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EFFICACY OF BIO-SYNTHESIZED SILVER NANOPARTICLES USING *ACANTHOPHORA SPICIFERA* TO ENCUMBER BIOFILM FORMATION

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Silver nanoparticles (AgNPs) are attracting attention due to their anti-microbial activity and consequently used in possible biomedical applications. The present study has evaluated the efficacy of biosynthesized AgNPs using *Acanthophora spicifera* against five prominent biofilm forming pathogens using microtitre plate (MTP) assay and anti-microbial assay. The outcome, displayed 94 % of impediment in recurrence of biofilm by AgNPs tested in 24 h treatment MTP assay. The formation of relatively large zone of inhibition confirms 100% effective anti-microbial potential of AgNPs against all five pathogens at 100 nM concentration. The spectrum and rapidity of action however varied between the bacterial species. Overall study makes an obvious futuristic application for biosynthesized AgNPs in preventing bio-medical devices from infection.

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Keywords: Silver nanoparticles, *Acanthophora spicifera*, Biofilm, Anti-microbial assay, Biomedical application

1. Introduction

Bacterial biofilm are ubiquitous in nature, forming complex colonies anchored to either biotic or abiotic surfaces. Scientists have found that almost 99% of world's bacteria exist as biofilms [1]. Biofilm formation is due to the aggregation of microbial cells, which produce a matrix of polymeric compounds called Extracellular Polymeric Substances (EPS). The composition of EPS depends on the type of microorganisms and environmental conditions [2]. Millions of bacteria encased in biofilm are the root cause of many serious infections such as cystic fibrosis, periodontal disease etc.

Microbial biofilms pose a serious threat to health of patients implanted with synthetic Biomedical Devices (BDs). Biofilms developed on the simple medical devices such as contact lenses, or on more complex materials such as central venous catheters, prosthetic heart valves, artificial hip prostheses and intrauterine devices [3, 4, 5, 6, 7] were capable to resist antibiotics and cause severe problems to the immune system. BDs are very important supporting tool for proper clinical management, which helps to improve the significance of patient's lives. BDs subjected to antibiotic therapy often fail to prove themselves from bacterial infections [8]. It has been identified that about 70% of bacteria were resistant to commercially available antibiotics [9]. Some of the well-known pathogenic microorganisms associated with biofilm formation on BDs include *Escherichia, Legionella, Staphylococcus, Streptococcus, Salmonella, Shigella* and *Vibrio* that

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plays a vital role in causing chronic diseases [10]. The infection is due to the surface modification of biofilm providing an ideal environment for the exchange of extra-chromosomal DNA, which is responsible for antibiotic resistance, virulence and increases their endurance resulting in drug resistant pathogens [11, 12].

Recent advancements in nanotechnology, has introduced fabricated nano-sized silver particles towards biomedical and industrial applications for its inhibitory effect against microbes [13]. Silver has been long acknowledged for its anti-bacterial properties, but its role in anti-microbial medical devices is continually a debated. Silver compounds (silver nitrate, silver chloride or silver oxide) are prioritized choice for infection-resistant coatings. AgNPs are used to treat human skin infections [14], used as arthroplasty [15] and as dental materials [16]. Added to this, AgNPs also has potent cytoprotective activity against HIV infected cells [17]. Physical and chemical methods are quite expensive and potentially toxic to environment compared to biological methods. Alternate biological sources include microorganisms, plant extract or plant biomass are typical used for biosynthesis of nanoparticles and are ecofriendly [18, 19, 20].

Seaweeds are rich source of various natural byproducts and are exploited for synthesize of nanoparticles to combat various biological diseases, which is now an emerging field of interest in scientific research [21, 22]. Studies related to *Acanthophora spicifera* (red algae) were few and disseminated about the ecological significances [23, 24], anti-microbial activity [25] and identification of polyenoic fatty acids. The present study, discloses anti-biofilm and anti-microbial potency of synthesized AgNPs fabricated by exploiting *A. spicifera* against five well-known pathogenic strains, whose biofilm formation plays a critical role in antibiotic resistance and pathogenesis. To our knowledge, this work has not yet been reported earlier in *A. spicifera*. This attempt is hence a feasible approach for green synthesis of AgNPs with greater competency against biofilm forming bacteria.

2. Experimental

2.1 Sample collection and Identification

The seaweed samples were collected along the East coast of Gulf of Mannar, Puthumadam, Tamil Nadu, India. The samples were immediately brought to lab, cleaned and washed with fresh and deionized water. The collected seaweed was identified as *Acanthophora spicifera,* which belongs to Rhodophyta*. A. spicifera* found near intertidal high-motion water and attached to hard substrates such as rocks, dead coral heads, and free-floating structures. After identification, seaweeds were shade dried for 15 days and grounded using mortar and pestle.

2.2 Preparation of *A. spicifera* extract (AE)

The AE was prepared using 100 mg of powdered *A. spicifera* taken in Erlenmeyer flask containing 250 ml of distilled water. The flask was heated to 60 °C for 20 min and then the extract was filtered using Whatman No.1 filter paper and refrigerated for further use.

2.3 Synthesis and characterization of AgNPs

For the synthesis of AgNPs, AE was heated with 1 mM Silver nitrate solution (Sigma Aldrich) at 60 °C for 20 min and color change from pale yellow to dark brown was visualized. Bioreduction of AgNPs was measured using UV-visible spectrophotometer (Model-UV-2450 Shimadzu). Later, the mixture was centrifuged at 12,000 rpm for about 20 min to separate the precipitate formed and then redispersed in deionized water of same volume. The above procedure was repeated three times for better separation of metal nanoparticles. The FTIR spectrum was recorded at a wavelength of 400 - 4000 nm^{-1} using RX 1 - One instrument in diffuse reflectance mode. The synthesized nanoparticles were characterized using Transmission Electron Microscope (TECNAI 10 PHILIPS) operated at a voltage of 80 KV prepared on carbon-coated copper grids.

2.4 Preparation of AgNPs nanomolar concentration

The nanomolar concentrations of AgNPs were determined previously in gold [26] and silver [27]. The same method was adopted to prepare nanomolar concentration (nM) of

nanoparticles in solution using deionized water. From the stock, different concentrations (from 10nM to 100nM) of AgNPs were prepared and used throughout the study.

2.5 Bacterial strains and growth conditions

Five pathogenic bacterial strains used in this study were sourced from MTCC, Indian Institute of Microbial Technology (IMTECH), Chandigarh, India. *Escherichia coli* (MTCC 1687), *Salmonella typhii* (MTCC 531), *Shigella flexneri* (MTCC 1457), *Staphylococcus aureus* (MTCC 96) and *Vibrio cholerae* (MTCC 3906). Cultures were seeded in Luria Bertani (LB) broth and incubated to ensure the exponential growth conditions throughout the microbiological studies.

2.6 Biofilm determination by Congo red agar (CRA)

Method based on Freeman *et al.* [28] with minor modification was followed to determine the biofilm formation, which requires LB agar with Congo red dye. Congo red dye was prepared separately as aqueous solution in a concentration of 0.8 g/l and autoclaved at 121 ºC for 15 min and added to the LB agar when cooled to 55 ºC. The bacterial strains were streaked and incubated for 24 – 48 h at 37 ºC. Appearance of black colonies confirms the presence of bacterial polysaccharide layer, which forms colored complexes with Congo red dye.

2.7 In vitro assay - biofilm formation using microtitre plate method (MTP)

It is a quantitative method proposed by Christensen et al. [29] and considered as the standard test to detect biofilm formation. This method was performed with slight modifications in the present study. Sterile, polystyrene, 96-well-flat bottom microtitre plates were used. In each well of MTP, 180 µl of LB broth and 10 µl of biofilm forming pathogenic cultures were added, followed by 10 µl of AgNPs prepared from nanomolar stock concentration (nM). Simultaneously, 10 µl of AE (without nanoparticles) was also added to the separate well with all five bacterial pathogens. Control (without AgNPs and AE) was also maintained to analyze the biofilm formation. The microtitre plate was incubated for 24 h at 37 °C. After incubation, contents in MTP were removed and the wells were washed with Phosphate Buffer Saline (PBS) of pH 7.2, fixed with 2% sodium acetate. Then the wells were stained with 0.1% crystal violet and excessive stain was removed using deionized water and kept for drying. After drying, 95% ethanol was added and optical density was measured using a microplate reader at 595 nm (BIORAD). The experiment was performed in triplicates and statistical analysis was done.

2.8 Anti-microbial activity by disc diffusion method

Anti-microbial activity of synthesized AgNPs was tested against *E. coli, S. typhii, S. flexneri, S. aureus* and *V. cholerae* by conventional disc diffusion method. Bacterial cultures were spread uniformly on LB agar plates and sterile discs (HIMEDIA) of 6 mm diameter were placed and loaded with 20 µl of AE and different molar concentrations of AgNPs (10nM, 40nM, 70nM and 100nM respectively). The plates were incubated for 24 h at 37 ºC and the zone of inhibition was measured. Quadrates of the experiment were performed to calculate the mean.

3. Results

3.1 AgNPs synthesis

The AgNPs was synthesized rapidly using *A. spicifera* in 20 min of reaction time without any incubation and change in color was observed representing the reduction of silver nitrate (Fig. 1). UV- visible spectroscopic measurement showed peak at 437nm (Fig. 2) due to excitation of Surface Plasmon Resonance (SPR). Intense FTIR bands were observed at 3351.28 cm^{-1}, 2633.71 cm^{-1}, 2083.50 cm^{-1}, 1637.18 cm^{-1}, 1082.87 cm^{-1} and 712.34 cm^{-1} (Fig. 3). Further characterization of AgNPs was analyzed using TEM to determine the size and shape of the nanoparticles. The AgNPs formed were predominantly spherical in the form of aggregates with average diameter of 48 nm (Fig. 4). The particles were stable for more than 3 months in room temperature without any special storage requirements.

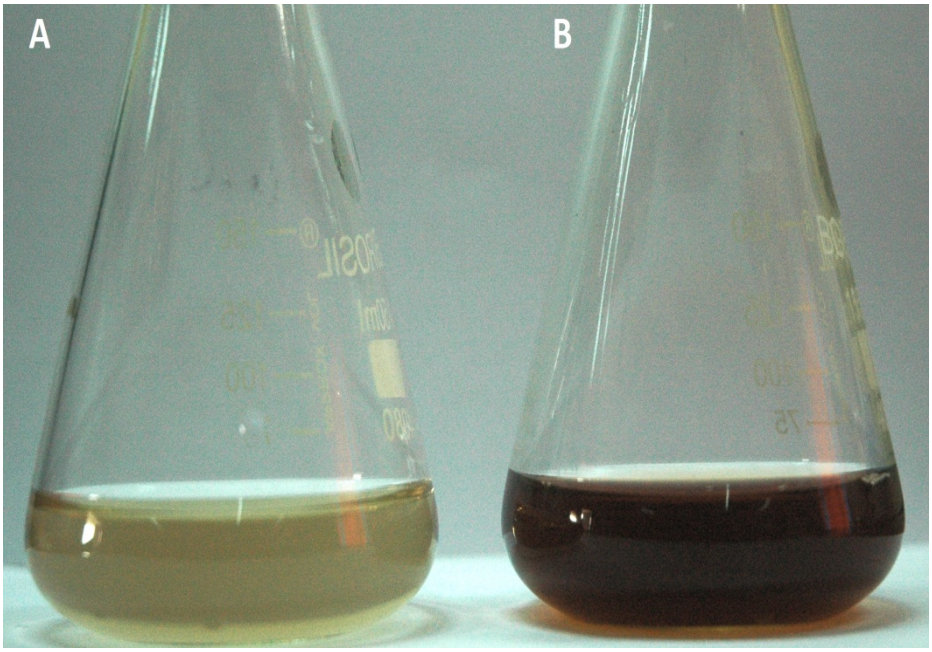

Fig. 1 Reduction of AgNO3 indicated by color formation. A) AE (A. spicifera extract) B) Biosynthesized AgNPs from A. spicifera

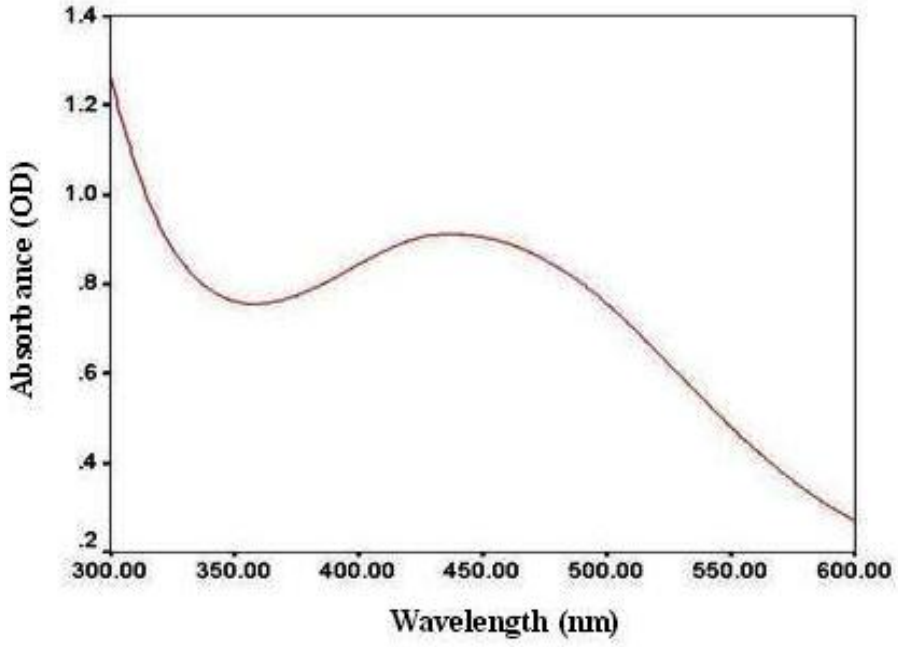

Fig. 2 UV-Spectrophotometric determination of AgNPs by forming peak at 437 nm

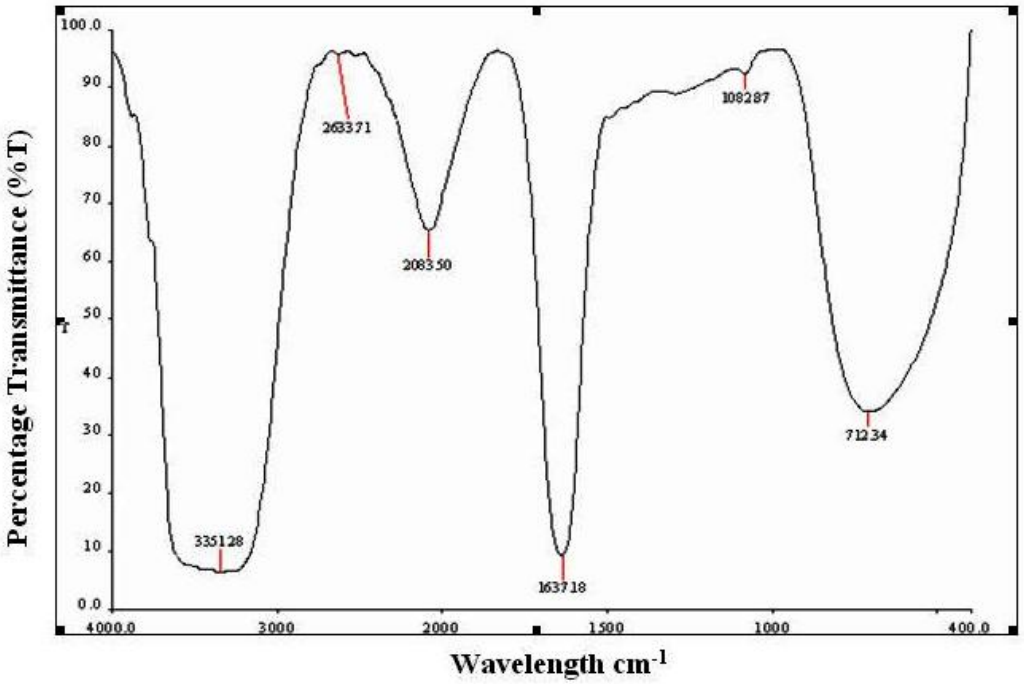

Fig. 3 FTIR spectrum of bio-synthesized AgNPs

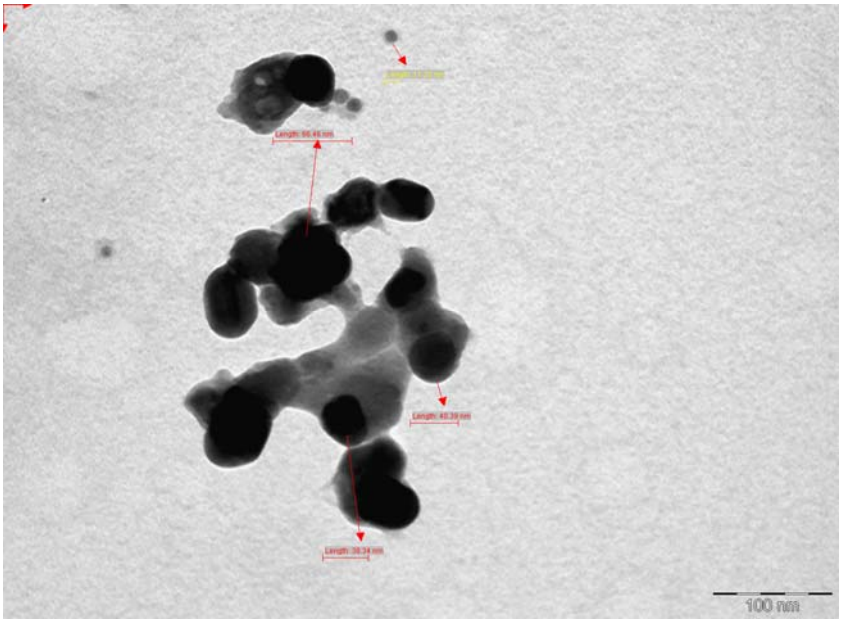

Fig. 4 TEM image of silver nanoparticles formed by reduction of Ag ions using A. spicifera observation of AgNPs synthesized by A. spicifera

3.2 Screening of biofilm forming bacteria

The biofilm formation was screened by Congo Red Agar (CRA) with five different pathogens and recognized as biofilm producers (Fig. 5). After 24 h the colonies were black in color with a dry crystalline uniformity. Based on the observation, *S. aureus* was the fastest biofilm forming pathogen, while *E. coli*, *S. typhii* were slow followed by *S. flexneri* and *V. cholerae*.

Fig. 5 Production of exopolysaccharide matrix by pathogens in Congo Red Agar (LB-CRA);A) E. Coli, B) S. typhii, C) S. flexneri, D) S. aureus, E) V. cholerae

3.3 Microtitre plate method (MTP)

The inhibition of AgNPs was recorded at 100 nM concentration. Treatment of AgNPs showed ~ 94 % of biofilm inhibition in all pathogenic strains (Fig. 6). Crystal violet plays an important role for quantifying the biofilm density. This data provides a valuable outline for the demonstration of AgNPs towards clinical trails at lower concentration. In addition, the recurrence of biofilm formation of all pathogens is totally reduced at 100 nM concentration in Congo Red Agar. (Fig. 7)

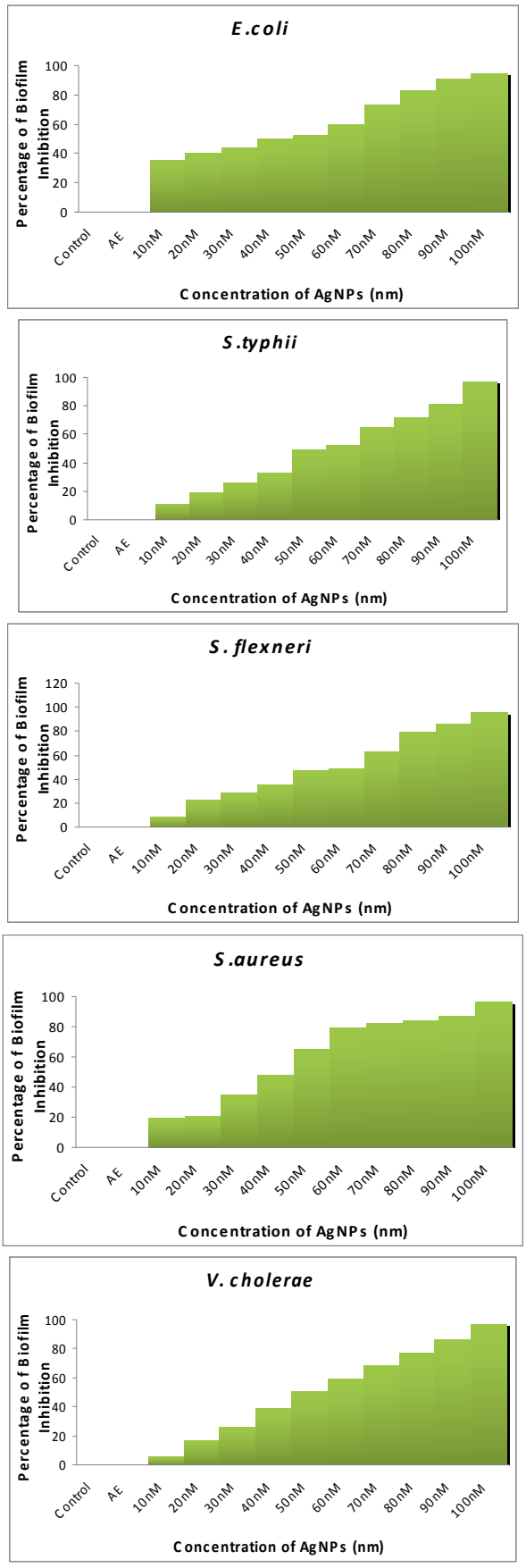

Fig. 6 Percentage of Biofilm Inhibition by AgNPs

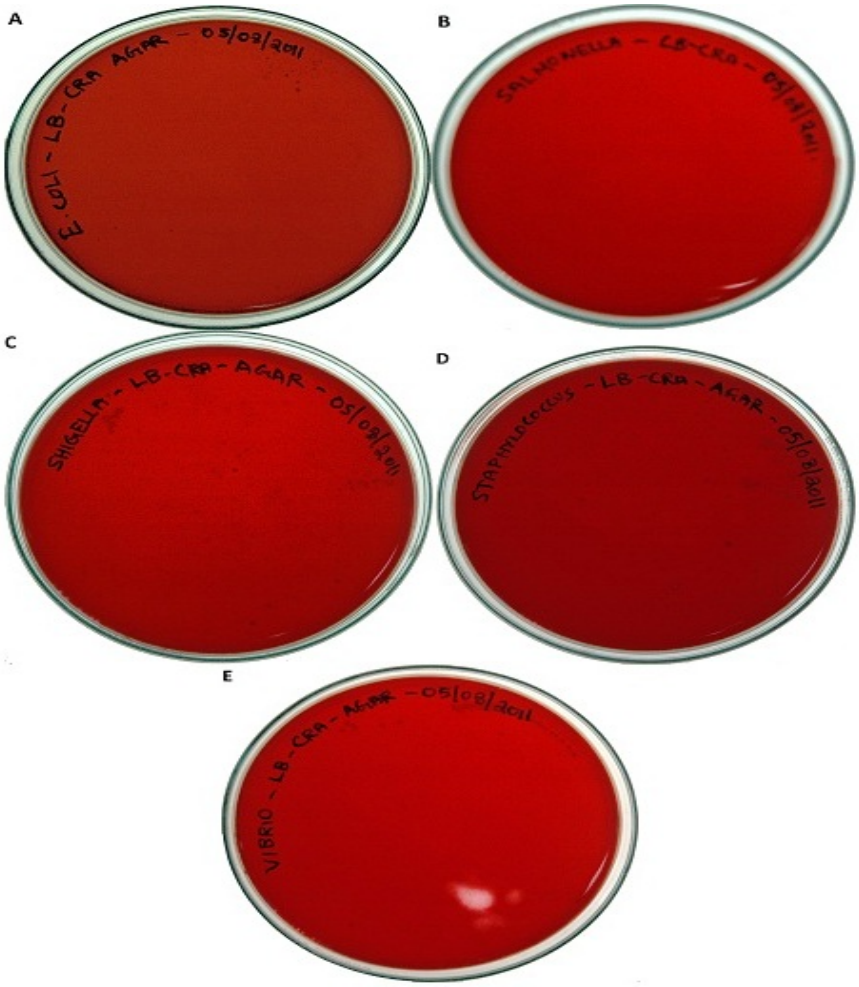

Fig. 7 Effect of AgNPs at 100 nM in Congo Red Agar (CRA) inhibiting 100 % of bacterial cultures. A) E. coli, B) S. typhii, C) S. flexneri, D) S. aureus, E) V. Cholerae

3.4 Anti-microbial assay

The bacterial growth inhibition was obviously greater in bio-synthesized AgNPs compared to AE alone. At 100 nM of AgNPs, the zone of inhibition were predominately greater in *E. coli* (14 mm), *S. typhii* (15 mm), *S. flexneri* (14.5 mm), *S. aureus* (14.5 mm) and *V. cholerae* (14.5 mm). At 40 nM concentration of AgNPs, 50% of bacterial growth was hampered (Fig. 7) and in case of AE no zone of inhibition was observed. The effective anti-microbial activity was observed in *S. typhii* and *S. flexneri* when compared to *S. aureus*, *V. cholerae* and *E. coli* (Table. 1).

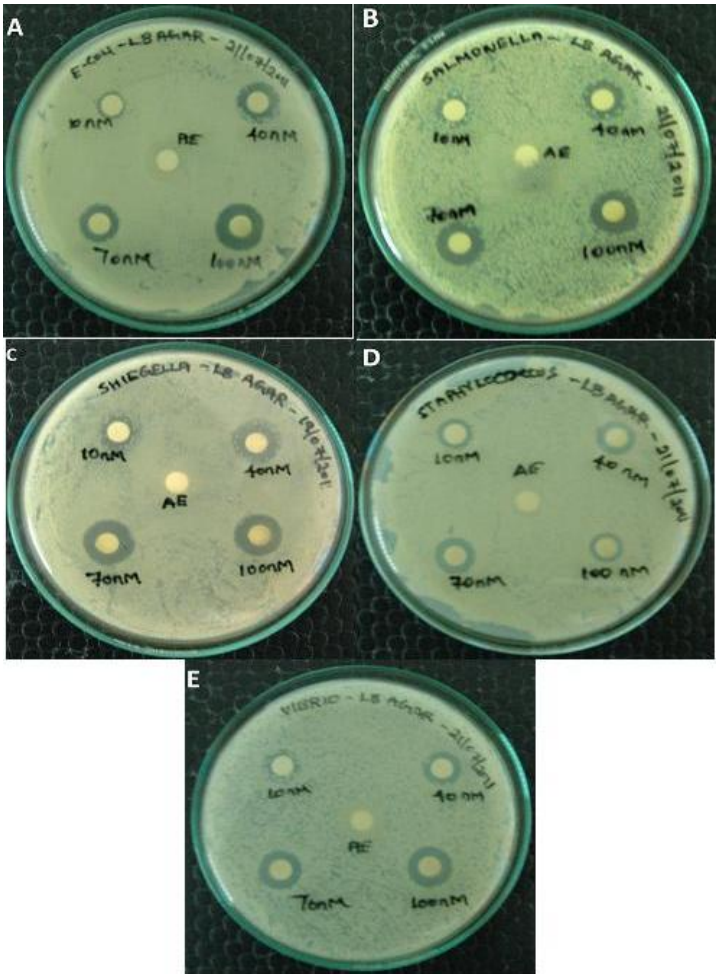

Fig. 8 Antimicrobial activity of AgNPs against pathogens at various nM concentration. A) E. coli, B) S. typhii, C) S. flexneri, D) S. aureus, E) V. Cholerae

Tab. 1 Zone of Inhibition (ZoI) in mm formed by A. spicifera (AE) and Silver nanoparticles (AgNPs) at various nM concentrations against biofilm forming pathogens. Results are mean ± SD (n = 4).

Strains used	**Zone of Inhibition (ZoI) in mm**				
	AE	**10 nM**	**40 nM**	**70 nM**	**100 nM**
E.coli	0	8.75 ± 0.5	9.75 ± 1.29	11 ± 1.41	14 ± 2
S. typhii	0	9.5 ± 0.57	10.75 ± 0.95	13 ± 1.15	15 ± 0
S. flexneri	0	9 ± 0	11.75 ± 0.95	13.5 ± 1.29	15 ± 0
S. aureus	0	9 ± 2.30	11.5 ± 1.73	11.25 ± 0.95	11.75 ± 0.5
V. cholerae	0	9.25 ± 0.5	12 ± 0.81	13 ± 1.41	12.75 ± 2.94

Diameter of disc = 6 mm

4. Discussion

Biofilms are notoriously difficult to eradicate and are often resistant to systemic antibiotic therapy [30]. Failure of available antimicrobials that combat biofilms upon implanted biomedical devices has stimulated the search of new drugs. Advancement in nanotechnology, has introduced AgNPs for its broad spectrum of antimicrobial activity against bacteria, fungi, protozoa and certain viruses. In the present study, AgNPs were synthesized from marine seaweed *Acanthophora spicifera* to delineate the effect of biofilm forming pathogens (*E.coli, S. typhii, S. flexneri, S. aureus and V. cholerea*). Appearance of the brown color provided a convenient visible signature during the formation of AgNPs using *A. spicifera*. The surface plasmon band in AgNPs solutions remain close to 437 nm throughout the reaction period indicates particles that are polydispersed [31]. The major FTIR bands at 3351.20 cm^{-1}, 2633.74 cm^{-1} and 712.34 cm^{-1} indicates the presence of alcohols and phenols (O-H); carboxylic acids and its derivatives (C=O) and Chloroalkanes (C-X) respectively as capping agents. TEM image showed relatively spherical shaped nanospheres formed with a diameter ranging between 11 to 66 nm and Singaravelu et al [21] acquired similar kinds of results in seaweed (*Sargassum wightii*).

Biofilm formation in five pathogenic strains was detected by growing them in LB agar with Congo red dye. The Congo red dye was used directly to identify the production of exopolysaccharide which is the essential requirement for biofilm formation. The colonies that were grown appear as dry crystalline black color colonies with the evidence of biofilm production. CRA test is simple, rapid and feasible when compared to other phenotypic tests and pose difficulty in identification when compared to molecular analysis of genes involved in biofilm formation [32]. According to Oliveria et al, [33] the presence of intercellular adhesion genes (*ica*ACD) results in the production of biofilm and cause recurrent infections with emergence of multidrug resistant pathogens. Although, the presence of interconnected water channels in biofilms, hiders the diffusion of drug inside the matrix [34]. Later, MTP assay was performed to instigate the effect of AgNPs against slim recurrence. The biofilm formation in *E.coli, S. typhii, S. flexneri S. aureus and V. cholerea* was well inhibited at 100 nM of AgNPs. The results resolve that the treatment of AgNPs can obstruct the growth as well as the synthesis of exopolysaccharide or slime formation. These nanoparticles diffuse directly into the exopolysaccharide layer through the pores that meant for nutrient transportation and acts as an anti-microbial agent [28]. Despite, the regulation of biofilm suppression, there were fluctuations in the inhibition rate at each nanomolar concentration for the stains used. Kalishwaralal et al. [27] with AgNPs made similar observations against biofilm producers in contact lens. Therefore, the efficacy of AgNPs toxicity towards bacteria may vary based on exposure time or bacterial species phenotype [35].

Disc diffusion assay showed higher zone of inhibition at 100 nM concentration (Fig. 7). The zone of inhibition increased linearly with increase in nanomolar concentrations of AgNPs. Hence, we confirmed bacterial growth inhibition is dose dependent [36]. In contrast, there was no inhibitory effect with AE in any of the aforesaid strains. The inhibitory action of AE may not be effective when compared to biosynthesized AgNPs because the treatment of AgNPs imparts toxicity to the bacteria and inactivates DNA replication [37]. Indulgences of proton motive force in membranes of E.coli have been studied when AgNPs are exposed in nanomoles concentration [38]. In addition, it was also reported that Ag+ ions binds to functional groups of proteins, resulting in protein denaturation [39].

5. Conclusion

The study reveals AgNPs synthesized from seaweed *A. spicifera,* displayed good anti-microbial efficacy as well as anti-biofilm potency against five biofilm pathogens at higher nanomolar concentrations. The exact mechanism by which the AgNPs mediate these processes is unknown and requires further investigations. However, it is believed that this process, disrupt the ability of the bacteria to form biofilms. Because of the results obtained, use of biosynthesized AgNPs from *A. spicifera* can be recommended for innovative application of sterile surface coatings for various bio-medical devices.

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References

[1] J.W. Costerton, K.J. Cheng, G.G. Geesey, T.I. Ladd, J.C. Nickel, M. Dasgupta, T.J. Marrie, Annual. Rev. Microbiol. **41,** 435–464 (1987).
[2] C. Mayer, R. Moritz, C. Kirschener, W. Borchard, R. Maibaum, J. Wingender, H.C. Flemming, Int. J. Biol. Macromol. **26,** 3-16 (1999).
[3] E. Anaissie, G. Samonis, K. Kontoyiannis, J. Costerton, U. Sabharwal, G. Bodey, I. Raad, Eur. J. Clin. Microbiol. Infect. Dis. **14**, 134-137 (1995).
[4] L. Ganderton, J. Chawla, C. Winters, Eur. J. Clin. Microbiol. Infect. Dis. **11**, 789-796 (1992).
[5] A.S. Wolf, D. Kreiger. Arch. Gynecol. **239**, 31-37 (1986).
[6] E.P.J.M. Everaert, B. Van de Belt-Gritter, H.C. Van der Mei, H.J. Busscher, G.J. Verkerke, F. Dijk, H.F. Mahieu, A. Reitsma, J. Mater. Sci- Mater Med. **9**, 147-157 (1998).
[7] I.S. Saidi, J.F. Biedlingmaier, P. Whelan, Otolaryngol Head Neck Surg. **120**, 621-627 (1999).
[8] J.M. Ghigo. Nature, **412**, 442-445 (2001).
[9] A.L. Demain, S. Sanchez, J. Antibiot, **62 (1)**, 5-16 (2009).
[10] R.M. Donlan. Emerg. Infect. Dis. **7**, 277-281(2001).
[11] R.M. Donlan. Clin. Infect. Dis. **33**, 1387-1392 (2002).
[12] M. Hausner, S. Wuertz. Appl. Environ. Microbiol. **65**, 3710-3713 (1999).
[13] C. Lok, C. HO, R. Chen, Q. He, W. Yu, H. Sun, P.K. Tam, J. Chiu, C. Che. J. Biol. Inorg. Chem. **12**, 527-534 (2007).
[14] M. Ip, S.L. Lui, V.K.M. Poon, I. Lung, A. Burd. J. Medical Microbiol. **55**, 59 (2006).
[15] V. Alt, T. Bechert, P. Steinrucke, M. Wagener, P. Seidel, E. Dingeldein, D. Scheddin, E. Domann, R. Schnettler. Der Orthopade. **33(8)**, 885-892 (2004).
[16] R.W.Y. Sun, R. Chen, N.P.Y. Chung, C.M. Ho, C.L.S. Lin, C.M. Che, Chem. Commun. (Camb.) 40 (2005) 5059-5061.
[17] M. Sastry, A. Ahmad, M.I. Khan, R. Kumar. Crit. Rev. Biotechnol. 25 (2005)
[18] D. Bhattacharya, G. Rajinder. Crit. Rev. Biotechnol. **25**, 199 (2005).
[19] P. Mohanpuria, N.K. Rana, S.K. Yadav. J. Nanopart. Res. **10**, 507 (2008).
[20] K. Govindaraju, S. Khallel Basha, V. Ganesh kumar, G. Singaravelu. Journ. of Mater. Science. **43**, 5115-5122 (2009).
[21] G. Singaravelu, J. Arockiyamari, V. Ganesh kumar, K. Govindaraju. Colloids and Surfaces B: Biointerfaces. **57**, 97-101 (2007).
[22] S.M. Phang. Aquat. Ecosyst. Health. **9(2)**, 1-21 (1986).
[23] S.M. Mijan Uddin, I. Ahamad, I. Asmida. Malays. Appl. Biol. **36(2)**, 69-73 (2007)
[24] R. Lavanya, N. Veerappan. Adv. In Biol. Res. **5**, 38-44 (2011).
[25] N. Bhaskar, T. Kinami, K. Miyashita, Si-Bum Park, Y. Endo, K. Fujimoto. Z.Naturforsch. **59c**, 310-314 (2004).
[26] X. Liu, M. Atwater, Q. Wang, J. Juo, Colloid and Surfaces. B.: Biointerfaces. **58**, 3-7 (2007).
[27] K. Kalishwaralal, S. Barathmanikanth, S. R. K. Pandian, V. Deepak, S. Gurunathan. Colloid and Surfaces. B: Biointerfaces. **79**, 340-344 (2010).
[28] D.J. Freeman, F.R. Falkiner, C.T. Keane. J. Clin. Pathol. **42,** 872-874 (1989).
[29] G.D. Christensen, W. A. Simpson, J. A. Yonger, L.M. Baddour, F.F. Barrett, D.M. Melton. J. Clin. Microbiol. **22**, 996-1006 (1985).
[30] K. Lewis. Antimicrob Agents and Chemother, **45**, 4285-4287 (2001)
[31] F. Fitzpatrick, H. Humphreys, P. O'Gara. J Clin Microbiol. **43**, 1973-1976
[32] A. Oliveira and R.S. Cunha, BMC Research Notes. **3**, 260 (2010)
[33] Oliveria et al

[34] A.R. Shahverdi, A. Fakhimi, H.R. Shahverdi, S.M. Minaian. Nanomedicine **3,** 168 (2007).
[35] E. Navarro, F. Piccapietra, B. Wagner, B, F. Marconi, R. Kaegi, N. Odzak, L. Sigg, R. Behra. Environ. Sci. Technol. **42(23)** 8959 – 8964 (2008).
[36] I. Sondi, B. Salopek-Sondi. J Colloid Interface Sci. **275**, 177 – 82 (2004).
[37] Q.L. Feng, J. Wu, G.Q. Chen, F.Z. Cui, T.M. Kim, J.O. Kim, J. Biomed. Mater. Res. **52**, 662 (2000).
[38] C.N. Lok, C.M. Ho, R. Chen, Q.Y. He, W.Y. Yu, H. Sun, P.K, Tam, J.F. Chiu, C.M. Che. J. Proteome Res. **5**, 916 (2006).
[39] J.A. Spadaro, T.J. Berger, S.D. Barranco, S.E. Chapin, R.O. Becker, Microb. Agents Chemother. **6**, 637 (1974).