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Biosynthesis and Characterization of Silver Nanoparticles using Aqueous Extract of Brown Algae and their Effect on Beneficial Soil Microbes

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Abstract: ***The present research work focused on the effect on soil microbes and silver nanoparticles were obtained by biosynthesise method using aqueous extract of Stoechospermum marginatum. Seaweed extract is used as a reducing agent of 1Mm silver nitrate solution for the synthesis of AgNPs. The periodical monitoring of reaction mixture was done using UV-vis spectroscopy at 416-591nm. The Transmission Electron Micrographs indicate the size of the NPs to be in the range of 50nm and the nanoparticles were spherical in shape. The Minimal inhibitory concentration (MIC) of AgNPs was found to be 2µml, 1.5µml, 1µml for all the microbes selected for the study. The zone of inhibition measured by agar well diffusion method. The effect of synthesized nanoparticles on soil microbes isolated from the garden soil Bacillus spp, Bacillus substilis, Staphyllococcus epidermidis, Serratia spp, Pseudomonas fluorescens, Pseudomonas spp, Aspergillus flavus, Aspergillus fumigates, Alternaria altermata, Cladosporium spp were studied, results showed the effective bactericidal and fungicidal activity against their microbes.***

Keywords: Antioxidant activity, FT-IR, Marine macroalgae – Stoechospermum marginatum (Ag). Kutz. , Silver nanoparticles; Soil microbes, TEM and UV- Vis.

I. INTRODUCTION

Nanoparticle research is currently an area of intense scientific research, due to a wide variety of potential applications in biomedical, optical, and electronic fields. In the awareness about developing new methods of synthesis of nanomaterial with green technologies is a challenge (Dhanalakshmi Aravinda et al., 2014). Consequently, the natural antioxidants are more acceptable than synthetic antioxidants as these antioxidants do not contain chemical contaminants and display a variety of beneficial functions. Thus, natural antioxidants are considered safe for use as ingredients in medicine, dietary supplements, nutraceuticals and cosmetics with the objective of improving consumer health, reducing the effects of harmful diseases and other broader aspects of immune system function (Shahidi, 2009).Nanotoxicological studies are intended to determine whether the toxic effects of Nanoparticles are having any serious threat to the environment and human beings. The toxicity of silver Nanoparticles affects many bacterial communities which are beneficial/harmful for both humans and environment(Crabtree et al., 2003). Silver Nanoparticles showed a desired toxicity towards beneficial soil microbes as well as humans and also very important about the safety of nano silver products (Stoimenov et al., 2002).

Silver nanoparticles have been widely used during the past few years in various applications due to their well-known effectiveness in biomedical (Cao et al., 2010) electronic (Mohan et al., 2007) catalysis (Feng et al., 2011) and optical applications (Hayward et al., 2000). In particular, the outstanding antimicrobial properties of Ag-NPs have led to the development of a wide variety of nanosilver products, including nanosilver-coated wound dressings, contraceptive devices, surgical instruments, and implants (Lohse and Murphy,2002; You et al.,2012).Apart from these antimicrobial activities, Ag-NPs are also known to possess antifungal, anti-inflammatory, antiviral, anti-angiogenesis, and antiplatelet properties (Monteiro et al.,2012; Martínez-Gutierrez et al., 2012). Additionally, more recent developments have seen Ag-NPs used in room spray, wallpaper gloves, laundry detergent, and wall paint formulations as well as in the textile industry for clothing manufacturing (Park et al., 2012; Gottesman et al., 2011). Beneficial bacteria are of vital importance to soil, plant and animal health. In order to detect the ecotoxicity of environmental chemicals, many studies have used bacteria and earthworms (Gooneratne et al., 2011; Pasco et al., 2011). We report here the biosynthesis of silver nanoparticles via a single –step reduction of silver ions using renewable and biodegradable seaweed extracts at room temperature without the use of any reducing or capping agents. Characterization of the synthesised nanoparticles utilizing UV-visible spectroscopy, Antioxidant activity, Fourier transform infrared spectroscopy analysis, and Transmission electron microscopy besides their effect on beneficial soil microbial isolates were reported.

II. MATERIALS AND METHODS

A. *Collection And Preparation Of Seaweeds For Analysis*

The brown algae Stoechospermum marginatum(Ag). Kutz (Phaeophyceae) was collected from punnakayal, Thoothukudi district. The collected sample were washed with sea water and immediately transported to a laboratory in polythene bags containing natural sea water to prevent evaporation. Algal material was washed with distilled water to remove the dust and soil. After cleaning, the fresh algae were shade dried at room temperature for a week. Collected seaweed was identified on the basis of pigmentation, morphology and authenticated by Dr.P. Anantharaman, Associate Professor CAS in Marine Biology, Annamalai University of Parangipettai, India. Dried seaweeds were powdered with the help of mixer grinder. Seaweed was collected during low tide in the forenoon during January 2016.

B. *Preparation Of Seaweed Extract*

The seaweed powder (5g) was soaked for 24h in 1L of sterile water. Then the crude extract was blended thoroughly and filtered using a Whatman No.1 filter (24µm) twice. The filtrate was used for further analysis.

C. *Synthesis Of Silver Nanoparticles*

In the seaweed extract, 1mM silver nitrate solution was added. The reduction of silver nitrate occurred within 10min which resulted in colour change (dark brown), as noted by visual observations indicating the formation of AgNPs. As per the absorption spectrum, this medium remained stable for more than 3 months. The absorbance of aliquots of the reaction solution was measured using a UV-2371 spectrophotometer operated at a resolution of 1nm (Kumar *et al.*, 2012).

D. *Antioxidant Activity*

Seaweeds contain many phytochemicals including compounds with antioxidant activity, which are mostly phenolic compound (Walailuck *et al.*, 2011). Compounds with antioxidant activity are mainly phenolic acids, flavonoids and polyphenols, so content of total phenol **(**Duan *et al.*, 2006), flavonoid **(**Zhinshen *et al.*, 1999), tannin (Julkunen-Titto, 1985) terpenoid (Ramani *et al.*, 2011 and Abdul Wadood *et al.*, 2013) and tocopherol (Rosenberg, 1992) were investigated in *Stoechospermum marginatum*and *Stm*-AgNPs (aqueous extract).

E. *Characterization of Ag-NPs*

The different techniques were used to characterized the *Stm*-AgNPs such as UV-vis (2371) used to know the band at nanometer, FT-IR (Fourier Transform Infrared spectroscopy) from (Systronics 166) type FTIR spectrometer which was used in the range 400 to 4000 cm^{-1} by KBr pellet method for extract powder and silver nanoparticles TEM (JEM 2100) analysis were carried out to confirm the image of specimen by magnified focus on imaging device.

F. *Isolation of soil microbes (robert et al., 1957; berg and ballin 1996)*

The isolation of microorganisms was carried out using a serial dilution technique. Aliquots of 100µL of different dilutions of garden soil were spread onto plates of nutrient agar medium for bacteria and potato dextrose agar for fungi. The plates were incubated at 28˚C for 5 days under aerobic conditions. Developed colonies were picked and isolated following morphological criteria. Purified isolates were obtained by repeatedly streaking colonies on a TSA (Trypticase soy agar medium) and observing them using light microscopy. The identification and classification of the colony morphotypes were achieved using five parameters: colony size, form, colour, texture and margin. The isolated bacterial and fungal colonies were identified by Dr. D. Arvind Prasanth, Assistant Professor of Microbiology, Periyar University, and Salem. The effect of synthesized nanoparticles on soil microbes were tested using these isolated microbial colonies.

G. *Antibacterial Assays*

Overnight cultures of microbial isolates were subcultured in the nutrient broth. Samples of 3ml of microbial culture were placed into test tubes and 1, 1.5 and 2µl of appropriate dilutions of *Stm*-AgNPs were added. After 24 h incubation at 37 °C, the optical density (OD_{520}) was measured using the spectrophotometer. The MIC (Minimum Inhibition Concentration) for growth was defined as the lowest concentration of NPs, which inhibited bacterial and fungal growth.

The antibacterial assays were done on the garden soil bacterial isolates by standard well method. Nutrient broth/agar medium was used to cultivate bacteria. Fresh overnight cultures of inoculums of each culture were spread on to nutrient agar plates using sterile

cotton swabs. The well made in the agar plate using cork borer. The silver nanoparticles along with the sample (2 µg) were poured over the well of inoculated plates followed by incubation overnight at 37º C. The antibacterial activity was assigned by measuring the diameter of the zone of inhibition around the well.

H. Agar well diffusion Assay For Fungi (Berg and Ballin 1996).

Antifungal activity *Stm*-AgNPs against garden soil fungal isolates were determined by using well agar diffusion method. Stock cultures were prepared and maintained in Potato dextrose agars were also done parallel. The plates were examined for evidence of zone inhibition, which appear as a clear around the well. The diameter of such zone of inhibition was measured using a meter ruler. Mean value was calculated by performing the experiments in triplicates.

I. Statistical Analysis

Results obtained in this study have been subjected to the following statistical analysis by using computer software. Standard deviations

III. RESULTS AND DISCUSSION

A. Synthesis And Characterization Of Silver Nanoparticles

Several reports have been employed for the synthesis of silver nanoparticles for its beneficial applications. Recently, seaweeds have been identified as the potential source for synthesizing nanoparticles Aki et al., 2008 have synthesized silver nanoparticles from Stoechospermum marginatum extract within 24 h of incubation time. Similarly, we have synthesized silver nanoparticles from the extract of Stoechospermum marginatum rapidly within 20 min. Endpoint with prominent color change (Plate.1) indicates the excitation of surface plasmon resonance due to reduction of silver nitrate (Harborn 1998). Longitudinal plasmon vibrations corresponding to silver nanoparticles were convincing with UV spectral peak at 477-455 nm (Fig. 1). This clearly indicates the interaction between silver ions and bio molecules present in the aqueous seaweed extract (Binupriya et al., 2010).

Plate 1: Digital photograph showing in the colour change of $AgNO_3$ on addition of aqueous extract of seaweeds.

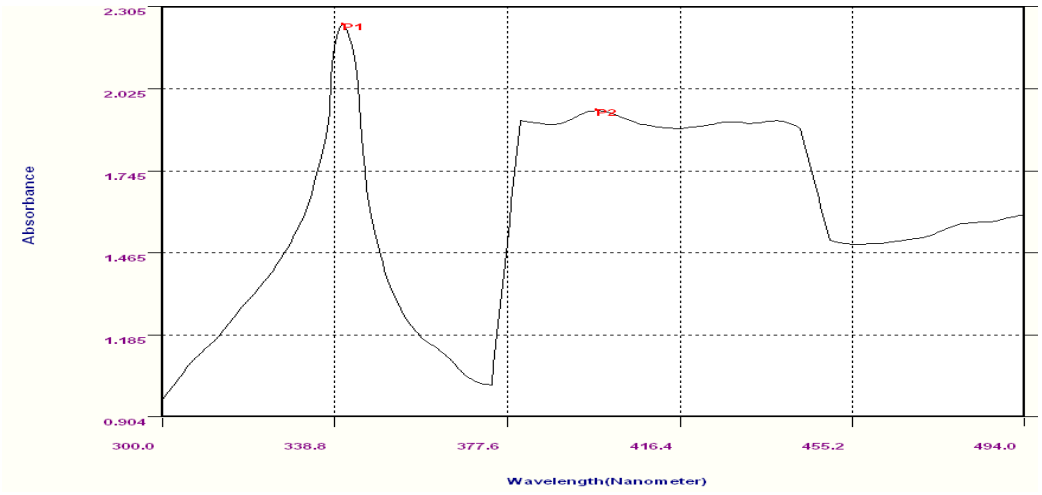
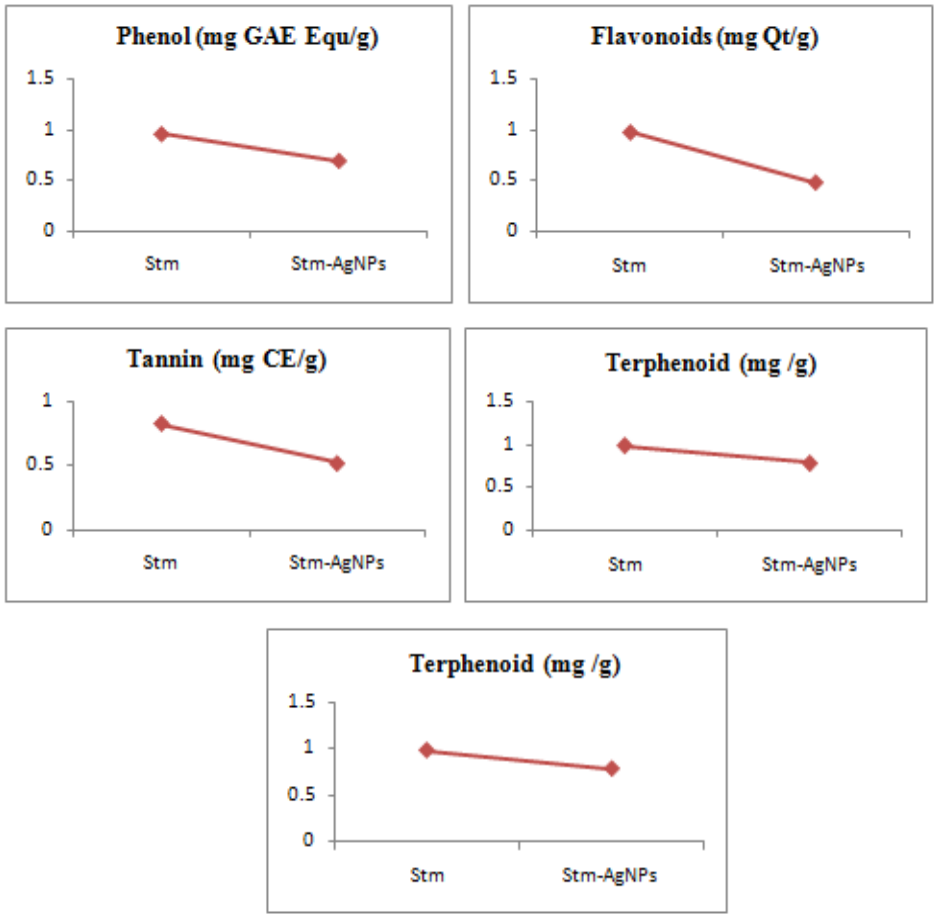


Fig. 1:UV-Visible spectra of the synthesised silver nanoparticles from the aqueous extracts of seaweed s*Stm*-AgNPs**.**

B. Antioxidant Activity

The antioxidant activity of total phenols, tannins, flavonoids, tocopherol and terpenoids contents of aqueous extracts of Stoechospermum marginatum and Stm-AgNPs presented in **(Fig.2)**, indicated these antioxidant content were predominantly higher in aqueous extract of Stoechospermum marginatum compared to the seaweed reduced Stm-AgNPs.Fresh algal specimens have been reported to contain several hydrophilic, but labile, antioxidant molecules such as L-ascorbate (Aguilera et al., 2002b; Burritt et al., 2002; Indergaard and Minsaas, 1991; Morgan et al., 1980) and GSH (Burritt et al., 2002; Kakinumaet al., 2001),

So our result revealed that the extract of marine seaweeds Stoechospermum marginatum, were capable of producing Ag nanoparticles extracellularly and these nanoparicles are quite stable in solution due to capping likely by the polyphenols present in the extract (Yvomic and Walsh et al., 2006: Indu and Seenivasan, 2013).Our result revealed seaweed extract itself is responsible for the majority of antioxidant activity and AgNPs is not contributing much to the antioxidant activity it is evidenced by (Abdul *et al.*, 2014).



Stm - Stoechospermum marginatum ; Stm-AgNPs

Fig. 2:Comparison of amount of antioxidant contents in seaweed and seaweed synthesis silver nanoparticles

C. Ft-Ir Analysis Of Silver Nanoparticles

FT-IR predicts the molecular configuration of different functional group present in the seaweed extract. Considerable absorption peak were found at712.63, 873.36, 1082.78, 1470.44, 1634.09, 3458.40 cm^{-1} respectively (**Fig. 3 (a)**. The peak corresponding to 3458.40 cm^{-1}indicates the presence of intermolecular N-H stretching vibration of amine, C=C (in ring) stretching vibration of arenes, and C-CH_3 bending of aldehydes and ketones groups present (Kasthuri et al., 2009 and Dovbshko et al., 1997). A small absorbtion peak formed at 1100.20, 1194.09, 1384.34, 1633.50, 3445.97 cm^{-1} **Fig. 3 (b)** is an attribute to stretching vibration modes

of C=C groups of alkene and N-H stretching vibration of amine, C=C (in ring) stretching vibration of arenes, and C-CH_3 bending of aldehydes and ketones is responsible for polysaccharides (Nyquist 2001 and Fujioka et al., 2004) respectively.

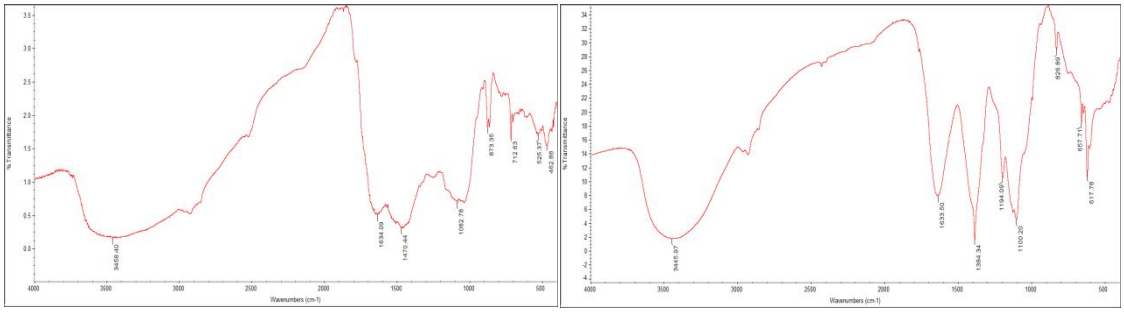


Fig. 3 (a) FT-IR spectra ofS. marginatum Fig. 3 (b) FT-IR spectra of Stm-AgNPs

D. TEM analysis

Transmission Electron microscope was recorded from drop coated film of the silver nanoparticle synthesized by using S. marginatumvividly describes the sperical shapes. The micrograph showed nanoparticle with size of ranging from 50 to100 nm (Plate 2). The strong interaction of biomolecules in the seaweed extract and surface of nanoparticles are found to be sufficient for formation of spherical shape (Bindhu and Umadevi 2015).

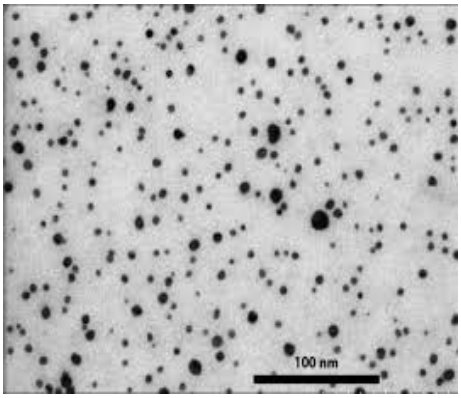


Plate 2: Transmission Electron Microscopy (TEM) micrographs of synthesised seaweeds

E. Effect of synthesised nanoparticles Stm-AgNPs on soil microbial isolates.

The effect of Stm-AgNPs on the microbial isolated from garden soil were studied. The isolation of microorganism was carried out using serial dilution technique. Aliquots of 100 µl of different dilution of garden soil were spread onto plates of nutrient agar medium for bacteria and potato dextrose agar for fungi. The plates were incubated at 28º C for 5 days under aerobic conditions. Developed colonies were picked and isolated based on morphological criteria and the isolated bacteria were sub-cultured as pure culture. Pure cultured microbes were identified as Bacillus spp, Bcillus subtilis, Staphylococcus epidermidis, Serratia spp, Pseudomonas spp, Pseudomonas fluorescens and isolated fungi were Aspergillus fumigates, Aspergillus flavus, Alternaria alternata, Cladosporium spp**.** The bacteria isolated from the garden soils are soil N-cycle, nitrifying bacteria. To study the effect of silver nanoparticles on soil microbes 2 type of in vitro assay were carried out they are calorimetric broth assay and agar well diffusion assay.

Table 1: Bactericidal activity of silver nanoparticles synthesised by *S. marginatum* against garden soil bacterial isolates (Zone of inhibition (mm).

Bacteria	Control			Stm-AgNPs
	W	AgNPs	Str	
Bacillus subtilis	NI	7	1 ± 0.076	NI
Bacillus spp	NI	NI	1 ±0.26	NI

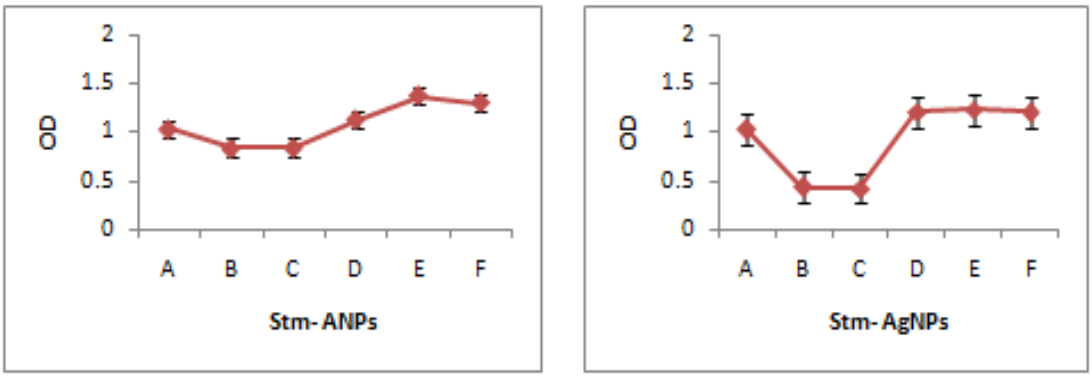
Serratia spp	NI	NI	8 ±0.097	NI
Staphylococcus epidermidis	NI	NI	NI	3 ± 0.10
Pseudomonas fluorescens	NI	NI	1 ± 0.021	5 ± 0.10
Pseudomonas spp	NI	NI	1 ± 0.120	1± 0.10

Values are mean of 3 replicate ± S W- Water; AgNPs - Nanoparticles synthesis; *Str* – Streptomycin; NI- No inhibition.

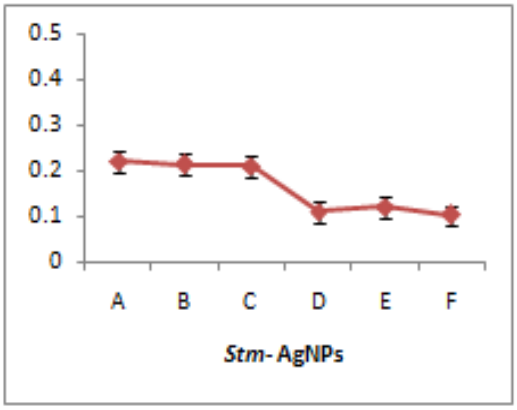
Table 2: Fungicidal activity of silver nanoparticles synthesised by S. marginatum against garden soil fungal isolates (Zone of inhibition (mm).

Fungi	Control			Stm-AgNPs
	W	AgNPs	Str	
Aspergillus flavus	3 ± 0.76	3 ±0.76	2 ±0.022	1± 0.063
Aspergillus fumigatus	NI	5 ± 0.06	2 ± 0.004	5 ± 0.063 2± 0.061
Alternaria alternata	NI	NI	2 ± 0.702	4± 0.042
Cladosporium spp	NI	NI	2 ± 0.032	5± 0.063

Values are mean of 3 replicate ± SDW- Water; AgNPs - Nanoparticles synthesis; *Str* – Streptomycin; NI- No inhibition



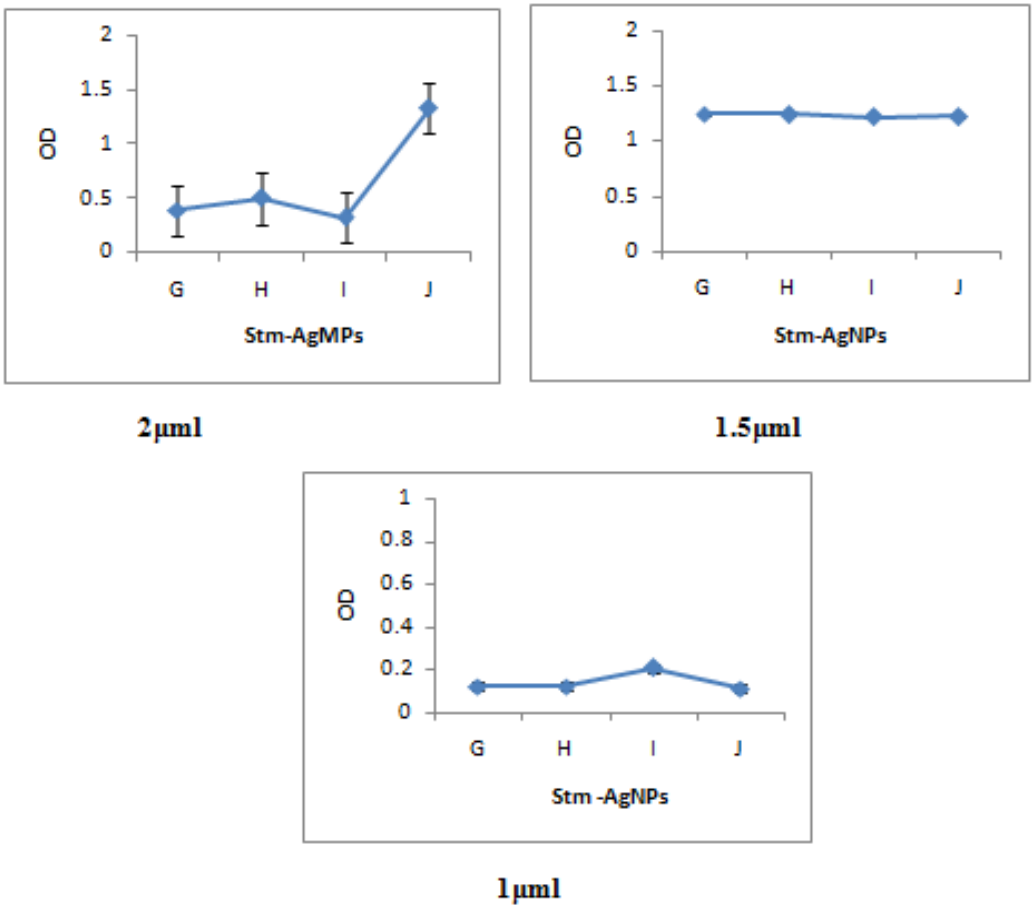
2µml **1.5µml**



1µml

Values are mean of 3 replicate ± SDA- Bacillus spp B- Bacillus subtilis C- Staphyllococcus epidermidis D- Serratia spp E- Pseudomonas fluorescens F- Pseudomonas spp

Fig. 4:Bactericidal activities of 2µml, 1.5µml and 1µml of .silver nanoparticles on bacterial isolates



2µml **1.5µml**

1µml

Values are mean of 3 replicate ± SDG- Aspergillus flavus H- Aspergillus fumigatus I- Alternaria altermat J- Cladosporium spp
Fig. 5: Fungicidal activities of 2µml, 1.5µml and 1µml of silver nanoparticles on fungal isolates.

In the present study, the effect of synthesized silver nanoparticles were tested against Bacillus spp, Bacillus substilis, Staphyllococcus epidermidis, Serratia spp, Pseudomonas fluorescens, Pseudomonas spp, Aspergillus flavus, Aspergillus fumigates, Alternaria altermata and Cladosporium spp with various concentrations (1, 1.5 and 2 µl) and the results are shown in (Table 1-2 and Fig. 4-5). The results with a maximum zone of inhibition was found in Pseudomonas fluorescens and Cladosporium spp(5 mm for l-2µl) and minimumactivity was noted in Pseudomonas spp and Aspergillus flavus (1,3 mm for 1-2µl). This enormous difference may be due to the susceptibility of the organism used in the current study. The algal synthesis of silver nanoparticles against the different the particular mechanisms happened in the silver nanoparticles against the bacterial culture is clearly known and small surface area containing nanoparticles having interaction like large surface area it may attach with the cell membrane of the bacteria and involves the process of upsetting the respiration and permeability (Kyung-Hwan et al., 2005). The adsorption on bacterial surface and intracellular enzyme activity is the main reason for the antibacterial reactions (Mritunjai et al., 2008). The nanoparticles get attached to the cell membrane and also penetrate inside the bacteria. When silver nanoparticles enter the bacterial cell, it forms a low molecular weight region in the center of the bacteria to which the bacteria conglomerates thus protecting the DNA from the silver ions. The nanoparticles preferably attack the respiratory chain cell division finally leading to cell death. The nanoparticles release silver ions in the bacterial cells, which enhance their bactericidal activity (Morones et al.,2005; Kvitek et al., 2008). Several studies propose that AgNPs may attach to the surface of the cell membrane disturbing permeability and respiration functions of the cell (Morones et al., 2005). It is also possible that AgNPs not only interact with the surface of membrane, but can also penetrate inside the bacteria (Sondi 2007).Thus, it is corroborated that the bactericidal effect of silver nanoparticles is size dependent (Raimondi et al., 2005; Morones et al., 2005). The soil microbes efficacy of the silver nanoparticles depend on the shapes of the nanoparticles also, this can be confirmed by studying the inhibition of bacterial growth by differentially shaped nanoparticles (Morones et al., 2005). The ability of silver nanoparticles to release silver ions is a key to their bactericidal activity. It can

interacting with functional groups and inactivates the proteins. It can damage bacterial cytoplasmic membranes, reduce the ATP synthesis and inhibit the bacterial DNA replication finally it causes cell death (Lansdown, 2002; Castellano *et al.*, 2007). The high specific surface-to volume ratio of silver nanoparticles increases their contact with microorganisms, promoting the dissolution of silver ions, thereby improving biocidal effectiveness.Hence the study on soil microbial toxicity of silver nanoparticle found to be valuable. Though the Nanoparticlesexhibit toxicity on the soil bacterial flora, the loss can be overcome by the regeneration. But usage of fewer amounts and less concentration of silver nanoparticle in agricultural field is always advisable (Raffi *et al.*, 2008).

IV. CONCLUSION

Silver nanoparticles were biologically synthesized by using marine brown algae. FTIR studies revealed the presence of alcoholic group, inferring that presence of phenolic compounds in the seaweed extracts responsible for the reduction silver into silver nanoparticles. These silver nanoparticles were found to be effective against the soil beneficial isolated from garden soil microbesBacillus spp, Bacillus substilis, Staphyllococcus epidermidis, Serratia spp**,** Pseudomonas fluorescens**,** Pseudomonas spp, Aspergillus flavus**,** Aspergillus fumigates**,** Alternaria altermataand Cladosporium spp.So some guidance is needed as to which precautionary measures are warranted in order to encourage the development of " green nanotechnologies" and their further innovative technologies, while at the same time minimizing the potential for adverse effects on human health and/or the environment. Thus there is urgent need for a systematic evaluation of the potential adverse effect of nanotechnology. It is therefore recommended that the ecotoxicological effect of nanoparticle be clarified before their application.

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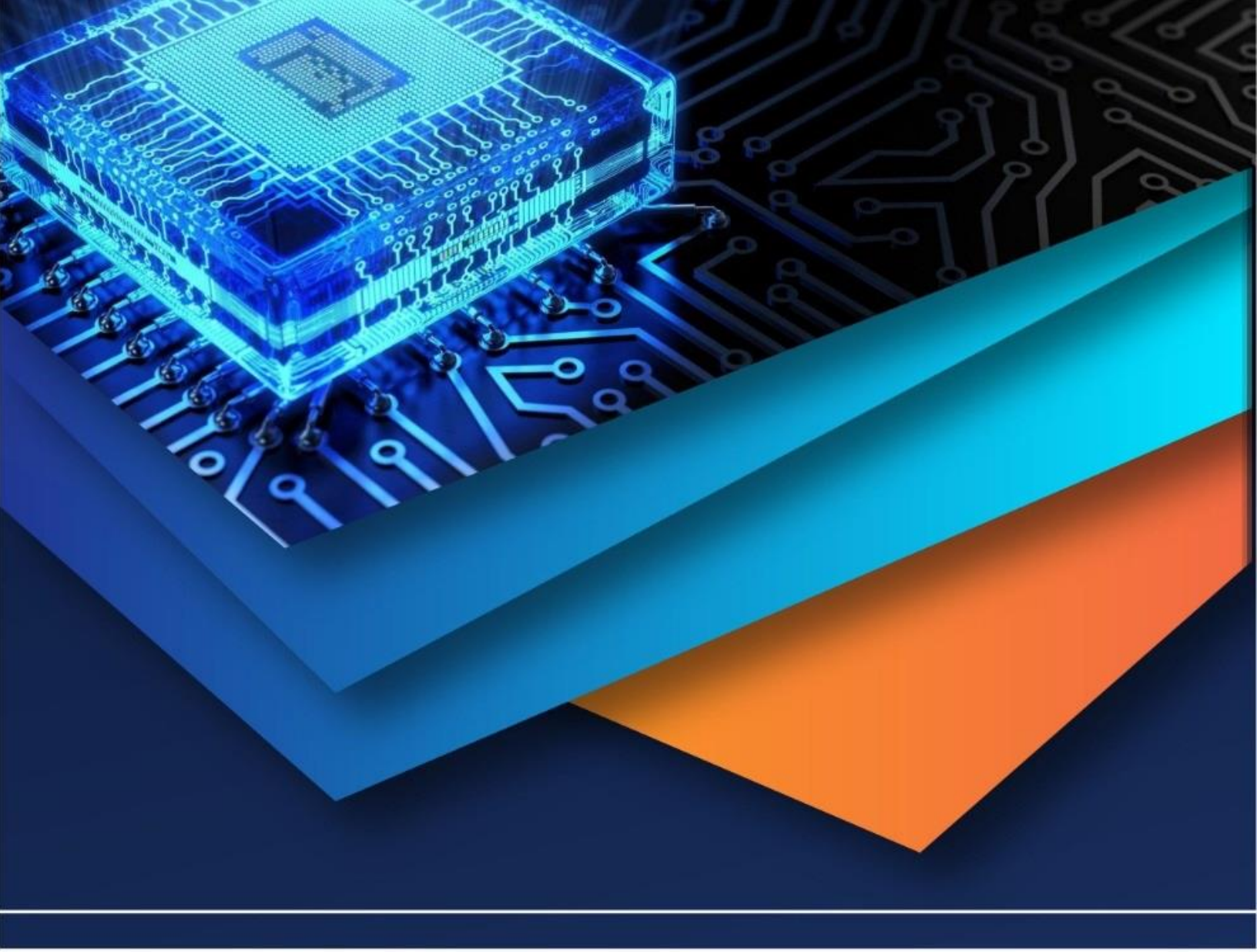

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