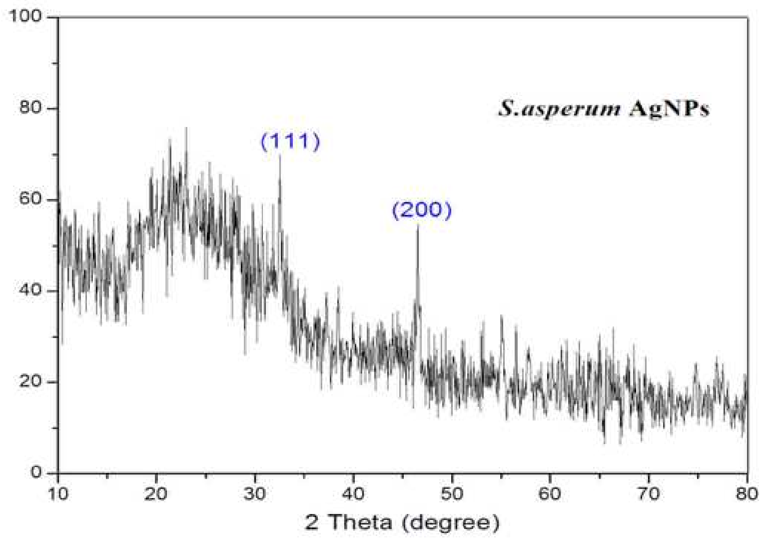

Fig. 10. XRD pattern of green synthesized Ag-NPs prepared from the chloroform: methanol (1:1 v/v) extract of *Spathoglossum asperum*

Conclusion

This study concludes that crude extracts obtained in chloroform: methanol (1:1 v/v) from the brown seaweeds *Hedophyllum sessile* and *Spatoglossum asperum* exhibit antimicrobial property against certain plant pathogens. These antimicrobial crude substances contain active groups related to terpenoids and phenols which are responsible for reducing silver nano particles. Bioactivity and XRD data support *Hedopyllum sessile* was more promising than *Spatoglossum asperum* for synthesizing silver nano particles.

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Author's Contributions

Annakodi Jothirethinam: Carried out the experimental works of this finding.

Sivanantham Prathiba: Carried out the experimental works of this finding.

Natarajan Shanthi: Carried out analysis of the samples, prepared the data and figures suite to article and contribute writing part of the paper.

Kulanthaiyesu Arunkumar: Mentor who design the research problem with aim and objectives and supervised the experiment and contributed writing part of the article as well.

Ethics

This research article is original work which is not published earlier. The corresponding author aware and confirmed that all the others have read and approved the article and no ethical issue involved.

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