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Antimicrobial and antioxidant activity of synthesized selenium nanoparticles against biofilm forming pseudomonas aeruginosa and staphylococcus aureus

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***Abstract*---**The pathogenicity of the bacteria is increasing at an alarming rate as they have developed several means to overcome the treatments done for their removal. The Biofilm formation is one of that major ways used by the bacteria to increase its sustainability. This biofilm provides rigidity and resistance to the bacteria against the antibiotics. There is a requirement of another approach to treat such pathogen forming this rigid barrier. Nanoparticles have proven to be effective against such type of barrier and have shown to be potent anti bio film agents. The present study aims on developing the selenium nanoparticles using the biological and chemical methods to act against the bio film forming bacteria that is *S. aureus* and *P. aeruginosa*. The biofilm forming bacteria were isolated from the sewage water sample on the selective media and further characterized through staining and biochemical test. The selenium nanoparticle was synthesized biologically using Emblica officinalis extract and chemically using ascorbic acid along with sodium selenite in both the cases. The synthesized nanoparticle was characterized by UV-visible spectra and its antibacterial activity was checked through 'Agar well diffusion' against the isolated bacteria. The antioxidant property of both nanoparticles was analyzed through FRAP assay. The conclusion derived from the study is that, selenium nanoparticle is found to be effective against the isolated biofilm forming microbes, it so acquires a wide application in the medical industries for the preparation of products against these microbes. The selenium nanoparticle also possesses good anti-oxidant property

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giving it a wider application in the pharma and cosmetic industries for development of antibacterial drugs and ointments respectively.

***Keywords*---**Selenium, Nanoparticles, Biofilm, Antioxidant.

Introduction

A biofilm is actually a polymeric mixture that consists of proteins, extracellular DNA and polysaccharides (Costerone and Stewart, 1999). The biofilm majority contains the mixed bacterial cells but it may also be composed of single type of bacterial cell (Donlan and Costerton, 2002). This structure provides several benefits to the organism forming it but, this is a major challenge for us as the biofilm of the microbes become more stable and efficient in their pathogenic behavior. The resistance of the bacteria to antibiotic is due to the biofilm present in persisted cells different from multidrug resistance (Lewis, 2001). The organisms which form biofilms are having anti resistance property against antibiotics, disinfectants and germicides. Over last several decades, extensive use of antibiotic therapy for analytic occurence due to bacterial and fungal contaminations is a menace for the reason of rising antimicrobial resistance (AMR) in micro organisms forming fastidious bio films on tissues and medical apparatus (Penesyan *et al.*, 2015). Antimicrobial drugs are proven to be less active or inefficacious rising to origination of long term diseases and rise in ill health and death rate. The approaches relying on the use of non-antibiotic antibacterial agents; has to be perfect to face the antibiotic resistance challenge (Beyth *et al.*, 2015). The use of nanoparticles is considered to be a novel approach amidst various methods for the prevention and eradication of biofilms (Gristina *et al.*, 2000). Nanotechnology is the field providing better options to treat such big problems. The current era focuses on the implementaion of metal ions and nano particles as a substitute to the utilization of organic amalgam as anti microbial agents (Lemire *et al.*, 2013).Nanoparticles possess potential antimicrobial activity because of their high surface area: ratio of their particles volume resulting in a high reactivity. A huge literature exists dealing with the antimicrobial activity of SNPs (Sondiand Salopek-Sondi, 2004; Fabrega*etal.*,2009; Martinez-Gutierrez*etal.*,2013). There are many other nanoparticles proven to be a potent bactericidal agent. For example, the antimicrobial activity of ZnO (Jones*etal.*, 2008) and TiO2 (Tsuang*etal.*, 2008) has been reported and also the selenium- (Tran and Webster, 2011) and tellurium-based (Mohanty *et al.*, 2014) nanomaterials revealed strong antimicrobial potential for a wide range of pathogenic strains. It gives way to the new perspective for these nanoparticles as coating agents in clinical gadgets and health-allied products to avert bacterial infections (Roe*etal.*, 2008). These potent agents are finding promising application in industrial field as a flair tool to contrast biofouling (Zhang *et al.*, 2012).

The selenium nanoparticles are preferred due to the important biological properties of selenium. Selenium exists in distinct oxidation state and possesses various characteristics. The metalloid is found as complex accompanying nutrient sulfides or Ag, Cu, Pb and Ni (Ohlendorf, 2003). It is a trace element found as seleno methionine, seleno cysteine and in various enzymes (Wang *et*

al., 2007). Furthermore, it contributes in glutathione peroxidase system (Wang*etal.*,2007; Zeng,2009) and in collaboration with vitamin E, it acts as an antioxidant to prevent noxious consequences of metabolites on tissues (Stolz *etal.*,1999;Weisburg*etal.*,1991).In addition, it is important in fertility of men, functioning of immune system, production of neuro transmitters and safeguard of malignity in such manner that its lacking escort multiple malfunctions including thyroid dysfunction (Saad *et al.*, 2009; Rayman, 2008). Majorfocus has been given to selenium nanoparticles due to the unique properties of selenium (Yang *et al.*, 2008).

Material and Methodology
Sample Collection

For the isolation of biofilm forming bacteria, sewage water sample was collected from the drainage system of Indira Nagar, Lucknow, Uttar Pradesh, India. The Emblica officinalis sample for the biosynthesis of nanoparticle was collected from local market.

Isolation of the Biofilm Forming Microbe

For the isolation of biofilm forming isolates, selective media was used. For isolating *Staphylococcus aureus* and *Pseudomonas aeruginosa*- Mannitol salt agar media and Cetrimide agar media, respectively was used. This waste water sample was serially diluted and then inoculated on the selective media plates by spread plate technique. The isolated colonies were then transferred to fresh media plates by 'streak plate technique' to obtain the pure culture.

Depiction of the Isolated Microbe

The isolated bacterial cultures were characterized on the basis of their morphology and biochemical behavior. The morphological identification of isolates was performed by 'Gram staining', while the biochemical behavior was determined by several biochemical test including, Methyl Red test, Voges Proskauer test, Indole test, Citrate utilization test, Urease test, Catalase test, Starch hydrolysis test and Sugar fermentation test. Apart from these characterizations the isolates were also tested for their biofilm forming property. The biofilm forming ability of isolated microbe was analyzed by the 'tube method'. For this, the isolates were grown for 72 hours in the nutrient broth. After incubation the broth was removed from the tube and its surface was flooded with 0.2% crystal violet stain and incubated for 10 minutes. Furthermore, excess stain was removed and the tube was rinsed twice by distilled water to observe the biofilm ring on the tube's inner surface.

Synthesis of the Selenium Nanoparticles

The selenium nanoparticle was synthesized by two different methods that is the chemical synthesis using Sodium Selenite and the biological synthesis using *Emblica officinalis* extract.

Biological Synthesis

For biological synthesis 30gm of the emblica officinalis pulp was mixed with 100ml of distilled water. This mixture was then ground into fine paste. It was further filtered using the whatman no.1filter paper and the filtrate was used for the next process. A flask containing 25ml of 5mM sodium selenite was kept on magnetic stirrer and to it the filtrate was added drop wise till there was a color change in the solution. In the next step, the mixture was kept in dark on rotatory shaker at room temp.

Chemical Synthesis

To perform the chemical synthesis of nanoparticle, 20ml of 100mM sodium selenite solution was kept on magnetic stirrer. The solution was loaded drop wise by 50 mM ascorbic acid solution till a color change was detected. The mixture was diluted with 20% dextrin and the content was centrifuge at 10000 rpm for 15 minutes to obtain the nanoparticles in the form of pellets. The pellets were collected, washed with distilled water and air dried.

Characterisation of the Nanoparticle

The synthesized nanoparticles were characterised by UV-Visible double spectrophotometer. The spectral range of 200- 400 nm was passed through the nanoparticles. The nanoparticles were dissolved in distilled water for which distilled water was kept as blank. The spectra were set and the graph of spectra was obtained.

Biological Activity Determination of the Nanoparticle

The synthesized nanoparticles from chemical as well as biological processes were further undertaken for the determination of their biological activity. They were studied for their antibacterial activity against the biofilm forming microbes isolated under the study as well as for the analysis of their antioxidant property.

Antibacterial test

Use of Agar well diffusion method was done to determine the antibacterial activity of selenium nanoparticle. The bacterial isolates for which the activity was determined includes the biofilm forming bacteria, *Staphylococcus aureus* and *Pseudomonas aeruginosa.* The isolates were inoculated on the 'Mueller-Hinton Agar' plates followed by well punctured on the plates which were loaded with different concentration of both the nanoparticles. The positive control used for *P. aeruginosa* and *S.aureus* includes Cefotaxime antibiotic (1000ppm) and Levoflaxin antibiotic (1000ppm), respectively.

Antioxidant Test

To determine the total antioxidant property of the synthesized nanoparticles, 'Ferric Reducing Antioxidant Power Assay (FRAP)' was used. Further the nanoparticles were taken in increasing order of concentration and into them 250

µl each of phosphate buffer (0.2 M; pH 6.6) and 1% potassium ferricyanide were added. Test tubes were vortexed at medium speed for 5 minutes to mix the components properly and kept for 20 minutes inside rotary shaker incubator.Further, 250 µl of 10% trichloro acetic acid was added to each test tube and mixed well. Each test tube was emptied in fresh Eppendorf tubes with proper labeling and was centrifuged at 8000 rpm for 5 minutes. Later, test tubes were washed and re-labelled for further use. Supernatants from Eppendorf tubes were collected in their respective labelled test tubes and the pellets were discarded. 250 µl deionized water was added in each of the test tubes to dilute the sample and increase the volume. 50 µl of 0.1% ferric chloride solution was add up to each test tube; the components were thoroughly mixed and allowed to stand for 10 minutes. The absorbance was taken with the help of spectro photo meter using 0.1% ascorbic acid as a reference standard.

The formation of Perl's Prussian blue color at 700 nm after 10 minutes determined the amount of Iron (II) ferricyanide complex. The concentration of antioxidant in selenium nanoparticle was calculated with the help of the formula derived from the standard curve of ascorbic acid.

Results and Discussion

Isolation of Biofilm Forming Bacteria

For the isolation purpose sewage water sample was inoculated on the culture media. After incubation yellow glistening colonies of S. aureus were observed on the Mannitol Salt Agar media while creamy white opaque colonies of P. aeruginosa were observed on the Cetrimide Agar media. The isolated colonies were then transferred onto a fresh media to obtain the pure culture of the isolates by streak plate method.

Characterization of isolated bacteria

The isolated biofilm forming bacteria were characterised morphologically by Gram's staining. The gram staining result showed gram negative rod shaped bacteria and gram positive chain forming cocci for isolate one and two, respectively. The isolates were further characterized by biochemical test, several of which were performed for the isolates according to Bergey's manual. The outcome of biochemical tests for the isolates is summarized in the table 1.

Table 1
Summarized result of biochemical test for the isolated microbe

S.NO	BIOCHEMICAL TEST NAME	SPECIES A	SPECIES B
1	Indole test	Negative	Negative
2	Methyl red test	Negative	Positive
3	Voges proskauer test	Negative	Negative
4	Citrate test	Positive	Positive
5	Catalase test	Positive	Positive
6	Urease test	Negative	Positive
7	Starch Hydrolysis test	Negative	Negative
8	Sugar Fermentation test		
	Glucose	Positive	Positive
	Sucrose	Positive	Positive
	Lactose	Positive	Negative

Synthesis of Selenium Nanoparticle

The nanoparticle synthesis process was carried out by biological method using aloe vera extract and chemical method using sodium selenite, and ascorbic acid. The color change after reaction indicated the successful synthesis of the nanoparticle. In case of biological synthesis, the color of Emblica officinalis extract changed from original light green to dark yellow after addition of sodium selenite under continuous mixing. Further color change was observed after incubation, which yielded the final orange color due to synthesized nanoparticle. During the chemical synthesis of sodium selenite solution, originally light pink in color, changed to orange after addition of ascorbic acid solution which further turned into dark brownish orange color following incubation. These color changes observed were due to the chemical reactions occurred during the synthesis of nanoparticles.

(1) A (1) B (1) C

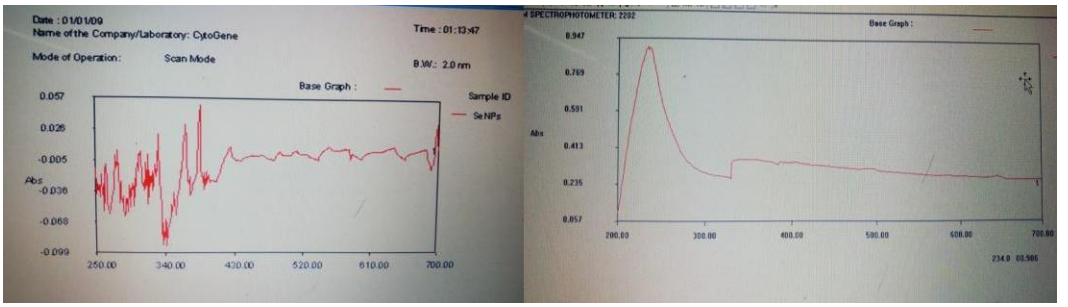
Fig 1: (a) Selenium nanoparticle synthesis by biological method (SNP 1) using the E. officinalis extract, (b) mixed with sodium selenite, (c) kept for incubation in dark

(2) A (2) B (2) C

Fig 2: Selenium nanoparticle synthesis by chemical method (SNP 2) with sodium selenite (A), mixed with ascorbic acid (B), kept for incubation in dark (C)

Characterization of Synthesized Nanoparticle

The amalgamated selenium nanoparticles were specified using the UV-Visible double beam spectrophotometer. For both the nanoparticles, UV-Visible spectra (200nm-700nm) range was taken. The presence of absorbance maxima peak at 234 nm indicated the presence of selenium nanoparticles in the synthesized material.



(3)A (3)B

Fig 3: UV-Visible spectra (200nm-700nm) of the (A) biologically syntheiszed selenium nanoparticle (SNP1) and (B) chemically synthesized nanoparticle (SNP2)

Biological Activity of Selenium Nanoparticle

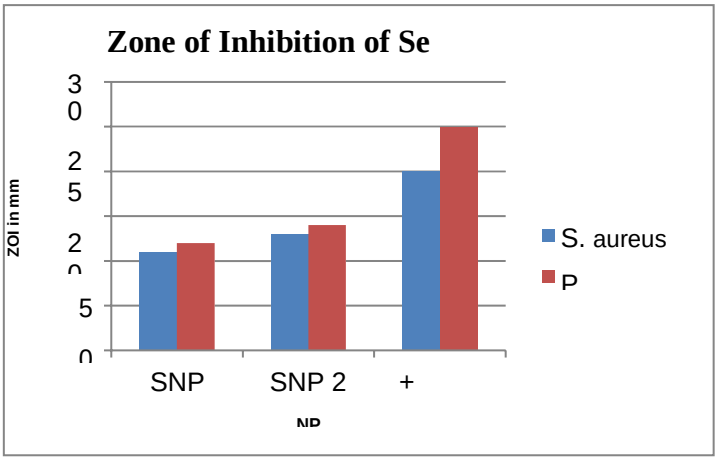
The sample of synthesized nanoparticles was observed to determine its biological activity. The nanoparticles were assayed for its antibacterial activity and antioxidant property. Both the nanoparticles were checked against the biofilm forming isolates of *Staphylococcus aureus* and *Pseudomonas aeruginosa.* Appropriate result was observed against biofilm forming bacteria in nanoparticle samples. The result of antibacterial test is provided in table 2 along with the graphical representation in graph1. The selenium nanoparticle of both origins was found to possess a good antioxidant property too. The antioxidant content was increasing accordingly with the increasing concentration of the nanoparticles. The concentration of antioxidant in the nanoparticle sample was calculated using standard curve of ascorbic acid. The result of antioxidant concentration is summarized in the table 3 along with standard graph 2 of ascorbic acid.

(4) A 4) B

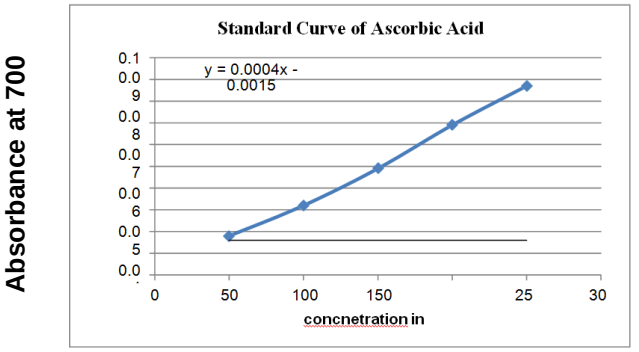
Fig 4: Antibacterial activity of selenium nanoparticle against (A) S. aureus and (B) P. aeruginosa

Table 2
Showing the Zone of inhibition in mm for the selenium nanoparticle against the biofilm forming bacteria; SNP1- biologically synthesized and SNP2- chemically synthesized

S. No.	Species	Zone of Inhibition (mm)		
		SNP 1	SNP 2	+ control
1.	*S. aureus*	11	13	20
2.	*P. aeruginosa*	12	14	25



Graph 1: Showing the result of the antibacterial activity of the selenium nanoparticle of both origins against the biofilm forming isolates



Graph 2: Showing the Standard curve of Ascorbic acid for the determination of antioxidant property of selenium nanoparticle

Table 3: Showing result of antioxidant concentration in the selenium nanoparticle; SNP1-biologically synthesized and SNP2- chemically synthesized

S. No	Sample volume	Absorbance at 700 nm		Conc. of antioxidant (µg/ml)	
		SNP 1	SNP 2	SNP 1	SNP 2
1	1ml	0.002	0.001	8.75	6.25
2	1.5ml	0.004	0.003	13.75	11.25
3	2ml	0.006	0.005	18.75	16.25
4	2.5ml	0.008	0.007	23.75	21.25
5	3.5ml	0.010	0.009	28.75	26.25

At present, it is familiar that either metals or metalloids in their ionic form inherit a solid anti microbial potency for microbial cells (Lemire *et al.*, 2013). Although, a diversity of nano structured metals have promising anti bacterial property. Nano materials possess variety of distinctive physical and chemical properties leading to higher potence as a biocide. This study is focused on the amalgam of the SeNPs by the biological and chemical method. Over past twenty years, many authors have increasingly made attempts to develop novel anti-microbial agents relying on metal/metalloid nano structured particle as a substitute plan to control the alarming inclnation in antibiotic resistance. The unfolding of these pathogenic circumstances give rise to further risks because of their course to high dosage of drug or added toxic medicinal treatments which may be cause of longer stay in clinic and an inclination in mortality rate (Ferri *et al.*, 2015). SeNPs either of biogenic origin or chemically amalgamated have been proven to own antibacterial and antibiofilm potential (Cihalova *et al.*, 2015; Cremonini *et al.*, 2016; Huang *et al.*, 2016). This study was carried out to execute antimicrobial activity of the selenium nanoparticle against the bio film producing bacteria. Tran and Webster reported the effect of Selanium Nanoparticles on *Staphylococcus aureus* ATCC25923.The observation indicates that Selanium Nanoparticles creates a 3 log inhibition of *Staphylococcus. aureus*

growth at concentrations between 7.8 and 31mg/L after 3h of exposure (Tran and Webster, 2011). Chudobova etal., described their chemically synthesized SeNPs with a higher anti-microbial activity than AgNPs, inducing a complete growth inhibition of *S. aureus* NCTC8511 at a concentration of 23.7mg/L (Chudobova *et al.*, 2014). In the present findings also the chemically synthesized nanoparticle is considered to be reasonably effectual for biofilm forming S. aureus and P. aeruginosa.

Conclusion

The study concludes the widespread applicability of selenium nanoparticles in various industries due to their potential effect against the biofilm forming bacteria. The nanoparticle from both the synthesis approach is found to have good antioxidant property and hence can be used for the preparation of several creams, and ointments by the cosmetic industries. They further act as potent anti-microbial agent and thus can be used in drugs formation against these microbes. Due to their potential effect against the biofilm forming bacteria, they can act as potent antimicrobial agents hence, can be used in drug formation against these microbes. The future prospects for the work could be in generating several other biological methods to synthesize the selenium nanoparticle as the demand of this generation is more towards the biological means rather than the chemical means, as there is less chance of any side effects for biological items. There is rising need in assessing these selenium nanoparticles against several other biofilm forming pathogenic bacteria.

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